

BIOSENSORS FOR MONITORING BIOMOLECULE LOCALIZATION AND TRAFFICKING IN CELLS

TECHNICAL FIELD

The present invention generally relates to the assessment/monitoring of the localization,
5 transport and trafficking of biomolecules such as proteins, for example cell surface receptor
endocytosis, recycling and intracellular trafficking of receptors and effectors.

BACKGROUND ART

Protein trafficking is an active process in which proteins are re-located from one region of
a cell to another. Membranes and their protein components are constantly being turned over
10 through a mechanism that has multiple components and pathways. One of the mechanisms of
modulating the activity of cell surface receptors, such as G protein-coupled receptors (GPCRs) and
the Epidermal Growth Factor receptor (EGFR), is through receptor endocytosis. For GPCRs,
ligand-induced receptor endocytosis can drive receptors removal from the PM through specialized
compartments like clathrin-coated vesicles, which involve the recruitment of the endocytic adaptor
15 β -arrestin to liganded receptors (Claing, Laporte et al. 2002). Internalizing receptors can be directed
into divergent lysosomal and recycling pathways, producing essentially opposite effects on the
strength and duration of cellular signaling via heterotrimeric G proteins, and can also promote
distinct signalling events from intracellular membranes through the signalling scaffolding of β -
arrestins (Hanyaloglu and von Zastrow 2008; Posner and Laporte 2010). Therapeutic advantages
20 have been proposed for drugs promoting the intracellular targeting of GPCR/ β -arrestin complexes,
while for some receptors their recycling to the PM is also essential for adequate maintenance of
physiological responses.

Thus, simple and reliable systems for monitoring receptor trafficking are key to study the
mechanism of receptor endocytosis and to develop efficient therapeutics acting on cell surface
25 receptors such as GPCRs. For instance the Angiotensin II type 1 receptor (AT1R) has attracted
significant attention for drug development, because of its involvement in the development of
cardiovascular diseases, including hypertension, hypertrophy, fibrosis and atherosclerosis
(Hunyady and Catt 2006), and because ligands, which have cardioprotective function can also
promote internalization of receptors and intracellular AT1R/ β -arrestin signalling complexes. Great
30 advantages can thus arise from developing assays efficiently assessing in a quantitative and high
efficiency manner drugs' propensity to induce the internalization of receptors such as GPCRs.

BRIEF DESCRIPTION OF DRAWINGS

In the appended drawings:

Figures 1A to 1F show the generation of Bioluminescence Resonance Energy Transfer (BRET)-based GPCR endocytosis sensor. **Figures 1A to 1C**: Three configurations of BRET-based sensors for assessing/monitoring GPCR endocytosis. To monitor the receptor (**Figure 1A**) and β -arrestin (**Figure 1C**) amounts at the plasma membrane, anchor rGFP at the plasma membrane by tagging an acylation moiety of lyn-kinase (MGCIKSKGKDS) in N-terminus of rGFP. **Figures 1B, 1C**: To examine targeting of either receptor (**Figure 1B**) or β -arrestin (**Figure 1C**) to the endosomes, FYVE domain of endofin (amino acids 739 to 806), which tethers the sensor in the endosomes, fused to the C-terminus of rGFP. HEK293SL cells expressing either lyn-rGFP (**Figure 1D**) or rGFP-endofinFYVE (**Figure 1E**) were subjected to confocal fluorescent microscopy. rGFP-endofinFYVE expressing cells were treated with 500 nM wortmannin for 40 min (**Figure 1E**, right panel). Scale bars, 10 μ m. **Figure 1F**: Simultaneous visualization of receptor, lyn-rGFP, and FYVE domain upon receptor endocytosis. HEK293SL cells were transiently transfected with B2R-CFP, lyn-rGFP, and mCherry-endofinFYVE. Top panel showed basal status and the bottom panel showed a bradykinin induced B2R endocytosis. Scale bars, 10 μ m.

Figures 2A to 2F show the dose and time-dependent AT1R endocytosis measured by BRET. **Figure 2A**: HEK293SL cells were transfected with AT1R-RlucII along with either lyn-GFP10 ($\square$) or lyn-rGFP ($\blacksquare$). Cells were incubated with various concentrations of AngII for 40 min then BRET was measured as described under "materials and methods". **Figure 2B**: HEK293SL cells were transfected with AT1R-RlucII along with either GFP10-endofinFYVE or rGFP-endofinFYVE. **Figure 2C**: HEK293SL cells were transfected with AT1R and β arr2-RlucII along with either GFP10-endofinFYVE or rGFP-endofinFYVE. AT1R-RlucII along with either lyn-rGFP (**Figure 2D**) or rGFP-endofinFYVE (**Figure 2E**) transfected cells were incubated in the absence or presence of 100 nM AngII at 37 °C for the indicated times then BRET was measured. The BRET ratio change is expressed as percentage of BRET ratio observed in the control (no AngII treatment) group. Data are represented as the means $\pm$ S.E. from 2-6 independent experiments. **Figure 2F**: Data in **Figures 2D** and **2E** were normalized to the maximal responses, respectively and plotted together.

Figures 3A to 3F show the effect of blocking receptor endocytosis and overexpression of β -arrestin2 on AngII-induced BRET changes. HEK293SL cells were transfected with AT1R-RlucII/lyn-rGFP (**Figure 3A**), AT1R-RlucII/rGFP-endofinFYVE (**Figure 3B**), or AT1R/ β arr2-RlucII/rGFP-endofinFYVE (**Figure 3C**) along with either pcDNA or dynamin K44A. Cells were

incubated in the absence (control, ■; DynK44A, O) or presence of 0.45 M sucrose (▲) for 20 min then stimulated with various concentrations of AngII for 40 min before BRET measurement. HEK293SL cells were transfected with AT1R-RLucII/ lyn-rGFP (**Figure 3D**) or AT1R-RLucII/ rGFP-endofinFYVE (**Figure 3E**) along with either pcDNA or β -arrestin2. **Figure 3F**: endocytosis of AT1R in the presence of the vesicle acidification inhibitors bafilomycin A (Baf) and Chloroquine (CQ). Cells were incubated in various concentrations of AngII for 40 min before BRET measurement. Values shown are the means $\pm$ S.E. from at least three independent experiments.

Figures 4A to 4E show dose-response curves obtained with the endocytosis BRET biosensors with various receptors. **Figure 4A**: HEK293SL cells were transfected with lyn-rGFP along with either AT1R-RLucII, B2R-RLucII, V2R-RLucII, or β_2 AR-RLucII. **Figure 4B**: HEK293SL cells were transfected with rGFP-endofinFYVE along with either AT1R-RLucII, B2R-RLucII, V2R-RLucII, or β_2 AR-RLucII. Cells were incubated with various concentrations of respective cognate ligand as described in the figure for 30 min (**Figure 4A**) or 40 min (**Figure 4B**) at 37°C then BRET were measured. **Figure 4C**: HEK293SL cells were transfected with β arr2-RLucII and rGFP-endofinFYVE along with either AT1R, B2R, V2R, β_2 AR, or FP receptor constructs. Cells were incubated with various concentrations of respective cognate ligand for 40 min before BRET measurement. The cognate ligands for each receptor were as follows: AT1R, AngII (squares); B2R, Bradykinin (BK, triangles); V2R, AVP (circles) or Oxytocin (OT, stars); β_2 AR, isoproterenol (ISO, inverted triangles); FP, PGF2 α (lozenges). Data for (**a**) to (**c**) are expressed as the means $\pm$ S.E. of 2-3 independent experiments. **Figure 4D**: Monitoring of EGFR endocytosis by BRET between RLucII-GRB2 and rGFP-endofinFYVE (GRB2 interacts with EGFR and participates in EGFR internalization). HEK293SL cells were transfected with EGFR along with RLucII-GRB2 and rGFP-endofinFYVE. Cells were incubated with various concentrations of EGF for 30 min at 37°C then BRET were measured. Results for **Figure 4D** are means $\pm$ SE of triplicates in a single representative experiment out of two independent experiments. **Figure 4E**: Assessment of Z'factors as an indication of robustness of the assays for High-Throughput Screening (HTS). HEK293 were co-transfected with AT1R-RLucII/lyn-rGFP, AT1R-RLucII/rGFP-endofinFYVE or AT1R/ β arr2-RLucII/ rGFP-endofinFYVE and plated in a 48-well plate and stimulated with 100 nM AngII at 37 °C for 20 min to allow receptor receptor disappearance from the plasma membrane and the accumulation of both receptor and β arrestin2 in endosomes. Cell surface receptor endocytosis was evaluated in BRET2. BRET values are expressed per well in the presented graphs and Z' factor evaluated over 0.64, 0.73 and 0.79 for the AngII-treated group, respectively, which indicates a robust assay for receptor internalization in endosomes.

Figures 5A to 5C show the monitoring of receptor recycling after ligand removal by endocytosis BRET assays. **Figure 5A**: HEK293SL cells were transfected with AT1R-RLucII along with lyn-rGFP. Cells were incubated in the absence (control) or presence of 100 nM AngII for 30 min then cells were washed and further incubated in the absence of AngII for 45 min. The BRET

ratio change is expressed as percentage of BRET ratio observed in the control (no AngII treatment) group. Data are represented as the means $\pm$ S.E. from four independent experiments.

Figure 5B: HEK293SL cells expressing lyn-rGFP along with either V2R-RlucII, B2R-RlucII, AT1R-RlucII, or β_2 AR-RlucII were subjected to the receptor recycling as described in **Figure 5A** with their cognate ligands, 100 nM AVP for V2R, 100 nM BK for B2R, 100 nM AngII for AT1R, and 1 μ M ISO for β_2 AR. Receptor recycling is expressed as a percent increase in the BRET ratio 45 min after ligand wash-out. All values are expressed as the means $\pm$ S.E. from 3-4 independent experiments.

Figure 5C: HEK293SL cells were transfected with AT1R-RlucII along with rGFP-endofinFYVE. Cells were incubated in the absence (control) or presence of 100 nM AngII for 30 min then cells were washed and further incubated in the absence of AngII for 45 min. The BRET ratio change is expressed as percentage of BRET ratio observed in the control (no AngII treatment) group. Data represent as the means $\pm$ S.E. from three independent experiments.

Figures 6A to 6F show the effects of AngII analogs on AT1R trafficking and sorting. **Figure 6A:** HEK293SL cells expressing AT1R-RlucII/rGFPendofinFYVE were incubated either with 100 nM AngII (squares), 100 nM SI (triangles), or 1 μ M DVG (circles) for indicated times then BRET were measured. BRET ratios were normalized to the maximal AngII response (60 min) as a 100% and the basal (no ligand) as a 0%. Data are expressed as the means $\pm$ S.E. from at least three independent experiments. HEK293SL cells were transfected with AT1R-RlucII/lyn-rGFP (**Figure 6B**), AT1R-RlucII/rGFP-endofinFYVE (**Figure 6C**) or AT1R/ β arr2-RlucII/rGFP-endofinFYVE (**Figure 6D**). Cells were incubated with various concentrations of AngII (squares), SI (triangles), or DVG (circles) for 30 min (**Figure 6B**) or 40 min (**Figures 6C, 6D**) before BRET measurement. Data are represented as the means $\pm$ S.E. from at least three independent experiments. **Figures 6E and 6F:** HEK293SL cells were transfected with AT1R-RlucII along with either rGFP-rab4 (**Figure 6E**) or rGFP-rab11 (**Figure 6F**). Cells were incubated either with 100 nM AngII (squares), 100 nM SI (triangles), or 1 μ M DVG (circles) for indicated times then BRET were measured. The BRET ratio change is expressed as percentage of BRET ratio observed in the control (no ligand treatment) group. Data represent the mean $\pm$ S.E. from three independent experiments.

Figures 7A and B show AT1R internalization accessed by intact cell [125 I]AngII-binding assay. **Figure 7A:** HEK293SL cells were transiently transfected either AT1R alone ($\square$), AT1R-RlucII alone ($\blacksquare$), or AT1R-RlucII along with lyn-rGFP ($\blacksquare$). The cells were incubated in the absence or presence of 100 nM AngII for 30 min at 37 $^{\circ}$ C then subjected to intact-cell [125 I]AngII-binding assay as describe under "materials and methods". **Figure 7B:** HEK293SL cells expressing AT1R-RlucII/Lyn-rGFP along with either pcDNA ($\square$), dynamin K44A ($\blacksquare$), or β -arrestin2 ($\blacksquare$) were incubated in the absence or presence of 100 nM AngII for 30 min at 37 $^{\circ}$ C then subjected to an intact-cell [125 I]AngII-binding assay as describe below (Example 1). Receptor endocytosis was expressed as

the percent loss of cell surface receptors. Data are represented as the means $\pm$ S.E. of three independent experiments.

Figure 8 shows the high basal endosomal localization of V2R. HEK293SL cells transiently expressing V2R-YFP along with mCherry-endofinFYVE, were subjected to a confocal microscopy.

5 Scale bar, 10 μ m.

Figure 9A shows the vesicular localization of rGFP-rab4 and rGFP-rab11 with mCherry-FYVE. HEK293SL cells were transfected either rGFP-rab4 (left) or rGFP-rab11 (right), then subjected to a confocal microscopy.

Figures 9B to 9D show the effect of $G\alpha_q$ inhibition on AngII-mediated AT1R internalization. **Figure 9B:** HEK293SL cells were transfected with AT1R-RlucII along with Lyn-rGFP. Cells were incubated in the absence (-Ubo) or presence of 100 nM Ubo (+Ubo) for 30 min then stimulated with various concentrations of AngII for 30 min before BRET measurement. **Figure 9C:** HEK293SL cells were transfected with AT1R-RlucII along with rGFP-FYVE. Cells were incubated in the absence (-Ubo) or presence of 100 nM Ubo (+Ubo) for 30 min then stimulated with various concentrations of either AngII, SI, or DVG for 40 min before BRET measurement. **Figure 9D:** Cells were transfected with AT1R-RlucII along with either rGFP-rab4 or rGFP-rab11. Cells were incubated in the absence (-Ubo) or presence of 100 nM Ubo (+Ubo) for 30 min then stimulated either with 100 nM AngII, 100 nM SI, or 1 μ M DVG for 10 min for the rGFP-rab4 and 30 min for the rGFP-rab11 then BRET were measured. The BRET ratio change is expressed as percentage of BRET ratio observed in the control (no ligand treatment) group. Data represent the mean $\pm$ S.E. from three independent experiments.

Figures 10A and 10B depict the principle of a BRET-based pharmacological chaperone (PC) assay and sequestration assay to assess functional rescue. **Figure 10A:** The pharmacological chaperone (PC) assay is based on relocalization of a pharmacological chaperone-rescued protein that would, otherwise, be retained in a different subcellular compartment. Relocalization detected and measured using BRET, preferentially with rGFP with a plasma-membrane targeting sequence (rGFP-CAAX, Lyn-rGFP or rGFP-PB; for a description, see **Figure 11D**). Misfolded receptors and channels such as hERG are retained in intracellular compartments and translocate to the plasma membrane upon pharmacological chaperone-mediated rescue. In this assay, the receptor is preferentially tagged with RlucII and the membrane with rGFP; the BRET signal is proportional to the density of the RlucII-tagged protein at the membrane. The misfolded protein could either be a mutant or the WT protein. For receptors that internalize upon agonist exposure, the functionality of the rescued receptor may then be assessed using the agonist-induced sequestration assay depicted in **Figure 10B** (which essentially corresponds to the BRET-based sensor depicted in **Figure 1A**). **Figure 10B:** principle of a BRET-based agonist-induced sequestration assay. A plasma membrane (PM) marker is tagged with a BRET acceptor such as GFP (G) and the PC-rescued receptor of interest (*e.g.*, a PC-rescued GPCR) is tagged with a BRET donor such as

RLuc (R). In the absence of agonist (left), the receptors are retained at the PM and co-localize with the BRET acceptor-tagged PM marker, thus resulting in a strong BRET acceptor signal. However, in the presence of an agonist (right), the receptors are internalized, thus decreasing the density of the BRET donor-tagged receptor at the PM, which results in a decrease in the BRET acceptor signal. This assay can be performed following PC-mediated cell-surface rescue of receptors, in the same well, thus in a homogenous assay that monitor two different aspects of the receptor biology.

Figures 11A to D show the constructs used to validate and optimize a sensor to detect pharmacological chaperone properties. **Figures 11A to C**: the chaperone-rescue assay was developed and tested with wild type (WT) and naturally-occurring substitutions of human GPCRs (Melanocortin receptor 4: hMC4R and the vasopressin receptor 2: hV2R) and a voltage-gated Potassium channel H2 (hERG) tagged with a BRET donor. The receptors were tagged in C-terminal with RlucII. The hERG channel was internally tagged with RlucII at the equivalent position of residue 379 and, the sequence from residues 373-379 was duplicated on each side of linker3 and linker 4 (see **Figure 11C**). Flexible linkers were used between the receptor/channel and the RlucII tag. The sequence of the linkers is indicated in **Figure 11A** for the MC4R constructs (Linker1), in **Figure 11B** for the V2R constructs (Linker2) and in **Figure 11C** for the hERG constructs (Linker3 & 4). **Figure 11D**: A BRET acceptor (rGFP) was tagged with different plasma-membrane or Golgi apparatus targeting sequences: in N-terminal with the palmitoylation & myristoylation signal sequence from the Lyn kinase (Lyn-), in C-terminal with the polybasic sequence and prenylation signal sequence from KRAS splice variant b (-CAAX), in C-terminal with the polybasic sequence from the human GRK5 (-PB), in C-terminal with the plasma-membrane targeting palmitoylation sequence and prenylation signal sequence from hRas, in C-terminal with the plasma-membrane targeting palmitoylation sequence from hRas and prenylation signal sequence from Ral1, in C-terminal with human Caveolin1 α (a marker of caveolae); and in N-terminal with the Golgi targeting sequence (residues 1-73) from human eNOS1. Linkers 5, 6 and 7 were used between the rGFP and the plasma-membrane targeting sequence: lyn, CAAX and PB, respectively. Linker 8 was used between rGFP and palmitoylation/prenylation sequence from hRAS (CAAX) and hRAS/Ral1(CAAX=CCIL), and between rGFP and Caveolin1 α . Linker 9 was used between the golgi targeting sequence from eNOS (1-73) and rGFP.

Figures 12A and B show the testing of different ratios (titration) of two forms of rGFP targeted to the plasma membrane. Titrations of BRET donor to acceptor and PC-rescue assay were performed on transfected cells (variable amount of rGFP construct + 24ng of receptor construct for 10 wells of a 96-well plate), following a 16h treatment with either a chaperone: (DCPMP (N-((2R)-3(2,4-dichloroPhenyl)-1-(4-(2-((1-methoxypropan-2-ylamino)methyl)phenyl)piperazin-1-yl)-1-oxopropan-2-yl)propionamide), 10 μ M) or vehicle (DMSO). HEK293 were transfected with hMC4R (R165Q)-RlucII construct and different quantities of rGFP-CAAX (**Figure 12A**); and rGFP-PB construct (**Figure 12B**). The BRET ratio is reported in function of GFP-

construct expression (evaluated in fluorescence) over RlucII construct expression (evaluated in bioluminescence).

Figures 13A to C show the cell surface expression and functional PC-mediated rescue of wt and mutant MC4R at different ratios of receptor and rGFP-CAAX. HEK293 were co-transfected with an rGFP-CAAX construct (72 ng of plasmid for 10 wells of a 96-well plate) and 3 different quantities (as indicated on the graphs: 6, 12 and 24 ng for 10 wells) of hMC4R wt-RlucII (**Figure 13A**); hMC4R (P299H)-RlucII (**Figure 13B**); and hMC4R (R165Q)-RlucII (**Figure 13C**). The PC-mediated rescue of cell surface expression and functionality (agonist-induced sequestration) was evaluated in BRET2, on transfected cells, following a 16h-treatment with either a chaperone: (DCPMP, 10 μ M; solid black and grey bars) or vehicle (DMSO; white bars). The grey bars represent data obtained from DCPMP-treated cells, exposed 1h to an agonist (alpha-MSH) to induce receptor sequestration. As expected, DCPMP-treatment induces an increase in cell surface expression, as revealed by an increase in BRET signal, compared to non-treated cells (with bars). Agonist-treatment induces sequestration as revealed by a decrease in BRET signal (grey bars) as compared to cells treated with DCPMP but not exposed to an agonist (black bars). The wt (**Figure 13A**) and R165Q mutant (**Figure 13C**) receptors were sensitive to both DCPMP and α -MSH (10 μ M, 1h at 37C) while the P299H mutant MC4R was not PC-rescued (**Figure 13B**). The optimal window for this assay is already obtained at 6ng of donor and, increasing the quantity of transfected donor construct did not lead to a measurable rescue of hMC4R (P299H).

Figure 13D shows polycistronic constructs encoding rGFP-CAAX(Kras) and either a WT or mutant hMC4R were transiently expressed in Hek293 cells. This figure shows that similar results for PC rescue of cell surface expression (white bars: DMSO vs. black bars: 10 μ M DCPMP) and functional rescue can be obtained, as measured by agonist-induced sequestration (+ alpha-MSH; grey bars), from polycistronic and non-polycistronic constructs (**Figures 13A-C**). Agonist-induced sequestration for cells not pretreated with a chaperone is presented (hashed-bars).

Figure 13E shows the PC-mediated rescue of V2R mutants known to be intracellularly retained, as evidenced by the increase in BRET at the plasma membrane. The PC-mediated rescue of cell surface expression was evaluated in BRET1, on transfected cells, following a 16h-treatment with either a chaperone: (SR121463, 10 μ M; solid black bars) or vehicle (DMSO; white bars).

Figures 14A and 14B show dose-response curves for 2 PC-mediated functional rescue of WT and mutant (R165Q) MC4R cell surface expression. HEK293 were co-transfected with an rGFP-CAAX construct and, the hMC4R wt-RlucII or hMC4R (R165Q)-RlucII constructs (72ng of rGFP construct+24ng of receptor construct for 10 wells of a 96-well plate). Dose-responses of PC-mediated rescue of cell surface expression, following a 16h-treatment with variable concentrations of DCPMP (**Figure 14A**) and with Compound 1 (**Figure 14B**), were evaluated in BRET2. Results obtained with hMC4R wt-RlucII (upper curves) or hMC4R (R165Q)-RlucII (lower curves) are

reported in function of the chaperone concentration expressed in a logarithmic scale. EC50 and other curve parameters are indicated below each graph.

Figures 15A to 15D show the assessment of Z'factor as an indication of robustness of the assay. HEK293 were co-transfected with an rGFP-CAAX construct and, the hMC4R wt-RlucII (**Figures 15A and 15B**) or hMC4R (R165Q)-RlucII (**Figures 15C and 15D**) constructs (72ng of rGFP construct + 24ng of receptor construct for 10 wells of a 96-well plate). Cell surface expression was evaluated in BRET2 in **Figures 15A and 15C** using coelenterazine 400a, and in BRET1 using coelenterazine H (**Figures 15B and 15D**) following a 16h-treatment with 10 μ M DCPMP (48 wells) vs. vehicle (DMSO) (48 wells). BRET values are expressed per well in the presented graphs and Z' factor evaluated over 0.63 with the hMC4R wt receptor and over 0.82 with the mutant R165Q mutant hMC4R, which indicates a robust assay with both receptors.

Figures 16A and 16B show the assessment of the impact of DMSO on the BRET-based cell surface expression assay. HEK293 were co-transfected with an rGFP-CAAX construct and, the hMC4R wt-RlucII (**Figure 16A**) or hMC4R (R165Q)-RlucII (**Figure 16B**) constructs (72ng of rGFP construct + 24ng of receptor construct for 10 wells of a 96-well plate). Cell surface expression was evaluated in BRET2, following a 16h-treatment with 10 μ M DCPMP (right bars) or vehicle (DMSO, left bars) in presence of an increasing concentration of DMSO (up to 3%) during the PC-treatment, in order to evaluate whether the BRET-based assay for cell surface evaluation is sensitive to different levels of DMSO. As presented, the results obtained indicate that this assay is resistant to at least 3% DMSO, which is compatible with HTS applications and characterization of compounds.

Figures 17A and 17B show PC-mediated rescue of MC4R and V2R expression in transfected and stable rGFP cell lines. HEK293 were co-transfected with an rGFP-CAAX construct (72 ng of plasmid for 10 wells of a 96-well plate) and 3 different quantities (as indicated on the graphs: 6, 12 and 24 ng for 10 wells) of hMC4R (R165Q)-RlucII (**Figure 17A**) or in hV2R (Y128S)-RlucII (**Figure 17B**). HEK293 cells selected for stably expressing different levels of rGFP-CAAX (low, medium (Med) & high (Hi)) were transfected with the same quantity of receptor constructs. The PC-mediated rescue of cell surface expression for MC4R was evaluated in BRET2, following a 16h-treatment with 10 μ M DCPMP, and for the V2R (Y128S)-RlucII expressing cells with the SR121463 chaperone (a known antagonist with inverse agonist and pharmacological chaperone properties; Serradeil-Le Gal C., *Cardiovasc Drug Rev.* 2001, 19(3): 201-14) at 10 μ M or vehicle (DMSO). The data is presented for the MC4R and V2R expressing cells as a % of BRET signal observed with cells treated with vehicle (DMSO). The presented data indicates that a better response can be obtained with stable cell lines expressing higher levels of rGFP-CAAX. The stable cell line expressing high levels of rGFP-CAAX (Stable:Hi) could be used to establish cell lines co-expressing a receptor-RlucII.

Figures 18A to 18E: PC-rescue assay for detecting ligands of hERG channel (a non-GPCR). Cell-surface expression and functional PC-mediated rescue of wt (**Figure 18A**) and mutant (G601S; **Figure 18B**) hERG at different ratios of hERG to rGFP-CAAX. HEK293 cells were co-transfected with an rGFP-CAAX construct (72 ng of plasmid for 10 wells of a 96-well plate) and 3 different quantities (as indicated on the graphs: 6, 12 and 24 ng for 10 wells) of hERG wt-RlucII (**Figure 18A**) and hERG (G601S)-RlucII (**Figure 18B**). The PC-mediated rescue of cell surface expression was evaluated in BRET2, following a 16h-treatment with either a chaperone: (Astemizole, 10 μ M; solid black bars) or vehicle (DMSO; white bars). Astemizole-treatment induces an increase in cell surface expression, as revealed by an increase in BRET signal, compared to vehicle-treated cells. The wt (**Figure 18A**) and G601S mutant (**Figure 18B**) hERG were both sensitive to a PC-treatment and were used to characterize ligands known to bind and act with different efficacy as chaperones on hERG (**Figures 18C and D**). In **Figure 18E**, robustness of the assay with the hERG (G601S)-RlucII construct was evaluated with a Z'factor. Cell surface expression was evaluated in BRET2, following a 16h-treatment with 10 μ M Astemizole (48 wells) vs. vehicle (DMSO) (48 wells). BRET values are expressed per well in the presented graphs and Z' factor evaluated at 0.622, which indicates a robust assay that would be amenable to high throughput screening application.

Figure 19A shows the configuration of a biosensor for monitoring β -arrestin recruitment to a GPCR at the plasma membrane. A BRET acceptor (e.g., rGFP, GFP10) is tagged with a PM targeting moiety (thus tethering the BRET acceptor at the PM), and a β -arrestin is tagged with a BRET donor (e.g., RlucII). In the presence of a GPCR agonist (represented by A), β -arr is recruited to the GPCR, thus increasing the concentration of RlucII- β -arr at the plasma membrane, which in turn results in an increase in energy transfer (BRET) between RlucII and the PM-tagged GFP.

Figures 19B and 19C show the increase in the BRET ratio for the recruitment of β -arrestin₁ and β -arrestin₂, respectively, at the class A GPCR β 2AR, for different PM-targeting moieties (Lyn, CAAX and PB-GRK5) and BRET acceptors (rGFP and GFP10), following stimulation with increasing doses of the agonist isoproterenol (iso). The β -arrestin-RlucII translocation sensor with rGFP-CAAX (squares) offers the best window with both receptors.

Figures 19D and 19E show the increase in the BRET ratio for the recruitment of β -arrestin₁ and β -arrestin₂, respectively, at the class B GPCR V₂R, for different PM-targeting moieties (Lyn, CAAX and PB-GRK5) and BRET acceptors (rGFP and GFP10), following stimulation with increasing doses of the agonist AVP.

Figure 19F shows the recruitment of β -arrestin₂ to β 2AR following stimulation with increasing doses of the agonist isoproterenol (iso), as assessed using different PM-targeting moieties (CAAX from Kras, CAAX from Hras, the plasma-membrane targeting palmitoylation sequence from hRas and prenylation signal sequence from Ral1 (CCIL) and the marker of the caveolae structures Caveolin1 α tagged with rGFP. The β -arrestin-RlucII translocation sensor with

rGFP-CAAX (squares) show an increase of density at the plasma-membrane. In contrast to the response obtained with the rGFP-CAAX markers, a stimulation of β 2AR lead to a decrease in density of β -arrestin₂ at the caveolae.

Figures 19G shows dose-response curves for translocation of β arrestin2 at the plasma membrane after AT1R stimulation. HEK293SL cells were transfected with AT1R and β arr2-RlucII along with either Lyn-rGFP, or rGFP-CAAX or GFP10-CAAX. Cells were incubated with various concentrations of AngII for 6 min at room temperature before BRET measurements. Data are expressed as percent basal BRET. Data are the means $\pm$ S.E. of 3 independent experiments.

Figures 19H-19J show the Z' factors obtained for the β arrestin₂-RlucII/rGFP-CAAX biosensor and receptors of **Figures 19C, 19E** and **19G**, respectively. This assay, to monitor receptor-mediated β arrestin recruitment, results in Z'factors of at least 0.74 (0.74, 0.80 and 0.838), which would be amenable to screening (including high-throughput screening) applications for both class A and B GPCRs.

Figures 20A and 20B show the AngII-dose dependent decrease in plasma PIP2 amount as detected by BRET between RlucII-PH(PLC δ 1) and rGFP-PH(PLC δ 1) or Lyn-rGFP or rGFP-CAAX. HEK293SL cells were transfected with AT1R and HA-RlucII-PH(PLC δ 1) along with either rGFP-PH(PLC δ 1), Lyn-rGFP, or rGFP-CAAX. Cells were incubated with various concentrations of AngII for 1 min at RT then BRET was measured. Results are means $\pm$ S.E. of triplicates in a single representative experiment.

Figure 21A shows the configuration of a unimolecular biosensor for monitoring β -arrestin recruitment to a GPCR at the plasma membrane. A BRET acceptor (*e.g.*, rGFP, GFP10) is tagged with a PM targeting moiety (thus tethering the construct at the PM) and a flexible linker is placed between the BRET acceptor and a BRET donor (*e.g.*, RlucII), which is attached to a β -arrestin. In the presence of a GPCR agonist (represented by A), β -arr is recruited to the GPCR, thus increasing the concentration of RlucII- β -arr at the plasma membrane, which in turn results in an increase in energy transfer (BRET) between RlucII and the PM-tagged GFP.

Figure 21B shows the BRET ratio using unimolecular biosensors with flexible linkers of different lengths to assess β -arrestin₂ recruitment to V₂R following stimulation with AVP.

Figures 21C to 21E show dose-response curves for the recruitment of β -arrestin₂ at different GPCRs (AT1R, V2R and β 2AR) using unimolecular biosensors.

Figure 22A shows a schematic representation of a unimolecular biosensor for measuring the translocation of the diacylglycerol- (DAG-) binding domain of PKCdelta (C1b) to the plasma-membrane. The biosensor comprises a PM-targeting domain/moiety (Mem), a BRET acceptor (*e.g.*, GFP10), a flexible linker, a BRET donor (*e.g.*, RLucII) and the DAG-binding domain of PKC δ , C1b. Upon activation of PLC, membrane PIP₂ is hydrolysed into IP₃ and DAG. The DAG enrichment causes the C1b domain to bind to the membrane, bringing the BRET acceptor (*e.g.*, GFP10) and BRET donor (*e.g.*, RLucII) closer to each other, inducing a higher BRET signal.

Figure 22B shows kinetics of DAG sensor activation following AT1R exposure to angiotensin II. AT1R stably expressing HEK293 cells were transfected with a construct encoding the unimolecular DAG sensor DNA and BRET. The BRET level was monitored every 4 s. AngII (final concentration of 100 nM) was added after 16 BRET measurements (64 s). Data are mean $\pm$ SD of triplicates of a representative experiment.

Figures 22C to 22E show dose-response curves obtained with the unimolecular DAG sensor representing the level DAG produced at the plasma membrane following activation of the Angiotensin II receptor (AT1R) with angiotensinII (ANGII) (**Figure 22C**), Prostaglandin F receptor (FP) with two natural ligands, prostaglandin 2 α (PGF2 α ; solid diamonds) and prostaglandin E2 (PGE2; open circles) (**Figure 22D**), Urotensin II receptor (GPR14) with urotensin II (UTII) (**Figure 22E**). The EC₅₀ values obtained for those ligands (ANGII= 5.3nM, PGF2 α =11nM, PGE2=90nM and UTII=1.7nM) are similar to the data already published for another related assay (calcium influx) and binding.

Figure 22F shows that the BRET response measured with the unimolecular DAG sensor reflects PLC activation and the concomitant production of diacyl glycerol. HEK293 cells transiently expressing the unimolecular DAG sensor were exposed to 5 μ M of m-3m3FBS, a direct activator of PLC (β 2, β 3, γ 1, γ 2, δ 1 isoforms), for the indicated time. The PLC activation lead to an increase in BRET, reflecting a sustained increase of DAG level at the plasma membrane.

Figures 22G and 22H show the robustness of the DAG biosensor. A Z-factor was determined for the DAG biosensor using HEK293 transiently expressing the urotensin-II (**Figure 23G**) or the prostaglandin F receptor (**Figure 23H**) along with the DAG biosensor. The cells were exposed to 100 nM of agonist (Uroll in **Figure 23G** or PGF2 α in **Figure 23H**) for x min prior to BRET measurements.

Figure 23A shows a schematic representation of a biosensor for measuring the translocation of the diacylglycerol- (DAG-) binding domain of PKCdelta (C1b) to the plasma-membrane. The biosensor comprises a PM-targeting domain/moiety attached to a BRET acceptor (e.g., rGFP) and a BRET donor (e.g., RLucII) linked to the DAG-binding domain of PKC δ , C1b. Upon activation of PLC, membrane PIP₂ is hydrolysed into IP₃ and DAG. The DAG enrichment causes the C1b domain to bind to the membrane, bringing the BRET acceptor (e.g., rGFP) and BRET donor (e.g., RLucII) closer to each other, inducing a higher BRET signal.

Figures 23B to 23D show dose-response curves for the recruitment of C1b at the plasma membrane following activation of the histamine H1 receptor (H1R) (**Figure 23B**), Bradykinin Receptor B2 (BKRB2) (**Figure 23C**), dopamine D2 receptor (D2R) (**Figure 23D**) and β 2AR (**Figure 23E**) using the DAG biosensor. Gq-coupled receptors (H1R and BKRB2) activation lead to a better signal than a Gi-coupled receptor (D2R) or a Gs-coupled receptor that essentially do not lead to a detectable response in absence of co-expression of G15, a G protein of the Gq family.

Figure 24A shows a schematic representation of a biosensor for measuring G protein translocation and activation. The biosensor comprises a PM-targeting domain/moiety (e.g., CAAX domain) attached to a BRET acceptor (e.g., rGFP) and a BRET donor (e.g., *RLucII*) attached to a protein G subunit, for example $G\gamma$ ($G\beta\gamma$ -sequestration based sensor) or $G\alpha$ ($G\alpha$ -sequestration based sensor). Upon activation of the GPCR by an agonist (A), the G protein subunit is released from the GPCR, thus reducing the amount/density of G protein subunit at the plasma membrane, leading to a lower BRET signal. A change in BRET could also reflect translocation from (decrease in BRET) or to (an increase in BRET) a subdomain of the membrane or sub-cellular compartment tagged with an rGFP-marker.

Figures 24B and 24C show the sequestration of various *RLucII*-tagged $G\gamma$ subunit from the plasma membrane (rGFP-CAAX Kras) in response to β 1AR (**Figure 24B**) or β 2AR (**Figure 24C**) stimulation with isoproterenol. Prior to the experiment, HEK293 cells were cotransfected with constructs encoding a β -adrenergic receptor, a WT $G\beta$ 1 subunit, an *RLucII*-tagged $G\gamma$ subunit (as indicated) and WT $G\alpha$ 15. The combination with *RLucII*- $G\gamma$ 1 subunit is giving the best window to establish dose-response curves for those 2 receptors.

Figure 24D shows a dose-response curve for the agonist-promoted *RLucII*-tagged $G\gamma$ 1 sequestration from rGFP-CAAX (Kras) following β 1AR (circles) and β 2AR (triangles) stimulation with isoproterenol of HEK293 cells transiently transfected with constructs encoding a β -adrenergic receptor, a WT $G\beta$ 1 subunit, an *RLucII*-tagged $G\gamma$ 1 subunit and WT $G\alpha$ 15. The observed EC_{50} are similar to the reported K_d of isoproterenol for those receptors.

Figure 24E shows dose-response curves for the agonist-promoted *RLucII*-tagged Gs sequestration from rGFP-CAAX Kras (circles), rGFP-CAAX Hras (squares) and rGFP-CAAX CCIL (triangles) following β 1AR stimulation with isoproterenol. The potency observed with the 3 PM-markers is spanning from 4.4nM (with rGFP-CAAX Kras) to 847nM (with rGFP-CAAX CCIL), indicating that the pharmacology of different ligands could be distinct in domains monitored with specific markers.

Figure 24F shows the kinetics of agonist-promoted *RLucII*-tagged Gs sequestration from rGFP-CAAX Kras (circles), rGFP-CAAX Hras (squares) and rGFP-CAAX CCIL (triangles) following β 1AR stimulation with 1 μ M isoproterenol for the indicated time. The maximal response is mostly reached within 5 min of stimulation as measured with the three PM-markers. The differences of EC_{50} in **Figure 24E** are thus not driven by differences in kinetics as the dose-response curves were establish at maximal response.

Figure 24G shows dose-response curves for the agonist-promoted *RLucII*-tagged G12 sequestration from rGFP-CAAX CCIL (triangles), rGFP-CAAX Hras (squares) and Golgi-rGFP (Golgi targetting domain of eNOS1; diamonds) following β 1AR stimulation with isoproterenol. The basal BRET indicates that G12 colocalized with the Golgi marker. However, most of the agonist-

induced translocation of G12 is observed using the PM-markers and only minimally from Golgi. These results show that both Gs and G12 can be observed following the stimulation of a receptor.

Figure 24H shows dose-response curves for the agonist-promoted *RLucII*-tagged Gq translocation to rGFP-CAAX Kras (circles), rGFP-CAAX Hras (squares), rGFP-CAAX CCIL (triangles) and Golgi-rGFP (Golgi targeting domain of eNOS1; diamonds) following the thromboxane A2 receptor isoform α (TpaR) stimulation with a prototypical agonist: U46619. The dose response curves were obtained from HEK293 cells transiently transfected with constructs encoding: TpaR, G α q pos118RLucII (RLucII inserted after residue 118 of G α q), WT G γ 5 and G β 1, pretreated or not for 20min with Ubo-Qic, a specific Gq inhibitor. The basal BRET indicates that Gq is mostly colocalized with rGFP-CAAX Kras (solid circles) and pretreatment with Ubo-Qic (open circles) further increase the density of Gq with this marker, blunting the window of response to U46619. The dose-response curves obtained with the other markers show an increase of density of Gq (an increase in BRET; solid squares, solid triangles and solid diamonds for rGFP-CAAX Hras, rGFP-CAAX CCIL and Golgi, respectively) only with cells not exposed to a Gq blocker. No response is observed with these markers with cells pretreated with the Gq inhibitor (open squares, open triangles and open diamonds for rGFP-CAAX Hras, rGFP-CAAX CCIL and Golgi, respectively). These results demonstrated that G protein translocation is linked, at least for Gq, to their activation and that it is possible to observe both sequestration or recruitment to subdomains, in response to an agonist stimulation.

Figure 25A shows a schematic representation of a biosensor for measuring Rho activation by the translocation of the Rho binding domain of Protein kinase N1 (PKN) to the plasma-membrane. The biosensor comprises a PM-targeting domain/moiety attached to a BRET acceptor (e.g., rGFP) and a BRET donor (e.g., *RLucII*) linked to the Rho binding domain of PKN. Upon G protein activation, a RhoGEF is recruited to an activated G α subunit such as of the Gq and G12/13 family or to the G $\beta\gamma$ released from the activated G α . This GEF activates a small G protein of the Rho family. Once activated, Rho recruits specific effectors with a domain that interact specifically with an activated Rho; PKN is one of those effectors. Based on this property, a sensor to monitor Rho activation was created by subcloning of PKN1 Rho-binding domain (CRIB) in an expression vector containing a BRET donor, *RLucII*, and by monitoring its translocation to the plasma membrane where the activated Rho is located. The translocation is bringing the BRET acceptor (e.g., rGFP) and BRET donor (e.g., *RLucII*) closer to each other, inducing a higher BRET signal.

Figure 25B shows dose-response curves for the agonist-promoted PKN-*RLucII* translocation to plasma membrane markers: rGFP-CAAX Kras (circles), rGFP-CAAX Hras (inverted triangles) and rGFP-CAAX (CCIL; triangles) following TpaR stimulation with an agonist (U46619). The dose response curves were obtained from HEK293 cells transiently transfected with

constructs encoding: TP α R, PKN-RLucII, and a plasma membrane rGFP-marker. TP α R is a prototypical Gq/12/13-coupled receptor known to activate RhoA.

Figure 25C shows kinetics of Rho sensor activation following AT1R exposure to angiotensin II. HEK293SL cells were transfected with constructs encoding AT1R along with PKN-crib-RLucII and rGFP-CAAX. Cells were first incubated in the absence or presence of 100 nM Ubo-Qic (a specific Gq inhibitor also known as FR900359) for 30 min before BRET measurements at every 2 sec. Tyrode (non-stimulated) or AngII (Stimulated; final concentration of 100 nM) were injected after 30s. Data are the average of duplicate reading at different time points of a representative experiment.

Figure 25D to 25F shows the impact of Gq inhibition on Rho activation by different AngII ligands. HEK293SL cells were transfected with constructs encoding AT1R along with PKN-CRIB-RLucII and rGFP-CAAX. Cells were incubated in the absence (solid line) or presence (dotted line) of 100 nM Ubo-Qic (Gq inhibitor) for 30 min, then stimulated with various concentrations of AngII or analogs for 4 min before BRET measurements. Data were normalized to the E_{max} of AngII. Data represent as the means $\pm$ S.E. from 3-4 independent experiments. Gq-activating ligands, such as AngII, hAngIII, SVdF, SBpa, hSarmesin, and SI showed a reduced efficacy and a rightward-shifted potency in the presence of ubo (**Figures 25D and E**). Blocking of Gq did not affect DVG, Saralasin, and TRV-mediated Rho activation since these ligands do not activate Gq. SII showed only changing EC₅₀ by ubo treatment, suggesting that SII weakly activates Gq/11.

Figure 25G shows the impact of Gq inhibition on Rho activation by AT1R and Acetylcholine receptors. HEK293SL Cells were transfected with constructs encoding AT1R along with PKN-CRIB-RLucII and rGFP-CAAX. Cells were incubated in the absence (control) or presence of 100 nM Ubo before being stimulated either with 100 nM AngII or 100 μ M carbachol (CCh) for 70s before BRET measurements. Results show that Ubo partially blocked AngII-mediated BRET increase and completely blocked CCh-mediated responses; suggesting that Gq plays a role in Rho activation by AngII and CCh. Data are mean $\pm$ SD of triplicates from a representative experiment.

Figure 25H shows the Effects on a Rho inhibitor of the Rho sensor activation. HEK293SL expressing AT1R, PKN-crib-RLucII and rGFP-CAAX, were incubated with 3 μ g/ml of C3 toxin (Rho inhibitor, Cytoskeleton, Inc.) in Tyrode for $\sim$ 4 hr at 37 $^{\circ}$ C then stimulated with 100 nM AngII for 70 s at RT. C3 toxin completely abolished agonist-mediated BRET increases, validating the sensor for monitoring Rho activity.

Figure 25I shows that PKN translocation to the plasma membrane is dependent on Rho activation. HEK293 cells transiently expressing TP α R and the PKN sensor (PKN-RLucII + CAAX-rGFP), were pretreated or not, overnight, with of a Rho inhibitor (CT04; Cytoskeleton, Inc) and exposed to 100nM of U46619 (TP α R agonist), 1 μ g/ml of Rho activator II (CN03; Cytoskeleton, Inc) or vehicle. The Rho inhibitor abolished the TP α R-mediated response while the Rho activator is inducing a response, validating this sensor for monitoring Rho activity.

Figures 26A and 26B show the BRET transfer obtained between RlucII and different BRET acceptors for unimolecular fusion constructs. The BRET signal obtained using rGFP was more than 10-fold higher than that obtained with typical BRET1 (Venus) and BRET2 (GFP2) acceptors. **Figure 26A** shows the difference of energy transfer when RlucII is paired with Venus, GFP2, and rGFP. **Figure 26B** shows the BRET ratio calculated from Venus-, GFP2- and rGFP-fused-constructs.

Figure 26C shows that the rGFP-enhanced BRET signal can be used to monitor BRET in microscopy, even for a density-BRET based assay such as the recruitment of beta-arrestin to the plasma membrane. The assay used in this experiment is similar to that presented in **Figure 19C** as measured using a plate reader. HEK293 cells were transiently transfected with constructs encoding the β 2AR, the β arrestin2-RlucII and the plasma membrane marker: rGFP-CAAX(Kras). Isoproterenol stimulation induced the increase of BRET signal level only at the plasma membrane, indicating that an increase in BRET signal is a reflection of β arrestin recruitment to the plasma membrane.

Figure 27A shows the results of a screening of modulators of AT1R endocytosis. Following transient transfection of AT1R-RlucII and rGFP-FYVE, HEK293 cells were dispensed to 384-well white tissue culture treated plate (Greiner) and grown for an additional 24h. Compounds are added using a 384 magnetic pintool (V&P scientific) at a final concentration of 15 μ M or 5 μ g/ml depending on the compound sub-library. For the agonist mode, compounds were incubated for 30 min at 37°C. GFP fluorescence was read using an Envision™ (Perkin-Elmer®) and coelenterazine 400a was added at a final concentration of 5 μ M using a multidrop 384 (Thermo-Scientific®). Cells were incubated at room temperature before reading the BRET signal (RLuc at 480 nm and rGFP at 530 nm). For the antagonist mode, compounds were incubated for 30 min at 37°C. Angiotensin II was added at 10 nM (EC_{80}) and incubated for an additional 30 minutes at 37°C. The rest of the assay was performed as the agonist mode. Data were analysed using ActivityBase (IDBS) and reported as % agonist or % inhibition based on the angiotensin II activation. Shown are respectively 30 and 42 compounds that act as potentiators (increased the signal over 100%) and inhibitors (blocked more than 50% the signal) for AT1R targeting to endosomes.

Figure 27B shows the effects of compound #21 in the screening on B2R and β arrestin2 endocytosis to endosomes, and **Figure 27C** shows the effects of compound #10 and #29 identified in the screening on B2R endocytosis to endosomes. One day before transfection, HEK293SL cells were seeded in 35 mm glass-bottom dishes at a density of 100,000 cells/dish. Cells were transfected with B2R-YFP + mCherry-FYVE (**Figure 27B left and middle and 27C**) or B2R + β arrestin2-YFP (**Figure 27C**). Forty-eight hours post-transfection, cells were serum starved for 30 min and pretreated with either vehicle (**Figure 27B left and 27C left**), compound 21 (**Figure 27B middle and right**), compound 10 (**Figure 27C middle**), or compound 29 (**Figure 27C right**) for 30

min at 37 °C. Then cells were stimulated with or without (non treated) bradykinin (1 μ M) for 15 min. Samples were analyzed on a Zeiss™ LSM-510 Meta laser scanning microscope using argon (514 nm) and HeNe I (543 nm) lasers, and images (2048x2048 pixels) were collected using a 63x oil immersion lens.

5 DISCLOSURE OF INVENTION

In the studies described herein, the present inventors have developed BRET-based biosensors that permit to assess/monitor the intracellular localization and trafficking (e.g., receptor internalization, recycling, exocytosis) of proteins, such as receptors and other proteins. Using GPCRs and an ion channel as models, the present inventors have developed sensitive means,
 10 based on the *renilla*'s BRET pair RLucII-rGFP, for real-time monitoring and pharmacological profiling of receptor and β -arrestin internalization and their trafficking into different cellular compartments, as well as for the identification of trafficking regulators. These sensors rely on changes in concentrations or densities of the donor relative to the acceptor at a given cellular localization or in a given cellular compartment, which is promoted by a modulator, independently of
 15 direct protein-protein interactions (as it is often the case for conventional BRET assays) it is more versatile and amenable to most proteins trafficking between different cellular localizations/compartments. It was found that the use of a *renilla*'s BRET pair, such as the representative RLucII/rGFP BRET pair, system gives very robust and reproducible response, which increases the dynamic range over ~5 to 10-fold compared to that of the traditional BRET1
 20 (Rluc/Venus) or BRET2 (RlucII/GFP10) pairs. In general, the dynamic range of the signal is very narrow using the Rluc/Venus pair (BRET ratios of 0.04-0.08), similar to the one obtained with the version of the biosensors using the RLucII/GFP10 pair (see Examples 3 and 12, **Figures 2A to 2C** and **19A to 19E**). This very shallow dynamic range greatly limits the analysis of subtle changes in receptor and effector trafficking and renders the assay inefficiently sensitive for high-throughput
 25 screening (HTS). The sensitive biosensors described herein may be useful for:

- *Real-time monitoring of cell surface receptor internalization and recycling (i.e. receptors returning to the plasma membrane after internalization):* They allow vetting removal of different receptors (e.g., GPCRs, RTKs) from the plasma membrane, and conversely, after inducing endocytosis, monitoring recycling of receptors through the regain of BRET signal
 30 at the PM following ligand removal. They also allow the study of regulation, the pharmacology and pathway-specificity of endocytosis of receptor trafficking.
- *Real-time monitoring of receptor and β -arrestin trafficking in different intracellular compartments.* They allow assessing the clathrin- and β -arrestin-dependent internalization of receptors (e.g., GPCRs) and the differential trafficking of receptor/ β -arrestin complexes
 35 into distinct cellular compartments such as the recycling endosomes (ENDs).

- *Pharmacological profiling of receptor (e.g., GPCR) and β -arrestin trafficking*: They allow assessing the propensity of ligands to regulate the trafficking of receptor/ β -arrestin complexes into distinct cellular compartments, and monitor the effects of drugs on both the initial internalization of receptor and the cycling of receptors (e.g., GPCRs) at the PM.
- 5 • *Identification of trafficking modulators through high-throughput screening (HTS)*: Because the assays are reproducible and sensitive, they allow high-throughput screening for identifying modulators of receptor (e.g., GPCR) and other protein trafficking. Proof-of-principle was provided with the AT1R, and the identification of new small molecule regulators of this receptor trafficking, and also other GPCRs like the B2R (Example 17).
- 10 • *Identification of agents (chaperones) capable of rescuing the expression of receptors*. The chaperone assay is independent on receptor signalling and is mostly a binding assay to detect ligands that stabilize or influence the conformation of a specific target (receptor). It is thus an interesting assay for screening orthosteric and allosteric ligands, in a signalling unbiased way. This binding assay could be further used to identify an “off-target” effect of an agent, i.e. to determine whether an agent identified against a particular target also cross-reacts with one or more additional targets (which could be assessed by determining whether the agent “rescues” these one or more additional targets using the biosensor described herein.
- 15 • *Monitoring/assessing the recruitment of proteins (e.g., adaptor or signalling proteins) to receptors (e.g., β -arrestin recruitment to GPCRs, G protein subunit sequestration, Grb2 recruitment to Receptor Tyrosine kinase (RTK), which reflects receptor activation*.
- 20

Because the different cellular compartment markers remain in their selective compartments (e.g. plasma membrane (PM) or endosomes (ENDs)), and because only the receptor (or other tagged proteins such as β arrestin, G protein subunits, effectors, etc.) moves from one compartment to the other upon modulation by a ligand (e.g., agonist stimulation, antagonist inhibition or pharmacological chaperones), it allows the tracking of trafficking proteins from the PM to ENDs, which may be revealed by a decrease (for the PM-rGFP/receptor-RlucII assay) and/or an increase (for the END-rGFP/receptor-RlucII assay) BRET signals, respectively. In addition, using the PM-rGFP/receptor-RlucII system, the receptor recycling can be monitored with ligand wash-off after its endocytosis. This assay is not limited to assessing endocytosis/recycling of receptors, but is also amenable to identification and characterisation of pharmacological chaperones (see Examples 7 to 11), and to also assess/monitor exocytosis and protein translocation processes. Therefore any type of protein movement (trafficking) between different intracellular compartments can be accessed quantitatively with high sensitivity using the biosensors described herein.

These biosensors can also be applied not only to protein (e.g., receptor, intracellular proteins) trafficking but also to monitoring any type of local concentration or density changes of

proteins and other biomolecules in the cells. It can be done in two ways: First, if the rGFP or the RlucII are tagged with specific intracellular organelle or cellular compartment markers, it may be possible to follow the protein of interest in different intracellular localization upon any specific condition. Second, the biosensors of the invention can be applied to monitoring local concentration or density of proteins, as well as the local density of lipids or other biomolecules (e.g., second messengers). The RLucII-rGFP pair was applied to detect membrane PI(4,5)P₂ generation using PLCδ1-PH domain (Example 13, **Figures 20A and B**). In the basal state, PLCδ1-PH-RlucII and PLCδ1-PH-rGFP (or rGFP fused to a PM targeting moiety such as Lyn or CAAX) are localized in the PM where PI(4,5)P₂ is located, so their local concentration is high enough to generate a BRET. When the phospholipase C (PLC) is activated, PI(4,5)P₂ is hydrolyzed, and the PLCδ1-PH domain tagged RlucII and rGFP diffuse into the cytosol reducing the local concentration of rGFP and RlucII, hence reducing the BRET signal. Similarly, the DAG-binding domain of PKCdelta (C1b) may be used to measure PIP₂ hydrolysis into IP₃ and DAG (**Figures 22A and 23A**). DAG enrichment causes the C1b domain to bind to the membrane, bringing the BRET acceptor (e.g., rGFP, linked to a PM targeting moiety) and BRET donor (e.g., RLucII, linked to C1b) closer to each other, inducing a higher BRET signal. With the same rationale, any kind of protein segregation also could be detected. In an embodiment, the trafficking is receptor internalization and/or recycling.

Accordingly, in a first aspect, the present invention provides biosensor for assessing the localization and/or trafficking of a protein/polypeptide of interest comprising: a first component comprising the protein/polypeptide of interest tagged with a *Renilla* green fluorescent protein (*Renilla* GFP) or a *Renilla* luciferase protein (*Renilla* Luc); a second component comprising a cellular compartment targeting moiety tagged with a *Renilla* GFP or a *Renilla* Luc; wherein if said protein/polypeptide of interest is tagged with said *Renilla* GFP, said cellular compartment targeting moiety is tagged with said *Renilla* Luc, and if said protein/polypeptide of interest is tagged with said *Renilla* Luc, said cellular compartment targeting moiety is tagged with said *Renilla* GFP.

The term "protein/polypeptide of interest" refers to any protein/polypeptide (native, mutated, soluble or membrane-bound) or fragments/portions thereof, whose localization, translocation and/or recruitment to one or more cellular compartments is to be assessed. The protein of interest may be, for example, a receptor, a protein recruited to, or sequestered away from, the plasma membrane upon receptor stimulation, a protein translocating to the nucleus, etc. In an embodiment, the protein of interest is a receptor (i.e., a protein found attached to or embedded within the plasma membrane). In an embodiment, the receptor is internalized upon ligand (e.g., agonist) binding. In an embodiment, the receptor is a G-protein coupled receptor (GPCR). "GPCR" refers to full length native GPCR molecules as well as mutant GPCR molecules. A list of GPCRs is given in Foord *et al* (2005) Pharmacol Rev. 57, 279-288, which is incorporated herein by reference, and an updated list of GPCRs is available in the IUPHAR-DB database (Harmar AJ, *et al.* (2009) IUPHAR-DB: the IUPHAR database of G protein-coupled receptors and ion channels.

Nucl. Acids Res. **37** (Database issue): D680-D685; Sharman JL, et al., (2013) IUPHAR-DB: updated database content and new features. *Nucl. Acids Res.* **41** (Database Issue): D1083-8).

In another embodiment, the receptor is an ion channel, for example a voltage-gated ion channel (e.g., a sodium, calcium, potassium channel). A list of ion channels is available in the IUPHAR-DB database (see references above).

In another embodiment, the protein/polypeptide of interest is an adaptor protein (e.g., a signal transducing adaptor protein) a variant/fragment thereof. Adaptor proteins are proteins that are accessory to main proteins in a signal transduction pathway, and contain a variety of protein-binding modules (e.g., SH2 and/or SH3 domains) that link protein-binding partners together and facilitate the creation of larger signaling complexes. These proteins usually lack any intrinsic enzymatic activity themselves, but instead mediate specific protein-protein interactions that drive the formation of protein complexes. Examples of adaptor proteins include MyD88, Grb2 and SHC1.

In another embodiment, the protein of interest is a β -arrestin, a β -arrestin variant, or an active portion/fragment thereof, for example β -arrestin-1 (RefSeq: NP_004032.2 for isoform 1; NP_064647.1 for isoform 2) or β -arrestin-2 (RefSeq: NP_004304.1 for isoform 1; NP_945355.1 for isoform 2; NP_001244257.1 for isoform 3; NP_001244258.1 for isoform 4; NP_001244259.1 for isoform 5; and NP_001244260.1 for isoform 6).

In another embodiment, the protein of interest is a G protein subunit, a G protein subunit variant, or an active portion/fragment thereof, e.g., a $G\alpha$, $G\gamma$ or $G\beta$ subunit or an active fragment thereof.

Thus, in another aspect, the present invention provides a biosensor for assessing G protein and/or GPCR activation, said biosensor comprising: a first component comprising a G protein subunit or an active fragment thereof tagged with a *Renilla* green fluorescent protein (*Renilla* GFP) or a *Renilla* luciferase protein (*Renilla* Luc); a second component comprising a PM targeting moiety tagged with a *Renilla* GFP or a *Renilla* Luc; wherein if said G protein subunit is tagged with said *Renilla* GFP, said PM targeting moiety is tagged with said *Renilla* Luc, and if said G protein subunit is tagged with said *Renilla* Luc, said PM targeting moiety is tagged with said *Renilla* GFP.

In another aspect, the present invention provides a biosensor for assessing whether a GPCR ligand modulates the activity of a G protein subunit, said biosensor comprising: a first component comprising said G protein subunit or an active fragment thereof tagged with a *Renilla* green fluorescent protein (*Renilla* GFP) or a *Renilla* luciferase protein (*Renilla* Luc); a second component comprising a PM targeting moiety tagged with a *Renilla* GFP or a *Renilla* Luc; wherein if said G protein subunit is tagged with said *Renilla* GFP, said PM targeting moiety is tagged with said *Renilla* Luc, and if said G protein subunit is tagged with said *Renilla* Luc, said PM targeting moiety is tagged with said *Renilla* GFP.

In an embodiment, said G protein subunit or active fragment thereof is tagged with said *Renilla* Luc, said PM targeting moiety is tagged with said *Renilla* GFP. In an embodiment, the G protein subunit is a $G\gamma$ subunit, e.g., $G\gamma 1$, $G\gamma 2$, $G\gamma 3$, $G\gamma 4$, $G\gamma 5$, $G\gamma 6$, $G\gamma 7$, $G\gamma 8$, $G\gamma 9$, $G\gamma 10$, $G\gamma 11$, $G\gamma 12$ or $G\gamma 13$. In another embodiment, the G protein subunit is a $G\alpha$ subunit, e.g., Gq, Gs, Gi1, Gi2, Gi3, Gt-cone, Gt-rod, Gt-gus, Gz, GoA, GoB, Golf, G11, G12, G13, G14, or G15/G16. In another embodiment, the G protein subunit is a $G\beta$, e.g., $G\beta 1$, $G\beta 2$, $G\beta 3$, $G\beta 4$ or $G\beta 5$ ($G\beta 5$ -S or $G\beta 5$ -L).

In another embodiment, the protein of interest is a protein that binds to DAG, or an active portion/fragment thereof, e.g., a phorbol esters/diacylglycerol binding domain (DAG-binding domain). In an embodiment, the DAG-binding domain is from PKC δ (C1b). Other proteins that comprise a DAG-binding domain (commonly referred to as C1 domain) include, for example AKAP13; ARAF; ARHGAP29; ARHGEF2; BRAF; CDC42BPA; CDC42BPB; CDC42BPG; CHN1; CHN2; CIT; DGKA; DGKB; DGKD; DGKE; DGKG; DGKH; DGKI; DGKK; DGKQ; DGKZ; GMIP; HMHA1; KSR1; KSR2; MYO9A; MYO9B; PDZD8; PRKCA; PRKCB1; PRKCD; PRKCE; PRKCG; PRKCH; PRKCI; PRKCN; PRKCQ; PRKCZ; PRKD1; PRKD2; PRKD3; RACGAP1; RAF1; RASGRP; RASGRP1; RASGRP2; RASGRP3; RASGRP4; RASSF1; RASSF5; ROCK1; ROCK2; STAC; STAC2; STAC3; TENC1; UNC13A; UNC13B; UNC13C; VAV1; VAV2 and VAV3.

In another embodiment, the protein of interest is PLC $\delta 1$ or an active portion/fragment thereof capable of binding to PI(4,5) P_2 , e.g., the pleckstrin homology (PH) domain of PLC $\delta 1$.

In another embodiment, the protein of interest is a protein that binds to a small GTPase (ExPASy ENZYME entry: EC 3.6.5.2). Small GTPases are a family of about 50 enzymes with a molecular mass of 21 kDa distantly related to the α subunit of G proteins, and which are involved in cell-growth regulation (Ras subfamily), membrane vesicle traffic and uncoating (Rab and ARF subfamilies), nuclear protein import (Ran subfamily) and organization of the cytoskeleton (Rho and Rac subfamilies). In an embodiment, the protein of interest is a protein that binds to one or more members of the Ras superfamily of small GTPases, e.g., Ras, Rho, Ran, Rab and Arf families of GTPases. The localization/translocation of such small GTPases may be assessed using a polypeptide comprising a Ras-binding domain (RBD), for example the RBD of RAF1 or a variant thereof that comprises an A85K substitution (which has a higher affinity for Ras). Other proteins that comprise a RBD include ARAF, BRAF, RGS12, RGS14, TIAM1 and PI3K. The protein of interest may thus comprise the entire/native sequence of a protein that binds to a small GTPase, or a variant of fragment thereof that maintains the ability to bind to a small GTPase.

In a further embodiment, the protein of interest is a protein that binds to one or more members of the Rho superfamily of small GTPases, Rho (A, B & C), Rac (rac1, 2, 3 or RhoG) or Cdc42 (Cdc42, RhoQ or RhoJ). In another embodiment, the protein of interest is a protein that binds to a Rho protein (RhoA, RhoB and/or RhoC, preferably RhoA), or an active fragment thereof, for example a Cdc42/Rac interactive binding (CRIB) domain. The CRIB domain (EMBL-

EBI/Interpro accession No. IPR000095) is a conserved region within the N-terminal portion of the GTPase binding domain (GBD, also called p21 binding domain, PBD) that is present in many putative downstream effectors of small GTPases (e.g., Cdc42p- and/or Rho-like small GTPases), and comprises about 15-16 amino acids. Proteins that comprise a CRIB domain include

5 mammalian activated Cdc42-associated kinases (ACKs), mammalian p21-activated kinases (PAK1 to PAK4), Rhotekin (RTKN), mammalian Wiskott-Aldrich Syndrome Proteins (WASPs), kinases of the protein kinase C superfamily, such as serine/threonine protein kinase N (PKN, also known as protein kinase C-related kinase, PRK). In an embodiment, the protein of interest comprises the CRIB domain of human PKN1 (Uniprot reference: Q16512-1) or PKN2 (Uniprot reference:

10 Q16513), preferably PKN1. The CRIB domain of human PKN1 comprises the sequence VQSEPRSWSLLEQLG (SEQ ID NO:40), which corresponds to residues 6-20 of native human PKN1 (Uniprot reference: Q16512-1).

Thus, in another aspect, the present invention provides a biosensor for assessing the activation of a small GTPase (e.g., Rho), said biosensor comprising: a first component comprising

15 a polypeptide comprising a domain that binds to said small GTPase (e.g., a CRIB domain) tagged with a *Renilla* green fluorescent protein (*Renilla* GFP) or a *Renilla* luciferase protein (*Renilla* Luc); a second component comprising a PM or endosomal targeting moiety tagged with a *Renilla* GFP or a *Renilla* Luc; wherein if said polypeptide comprising a domain that binds to said small GTPase (e.g., a CRIB domain) is tagged with said *Renilla* GFP, said PM or endosomal targeting moiety is

20 tagged with said *Renilla* Luc, and if said polypeptide comprising a domain that binds to said small GTPase (e.g., a CRIB domain) is tagged with said *Renilla* Luc, said PM or endosomal targeting moiety is tagged with said *Renilla* GFP. In an embodiment, the small GTPase is a Rho protein (e.g., RhoA). In an embodiment, the domain that binds to the small GTPase is a CRIB domain, such as the CRIB domain of human PKN1. In an embodiment, the second component comprises a

25 PM targeting moiety.

The term *Renilla luciferase* as used herein refers to an oxidative enzyme used in bioluminescence and that is derived from an organism of the genus *Renilla*, such as *Renilla reniformis* or *Renilla mulleri*. It includes the native luciferase from a *Renilla* organism, or variants thereof, for example the native form (in terms of amino acid sequence) of *Renilla reniformis*

30 luciferase (Rluc) or variants thereof such as RlucII or Rluc8. The term "RlucII" refers to a mutant form of *Renilla reniformis* luciferase that comprises the following amino acid substitutions: A55T, C124A and M185V relative to a native *Renilla* luciferase. In an embodiment, the RlucII comprises the sequence depicted in Example 1 (SEQ ID NO:10). The term "Rluc8" refers to a mutant form of *Renilla reniformis* luciferase that comprises the following amino acid substitutions: A55T, C124A,

35 S130A, K136R, A143M, M185V, M253L, and S287L relative to a native *Renilla* luciferase. The amino acid sequence of native *Renilla mulleri* luciferase is disclosed in GenBank accession No. AAG54094.1.

The term “*Renilla* GFP” refers to a green fluorescent protein that is derived from organisms of the genus *Renilla*, such as *Renilla reniformis* or *Renilla mulleri*. It includes the native GFP from a *Renilla* organism, or variants thereof. In an embodiment, the *Renilla* GFP is a *Renilla reniformis* GFP (referred to herein as “rGFP”), in a further embodiment, the native form (in terms of amino acid sequence) of *Renilla reniformis* GFP. In an embodiment, the rGFP comprises the sequence depicted in Example 1 (SEQ ID NO:11). The amino acid sequence of native *Renilla mulleri* GFP is disclosed in GenBank accession No. AAG54098.1. The nucleic acid sequence of the *Renilla* luciferase and/or *Renilla* GFP may be codon-optimized for expression in human cells (i.e. “humanized”, see, e.g., WO 2002057451 for a humanized version of *Renilla mulleri* GFP).

Resonance energy transfer (abbreviated RET) is a mechanism describing energy transfer between two chromophores, having overlapping emission/absorption spectra. When the two chromophores (the “donor” and the “acceptor”), are within a short distance (e.g., 10-100 Angstroms) of one another and their transition dipoles are appropriately oriented, the donor chromophore is able to transfer its excited-state energy to the acceptor chromophore through non-radiative dipole-dipole coupling. Bioluminescence Resonance Energy Transfer (BRET) is based on the non-radiative transfer of energy between a donor bioluminophore (bioluminescent enzyme such as *renilla* luciferase) and an acceptor fluorophore (e.g., *renilla* GFP).

The term “cellular compartment targeting moiety” refers to a biomolecule, preferably a polypeptide or peptide, which, when attached to the *Renilla* GFP or *Renilla* Luc (as a fusion protein, for example), targets them to a particular compartment, organelle or localization within the cell, such as for example the plasma membrane (or a particular subdomain of the plasma membrane, such as lipid rafts), the endosomes (e.g. early and/or late endosomes), the lysosomes, the phagosomes, the ribosomes, the mitochondria, the endoplasmic reticulum, the Golgi apparatus, the nucleus, etc., thereby increasing the effective concentration of the *Renilla* GFP or *Renilla* Luc. Such markers are typically proteins (or suitable fragments thereof) that are normally found at high levels in the targeted particular compartment. Peptides that target proteins to specific compartment, organelle or localization within the cell are known in the art and include endoplasmic reticulum (ER) signal peptide or ER-retrieval sequence, nuclear localization signal (NLS) peptide, and mitochondrial localization signal (MLS) peptide, for example.

In an embodiment, the cellular compartment targeting moiety is a plasma membrane (PM) targeting moiety. Any moiety capable of recruiting the *Renilla* GFP or *Renilla* Luc to the PM may be used in the biosensors. The *Renilla* GFP or *Renilla* Luc may thus be fused to any protein found at the plasma membrane (e.g., receptors or any other protein found at the PM), or fragments thereof. An example of such proteins is Caveolin-1, which the main component of the caveolae (a type of lipid raft that correspond to small (50-100 nm) invaginations of the plasma membrane) found in many cell types. Two isoforms of Caveolin-1, generated by alternative splicing of the CAV1 gene, have been identified: Caveolin-1 α (comprising residues 2-178) and Caveolin-1 β (corresponding to

the 32-178 sequence). Other examples of such moiety include peptides/polypeptides comprising a signal sequence for protein lipidation/fatty acid acylation, such as myristoylation, palmitoylation and prenylation, as well as polybasic domains. Several proteins are known to be myristoylated, palmitoylated and/or prenylated (e.g., protein kinases and phosphatases such as Yes, Fyn, Lyn, Lck, Hck, Fgr, G α proteins, nitric oxide synthase, ADP-ribosylation factors (ARFs), calcium binding proteins and membrane or cytoskeleton-associated structural proteins such as MARCKS (see, e.g., Wright et al., *J Chem Biol.* Mar 2010; 3(1): 19-35; Alcart-Ramos et al., *Biochimica et Biophysica Acta (BBA) – Biomembranes*, Volume 1808, Issue 12, December 2011, Pages 2981-2994), and thus the myristoylation, palmitoylation and prenylation signal sequences from any of these proteins may be used in the biosensor. In an embodiment, the myristoylation and/or palmitoylation sequence is from the Lyn kinase.

In an embodiment, the PM membrane targeting moiety comprises a CAAX motif (C is cysteine residue, AA are two aliphatic residues, and X represents any amino acid. CAAX motifs are found in “CAAX proteins” that are defined as a group of proteins with a specific amino acid sequence at C-terminal that directs their post translational modification. CAAX proteins encompass a wide variety of molecules that include nuclear lamins (intermediate filaments) such as prelamin A, lamin B1 and lamin B2, Ras and a multitude of GTP-binding proteins (G proteins) such as Ras, Rho, Rac, and Cdc42, several protein kinases and phosphatases, etc. (see, e.g., Gao et al., *Am J Transl Res.* 2009; 1(3): 312–325). The proteins that have a CAAX motif or box at the end of the C-terminus typically need a prenylation process before the proteins migrate to the plasma membrane or nuclear membrane and exert different functions. In an embodiment, the CAAX box is derived from a human RAS family protein, for example HRAS, NRAS, Ral-A, KRAS4A or KRAS4b. The last C-terminal residues of RAS, NRAS, KRAS4A or KRAS4b (referred to as the hypervariable region or HVR) are depicted below, with the putative minimal plasma membrane targeting region in italics and the CAAX box underlined (see, e.g., Ahearn et al., *Nature Reviews Molecular Cell Biology* **13**: 39-51, January 2012): HRAS: KLNPPDESGPGCMSCKCVLS; (SEQ ID NO:33); NRAS: KLNSSDDGTQGCMGLPCVVM; (SEQ ID NO:34); KRAS4A: KISKEEKTPGCVKIKKCIIM; (SEQ ID NO:35); KRAS4b: KMSKDGGKKKKKSKTKCVIM; (SEQ ID NO:36); Ral-A/Ral1: KNGKKKRKSLAKRIRERCCIL (SEQ ID NO:37).

In an embodiment, the PM targeting moiety comprises the sequence GKKKKKKSKTKCVIM (SEQ ID NO:7) from KRAS4b. In another embodiment, the PM targeting moiety comprises the the plasma-membrane targetting palmitoylation sequence from hRas and prenylation signal sequence from Ral-A/Ral1 (sequence: CMSCKCCIL, SEQ ID NO:43).

Several proteins also contain a non-lipid, polybasic domain that targets the PM such as Ras small GTPases, phosphatase PTEN, nonreceptor tyrosine kinase Src, actin regulators WASP and MARCKS, and G protein-coupled receptor kinases (GRKs) such as GRK5. In an embodiment,

the polybasic domain is from GRK5, and comprises the sequence SPKKGLLQRLFKRQHQNNSKS (SEQ ID NO:8).

In a particular aspect, the present invention provides a biosensor comprising: a cell or membrane preparation comprising: (i) a first component comprising a β -arrestin tagged with a *Renilla* GFP or a *Renilla* Luc; (ii) a second component comprising a plasma membrane (PM) targeting moiety tagged with a *Renilla* GFP or a *Renilla* Luc; and a GPCR; wherein if said β -arrestin is tagged with said *Renilla* GFP, said PM targeting moiety is tagged with said *Renilla* Luc, and if said β -arrestin is tagged with said *Renilla* Luc, said PM targeting moiety is tagged with said *Renilla* GFP. Such biosensor may be useful to monitor/measure the recruitment of a β -arrestin to a GPCR located at the plasma membrane.

In an embodiment, the cellular compartment targeting moiety is an endosomal targeting moiety. Several endosomal targeting moieties/markers are known in the art and include the Rab family of proteins (RAB4, RAB5, RAB7, RAB9 and RAB11), mannose 6-phosphate receptor (M6PR), caveolin-1 and -2, transferrin and its receptor, clathrin, as well as proteins comprising a FYVE domain such as early endosome autoantigen 1 (EEA1), Rabenosyn-5, Smad anchor for receptor activation (SARA), Vps27p and Endofin. Some markers are more specific to early endosomes (e.g., RAB4, Transferrin and its receptor, and proteins comprising a FYVE domain), others are more specific to late endosomes (e.g., RAB7, RAB9, and M6PR) and others are more specific to recycling endosomes (e.g., RAB11, RAB4). Thus, these proteins or suitable fragments thereof may be fused to *Renilla* Luc or *Renilla* GFP to link/target them to an endosomal localization.

In an embodiment, the endosomal targeting moiety comprises a FYVE domain. The FYVE domain is defined by the three conserved elements: the N-terminal WxxD, the central RR/KHHCR, and the C-terminal RVC motifs. In an embodiment, the endosomal targeting moiety comprises the FYVE domain of Endofin, for example about residues 739 to 806 human Endofin.

In an embodiment, the cellular compartment targeting moiety is a lysosomal targeting moiety, such as for example LAMP1 and LAMP2. Thus, these proteins or suitable fragments thereof may be fused to *Renilla* Luc or *Renilla* GFP to link/target them to a lysosomal localization.

In an embodiment, the cellular compartment targeting moiety is a peroxisomal targeting moiety, such as for example PMP70, PXMP2 and Catalase. Thus, these proteins or suitable fragments thereof may be fused to *Renilla* Luc or *Renilla* GFP to link/target them to a peroxisomal localization.

In an embodiment, the cellular compartment targeting moiety is an autophagosomal targeting moiety, such as for example ATG (AuTophagy related) family proteins (ATG4, ATG5, ATG16, ATG12, see Lamb *et al.*, *Nature Reviews Molecular Cell Biology* 14, 759–774 (2013)), LC3A/B and SQSTM1/p62. Thus, these proteins or suitable fragments thereof may be fused to *Renilla* Luc or *Renilla* GFP to link/target them to an autophagosomal localization.

In an embodiment, the cellular compartment targeting moiety is a ribosome targeting moiety. Several endosomal targeting moieties/markers are known in the art and include the Ribosomal Proteins (L7a, S3 and S6). Thus, these proteins or suitable fragments thereof may be fused to *Renilla* Luc or *Renilla* GFP to link/target them to a ribosomal localization.

5 In an embodiment, the cellular compartment targeting moiety is an endoplasmic reticulum (ER) targeting moiety. Several ER targeting moieties/markers are known in the art and include ERp72, ERp29, Protein disulphide isomerase (PDI), HSP70 family proteins such as GRP78 (HSPA5), GRP94 (HSP90B1) and GRP58 (PDIA3), Calnexin and Calreticulin. Thus, these proteins or suitable fragments thereof may be fused to *Renilla* Luc or *Renilla* GFP to link/target them to an
10 ER localization.

In an embodiment, the cellular compartment targeting moiety is a Golgi targeting moiety. Several Golgi targeting moieties/markers are known in the art and include eNOS (e.g., the N-terminal portion thereof, J. Liu *et al.*, Biochemistry, 35 (1996), pp. 13277-13281), GM130, Golgin-97, the 58K protein, Trans-Golgi network membrane protein 2 (TGOLN2), TGN46, TGN38,
15 Mannosidase 2, Syntaxin 6, GM130 (GOLGA2), Golgin-160, Membrin (GS27), GS28, Coatomer proteins, Rbet1 and RCAS1. Thus, these proteins or suitable fragments thereof may be fused to *Renilla* Luc or *Renilla* GFP to link/target them to a Golgi apparatus localization. In an embodiment, the Golgi targeting moiety the N-terminal portion of a human eNOS protein, for example residues 1 to 73 of human eNOS1 (SEQ ID NO: 42).

20 In an embodiment, the cellular compartment targeting moiety is a mitochondria targeting moiety. Several mitochondria targeting moieties/markers are known in the art and include AIF, COX IV, Cytochrome C, hexokinase I, SOD1, SDHA, Pyruvate dehydrogenase, VDAC, TOMM22, UCP1, UCP2, UCP3, PHB1 Galpha12 (or the N-terminal portion thereof; Andreeva *et al.*, FASEB J. 2008 Aug;22(8):2821-31. Epub 2008 Mar 26), a protein of the Bcl-family member or a fragment
25 thereof, for example a fragment of Bcl-XL (RKGQERFNRWFLTGMTVAGVVLLGSLFSRK, SEQ ID NO:87, Mossalam *et al.*, Mol Pharm. 2012 May 7; 9(5): 1449-1458). Thus, these proteins or suitable fragments thereof may be fused to *Renilla* Luc or rGFP to link/target them to a mitochondrial localization. The nuclear targeting moiety may also comprise a mitochondrial targeting signal, which is a 10-70 amino acid long peptide that directs newly synthesized proteins
30 to the mitochondria. It is found at the N-terminus and consists of an alternating pattern of hydrophobic and positively charged amino acids to form an amphipathic helix. Mitochondrial targeting signals can contain additional signals that subsequently target the protein to different regions of the mitochondria, such as the mitochondrial matrix.

In an embodiment, the cellular compartment targeting moiety is a nuclear targeting
35 moiety. Several nuclear targeting moieties/markers are known in the art and include Lamin A/C, Nucleoporins (NUP), ASHL2, ESET, Histones, LSD1, DNA repair enzymes such as PARP, and P84/THOC1. Thus, these proteins or suitable fragments thereof may be fused to *Renilla* Luc or

Renilla GFP to link/target them to a nuclear localization. The nuclear targeting moiety may also comprises a nuclear localization signal or sequence (NLS), which is an amino acid sequence that tags a protein for import into the cell nucleus by nuclear transport. Typically, this signal consists of one or more short sequences of positively charged lysines or arginines exposed on the protein surface. The best characterized transport signal is the classical NLS (cNLS) for nuclear protein import, which consists of either one (monopartite) or two (bipartite) stretches of basic amino acids. Monopartite cNLSs are exemplified by the SV40 large T antigen NLS (¹²⁶PKKKRRV¹³²) (SEQ ID NO:38) and bipartite cNLSs are exemplified by the nucleoplasmin NLS (¹⁵⁵KRPAATKKAGQAKKK¹⁷⁰) (SEQ ID NO:39).

In an embodiment, the cellular compartment targeting moiety is a nuclear export sequence (NES). NES is a short amino acid sequence (typically 4 hydrophobic residues) in a protein that targets it for export from the cell nucleus to the cytoplasm through the nuclear pore complex using nuclear transport. The sequence of such NES may be for example LxxxLxxLxL, where "L" is a hydrophobic residue (often leucine) and "x" is any other amino acid. In proteins that are translocated from cytosol to nucleus (such as ERK or MDM2), a decrease in the BRET signal is detected using an NES moiety.

In an embodiment, the cellular compartment targeting moiety is a cytoskeleton targeting moiety, for example actin or a fragment thereof, or a protein comprising an actin-binding domain (ABD), such as the N-terminal F-actin binding domain of Inositol-1,4,5-trisphosphate-3-kinase-A (ITPKA) (Johnson and Schell, *Mol. Biol. Cell* December 15, 2009 vol. 20 no. 24 5166-5180). In an embodiment, the cytoskeleton targeting moiety is a peptide comprising the sequence MGVADLIKKFESISKEE (SEQ ID NO: 88) ("Lifeact", Riedl *et al.*, *Nat Methods*. 2008 Jul; 5(7): 605)

In another aspect, the present invention provides a biosensor for assessing a modulation (increase or decrease) in the amount of a biomolecule at a cellular compartment between a first and a second condition, said biosensor comprising: a first component comprising a *Renilla* green fluorescent protein (*Renilla* GFP) tagged with a protein marker that binds to said biomolecule; and a second component comprising a *Renilla* luciferase protein (*Renilla* Luc) tagged with said protein marker.

In another aspect, the present invention provides a biosensor for assessing a modulation (increase or decrease) in the amount of a biomolecule at a cellular compartment between a first and a second condition, said biosensor comprising: a first component comprising a protein marker that binds to said biomolecule tagged with a *Renilla* GFP or *Renilla* Luc; and a second component comprising a cellular compartment targeting moiety tagged with a *Renilla* GFP or *Renilla* Luc; wherein if said protein marker is tagged with *Renilla* GFP, said cellular compartment targeting moiety is tagged with *Renilla* Luc and vice-versa.

Such biosensors may be used in a method for assessing a modulation in the amount of a biomolecule at a cellular compartment between a first and a second condition, *e.g.*, in the

presence and absence of an agent. If the agent increase the amount of biomolecule at the cellular compartment (e.g., PM) the BRET signal will be increased in the presence of the agent and vice-versa.

The protein marker may be any protein or fragment thereof that binds to said biomolecule, and thus whose concentration or density at said cellular compartment is dependent on the concentration or density of said biomolecule (e.g., a second messenger, including cyclic nucleotides such as cAMP and cGMP, IP₃, DAG, PIP₃, Ca²⁺ ions) at said cellular compartment. For example, PLCδ1 localization at the PM is dependent on the presence of PIP₂ and/or PIP₃. If the concentration of PI(4,5)P₂ at the PM decreases (which occurs when phospholipase C (PLC) is activated because PI(4,5)P₂ is hydrolyzed), PLCδ1 diffuse into the cytosol reducing its concentration/density at the PM. Thus, the concentration/density of PLCδ1 (or a fragment thereof that binds to PIP₂ and/or PIP₃, such as its PH domain) at the PM, which may be measured by BRET using *Renilla* Luc- and *Renilla* GFP-tagged PLCδ1 (or a fragment thereof, e.g., SEQ ID NO:25), or with a *Renilla* Luc or GFP-tagged PLCδ1 and a *Renilla* Luc or GFP-tagged PM-targeting moiety, may be used as an indicator of the concentration or density of the biomolecule at the PM. Similarly, the PH domain and Phox homology domain (PX domain) of certain proteins, (ex: akt and PLD1) interact with PIP₃, thus a protein marker comprising a PH or PX domain selective for PIP₃ binding, could be used to as an indicator of the concentration or density of PIP₃ at the PM. Another example is the C1 domain (also known as phorbol esters/diacylglycerol binding domain, which is found for example in the N-terminal portion of protein kinase. Also, PLCγ1 can bind to different phospholipids including PIP₃. The C1 domain binds to diacylglycerol (DAG), and thus a protein marker comprising a C1 domain could be used to as an indicator of the concentration or density of DAG at the PM. Thus, any protein or protein domain capable of binding to a biomolecule such as a second messenger and whose concentration or density at said cellular compartment is dependent on the concentration or density of said biomolecule could be used in such biosensor.

The term “biomolecule” refer to any molecule that may be produced by or present in a cell, for example a protein, a peptide, an amino acid, a nucleic acid (DNA or RNA), a lipid or fatty acid, a phospholipid, a sugar (polysaccharide), or any other compound such as ATP, AMP, ADP, histamine, etc. In an embodiment, the biomolecule is a second messenger (i.e. a molecules that relay signals received at receptors on the cell surface to target molecules in the cytosol and/or nucleus), e.g., Cyclic AMP, Cyclic GMP, Inositol Triphosphate (IP₃), phosphatidylinositols (e.g., Phosphatidylinositol 4,5-bisphosphate or PIP₂, Phosphatidylinositol 3,4,5-triphosphate or PIP₃, Diacylglycerol (DAG), Ca²⁺. In an embodiment, the biomolecule is a hydrophobic molecule (e.g., a phospholipid) found at the PM, such as diacylglycerol and phosphatidylinositols.

The variant as used herein refers to a protein/polypeptide having has an identity or similarity of at least 60% with a reference (e.g., native) sequence and retains a desired activity thereof, for example the capacity to bind to a target protein and/or to translocation to a cellular

compartment. In further embodiments, the variant has a similarity or identity of at least 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% with a reference (e.g., native) sequence and retains a desired activity thereof. "Similarity" and "identity" refers to sequence similarity/identity between two polypeptide molecules. The similarity or identity can be determined by comparing each position in the aligned sequences. A degree of similarity or identity between amino acid sequences is a function of the number of matching or identical amino acids at positions shared by the sequences. Optimal alignment of sequences for comparisons of similarity or identity may be conducted using a variety of algorithms, such as the local homology algorithm of Smith and Waterman, 1981, Adv. Appl. Math 2: 482, the homology alignment algorithm of Needleman and Wunsch, 1970, J. Mol. Biol. 48: 443, the search for similarity method of Pearson and Lipman, 1988, Proc. Natl. Acad. Sci. USA 85: 2444, and the computerized implementations of these algorithms (such as GAP, BESTFIT, FASTA and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, Madison, Wis., U.S.A.). Sequence similarity or identity may also be determined using the BLAST algorithm, described in Altschul et al., 1990, J. Mol. Biol. 215: 403-10 (using the published default settings). Software for performing BLAST analysis may be available through the National Center for Biotechnology Information web site (<http://www.ncbi.nlm.nih.gov/>).

The *Renilla* Luc or *Renilla* GFP may be fused N-terminal, within or C-terminal relative to the cellular compartment targeting moiety. In an embodiment, the cellular compartment targeting moiety is a PM targeting moiety, and it is fused to the N-terminal end of said *Renilla* Luc or said *Renilla* GFP. In an embodiment, the cellular compartment targeting moiety is an endosomal targeting moiety, and it is fused to the C-terminal end of said *Renilla* Luc or said *Renilla* GFP.

The *Renilla* Luc or *Renilla* GFP may be fused N-terminal, within (see, e.g., G α subunit with internal RlucII described in the examples), or C-terminal relative to the protein of interest. In an embodiment, the *Renilla* Luc or *Renilla* GFP is fused to the N-terminal end of the protein of interest. In another embodiment, the *Renilla* Luc or *Renilla* GFP is fused to the C-terminal end of the protein of interest.

In an embodiment, the protein of interest is tagged with a *Renilla* Luc and the cellular compartment marker is tagged with a *Renilla* GFP.

Other domains or linkers may be present at the N-terminal, C-terminal or within the above-noted first and/or second components. In embodiments, the *Renilla* Luc or *Renilla* GFP may be covalently linked to the protein of interest or the cellular compartment targeting moiety either directly (e.g., through a peptide bond) or "indirectly" via a suitable linker moiety, e.g., a linker of one or more amino acids (e.g., a polyglycine linker) or another type of chemical linker (e.g., a carbohydrate linker, a lipid linker, a fatty acid linker, a polyether linker, PEG, etc. In an embodiment, one or more additional domain(s) may be inserted before (N-terminal), between or after (C-terminal) the components defined above. In an embodiment, the *Renilla* Luc and/or *Renilla*

GFP are covalently linked through a peptide bond to the protein of interest and/or the cellular compartment targeting moiety. In an embodiment, a peptide linker is present between *Renilla* Luc or *Renilla* GFP and the protein of interest or the cellular compartment targeting moiety. In embodiments, the linker comprises about 4 to about 50 amino acids, about 4 to about 40, 30 or 20 amino acids, or about 5 to about 15 amino acids, e.g., 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids. In a further embodiment, the linker is one of the linker described in Example 1 below and/or **Figures 11A-11D**).

In an embodiment, the first and second components are linked together to provide a unimolecular biosensor. The first and second components are covalently attached by a linker, preferably a flexible polypeptide linker. In an embodiment, the flexible polypeptide linker has a length corresponding to the length of a random amino acid sequence of about 50 to about 500-1000 amino acids, for example corresponding to the length of a random amino acid sequence of about 100 to about 400-500 amino acids, preferably about 200-400 amino acids, for example about 300. In a further embodiment, the flexible linker comprises a random amino acid sequence of about 50 to about 500-1000 amino acids, for example a random amino acid sequence of about 100 to about 400-500 amino acids, preferably a random amino acid sequence of about 200-400 amino acids, for example about 300 amino acids. Methods for designing flexible amino acid linkers, and more specifically linkers with minimal globularity and maximal disorder, are known in the art. This may be achieved, for example, using the Globplot 2.3 program. The sequence may be further optimized to eliminate putative aggregation hotspots, localization domains, and/or interaction and phosphorylation motifs. Such a unimolecular biosensor allows the assessment of BRET in intact cells as well as in membrane preparations.

In another aspect, the present invention provides a nucleic acid encoding the above-defined first and/or second component(s). In an embodiment, the nucleic acid is present in a vector/plasmid, in a further embodiment an expression vector/plasmid. Such vectors comprise a nucleic acid sequence capable of encoding the above-defined first and/or second component(s) operably linked to one or more transcriptional regulatory sequence(s), such as promoters, enhancers and/or other regulatory sequences. In an embodiment, the nucleic acid encodes the first and second components (polycistronic construct).

The term "vector" refers to a nucleic acid molecule, which is capable of transporting another nucleic acid to which it has been linked. One type of preferred vector is an episome, i.e., a nucleic acid capable of extra-chromosomal replication. Preferred vectors are those capable of autonomous replication and/or expression of nucleic acids to which they are linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors". A recombinant expression vector of the present invention can be constructed by standard techniques known to one of ordinary skill in the art and found, for example, in Sambrook *et al.* (1989) in *Molecular Cloning: A Laboratory Manual*. A variety of

strategies are available for ligating fragments of DNA, the choice of which depends on the nature of the termini of the DNA fragments and can be readily determined by persons skilled in the art. The vectors of the present invention may also contain other sequence elements to facilitate vector propagation and selection in bacteria and host cells. In addition, the vectors of the present invention may comprise a sequence of nucleotides for one or more restriction endonuclease sites. Coding sequences such as for selectable markers and reporter genes are well known to persons skilled in the art.

A recombinant expression vector comprising a nucleic acid sequence of the present invention may be introduced into a cell (a host cell), which may include a living cell capable of expressing the protein coding region from the defined recombinant expression vector. The living cell may include both a cultured cell and a cell within a living organism. Accordingly, the invention also provides host cells containing the recombinant expression vectors of the invention. The terms "cell", "host cell" and "recombinant host cell" are used interchangeably herein. Such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

Vector DNA can be introduced into cells via conventional transformation or transfection techniques. The terms "transformation" and "transfection" refer to techniques for introducing foreign nucleic acid into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, electroporation, microinjection and viral-mediated transfection. Suitable methods for transforming or transfecting host cells can for example be found in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory manuals. "Transcriptional regulatory sequence/element" is a generic term that refers to DNA sequences, such as initiation and termination signals, enhancers, and promoters, splicing signals, polyadenylation signals which induce or control transcription of protein coding sequences with which they are operably linked. A first nucleic acid sequence is "operably-linked" with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably-linked to a coding sequence if the promoter affects the transcription or expression of the coding sequences. Generally, operably-linked DNA sequences are contiguous and, where necessary to join two protein coding regions, in reading frame. However, since for example enhancers generally function when separated from the promoters by several kilobases and intronic sequences may be of variable lengths, some polynucleotide elements may be operably-linked but not contiguous.

In another aspect, the present invention provides a kit comprising a first nucleic acid encoding the first component and a second nucleic acid encoding the second component.

In another aspect, the present invention provides a cell comprising or expressing the above-defined first and/or second component(s). In an embodiment, the cell has been transfected or transformed with a nucleic acid encoding the above-defined first and/or second component(s). The invention further provides a recombinant expression system, vectors and cells, such as those described above, for the expression of the first and/or second component(s) of the invention, using for example culture media and reagents well known in the art. The cell may be any cell capable of expressing the first and second component(s) defined above. Suitable host cells and methods for expression of proteins are well known in the art. Any cell capable of expressing the component(s) defined above may be used. For example, eukaryotic host cells such as mammalian cells may be used (e.g., rodent cells such as mouse, rat and hamster cell lines, human cells/cell lines). In another embodiment, the above-mentioned cell is a human cell line, for example an embryonic kidney cell line (e.g., HEK293 or HEK293T cells).

In an embodiment, the above-mentioned biosensor comprises a cell comprising or expressing the first and second components. In another embodiment, the above-mentioned biosensor comprises a membrane preparation comprising the first and second components.

In another aspect, the present invention provides a method for comparing the trafficking of a protein of interest in a cell under a first and a second condition, said method comprising: measuring the BRET signal in the biosensor defined herein under said first condition; and measuring the BRET signal in the biosensor defined herein under said second condition; wherein a difference in said BRET signal between said first and second conditions is indicative of a difference in the trafficking of said protein of interest under said first and second conditions. In an embodiment, the first condition is no activation and the second condition is activation (e.g., using an agonist) or vice-versa. In another embodiment, the first condition is no inhibition and the second condition is inhibition (e.g., using an antagonist) or vice-versa.

In another aspect, the present invention provides a method for determining whether an agent modulates (increases or decreases) the density or concentration of a protein of interest at a cellular compartment, said method comprising: measuring the BRET signal in the biosensor defined herein in the presence and absence of said agent; wherein a difference in said BRET signal in the presence of said agent relative to the absence thereof is indicative that said agent modulates (increases or decreases) the density or concentration of said protein of interest at the cellular compartment. An increase in the BRET signal being indicative that the agent increases the density or concentration of said protein of interest at the cellular compartment, whereas a decrease in the BRET signal being indicative that the agent decreases the density or concentration of said protein of interest at the cellular compartment.

Methods and devices to measure the BRET signal are well known in the art. The BRET signal may be measured, for example, by determining the intensity of the *Renilla* GFP signal (light intensity), and/or by calculating the ratio of the signal or light intensity emitted by the *Renilla* GFP

over the signal or light intensity emitted by the *Renilla* Luc (BRET ratio). The BRET signal may be measured using a microplate reader or microscope with a suitable filter set for detecting the *Renilla* luciferase (donor) and/or rGFP (acceptor) light emissions.

By choosing an appropriate cellular compartment targeting moiety, it is possible to assess/monitor the trafficking of a protein of interest to any cellular compartment (PM, ER, Golgi, mitochondria, endosomes, etc.). For example, to determine whether a given condition or an agent affects the trafficking of a protein of interest to the mitochondria, a biosensor comprising a mitochondrial targeting moiety tagged with *Renilla* GFP or *Renilla* Luc may be used. An increase in the BRET signal in the presence of the agent or under the given condition (relative to the absence of the agent or to a different condition) is indicative of the "recruitment" of the protein of interest to the mitochondria (i.e., an increase in the concentration/density of the protein of interest at the mitochondria). In contrast, a decrease in the BRET signal in the presence of the agent or under the given condition (relative to the absence of the agent or to a different condition) is indicative of a decrease in the concentration/density of the protein of interest at the mitochondria. Using suitable cellular compartment targeting moieties, a similar approach may be used to study the trafficking of proteins to different cellular compartments.

In an embodiment, the method comprises determining whether an agent or condition induces (i.e. increases) the trafficking of a cell surface receptor of interest in an endosomal compartment (i.e., increases the concentration/density of the protein of interest in the endosomes),

Accordingly, in another aspect, the present invention provides a method for comparing the trafficking of a cell surface receptor of interest at an endosomal compartment, said method comprising: measuring the BRET signal in the biosensor comprising an endosomal targeting moiety as defined herein under said first condition; and measuring the BRET signal in the biosensor comprising an endosomal targeting moiety as defined herein under said second condition; wherein a difference in the BRET signal between said first and second conditions is indicative of a difference in the trafficking of said protein of interest at said endosomal compartment under said first and second conditions.

In another aspect, the present invention provides a method for determining whether an agent induces (i.e. increases) the trafficking of a cell surface receptor of interest in a cell at an endosomal compartment, said method comprising: measuring the BRET signal in the biosensor comprising an endosomal targeting moiety, preferably an endosomal targeting moiety comprising a FYVE domain (e.g., the FYVE domain of Endofin) as defined herein in the presence and absence of said agent; wherein a higher BRET signal in the presence of said agent relative to the absence thereof is indicative that said agent induces (i.e. increases) the trafficking of a cell surface receptor of interest in a cell in said endosomal compartment (i.e. increase the concentration/density of the protein of interest in the endosomes).

As shown in the experiments described herein, it is possible to assess/monitor the trafficking of a protein across the endosomal pathway, for example by using a plurality of biosensors, each comprising a different endosomal targeting moiety (e.g., a first biosensor comprising a targeting moiety for the early endosomes and a second biosensor comprising a targeting moiety for the late endosomes).

In another aspect, the present invention provides a method for comparing the internalization of a cell surface receptor of interest in a cell under a first and a second condition, said method comprising: measuring the BRET signal in the biosensor comprising a PM targeting moiety as defined herein under said first condition; and measuring the BRET signal in the biosensor comprising a PM targeting moiety as defined herein under said second condition; wherein a difference in said BRET signal between said first and second conditions is indicative of a difference in the internalization of said cell surface receptor of interest under said first and second conditions.

In another aspect, the present invention provides a method for determining whether an agent induces the internalization and/or sequestration of a cell surface receptor of interest in a cell, said method comprising: measuring the BRET signal in the biosensor comprising a PM targeting moiety as defined herein in the presence and absence of said agent; wherein a lower BRET signal in the presence of said agent relative to the absence thereof is indicative that said agent induces the internalization and/or sequestration of the cell surface receptor of interest.

The biosensors described herein further permit to determine whether internalized receptors are recycled back at the cell surface, and if so to assess the kinetics of receptor recycling.

In another aspect, the present invention provides a method for monitoring the recycling of an internalized receptor of interest at the cell surface, said method comprising: (a) contacting the biosensor comprising a PM targeting moiety as defined herein in the presence of a ligand that induces the internalization of said receptor; (b) measuring a first BRET signal in the biosensor; (c) washing said biosensor to remove said ligand; (d) measuring a second BRET signal in the biosensor after said washing; and (e) determining the recycling of an internalized receptor of interest at the cell surface by comparing said first and second signals, wherein a higher second BRET signal relative to said first BRET signal is indicative of recycling of the internalized receptor of interest at the cell surface.

In another aspect, the present invention provides a method for monitoring the recycling of an internalized receptor of interest at the cell surface, said method comprising: (a) contacting a first and a second biosensor comprising a PM targeting moiety as defined herein in the presence of a ligand that induces the internalization of said receptor; (b) measuring a BRET signal in the first biosensor after said contacting; (c) washing said second biosensor to remove said ligand; (d) measuring a BRET signal in the second biosensor after said washing; and (e) determining the

recycling of an internalized receptor of interest at the cell surface by comparing the BRET signal in the first and second biosensors, wherein a higher BRET signal in said second biosensor relative to said first biosensor is indicative of recycling of the internalized receptor of interest at the cell surface.

5 In an embodiment, the method further comprises repeating steps (d) and (e) at different times after washing to study the kinetics of recycling of the internalized receptor of interest.

 In another aspect, the present invention provides a method for monitoring a modulation of G protein and/or GPCR activity between a first condition and a second condition, said method comprising: measuring the BRET signal in the biosensor for monitoring G protein and/or GPCR
10 modulation as defined herein under said first condition; and measuring the BRET signal in the biosensor for monitoring G protein and/or GPCR modulation as defined herein under said second condition; wherein a difference in the BRET signal between said first and second conditions is indicative of a modulation of G protein and/or GPCR activity between said first and second conditions.

15 In an embodiment, the first condition is absence of a test compound (e.g., putative inhibitor or agonist) and the second condition is presence of a test compound, or vice-versa. A lower BRET signal in the presence of the test compound is indicative that the test compound is an agonist.

 In another aspect, the present invention provides a method for determining whether a
20 GPCR ligand modulates the activity of a G protein subunit of interest, said method comprising: measuring the BRET signal in the biosensor for monitoring G protein and/or GPCR modulation as defined herein in the presence or absence of said GPCR ligand; wherein a difference in the BRET signal in the presence vs. absence of said GPCR ligand is indicative that said GPCR ligand modulates the activity of the G protein subunit of interest.

25 In another aspect, the present invention provides a method for monitoring a modulation of the activity of a small GTPase between a first condition and a second condition, said method comprising: measuring the BRET signal in the biosensor for assessing the activation of a small GTPase as defined herein under said first condition; and measuring the BRET signal in the biosensor for assessing the activation of a small GTPase as defined herein under said second
30 condition; wherein a difference in the BRET signal between said first and second conditions is indicative of a modulation of the activity of a small GTPase between said first and second conditions.

 In an embodiment, the first condition is absence of a test compound (e.g., putative inhibitor or agonist) and the second condition is presence of a test compound, or vice-versa. A
35 higher BRET signal in the presence of the test compound is indicative that the test compound is an agonist (recruitment of the small GTPase at the PM or endosomes).

In another aspect, the present invention provides a method for determining whether a test agent modulates the activity of a small GTPase (e.g., a Rho protein), said method comprising: measuring the BRET signal in the biosensor for assessing the activation of a small GTPase as defined herein in the presence or absence of said test agent; wherein a difference in the BRET signal in the presence vs. absence of said test agent is indicative that said test agent modulates the activity of said small GTPase.

Using the biosensors described herein, it is also possible to assess/monitor the "rescue" of a protein of interest (for example, a defective protein that does not properly exit from the ER) by a pharmacological chaperone (PC). The term "pharmacological chaperone" ("PC") as used herein refers to a molecule that binds to a protein (e.g., a receptor) and has one or more of the following effects: (i) enhancing the formation of a stable molecular conformation of the protein; (ii) enhances proper trafficking of the protein from the ER to another cellular location, preferably a native cellular location, i.e., preventing ER-associated degradation of the protein; (iii) preventing aggregation of conformationally unstable, i.e., misfolded proteins; (iv) restoring or enhancing at least partial wild-type function, stability, and/or activity of the protein and/or (v) inducing a different folding of the protein. Thus, a pharmacological chaperone for a protein is a molecule that binds to the protein, resulting in proper folding, trafficking, non-aggregation, and/or activity of the protein, and/or to modulate the folding of the protein (inducing a folding of the protein that is different than the folding in the absence of the chaperone).

It has previously been shown that small molecule inhibitors of enzymes associated with lysosomal storage disorders (LSDs) can both rescue folding and activity of the mutant enzyme, and enhance folding and activity of the wild-type enzyme (see U.S. Patent Nos. 6,274,597; 6,583,158; 6,589,964; 6,599,919; and 6,916,829). In particular, it was discovered that administration of small molecule derivatives of glucose and galactose, which were specific competitive inhibitors of mutant enzymes associated with LSDs, effectively increased *in vitro* and *in vivo* stability of the mutant enzymes and enhanced the mutant enzyme activity. The original theory behind this strategy is as follows: since the mutant enzyme protein folds improperly in the ER (Ishii et al., Biochem. Biophys. Res. Comm. 1996; 220: 812-815), the enzyme protein is retarded in the normal transport pathway (ER → Golgi apparatus → endosome → lysosome) and rapidly degraded. Therefore, a compound which stabilizes the correct folding of a mutant protein will serve as an active site-specific chaperone for the mutant protein to promote its smooth escape from the ER quality control system. Enzyme inhibitors occupy the catalytic center, resulting in stabilization of enzyme conformation in cells and in animals. These specific chaperones were designated "active site-specific chaperones (ASSCs)" since they bound in the active site of the enzyme.

In addition to rescuing the mutant enzymes, the ASSCs enhance ER secretion and activity of recombinant wild-type enzymes. An ASSC facilitates folding of overexpressed wild-type enzyme, which is otherwise retarded in the ER quality control system because overexpression and

over production of the enzyme exceeds the capacity of the ER and leads to protein aggregation and degradation. Thus, a compound that induces a stable molecular conformation of an enzyme during folding serves as a "chaperone" to stabilize the enzyme in a proper conformation for exit from the ER. As noted above, for enzymes, one such compound unexpectedly turned out to be a competitive inhibitor of the enzyme.

In addition to the LSDs, a large and diverse number of diseases are now recognized as "conformational diseases" that are caused by adoption of non-native protein conformations, which may lead to retardation of the protein in the ER and ultimate degradation of the proteins (Kuznetsov et al., N. Engl. J. Med. 1998; 339:1688-1695; Thomas et al., Trends Biochem. Set 1995; 20:456-459; Bychkova et al., FEBS Lett. 1995; 359:6-8; Brooks, FEBS Lett. 1997; 409:115-120). For example, small synthetic compounds were found to stabilize the DNA binding domain of mutant forms of the tumor suppressor protein p53, thereby allowing the protein to maintain an active conformation (Foster et al., Science 1999; 286:2507-10). Synthesis of receptors has been shown to be rescued by small molecule receptor antagonists and ligands (Morello et al., J Clin. Invest. 2000; 105: 887-95; Petaja-Repo et al., EMBO J. 2002; 21: 1628-37). Even pharmacological rescue of membrane channel proteins and other plasma membrane transporters has been demonstrated using channel-blocking drugs or substrates (Rajamani et al., Circulation 2002; 105:2830-5; Zhou et al., J Biol. Chem. 1999; 274:31123-26; Loo et al., J. Biol. Chem. 1997; 272: 709-12; Pedemonte et al., J. Clin. Invest. 2005; 115: 2564-71). Thus, the biosensors described herein may be useful to identify chaperones that rescue the expression and/or proper maturation of proteins, and in turn which may be useful for the treatment of diseases associated with defects in the expression and/or proper maturation of one or more proteins, as described above.

In another aspect, the present invention provides a method for determining whether an agent acts as a pharmacological chaperone for a receptor of interest, said method comprising:

providing a biosensor comprising: a cell comprising: said receptor of interest tagged with a *Renilla* GFP or a *Renilla* Luc, preferably a *Renilla* Luc; and a plasma membrane (PM) targeting moiety tagged with *Renilla* GFP or a *Renilla* Luc, preferably a *Renilla* GFP; wherein if said receptor is tagged with said a *Renilla* GFP, said PM targeting moiety is tagged with said *Renilla* Luc, and if said receptor is tagged with said *Renilla* Luc, said PM targeting moiety is tagged with said a *Renilla* GFP; and

measuring the BRET acceptor signal in the presence and absence of said agent; wherein an increase in the BRET signal in the presence of said agent relative to the absence thereof is indicative that said agent acts as a pharmacological chaperone for said receptor.

In another aspect, the present invention provides a method for determining whether an agent acts as a pharmacological chaperone for a protein of interest, said method comprising:

providing a biosensor comprising: a cell comprising: said protein of interest tagged with a *Renilla* GFP or a *Renilla* Luc; and an endoplasmic reticulum (ER) targeting moiety tagged with a

rGFP or a *Renilla* Luc; wherein if said protein is tagged with said *Renilla* GFP, said ER targeting moiety is tagged with said *Renilla* Luc, and if said protein is tagged with said *Renilla* Luc, said ER targeting moiety is tagged with said *Renilla* GFP; and

measuring the BRET acceptor signal in the presence and absence of said agent;

5 wherein a decrease in the BRET signal in the presence of said agent relative to the absence thereof is indicative that said agent acts as a pharmacological chaperone for said receptor.

The above-mentioned method may be performed using a native protein/receptor, or a mutated receptor, as shown in the experiments described herein. The experiments described herein further shows that the biosensors are suitable to measure rescue of a GPCR as well as of a
 10 non-GPCR receptor (a voltage-dependent potassium channel), providing evidence that they may be used to monitor the rescue of any protein or receptor. In an embodiment, the protein is a native GPCR or a mutated GPCR. In a further embodiment, the GPCR is a native melanocortin-4 receptor (MC4R) or a mutated MC4R. In an embodiment, the mutated MC4R contains one or more mutations that result in reduced or improper intracellular folding of the MC4R polypeptide.
 15 Exemplary mutations are as follows: P78L, R165Q, R165W, I125K, C271Y, A175T, I316L, I316S, I317T, N97D, G98R, N62S, C271R, S58C, N62S, N97D, Y157S, I102S, L106P, L250Q, Y287X, P299H, S58C, CTCT at codon 211, and TGAT insertion at codon 244. In another embodiment, the GPCR is a native V2R or a mutated V2R. In a further embodiment, the mutated V2R comprises a Y128S or W164S substitution. In another embodiment, the protein is an ion channel, a native ion
 20 channel or a mutated ion channel, in a further embodiment a voltage-gated potassium channel, such as hERG.

In an embodiment, the above method for determining whether an agent acts as a pharmacological chaperone further comprises determining whether the rescued protein/receptor is functional, e.g., using a ligand.

25 In another aspect, the present invention provides a method for determining whether an agent induces the recruitment of a β -arrestin at the plasma membrane (PM), said method comprising:

providing a biosensor comprising a cell or membrane preparation comprising: said β -arrestin tagged with a *Renilla* GFP or a *Renilla* Luc), preferably a *Renilla* Luc; a plasma membrane
 30 (PM) targeting moiety tagged with a *Renilla* GFP or a *Renilla* Luc, preferably a *Renilla* GFP; and a GPCR; wherein if said β -arrestin is tagged with said *Renilla* GFP, said PM targeting moiety is tagged with said *Renilla* Luc, and if said β -arrestin is tagged with said *Renilla* Luc, said PM targeting moiety is tagged with said *Renilla* GFP; and

measuring the BRET acceptor signal in the presence and absence of said agent;

35 wherein an increase in the BRET signal in the presence said agent relative to the absence thereof is indicative that said agent induces the recruitment of said β -arrestin at the PM.

The above-mentioned methods comprise contacting the biosensor with a substrate for a *Renilla* Luc, such as a luciferin, to produce energy (in the form of light) that will be accepted by (excite) the rGFP. Non-limiting examples of luciferins include D-luciferin, imidazopyrazinone-based compounds such as coelenterazine (coelenterazine 400A (DeepBlueC™), coelenterazine H and analogues of e-Coelenterazine such as Prolume Purple™ from Nanolight™), ViviRen™ (from Promega), Latia luciferin ((*E*)-2-methyl-4-(2,6,6-trimethyl-1-cyclohex-1-yl)-1-buten-1-ol formate), bacterial luciferin, Dinoflagellate luciferin, etc. In an embodiment, the substrate is coelenterazine 400A, coelenterazine H or Prolume Purple™.

As used herein, the term “agent” is used to refer to any molecule, for example, protein, oligopeptide, small organic molecule, polysaccharide, polynucleotide, and the like, to be tested for bioactivity. Such agents can be obtained from any number of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means.

Positive controls and negative controls may be used in the methods/assays. Control and test samples may be performed multiple times to obtain statistically significant results.

In an embodiment, the above-mentioned methods are high-throughput methods (high-throughput screening, HTS). The term “high-throughput screening” (HTS) as used herein refers to a method that allow screening rapidly and in parallel large numbers of compounds (hundreds, thousands) for binding activity or biological activity against target molecules. Such HTS methods are typically performed in microtiter plates having several wells, for example 384, 1536, or 3456 wells. For HTS, it is important that the readout signal be detected with high sensitivity, accuracy and reproducibility.

MODE(S) FOR CARRYING OUT THE INVENTION

The present invention is illustrated in further details by the following non-limiting examples.

Example 1: Materials and Methods

Materials. Angiotensin II (AngII; [Asp-Arg-Val-Tyr-Ile-His-Pro-Phe], SEQ ID NO:), poly-ornithine, poly-D-lysine, isoproterenol, arginine-vasopressin (AVP), bradykinin, were from Sigma®. Prostaglandin F2α (PGF2α), Prostaglandin E2 and u46619 were from Cayman Chemical® (Ann Arbor, MI). [Sar¹, Ile⁸]-AngII (SI) and [Asp¹, Val⁵, Gly⁸]-AngII (DVG) [Sar¹-Val⁵-D-Phe⁸] AngII (SVdF) and [Sar¹-D-Ala⁸] AngII (TRV120027), were synthesized at the Université de Sherbrooke (Canada, QC). UBO-QIC was obtained from Institute for Pharmaceutical Biology of the University

of Bonn (Germany). Iodine-125 was obtained from PerkinElmer®. Dulbecco's modified Eagles medium (DMEM), fetal bovine serum, OPTI-MEM®, and other cell culture reagents were purchased from Invitrogen®. Coelenterazine 400a, Coelenterazine H and Prolume Purplel were purchased from either Goldbio®, Biotium or Nanolight® Technology. Polyethylenimine (PEI; 25 kDa linear; was purchased from Polysciences (Warrington, PA, USA). Salmon sperm DNA was purchased from Lifetechnologies (ThermoFisher). Phusion DNA polymerase was from Thermo Scientific®. Restriction enzymes and T4 DNA ligase were obtained from NEB®.

Plasmids and constructions. For the construction of the lyn-GFP10, the coding sequence of the first 11 residues (MGCIKSKGKDS, SEQ ID NO: 1) of the human Lyn-kinase and the full coding region of GFP10 were synthesized at GeneScript® (Piscataway, NJ) and subcloned into pcDNA 3.1/zeo (-) using infusion (Clontech®, CA). The lyn-rGFP was generated by replacing the coding sequence of GFP10 in the lyn-GFP10 construct by the humanized rGFP, which was generated by PCR amplification. StreptagII-fused GFP10 was synthesized at GenScript® and subcloned into pcDNA3.1/zeo(-) (STII-GFP10). The FYVE domain of the human endofin (amino acids 739-806), was synthesized at Bio Basic® Inc. (Ontario, Canada) and subcloned into the STII-GFP10 construct in-frame (GFP10-endofinFYVE). rGFP-endofinFYVE was generated by inserting the FYVE domain of GFP10-endofinFYVE into a vector containing humanized rGFP in pcDNA3.1(+) in-frame. rGFP-rab4 and rGFP-rab11 were generated by replacing the FYVE domain in rGFP-endofinFYVE with PCR amplified rab4 and rab11 coding sequences, respectively. To generate RlucII fused AT1R, the human AT1R coding sequences containing a signal peptide and Flag sequence were PCR amplified and subcloned into in frame in pcDNA3.1/hygro(+) also containing the RlucII via NheI and HindIII sites. Plasmids encoding human β arr2-RlucII has been previously described (Quoyer, Janz et al. 2013). RlucII-tagged receptors were obtained by PCR using published constructs of MC4R-Venus constructs (P. René et al. J Pharmacol Exp Ther. 2010 Dec;335(3):520-32) and hV2R wt (Morello, J.P., et al., J Clin Invest, 2000. **105**(7): p. 887-95). RlucII-tagged receptors were obtained by PCR using plasmids encoding hERG, a generous gift from D. Deblois (Université de Montréal, Montréal, Canada). *Renilla reniformis* GFP (rGFP) constructs were obtained by PCR from the synthesized coding sequence (from GenScript, USA). *PH domain tagged RlucII and rGFP:* PH domain of PLC δ 1 was PCR amplified using PLC δ 1 image clone (IMAGE:5769665) as a template. The PCR product was used to replace endofinFYVE domain in GFP10-endofinFYVE by subcloning into XbaI and HindIII sites. The PH domain of GFP10-PH(PLC δ) was inserted either into a vector containing humanized rGFP in pcDNA3.1(+) or a vector containing HA-RlucII in pcDNA3.1(+) in-frame (rGFP-PH(PLC δ 1) and HA-RlucII-PH(PLC δ 1), respectively). *hMC4R-RlucII:* Plasmids encoding the fusion protein *hMC4Rwt-RlucII*, *hMC4R (R165Q)-RlucII* and *hMC4R-(P299H)-RlucII* were obtained by PCR amplification of MC4R from MC4R-venus constructs and, subcloned in frame at the N-terminus to the humanized *Renilla* luciferase II (hRlucII) sequence (a variant of the hRluc previously reported (Leduc, Breton

et al. 2009)) into pcDNA3.1 RlucII vector (linker sequence: VGGGGSKLPAT, SEQ ID NO:2). *hV2R-RlucII*: The V2R substitution Y128S was created using the site-directed mutagenesis with the Quick Change™ mutation kit (Agilent Technologies, Santa-Clara, USA). Plasmids encoding the fusion protein *hV2R wt-RlucII* and *hV2R (Y128S)-RlucII* were obtained by PCR amplification of V2R coding sequence, subcloned in frame at the N-terminus to the hRlucII sequence into pcDNA3.1 RlucII vector (linker sequence: GGSGLKLPAT, SEQ ID NO:3). *hERG-RlucII*: Plasmids encoding the fusion protein *hERG wt-RlucII* and *hERG (G601S)-RlucII* were obtained by PCR amplification of 3 fragments encoding: residues 1-379 of hERG, 373-1159 of hERG and the RlucII, and subcloned by Gibson Assembly (New England Biolabs) pcDNA3.1 (+) vector (linker at the N-terminal of RlucII: NAAIRSGG, SEQ ID NO:4 and at the C-terminal of RlucII: GGNAAIRS, SEQ ID NO:5). *rGFP-CAAX*: Plasmid encoding the fusion protein *rGFP-CAAX* was obtained by PCR amplification of rGFP coding sequence with a reverse primer encoding a linker (sequence: GSAGTMASNNTASG, SEQ ID NO:6) and the plasma-membrane targeting polybasic sequence and prenylation signal sequence from KRAS splice variant b: -GKKKKKKSKTKCVIM (named: CAAX, SEQ ID NO:7). The CAAX plasma-membrane targeting sequence is in frame at the C-terminus of the rGFP coding sequence. The PCR fragment is sub-cloned into pcDNA3.1 (+) vector. *rGFP-PB*: Plasmids encoding the fusion protein *rGFP-PB* was obtained by replacing the CAAX motif of rGFP-CAAX by the GRK5-plasma membrane targeting domain (PB; sequence: SPKKGLLQRLFKRQHQNNSKS, SEQ ID NO:8) using PCR amplification and Gibson assembly. The complete vector pCDNA 3.1 (+) rGFP-CAAX is amplified by PCR using oligos encoding PB. The PCR reaction product is digested with DpnI, purified and recircularized in a Gibson assembly reaction. *Cloning of RlucII-GRB2*: The coding sequence of human GRB2 variant1 was PCR-amplified and subcloned at the C-terminus of RlucII in the vector pCDNA3.1 (+) RlucII with a small flexible linker (sequence: GSAGT, SEQ ID NO:9) between GRB2 and RlucII. All the PCR were done by using the Phusion® DNA polymerase. All constructs were verified by DNA sequencing prior to use.

Cell culture and Transient Transfection. Human embryonic kidney 293 (HEK293) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 unit/ml penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO₂. HEK293SL cells were cultured in DMEM supplemented with 5% fetal bovine serum and 20 µg/ml gentamycin. Cells were grown at 37 °C in 5% CO₂ and 90% humidity.

Transfections using calcium phosphate: HEK293SL cells were transfected using a calcium phosphate method (Fessart, Simaan et al. 2005). Cells were seeded at a density of ~7.5x10⁵ per 100 mm dishes a day before transfection and transfection was carried out as described previously (Fessart, Simaan et al. 2005). After 18 h of transfection, the medium was replaced, and the cells were divided for subsequent experiments. All assays were performed 48 h after transfection.

Transfection using Poly(ethylenimine) (PEI): Two days before the experiments, HEK293 cells from a 6-well plate were washed with PBS containing no calcium or magnesium, detached and transfected with the indicated plasmids using PEI as a transfecting agent (at a ratio of 3 to 1, PEI/DNA) and then directly seeded in 96-well plates pre-treated with poly-L-ornithine hydrobromide at a density of 35 000 cells per well.

Stable rGFP-CAAX cell lines. HEK293 cells from a 6-well plate were washed with Phosphate Buffered Saline (PBS) and transfected with 1.2 ug of rGFP-CAAX encoding construct/well using poly-ethylenimine 25-kDa linear (PEI) as a transfecting agent (at a ratio of 3 to 1, PEI/DNA) (Hamdan, Rochdi *et al.* 2007). The rGFP-CAAX construct also encodes for the hygromycin resistance, and transfected cells were seeded in T75 dishes and selection (hygromycin at 100 µg/ml) was maintained for 4 weeks and hygromycin-resistant cells were FACS-sorted against GFP fluorescence, in populations expressing different levels of rGFP-CAAX.

BRET measurements for Figures 1B to 9D, 25D to 25F. The following day of transfection, cells were detached and replated onto poly-ornithine coated white 96- well plate at a density of ~25,000 cells per well. The next day, cells were washed once with pre-warmed Tyrode's buffer (140 mM NaCl, 2.7 mM KCl, 1 mM CaCl₂, 12 mM NaHCO₃, 5.6 mM D-glucose, 0.5 mM MgCl₂, 0.37 mM NaH₂PO₄, 25 mM HEPES, pH 7.4), and then stimulated with either various concentrations of ligands in Tyrode's buffer for the indicated time, or single concentration of ligands for various times at 37°C. For recycling experiment, after stimulating the cells with the ligands for 30 min at 37°C, they were washed either three times with ice-cold Tyrode's buffer or once with Tyrode's buffer/three times with acid (50 mM sodium citrate, pH 4.0)/two times with Tyrode's buffer. All the washing steps were performed on ice. Cells were then further incubated with Tyrode's buffer at 37°C in a water bath for 45 min. The cell-permeable substrate, coelenterazine 400a was added at a final concentration of 5 µM in Tyrode's buffer 3~4 min before BRET measurements. Measurements were performed by using Synergy2 (BioTek®) microplate reader with a filter set of 410 ± 80 nm and 515 ± 30 nm for detecting the RlucII *Renilla luciferase* (donor) and GFP10 or rGFP (acceptor) light emissions, respectively. The BRET signal was determined by calculating the ratio of the light intensity emitted by the GFP10 or rGFP over the light intensity emitted by the RlucII. All the BRET measurements were performed in triplicate at 37 °C.

BRET assay for evaluation of PC rescue of cell surface expression and functionality (sequestration assay). In **Figures 12A to 18E**, Hek293 cells were transiently transfected using PEI as described in this section. The DNA transfected per well of a 96-well plate is as follow: in **Figures 12A and 12B**, with 2.4ng of hMC4R-RlucII encoding construct and an increasing quantity of rGFP-CAAX (Kras) up to 9.6ng for **Figure 12A** and for **12B**, rGFP-PB up to 9.6ng; in **Figures 13A to 13C** with 0.6, 1.2 or 2.4ng of hMC4R-RlucII and 4.8ng of rGFP-CAAX (Kras); in **Figure 13D** with 2.4ng of polycistronic rGFP-CAAX(Kras)/MC4R-RlucII construct; in **Figure 13E** with

1.2ng of hV2R-RlucII and 4.8ng of rGFP-CAAX (Kras); in **Figures 14A to 16B** with 2.4ng of hMC4R-RlucII and 7.2ng of rGFP-CAAX (Kras); Hek293 cells stably expressing rGFP-CAAX(Kras) were transfected with 0.6, 1.2 or 2.4ng of hMC4R-RlucII; in **Figure 17A** or 0.6, 1.2 or 2.4ng of hV2R_Y128S-RlucII; in **Figure 17B**; in **Figures 18A and 18B** with 0.6, 1.2 or 2.4 ng of hERG-RlucII and 4.8 ng of rGFP-CAAX (Kras); and in **Figures 18C-18E** with 0.6 ng of hERG-RlucII and 7.2 ng of rGFP-CAAX (Kras). Transfected cells seeded in 96-well plates were treated with a pharmacological chaperone (for MC4R: DCPMP(N-((2R)-3(2,4-dichloroPhenyl)-1-(4-(2-((1-methoxypropan-2-ylamino)methyl)phenyl) piperazin-1-yl)-1-oxopropan-2-yl)propionamide) or Compound 1; for V2R: SR121463; for hERG: Astemizole, Cisapride, Quinidine, Diltiazem, Amiodarone and Acetaminophen) or vehicle for 16h-18h, as indicated in each figure, prior to the BRET assay performed 2-day post-transfection. For the BRET assay, cells were washed once with PBS and left in Tyrode's buffer. The cells were then optionally treated for MC4R with 10 μ M of α -MSH for an hour at 37°C to evaluate PC-rescue of functionality as a function of agonist induced sequestration of receptors that were expressed at the cell surface (**Figure 13**). The Rluc substrate, Coel-400a (for BRET2 experiments) or coelenterazine H (for BRET1 experiments, **Figure 13E and 15B and 15D**), was added at a final concentration of 2.5 μ M and cells were further incubated for an additional 5 minutes. BRET values were then collected using a Mithras LB940 Multimode Microplate Reader, equipped with the following filters for BRET2: 400nm $\pm$ 70nm (energy donor) and 515nm $\pm$ 20nm (energy acceptor) and for BRET1: 480nm $\pm$ 20nm (energy donor) and 530nm $\pm$ 20nm (energy acceptor). BRET values were determined by calculating the ratio of the light emitted by the acceptor over the light emitted by the RlucII.

β arrestin recruitment to plasma membrane using rGFP-markers: For **Figures 19B to 19D**, HEK293 cells were transfected with PEI, as described previously, with 3ng of either β arrestin1-RlucII (**Figures 19B & 19D**) or β arrestin2-RlucII (**Figures 19C & 19E**) + 4.8ng of PM-marker (rGFP-CAAX=red triangles, GFP10-CAAX=circles, rGFP-PB=green triangles & Lyn-rGFP=squares) + 10ng V2R (**Figures 19D & 19E**) or 40 ng β 2AR (**Figures 19B & 19C**) per well of a 96-well plate. 48h post-transfection, cells were washed and stimulated for 10 min with the indicated doses at 37°C. Coel-400a was then added at a final concentration of 2.5 μ M and incubated for an additional 5 min. BRET was measured at 37°C, using a Tristar Microplate Reader (Berthold Technologies). Data was normalized as a ratio of the max response obtained with the GFP10-CAAX (Kras) construct. For **Figure 19F**, a transfection mix of 200ng of β 2AR, 20 ng β -arrestin2-RlucII, 800 ng rGFP-CAAX, complemented to 2 μ g with ssDNA and PEI at a ratio of PEI:DNA of 3:1, is added to 3 ml of Hek293SL (350,000 cells/ml). Cells were seeded on poly-D-lysine pretreated plates. 48 h post-transfection, cells were washed and preincubated in Tyrode + 1mM CaCl₂ at 37°C for 60 min then treated with the indicated doses of Isoproterenol for 2min at 37°C. Coel-400a was then added at a final concentration of 2.5 μ M and incubated for an additional 6 min. BRET was measured at 37°C, using a Tristar Microplate Reader (Berthold Technologies).

For **Figures 19C, 19E, 19H and 19I**, HEK293 cells were transfected with PEI, as described previously with 3ng of β arrestin2-RlucII (**Figures 19C & 19E**) + 4.8ng of rGFP-CAAX (Kras) + 10ng V2R (**Figure 19I**) or 40 ng β 2AR (**Figures 19H**) per well of a 96-well plate. 48h post-transfection, cells were washed and half of a 96-well plate stimulated for 10min with 100nM AVP (for **Figure 19H**) or with isoproterenol at 1 μ M (for **Figure 19H**) and the other half of the plate with vehicle, at 37⁰C. Coel-400a was then added at a final concentration of 2.5 μ M and incubated for an additional 5 min. BRET was measured at 37⁰C, using a Tristar Microplate Reader (Berthold Technologies). Z'-factor values were calculated as described by Zhang *et al.* (Zhang, Chung *et al.* 1999). For **Figures 19J and 19G**, Hek293SL cells were seed at 100 mm dish and then next day the cells were transfected with 90 ng of β arrestin2-RlucII and 480 of rGFP-CAAX (Kras) along with 600ng AT1R (**Figure 19J**) with a calcium phosphate method, as described previously. 24h after transfection, cells were replated onto 96-well plate then next day, cells were washed and half of a 96-well plate stimulated for 6 min with 100 nM AngII (**Figure 19J**) and the other half of the plate with vehicle, at room temperature before BRET measurements. (**Figure 19G**) HEk293SL cells were transfected with AT1R (600 ng) and β arrestin2-RlucII (90 ng) along with either Lyn-rGFP (480 ng), rGFP-CAAX (480 ng), or GFP10-CAAX (480 ng) in 100 mm dishes. Next day, the cells were replated onto 96-well plates. 48h post-transfection, the cells were stimulated various concentrations of AngII for 6 min before BRET measurements. Coelenterazine 400a (final concentration of 5 μ M) was added after 2 min of AngII stimulation. BRET was measured at room temperature, using a Synergy2 (BioTek[®]) microplate reader. Z'-factor values were calculated as described by Zhang *et al.* (Zhang, Chung *et al.* 1999).

β arrestin recruitment unimolecular sensor: for **Figure 21B**, 200 ng of V2R-pRK5, 50 ng of β 2AR unimolecular sensor with different linkers, complemented to 1 μ g with ssDNA and PEI at a ratio of PEI:DNA of 3:1, was added to 1.2 ml of HEK293SL. Cells were seeded on poly-D-lysine pretreated plates. 48h post-transfection, cells were washed and preincubated in Tyrode +1 mM CaCl₂ at 37⁰C for 60 min then treated with AVP (100 nM) for 10 min at 37⁰C. Coelenterazine 400a (coel-400a) (Biotium) was added at a final concentration of 2.5 μ M and, incubated for an additional 5 min. BRET ratios were measured at 37⁰C, using Mithras LB940 Multimode Microplate Reader (Berthold Technologies). For **Figure 21C**, 1x transfection: 400 ng sphAT1R, 200 ng of β 2AR unimolecular sensor with a 200 residues-long linker, complemented to 4 μ g with ssDNA and PEI at a ratio of PEI:DNA of 3:1, was added to 7ml ml of HEK293SL. Cells were seeded on poly-D-lysine pretreated plates. 48h post-transfection, cells were washed and preincubated in Tyrode +1 mM CaCl₂ at 37⁰C for 60 min then treated with different concentrations of ligand for 5 min at 37⁰C. Coelenterazine 400a (coel-400a) (Biotium) was added at a final concentration of 2.5 μ M and incubated for an additional 5 min. BRET ratios were measured at 37⁰C, using Mithras LB940 Multimode Microplate Reader (Berthold Technologies).

Unimolecular DAG sensor. For **Figures 22B and C**, HEK293SL cells stably expressing hAt1AR (~50 fmol/mg) were cultured in DMEM supplemented with 10% FBS and 20 µg/ml gentamycin and seeded at a density of 75,000 cells/100mm dishes and were transiently transfected the next day with 150ng of construct encoding for the DAG unimolecular sensor, using calcium phosphate method as described previously. 48h post-transfection, cells were washed and Coel-400a was added to a final concentration of 5µM and incubated 3min. For **Figure 22B**, BRET was measured every 4 sec, AngII is then added at 64 sec for a final concentration of 100nM and, kinetics of agonist-promoted stimulation evaluated. Data are mean ± SD of triplicates of a representative experiment. For **Figure 22C**, cells were stimulated with the indicated concentrations of AngII for 1min prior to BRET measurements. Data are mean ± S.E. of six independent experiments. For **Figure 22D and E**, Hek293SL cells were transfected with FuGENE HD, according to Roche's protocol with a ratio of 2:1 fugene:DNA. 10ng of construct encoding the unimolecular sensor and 400ng for the receptor (**Figure 22D**, FP and in **2E**, GPR14) complemented to 1µg with ssDNA, were transfected per well of a 6well plate. 48h post-transfection, cells were washed, incubated in Tyrode's buffer for 1h. Cells were then stimulated with the indicated doses with their respective ligands (in **Figure 22D**, with PGF2α and PGE2 and in **22E**, with Urotensin II) for 1min then Coel-400a was added at a final concentration of 2.5µM for an additional 5min. For **Figure 22F**, cells were transfected as in **Figure 22D** but with just the unimolecular DAG sensor encoding construct. 48h post-transfection, cells were washed, incubated in Tyrode's buffer for 1h. Coel-400a was added at a final concentration of 2.5µM for an additional 5min. Cells were then stimulated with 5µM m-3m3FBS or just vehicle for the indicated time. For **Figures 22G and 22H**, cells were transfected as in **Figures 22E and 22D**, respectively. 48h post-transfection, cells were washed, incubated in Tyrode's buffer for 1h. Half of the wells of a 96-well plate were stimulated with 100nM of ligands (in **Figure 22H**, with PGF2α and in **22G**, with Urotensin II) for 1min and the other half with vehicle. Coel-400a was then added at a final concentration of 2.5µM for an additional 5min incubation. For **Figures 22D to 22H**, the BRET ratios were measured at 37°C, using Mithras LB940 Multimode Microplate Reader (Berthold Technologies).

DAG sensor based on C1b recruitment to rGFP-markers. For **Figures 23B to D**, HEK293 cells were transfected using PEI, as already described, with 100ng of RlucII-C1b, 500ng of rGFP-CAAX (Kras) and 100ng of either human histamine type 1 (H1R, Gq-coupled receptor, human Bradykinin type 2 (BKRB2, Gq-coupled receptor), human dopamine type 2 (D2R, Gi-coupled receptor used as negative control) receptors and complemented to 1µg with ssDNA. 48h post-transfection, cells were washed and incubated for 1h at RT in Tyrode's buffer. Cells were incubated 5min with the indicated doses of their respective agonist (Histamine for H1R, Kallidin for BKRB2 and Dopamine for D2R). Prolume Purple™ was then added at 2 µM final for an additional 5min. BRET measurements were done using a Synergy Neo Multi-Mode Microplate Reader

(BioTek Instruments, Inc). For **Figure 23E**, 100 ng of $\beta 2AR$, 20 ng RlucII-C1b, 400 ng of rGFP-CAAX (Kras), and either 100ng of WT $G\alpha 15$ or 100ng of empty vector (Mock), complemented to 1 μg with ssDNA, and PEI at a ratio of PEI:DNA of 3:1, is added to 1.2 ml of Hek293SL (350 000cells/ml). Cells were seeded on poly-D-lysine pretreated plates. 48h post-transfection, cells were washed and preincubated in Tyrode +1 mM $CaCl_2$ at 37°C for 60 min. Coelenterazine 400a was then added at a final concentration of 2.5 μM and incubated for 6 min. Cells were then treated with the indicated doses of Isoproterenol, for 1min. BRET was measured at 37°C, using a Tristar Microplate Reader (Berthold Technologies).

Sensor based on Gprotein translocation: For **Figures 24B to D**, 100 ng of HA- $\beta 1AR$ or HA- $\beta 2AR$, 5 ng of the indicated RlucII- $G\gamma$, 100 ng of WT $G\alpha 15$, 100 ng of WT $G\beta 1$, 200 ng of rGFP-CAAX (Kras), complemented to 1 μg with ssDNA, and PEI at a ratio of PEI:DNA of 3:1, is added to 1.2 ml of Hek293SL (350,000 cells/ml) (for **Figures 24B & C**) or 2x to 3 ml of HEK293SL (350,000 cells/ml) (for **Figure 24 D**). Cells were seeded on poly-D-lysine pretreated plates. 48 h post-transfection, cells were washed and preincubated in Tyrode +1 mM $CaCl_2$ at 37°C for 60 min. Coelenterazine 400a was then added at a final concentration of 2.5 μM and incubated for 6 min. Cells were then treated with either 1 μM (**Figures 24B and C**) or the indicated doses (**Figure 24D**) of Isoproterenol, for 2min. BRET was measured at 37°C, using a Tristar® Microplate Reader (Berthold Technologies). For **Figures 24E to G**, 100 ng of HA- $\beta 1AR$, 30 ng of $G\alpha s$ pos67RlucII or $G\alpha 12$ pos84RlucII, 100ng of WT $G\gamma 5$, 100 ng of WT $G\beta 1$, 400 ng of rGFP-CAAX or Golgi marker (eNOS(1-73)-rGFP), complemented to 1 μg with ssDNA, and PEI at a ratio of PEI:DNA of 3:1, is added 2x to 3 ml of HEK293SL (350,000 cells/ml). Cells were seeded on poly-D-lysine pretreated plates. 48h post-transfection, cells were washed and preincubated in Tyrode +1 mM $CaCl_2$ at 37°C for 60 min. Prolume Purple™ was then added at a final concentration of 2 μM and incubated for 6 min. Cells were then treated or not with either 1 μM for the indicated time (**Figure 24F**) or the indicated doses (**Figures 24E & G**) of Isoproterenol, for 4min. BRET was measured at 37°C, using a Tristar® Microplate Reader (Berthold Technologies). For **Figure 24H**, 200ng of TP α R, 30ng of $G\alpha q$ pos118RlucII, 100ng of WT $G\gamma 5$, 100ng of WT $G\beta 1$, 400ng of rGFP-CAAX or Golgi marker (eNOS(1-73)-rGFP), complemented to 1 μg with ssDNA, and PEI at a ratio of PEI:DNA of 3:1, is added to 1.2 ml of HEK293SL (350,000 cells/ml). Cells were seeded on poly-D-lysine pretreated plates. 48h post-transfection, cells were washed and preincubated in Tyrode + 1mM $CaCl_2$ at 37°C for 60min. Incubated or not with 100nM of Ubo-Qic for 20min. Cells were then treated for the indicated doses of U46619, for 6min. Coel-400a was then added at a final concentration of 2.5 μM and incubated for an additional 5 min. BRET was measured at 37°C, using a Tristar Microplate Reader (Berthold Technologies).

PKN-based RhoA activation assay. For **Figures 25B-D**, HEK293SL cells were grown in DMEM supplemented with 6% fetal bovine serum (FBS) and 20 $\mu g/ml$ gentamycin, at 37°C. Cells were seeded at a density of 7.5×10^5 cells per 100 mm dishes and were transiently transfected the

next day with constructs encoding AT1R (3 μ g) along with PKN-crib-RlucII (90ng) and rGFP-CAAX (480ng) using calcium phosphate method as described previously. After 24 h, cells were detached and seeded onto poly-ornithine-coated 96-well white plates at a density of 25 000 cells per well in media. The next day, cells were washed once with Tyrode's buffer and left in 80 μ l of Tyrode's buffer at 37°C. When indicated, cells were treated with Ubo-Qic 100nM for 30min or C3 toxin for 3 μ g/ml (in **Figure 25I**), 4hours at 37°C. Cell stimulation and BRET measurements were done at RT. BRET signals were monitored by addition of Coel-400a to a final concentration of 5 μ M using a Synergy2 (BioTek®) microplate reader. Filter set was 410 $\pm$ 80nm and 515 $\pm$ 30nm for detecting the RlucII *Renilla* luciferase (donor) and rGFP (acceptor) light emission. For **Figure 25B**, a transfection mix of 200ng of TP α R, 20ng PKN-RlucII, 600ng CAAX-rGFP, complemented to 2 μ g with ssDNA and PEI at a ratio of PEI:DNA of 3:1, is added to 3ml of Hek293SL (350 000cells/ml). Cells were seeded on poly-D-lysine pretreated plates. 48 h post-transfection, cells were washed and preincubated in Tyrode +1 mM CaCl₂ at 37°C for 30 min then with Coel-400a at a final concentration of 2.5 μ M and, incubated for 6 min. Cells were stimulated for 2min with the indicated doses of U46619. BRET was measured at 37°C, using a Tristar Microplate Reader (Berthold Technologies) equipped with BRET400-GFP2/10 filter set (acceptor, 515 $\pm$ 20nm; and donor, 400 $\pm$ 70nm filters). For **Figures 25I** and **J**, a transfection mix of 200ng of TP α R, 20ng PKN-RlucII, 600ng CAAX-rGFP, complemented to 2 μ g with ssDNA and PEI at a ratio of PEI:DNA of 3:1, is added to 3ml of Hek293SL (350,000 cells/ml). Cells were seeded on poly-D-lysine pretreated plates. 24h post-transfection, the Rho inhibitor (CT04; Cytoskeleton, Inc) was added when indicated, overnight at final concentration 2 μ g/ml. 48 h post-transfection, cells were washed and preincubated in Tyrode +1mM CaCl₂ at 37°C for 60min then treated, as indicated, with 100nM of U46619 or 1 μ g/ml of Rho activator II (CN03; Cytoskeleton, Inc) for 1 min at 37°C. Coel-400a was then added at a final concentration of 2.5 μ M and incubated for an additional 5 min. BRET was measured at 37°C, using a Tristar Microplate Reader (Berthold Technologies).

Intact cell [¹²⁵I]-AngII binding. [¹²⁵I]-AngII was prepared with the Iodogen method, and its specific radioactivity was determined from self-displacement and saturation experiments as previously described (Zimmerman, Beautrait et al. 2012) The density of cell surface receptors was evaluated with binding assays at 4 °C using [¹²⁵I]-AngII as tracer. HEK293SL cells expressing either AT1R or AT1R-RlucII were seeded 1 day after transfection at a density of ~120,000 cells per well in poly-ornithine coated 24-well plates. The following day, cells were washed once with pre-warmed DMEM with 20 mM HEPES (DMEM-H) and then incubated in the absence or presence of 100 nM AngII in DMEM-H for 30 min at 37 °C. The plates were quickly washed three times with ice-cold acid (50 mM sodium citrate, pH 4.0) for 5 min each on ice to stop the stimulation and remove both the remaining surface bound and unbound AngII ligand. To remove and neutralize the residual acid, cells were further washed twice with ice-cold Tyrode's buffer. Cells were then incubated with 0.5 ml of [¹²⁵I]-AngII (~250,000 cpm) in the binding buffer (0.2 % BSA, 50 mM Tris,

100 mM NaCl₂, 5 mM MgCl₂, pH 7.4) at 4°C overnight. Nonspecific binding was determined in the presence of 1 μM AngII. Next day, the cells were washed three times with ice-cold PBS with calcium and magnesium, and 0.5 ml of 0.5 N NaOH/0.05% SDS was added. Radioactivity was counted using a PerkinElmer Wizard® 1470 automatic γ-counter. Protein amounts were measured by Bio-rad® Protein Assay kit according to the manufacture's instruction with some modifications. Briefly, the cells were treated same as above except incubation without radiolabelled AngII, and then after washing, add 2 ml of diluted Protein assay reagent instead of NaOH/SDS. After mixing by pipetting, the samples were transferred to plastic cuvettes and measured absorbance at 595 nm.

Confocal microscopy. One day before transfection, HEK293SL cells were seeded in 35 mm glass-bottom dishes at a density of 100,000 cells/dish. Cells were transfected with B2R-CFP, LYN-rGFP and mCherry-endofinFYVE. Forty-eight hours post-transfection, cells were serum starved for 30 min, either left untreated (non treated) or treated with bradykinin (1μM) for 15 min. Samples were analyzed on a Zeiss LSM-510 Meta laser scanning microscope using argon (514 nm) and HeNe I (543 nm) lasers, and images (2048x2048 pixels) were collected using a 63x oil immersion lens. For detecting CFP and GFP, UV and argon lasers were used with 405 nm and 514 nm excitation, and either BP 420-480 nm or BP 505-550 nm emission filters, respectively. For mCherry detection, a HeNe I laser was used with 543 nm excitation and LP 560 nm emission filter sets.

BRET microscopy/imaging. HEK293S cells were cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin and 0.1 mg/ml streptomycin and plated on poly-D-lysine coated glass-bottom 35 mm culture dishes at the density of 1-2x10⁵ cells/dish. On the next day, cells were transfected with RlucII-fused (BRET donor) and rGFP-fused (BRET acceptor) constructs using Xtreme GENE HP reagent (Roche) using 1 μg DNA and 3 μl reagent per dish according to the manufacturer protocol. For **Figures 26A and 26B** (luminescence spectrum measurement), cells were transfected with 100 g/dish of RLucII N-terminally fused to Venus, GFP2 or rGFP. Cells are detached from the culture surface by adding 1ml of PBS supplemented with 5 mM EDTA, and re-suspended to PBS. As a luciferase substrate, 1μM of Prolume purple (Nanolight Technology) was added and the luminescence spectrum was obtained with Synergy Neo plate reader (BioTek) 2min after the addition of the substrate. For Figure 26C (luminescence microscopy), cells were transfected with 100ng/dish of HA-β2AR, 50ng/dish or βarrestin2-RlucII and 500ng/dish of rGFP-CAAX were transfected. Cells were washed once with 1 ml of modified Hank's balanced salt solution (138mM NaCl, 5.33mM KCl, 0.44mM KH₂PO₄, 0.33mM Na₂HPO₄, 4.16mM NaHCO₃, 1.0mM CaCl₂, 1.0mM MgCl₂, 10mM HEPES, pH 7.4) and set on the microscope. 10μM of Prolume Purple (NanoLight Technology) was added to the dish. BRET images were obtained using Nikon® Ti-U microscope equipped with 60x objective (Apochromat TIRF, NA 1.49, Nikon) and imaging camera (PIXIS1024, Princeton instruments) with filter changer (Lambda 10-2, Sutter instrument).

Immediately after the addition of coelenterazine, camera shutter was closed and a blank image was acquired for 90sec. Then images were acquired with filters corresponding to BRET donor (410/80nm) and BRET acceptor (480LP or 510/40 nm) wavelength for 90 sec each. Images were captured every 5 min, and blank image values were subtracted from the corresponding pixels of BRET donor and acceptor images in order to remove photon counts deriving from dark current and sampling noises of the camera. For each time points, BRET ratio images were generated using pixel arithmetic functions of MetaMorph software version 7.8 (Molecular Devices) as follows; Pixel hue : BRET level calculated by dividing the counts of acceptor images with donor images, and allocated to default rainbow hue (lowest (typically 0.0) in purple and highest (typically 2.0) in red). Pixel brightness: the value of donor images with auto brightness.

Z'-factors determination. BRET1 and BRET2 assays were performed on cells cotransfected with rGFP-CAAX construct and either the hMC4R wt-RlucII or hMC4R (R165Q)-RlucII construct (as indicated in **Figures 15A to 15D**), with half of the 96-well plate treated with the pharmacological chaperone (10uM DCPMP) and the second half of the plate treated with the corresponding vehicle (DMSO). Z'-factor values were calculated as described by Zhang *et al.* (Zhang, Chung et al. 1999). A Z'-factor over 0.4 is considered a robust assay.

Evaluation of resistance to DMSO. Ligands and compound-libraries are often dissolved in DMSO. To evaluate whether the BRET-based assay for cell surface evaluation is sensitive to concentrations of DMSO usually reached with dose-response curves of ligands selected from a compound-library, transfected cells were DCPMP-treated at 10uM or with vehicle (DMSO) in well containing different concentrations of DMSO, as indicated in **Figure 16**. BRET values were then obtained as previously described.

Data Analysis. Estimation of the $t_{1/2}$ and the EC_{50} values for ligand-mediated endocytosis were calculated using the GraphPad® Prism curve fitting program. The curves presented throughout this study, representing the best fits, and were generated using this GraphPad® Prism program as well.

Sequences: The amino acid sequences of polypeptides and constructs used herein are depicted below.

RLucII

MTSKVYDPEQRKRMITGPQWWARCKQMNVLDSFINYYDSEKHAENAVIFLHGNATSSYLWRHV
PHIEPVARCIIPDLIGMGKSGKSGNGSYRLLDHYKYLTAWFELLNLPKKIIFVGHDWGAALAFHYSY
EHQDKIKAIVHAESVVDVIESWDEWPDIEEDIALIKSEEGEKMVLENNFFVETVLP SKIMRKLEPEEF
AAYLEPFKEKGEVRRPTLSWPREIPLVKGGKPDVVQIVRNYNAYLRASDDL PKMFIESDPGFFSNA
IVEGAKKFPNTEFVKVKGLHFSQEDAPDEM GKYKSFVERVLKNEQ (SEQ ID NO:10)

rGFP : *Renilla reniformis* green fluorescent protein

MDLAKLGLKEVMPTKINLEGLVGDHAFSMEGVGEGNILEGTQEVKISVTKGAPLPFAFDIVSVAFS
YGNRAYTGYPEEISDYFLQSFPEGFTYERNIRYQDGGTAIVKSDISLEDGKFIVNVDFKAKDLRRM

GPVMQQDIVGMQPSYESMYTNVTSVIGECIIAFKLQTGKHFTYHMRTVYKSKKPVETMPLYHFIQH
RLVKTNVDTASGYVQHETAIAAHSTIKKIEGSLP (SEQ ID NO:11)

GFP10

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTCLKFICTTGKLPVPWPPTLVTTLS
5 YGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDF
KEDGNILGHKLEYNYPHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLP
DNHYLFTQSALS KDPNEKRDHMLLEFVTAAGITLGMDELYK (SEQ ID NO:12)

hMC4R WT: human wild-type Melanocortin 4 receptor

MVNSTHRGMHTSLHLWNRSSYRLHSNASESLGKGYSDDGGCYEQLFVSPEVFVTLGVISLLENILVI
10 VAIKKNLHSPMYFFICSLAVADMLVSVSNGSETIVITLLNSTDTDAQSFTVNIDNVIDSVICSSLLA
SICSLLSIAVDRYFTIFYALQYHNIMTVKRVGIIISCIWAACVSGILFIIYSDSSAVIICLITMFFTMLALM
ASLYVHMFLMARLHIKRIAVLPGTGAIKQGANMKGAITLTILIGVFVVCWAPFFLHLIFYISCPQNPYC
VCFMSHFNLYLILMCNSIIDPLIYALRSQELRKTKEIICCYPLGGLCDLSSRY (SEQ ID NO:13)

hMC4R (R165Q): mutant R165Q-hMC4R, intracellularly retained and PC-rescuable

MVNSTHRGMHTSLHLWNRSSYRLHSNASESLGKGYSDDGGCYEQLFVSPEVFVTLGVISLLENILVI
15 VAIKKNLHSPMYFFICSLAVADMLVSVSNGSETIVITLLNSTDTDAQSFTVNIDNVIDSVICSSLLA
SICSLLSIAVDRYFTIFYALQYHNIMTVKRVGIIISCIWAACVSGILFIIYSDSSAVIICLITMFFTMLAL
MASLYVHMFLMARLHIKRIAVLPGTGAIKQGANMKGAITLTILIGVFVVCWAPFFLHLIFYISCPQNP
YCVCFMSHFNLYLILMCNSIIDPLIYALRSQELRKTKEIICCYPLGGLCDLSSRY (SEQ ID NO:14)

20 hMC4R (P299H): mutant P299H-hMC4R, intracellularly retained and mostly not PC-rescuable

MVNSTHRGMHTSLHLWNRSSYRLHSNASESLGKGYSDDGGCYEQLFVSPEVFVTLGVISLLENILVI
VAIKKNLHSPMYFFICSLAVADMLVSVSNGSETIVITLLNSTDTDAQSFTVNIDNVIDSVICSSLLA
SICSLLSIAVDRYFTIFYALQYHNIMTVKRVGIIISCIWAACVSGILFIIYSDSSAVIICLITMFFTMLALM
ASLYVHMFLMARLHIKRIAVLPGTGAIKQGANMKGAITLTILIGVFVVCWAPFFLHLIFYISCPQNPYC
25 VCFMSHFNLYLILMCNSIIDPLIYALRSQELRKTKEIICCYPLGGLCDLSSRY (SEQ ID NO:15)

hV2R WT: human wild type Vasopressin 2 receptor

MLMASTTSAPVGHPSLPSLPSNSSQERPLDTRDPLLARAELALLSIVFVAVALSNGLVLAALARRG
RRGHWAPIHVFIGHLCLADLAVALFQVLPQLAWKATDRFRGPDALCRAVKYLQMVGMYASSYMIL
AMTLDRHRAICRPMLAYRHGSGAHWNRPVLVAVAFSLLSLPQLFIFAQRNVEGGSGVTDCWAC
30 FAEPWGRRTYVTWIALMVFAPTLGIAACQVLIFREIHASLVGPSPERPGRRRGRRTGSPGEGA
HVSAAVAKTVRMTLVIVVVYVLCWAPFFLVQLWAAWDPEAPLEGAPFVLLMLLASLNSCTNPWIY
ASFSSSVSSELRSLLCCARGRTPPSLGPQDESCTTASSSLAKDTSS (SEQ ID NO:16)

hV2R (Y128S): mutant Y128S-hV2R, intracellularly retained and PC-rescuable

MLMASTTSAPVGHPSLPSLPSNSSQERPLDTRDPLLARAELALLSIVFVAVALSNGLVLAALARRG
35 RRGHWAPIHVFIGHLCLADLAVALFQVLPQLAWKATDRFRGPDALCRAVKYLQMVGMYASSSMIL
AMTLDRHRAICRPMLAYRHGSGAHWNRPVLVAVAFSLLSLPQLFIFAQRNVEGGSGVTDCWAC
FAEPWGRRTYVTWIALMVFAPTLGIAACQVLIFREIHASLVGPSPERPGRRRGRRTGSPGEGA

HVSAAVAKTVRMTLVIVVVYVLCWAPFFLVQLWAAWDPEAPLEGAPFVLLMLLASLNSCTNPWIY
ASFSSSVSSELRSLLCCARGRTPPSLGPQDESCTTASSSLAKDTSS (SEQ ID NO:17)

hERG WT: human wild type voltage-gated Potassium channel H2

MPVRRGHVAPQNTFLDTIIRKFEGQSRKFIIANARVENCAVIYCNDGFCELCGYSTRAEVMQRPCTC
5 DFLHGPRTQRRAAAQIAQALLGAEERKVEIAFYRKDGSCFLCLVDVVPVKNEGDGAVIMFILNFEVV
MEKDMVGSPAHDNTNHRGPPTSWLAPGRAKTFRLKLPALLALTARESSVRSGGAGGAGAPGAVV
VDVDLTPAAPSSSESLALDEVTAMDNHVAGLGPAEERRALVGPSPPRSAPGQLPSPRAHSLNPD
ASGSSCSLARTRSRESCASVRRASSADDIEAMRAGVLPPPPRHASTGAMHPLRSGLLNSTSDSD
LVRYRTISKIPQITLNFVDLKGDPFLASPTSDREIAPKIKERTHNVTQVLSLGADVLPEYKLQA
10 PRIHRWTILHYSPPFKAVWDWLILLVIYTAVFTPYSAAFLLKETEEGPPATECGYACQPLAVVDLVD
IMFIVDILINFRTTYVNANEEVVSHPGRIAVHYFKGWFLIDMVAAIPFDLLIFGSGSEELIGLLKTARLL
RLVRVARKLDRYSEYGA AVLFLMCTFALIAHWLACIWIYAIGNMEQPHMDSRIGWLHNLGDQIGK
PYNSSGLGGPSIKDKYVTALYFTFSSLTSGVFGNVSPNTNSEKIFSICVMLIGSLMYASIFGNVSAII
QRLYSGTARYHTQMLRVREFIRFHQIPNPLRQRLEEYFQHAWSYTNGIDMNAVLKGFPECLQADI
15 CLHLNRSLLQHCKPFRGATKGCLRALAMKFKTTHAPPGDTLVHAGDLLTALYFISRGSIEILRGDVV
VAILGKNDFIGEPLNLYARPGKSNGDVRLTYCDLHKIHRDDLLEVLDMYPEFSDFHWSSLEITFNL
RDTNMIPGSPGSTELEGGFSRQQRKRKLSFRRRTDKDTEQPGEVSALGPGRAGAGPSSRGRPGG
PWGESPPSSGPSSPESEDEGPGRSSSPLRLVPFSSPRPPGEPGGEPLMEDCEKSSDTCNPLS
GAFSGVSNIFSFWDGRGRQYQELPRCPAPTPLLNIPLSSPGRPRGDVESRLDALQRQLNRLE
20 TRLSADMATVLQLLQRQMTLVPPAYSAVTTPGPGPTSTSPLLPVSPPLTLTDSLSQVSQFMACE
ELPPGAPELPQEGPTRRLSLPGQLGALTSQPLHRHGSDPGS (SEQ ID NO:18)

hERG (G601S): mutant G601S-hERG, intracellularly retained and PC-rescuable

MPVRRGHVAPQNTFLDTIIRKFEGQSRKFIIANARVENCAVIYCNDGFCELCGYSTRAEVMQRPCTC
DFLHGPRTQRRAAAQIAQALLGAEERKVEIAFYRKDGSCFLCLVDVVPVKNEGDGAVIMFILNFEVV
25 MEKDMVGSPAHDNTNHRGPPTSWLAPGRAKTFRLKLPALLALTARESSVRSGGAGGAGAPGAVV
VDVDLTPAAPSSSESLALDEVTAMDNHVAGLGPAEERRALVGPSPPRSAPGQLPSPRAHSLNPD
ASGSSCSLARTRSRESCASVRRASSADDIEAMRAGVLPPPPRHASTGAMHPLRSGLLNSTSDSD
LVRYRTISKIPQITLNFVDLKGDPFLASPTSDREIAPKIKERTHNVTQVLSLGADVLPEYKLQA
PRIHRWTILHYSPPFKAVWDWLILLVIYTAVFTPYSAAFLLKETEEGPPATECGYACQPLAVVDLVD
30 IMFIVDILINFRTTYVNANEEVVSHPGRIAVHYFKGWFLIDMVAAIPFDLLIFGSGSEELIGLLKTARLL
RLVRVARKLDRYSEYGA AVLFLMCTFALIAHWLACIWIYAIGNMEQPHMDSRIGWLHNLGDQIGK
PYNSSSLGGPSIKDKYVTALYFTFSSLTSGVFGNVSPNTNSEKIFSICVMLIGSLMYASIFGNVSAIIQ
RLYSGTARYHTQMLRVREFIRFHQIPNPLRQRLEEYFQHAWSYTNGIDMNAVLKGFPECLQADICL
HLNRSLLQHCKPFRGATKGCLRALAMKFKTTHAPPGDTLVHAGDLLTALYFISRGSIEILRGDVVVA
35 ILGKNDFIGEPLNLYARPGKSNGDVRLTYCDLHKIHRDDLLEVLDMYPEFSDFHWSSLEITFNLRD
TNMIPGSPGSTELEGGFSRQQRKRKLSFRRRTDKDTEQPGEVSALGPGRAGAGPSSRGRPGGPW
GESPPSSGPSSPESEDEGPGRSSSPLRLVPFSSPRPPGEPGGEPLMEDCEKSSDTCNPLSGAF

SGVSNIFSFWGDSRGRQYQELPRCPAPTPSLLNIPLSSPGRPRRGDVESRLDALQRQLNRLETRL
SADMATVLQLLQRQMTLVPPAYSAVTTGPGPTSTSPLLPVSPPLTLTLDLSQVSQFMACEELP
PGAPELPQEGPTRRLSLPGQLGALTSQPLHRHGSDPGS (SEQ ID NO:19)

Lyn: palmitoylation & myristoylation signal sequence from the Lyn kinase

5 MGCIKSKGKDS (SEQ ID NO:1)

CAAX-Kras: plasma-membrane targeting polybasic sequence and prenylation signal sequence from kRas splice variant b

GKKKKKKSKTKCVIM (SEQ ID NO:7)

PB: plasma-membrane targeting polybasic sequence from the human GRK5

10 SPKKGLLQRLFKRQHQNNSKS (SEQ ID NO:8)

endofin's FYVE domain

QKQPTWVPDSEAPNCMNCQVKFTFTKRRHHCRACGKVFCGVCCNRKCKLQYLEKEARVCVVCY
ETISK (SEQ ID NO:20)

Rab4

15 MSETYDFLFKFLVIGNAGTGKSCLLHQFIEKKFKDDSNHTIGVEFGSKIINVGGKYVKLQIWDTAGQ
ERFRSVTRSYRGAAGALLVYDITSRETYNALTNLWTDARMLASQNIIVILCGNKKDLADREVTF
EASRFAQENELMFLETSGENVEEAFVQCARKILNKIESGELDPERMGSIGYGDAAALRQLRSP
RRAQAPNAQECGC (SEQ ID NO:21)

Rab11

20 MGTRDDEYDYLKVVLLIGDSGVGKSNLLSRFTRNEFNLESKSTIGVEFATRSIQVDGKTIKAQIWD
AGQERYRAITSAYYRGAVGALLVYDIKHLTYENVERWLKELRDHADSNIIVMLVGNKSDLRHLRA
VPTDEARAFAEKNGLSFIETSALDSTNVEAAFQITLTIYRIVSQKQMSDRRENDMSPSNNVPIHV
PPTTENKPKVQCCQNI (SEQ ID NO:22)

signal peptide-Flag-human AT1R (spFlag-AT1R)

25 MKTIIALSYIFCLVFADYKDDDDAMILNSSTEDGIKRIQDDCPKAGRHNYIFVMIPTLYSIIFVVGIFGN
SLVVIVIFYFMKLKTVASVFLNLALADLCFLTLPLWAVYTAMEYRWPFNGNYLCKIASASVSFNLYA
SVFLLTCLSIDRYLAIVHPMKSRRLRRTMLVAKVTCIIWLLAGLASLPPIIHRNVFFIENTNITVCAFH
ESQNSTLPIGLGLTKNILGFLFPFLIILTSYTLIWKALKKAYEIQKNKPRNDDIFKIIMAIVLFFFFSWIPH
QIFTFLDVLILGLIIRDCRIADIVDTAMPITICIAFYNNCLNPLFYGFLGKKFKRYFLQLLKYIPPKAKSH
30 SNLSTKMSTLSYRPSDNVSSSTKKPAPCFEVE (SEQ ID NO:23)

hGRB2 v1; human GRB2 variant 1

MEAIKYDFKATADDELSFKRGDILKVLNEECDQNWYKAELNGKDGFIKPNYIEMKPHFPFGNDVQ
HFKVLRDGAGKYFLWVVKFNSLNLVDYHRSTSISRNNQIFLRDIEQVPQQPTYVQALFDFDPQE
DGELGFRRGDFIHVMDNSDPNWWKGACHGQTGMFPRNYVTPVNRNV (SEQ ID NO:24)

35 PH domain of PLCδ1

DSGRDFLT LHGLQDDEDLQALLKGSQLLKVKSSSWRRERFYKLQEDCKTIWQESRKVMRTPESQ
LFSIEDIQEVRMGHRTEGLEKFARDVPEDRCFSIVFKDQRNTLDLIAPSPADAQHWVLGLHKIIHHS
GSMDQRQKLQHWIHSCLRKADKNKDNKMSFKELQNFLKELNI (SEQ ID NO:25)

HA tag

5 MYPYDVPDYA (SEQ ID NO:26)

Residues 1-73 of human eNOS1

MGNLKSVAQEPGPPCGLGLGLGLGCGKQGPATPAPEPSRAPASLLPPAPEHSPPSSPLTQPPE
GPKFPRVKN (SEQ ID NO:42)

Calveolin1 α

10 MSGGKYVDSEGHLYTVPIREQGNIYKPNKAMADELSEKQVYDAHTKEIDLVRDPKHLNDDVVK
IDFEDVIAEPEGTHSFDGIWKASFTTFTVTKYWFYRLLSALFGIPMALIWGIYFAILSFLHIWAVPCI
KSFLIEIQCISRVYSIYVHTVCDPLFEAVGKIFSNVRINLQKEI (SEQ ID NO:44)

Linker1: Linker sequence between the hMC4R and RlucII

VGGGGSKLPAT (SEQ ID NO:2)

15 Linker2: Linker sequence between the hV2R and RlucII

GGGSLKLPAT (SEQ ID NO:3)

Linker3: Linker sequence in N-terminal of RlucII, following residue 379 of hERG

NAAIRSGG (SEQ ID NO:4)

Linker4: Linker sequence in N-terminal of RlucII, preceding residue 373 of hERG

20 GGNAAIRS (SEQ ID NO:5)

Linker5: Linker between Lyn's plasma-membrane targeting sequence (Lyn) and rGFP

LSNAT (SEQ ID NO:27)

Linker6: Linker between rGFP and polybasic/prenylation sequence from kRAS (CAAX)

GSAGTMASNNTASG (SEQ ID NO:28)

25 Linker7: Linker between rGFP and polybasic sequence from GRK5 (PB):

GGGSLKLPAT (SEQ ID NO:3)

Linker8: Linker between rGFP and palmitoylation/prenylation sequence from hRAS (CAAX) and
hRAS/Ral1(CAAX=CCIL), between rGFP and Caveolin1 α , and between RlucII and GRB2::

GSAGT (SEQ ID NO:9)

30 Linker9: Linker between Golgi targeting sequence from eNOS (1-73) and rGFP

GSNAT (SEQ ID NO:41)

Linker10: between (i) rGFP and (ii) endofin's FYVE domain, Rab4 or Rab11

GGGGSGSGGLE (SEQ ID NO:29)

Linker11: between spFlag-AT1R and RlucII

35 GGSGGKLPAT (SEQ ID NO:30)

Linker12: between RlucII and PH domain of PLC δ 1

GNASGTGSGGSGSGGLE (SEQ ID NO:31)

Linker13: between rGFP and PH domain of PLC δ 1

GSGGSGSGGLE (SEQ ID NO:29)

Linker14: between HA tag and RlucII

SNAKL (SEQ ID NO:32)

- 5 hV2R (W164S): mutant W164S-hV2R, intracellularly retained and PC-rescuable

MLMASTTSAPVGHPSLPSLPSNSSQERPLDTRDPLLARAELALLSIVFVAVALSNGLVLAALARRG
RRGHWAPIHVFIGHLCLADLAVALFQVLPQLAWKATDRFRGPDALCRAVKYLQVMGYASSYMIL
AMTLDRHRAICRPMLAYRHGSGAHWNRPVLVASAFSLLLSLPQLFIFAQRNVEGGSGVTDCWAC
FAEPWGRRTYVTWIALMVFVAPTLGIAACQVLIFREIHASLVPGPSEPPGGRRRGRRTGSPGEGA
10 HVSAAVAKTVRMTLVIVVVYVLCWAPFFLVQLWAAWDPEAPLEGAPFVLLMLLASLNSCTNPWIY
ASFSSSVSSELRSLLCCARGRTPPSLGPQDESCTTASSSLAKDTSS (SEQ ID NO:46).

CAAX (Hras): plasma-membrane targetting palmitoylation sequence and prenylation signal
sequence from hRas

CMSCKCVLS (SEQ ID NO:47)

- 15 CAAX (CCIL): plasma-membrane targetting palmitoylation sequence from hRas and prenylation
signal sequence from Ral1

CMSCKCCIL (SEQ ID NO: 43)

hMC4R N62S mutant Melanocortin 4 receptor, intracellularly retained and PC-rescuable

- 20 MVNSTHRGMHTSLHLWNRSSYLHNSASESLGKGYSDDGGCYEQLFVSPEVFVTLGVISLLESILVI
VAIAKNKNLHSPMYFFICSLAVADMLVSVSNGSETIVITLLNSTDTDAQSFTVNIDNVIDSVICSSLLA
SICSLLSIAVDYFTIFYALQYHNIMTVKRVGIIISCIWAACVSGILFIIYSDSSAVIICLITMFFTMLALM
ASLYVHMFLMARLHIKRIAVLPGTGAIKQGANMKGAITLTILIGVFVVCWAPFFLHLIFYISCPQNPYC
VCFMSHFNLYLILMCNSIIDPLIYALRSQELRKTKEIICCYPLGGLCDLSSRY (SEQ ID NO:48)

hMC4R R165W mutant Melanocortin 4 receptor, intracellularly retained and PC-rescuable

- 25 MVNSTHRGMHTSLHLWNRSSYLHNSASESLGKGYSDDGGCYEQLFVSPEVFVTLGVISLLENILVI
VAIAKNKNLHSPMYFFICSLAVADMLVSVSNGSETIVITLLNSTDTDAQSFTVNIDNVIDSVICSSLLA
SICSLLSIAVDYFTIFYALQYHNIMTVKRVGIIISCIWAACVSGILFIIYSDSSAVIICLITMFFTMLAL
MASLYVHMFLMARLHIKRIAVLPGTGAIKQGANMKGAITLTILIGVFVVCWAPFFLHLIFYISCPQNP
YCVCFMSHFNLYLILMCNSIIDPLIYALRSQELRKTKEIICCYPLGGLCDLSSRY (SEQ ID NO:49)

- 30 Unimolecular DAG sensor

MGCIKSKGKDSLSNAMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTCLKFICTT
GKLPVPWPTLVTTLSYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKF
EGDTLVNRIELKGIDFKEDGNILGHKLEYNYPHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADH
YQQNTPIGDGPVLLPDNHYLFTQSALS KDPNEKRDHMLLEFVTAAGITLGMDELYKGTGTAAKE
35 GEKQKGAMQPSEQQRGKEAQKEKNGKEPNRPEQPKPAKVEQQEPEERPKREPMQLEPAE
SAKQGRNLPQKVEQGEERPQEADMPGQAQSAMRPQLSNSEEGPARGKPAPEEPDEQLGEPEE
AQGEHADEPAPSKPSEKHMVPQMAEPEKGEEAREPQGAEDKPAPVHKPKKEEPQRPNEEKAPK

PKGRHVGRQENDDSAKGPEPGRPDRCGKEKEPEEEPAQGHSLPQEPEPMRPKPEVRKKPHP
 GASPHQVSDVEDAKGPERKVNPMEGEESAKQAQQEGPAENDEAERPERPASGGAREAMTSKV
 YDPEQRKRMITGPQWWARCKQMNVLDSFINYYDSEKHAENAVIFLHGNATSSYLWRHVVPHIEP
 VARCIIPDLIGMGKSGKSGNGSYRLLDHYKYLTAWFELLNLPKKIIFVGHWDGAALAFHYSYEHQD
 5 KIKAIVHAESVVDVIESWDEWPDIEEDIALIKSEEGERKMLENNFFVETVLP SKIMRKLEPEEFAAYL
 EPFKEKGEVRRPTLSWPREIPLVKGGKPDVVQIVRNYNAYLRASDDLPMFIESDPGFFSNAIVEG
 AKKFPNTEFVKVKGHLHFSQEDAPDEMCKYIKSFVERVLKNEQSGSGFNIDMPHRFKVHNYMSP
 TFCDHCGSLLWGLVKQGLKCEDCGMNVHHKCREKVANLCG (SEQ ID NO:50)

Unimolecular β arrestin1 sensor

10 MGCIKSKGKDSL SNAMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLT LKFICTT
 GKLPVPWPPTLVTTLSYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKF
 EGDTLVNRIELKGIDFKEDGNILGHKLEYNYPHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADH
 YQQNTPIGDGPVLLPDNHYLFTQSALSKDPNEKRDHMLLEFVTAAGITLGMDELYKGTGTAAKE
 GEKQKGAMQPSEQQRGKEAQKEKNGKEPNRPEQPKPAKVEQQEDEPEERPKREPMQLEPAE
 15 SAKQGRNLPQKVEQGEERPQEADMPGQAQSAMRPQLSNSEEGPARGKPAPEEPDEQLGEPEE
 AQGEHADEPAPSKPSEKHMVPQMAEPEKGEEAREPQGAEDKPAPVHKPKKEEPQRPNEEKAPK
 PKGRHVGRQENDDSAKGPEPGRPDRCGKEKEPEEEPAQGHSLPQEPEPMRPKPEVRKKPHP
 GASPHQVSDVEDAKGPERKVNPMEGEESAKQAQQEGPAENDEAERPERPASGGAREAMTSKV
 YDPEQRKRMITGPQWWARCKQMNVLDSFINYYDSEKHAENAVIFLHGNATSSYLWRHVVPHIEP
 20 VARCIIPDLIGMGKSGKSGNGSYRLLDHYKYLTAWFELLNLPKKIIFVGHWDGAALAFHYSYEHQD
 KIKAIVHAESVVDVIESWDEWPDIEEDIALIKSEEGERKMLENNFFVETVLP SKIMRKLEPEEFAAYL
 EPFKEKGEVRRPTLSWPREIPLVKGGKPDVVQIVRNYNAYLRASDDLPMFIESDPGFFSNAIVEG
 AKKFPNTEFVKVKGHLHFSQEDAPDEMCKYIKSFVERVLKNEQSGSAGTAGDKGTRVFKKASPN
 GKLTVYLGKRDFVDHIDLVDVPDGVVLVDPEYLKERRVYVTLTCAFRYGRELDVLGLTFRKDLFV
 25 ANVQSFPPAPEDKKPLTRLQERLIKKLGEHAYPFTFEIPP NLPCSVTLQPGP EDTGKACGV DYEYK
 AFCAENLEEKIHKRNSVRLVIRKVQYAPERPGPQPTAETTRQFLMSDKPLHLEASLDKEIYYHGEPI
 SVNHVHTNNTNKT VKKIKISVRQYADICLFNTAQYKCPVAMEEADDTVAPSSTFCKVYTLTPFLAN
 NREKRGLALDGKLGKHDNTLASSTLLREGANREILGIIVSYKVKVLVSRGGLLGDLASSDVAVEL
 PFTLMHPKPKEEPHREVPENETPVDTNLIELDTNDDDIVFEDFARQRLKGMKDDKEEEEDGTGS
 30 PQLNNR (SEQ ID NO:51)

Unimolecular β arrestin2 sensor

MGCIKSKGKDSL SNAMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLT LKFICTT
 GKLPVPWPPTLVTTLSYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKF
 EGDTLVNRIELKGIDFKEDGNILGHKLEYNYPHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADH
 35 YQQNTPIGDGPVLLPDNHYLFTQSALSKDPNEKRDHMLLEFVTAAGITLGMDELYKGTGTAAKE
 GEKQKGAMQPSEQQRGKEAQKEKNGKEPNRPEQPKPAKVEQQEDEPEERPKREPMQLEPAE
 SAKQGRNLPQKVEQGEERPQEADMPGQAQSAMRPQLSNSEEGPARGKPAPEEPDEQLGEPEE

AQGEHADEPAPSKPSEKHMVPQMAEPEKGEEAREPQGAEDKPAPVHKPKKEEPQRPNEEKAPK
 PKGRHVGRQENDDSAGKPEPGRPDRKGKEKEPEEEPAQGHSLPQEPEPMPRPKPEVRKKPHP
 GASPHQVSDVEDAKGPERKVNPMEGEESAKQAQGEPAENDEAERPERPASGGAREAMTSKV
 YDPEQRKRMITGPQWWARCKQMNVLDSFINYYDSEKHAENAVIFLHG NATSSYLWRHVPHIEP
 5 VARCIIPDLIGMGKSGKSGNGSYRLLDHYKYLTAWFELLNLPKKIIFVGHWDGAALAFHYSYEHQD
 KIKAIVHAESVVDVIESWDEWPDIEEDIALIKSEEGEKMVLENNFFVETVLPSKIMRKLEPEEFAAYL
 EPFKEKGEVRRPTLSWPREIPLVKGGKPDVVQIVRNYNAYLRASDDLPMFIESDPGFFSNAIVEG
 AKKFPNTEFVKVKGLHFSQEDAPDEMCKYIKSFVERVLKNEQSGSAGTAGEKPGTRVFKKSSP
 NCKLTVYLGRDFVDHLDKVDVDPVGVVLDVDPYLDKDRKVFVTLTCAFRYGREDLDVLGLSFRKDL
 10 FIATYQAFPPVPNPPRPPTRLQDRLLRKLGHQHAHPFFFTIPQNLPCSVTLQPGPEDTGKACGVDFE
 IRAFCAKSLEEKSHKRN SVRLVIRKVQFAPEKPGPQPSAETTRHFLMSDRSLHLEASLDKELYH
 EPLNVNVHVTNNSTKT VKKIKVSVRQYADICLFSTAQYKCPVAQLEQDDQVSPSSTFCKVYTITPLL
 SDNREKRGALDGLKHEDTNLASSTIVKEGANKEVLGILVSYRVKVKLVVSRGGDVSVELPFVLM
 HPKPHDHIPLPRPQSAAPETDVPVDTNLIEFD TNYATDDDIFEDFARLRLKGMKDDDYDDQLC(S
 15 EQ ID NO:52)

Human G α 12 subunit with an RlucII inserted at position 84:

MSGVVRTL SRCLLPAEAGGARERRAGSGARDAEREARRRSRDIDALLARERRAVRRLVKILLGA
 GESGKSTFLKQMRIIHGREGSGGGGSM TSKVYDPEQRKRMITGPQWWARCKQMNVLDSFINYY
 DSEKHAENAVIFLHGNAASSYLWRHVPHIEPVARCIIPDLIGMGKSGKSGNGSYRLLDHYKYLT
 20 WFELLNLPKKIIFVGHWDGAALAFHYSYEHQDKIKAIVHAESVVDVIESWDEWPDIEEDIALIKSEEG
 EKMVLENNFFVETVLPSKIMRKLEPEEFAAYLEPFKEKGEVRRPTLSWPREIPLVKGGKPDVVQIV
 RNYNAYLRASDDLPMFIESDPGFFSNAIVEGAKKFPNTEFVKVKGLHFSQEDAPDEMCKYIKSFV
 ERVLKNEQSGGGGSGTDFDQKALLEFRDTIFDNILKGSRLVDARDKLGIPWQYSENEEHGMFLMA
 FENKAGLPVEPATFQLYVPALSALWRDSGIREAFSRRSEFQLGESVKYFLDNLD RIGQLEYMPTE
 25 QDILLARKATKGIVEHDFVIKKIPFKMVDVGGQRSQRQKWFQCFDGITSILFMVSSSEYDQVL MED
 RRTNRLVESMNIFETIVNNKLFFNVSIILFLNKMDLLVEKVKT VSIKKHFPDFRGDPHRLEDVQRYLV
 QC FDRKRRNRSKPLFHHTTAIDTENVRVFVHAVKD TILQENLKDIMLQ (SEQ ID NO:53)

Human G α q subunit with an RlucII inserted at position 118:

MTLESIMACCLSEEAKEARRINDEIERQLRRDKRDARRELKLLLLGTGESGKSTFIKQMRIIHGSGY
 30 SDEDKRGFTKL VYQNI FTAMQAMIRAMDTLKIPYKYEHNKAHAQLVREVDVNAAIRSTRMTSKVYD
 PEQRKRMITGPQWWARCKQMNVLDSFINYYDSEKHAENAVIFLHGNAASSYLWRHVPHIEPVA
 RCIIPDLIGMGKSGKSGNGSYRLLDHYKYLTAWFELLNLPKKIIFVGHWDGAALAFHYSYEHQDKIK
 AIVHAESVVDVIESWDEWPDIEEDIALIKSEEGEKMVLENNFFVETVLPSKIMRKLEPEEFAAYLEPF
 KEKGEVRRPTLSWPREIPLVKGGKPDVVQIVRNYNAYLRASDDLPMFIESDPGFFSNAIVEGAKK
 35 FPNTEFVKVKGLHFSQEDAPDEMCKYIKSFVERVLKNEQCTNAAIRSEKVS AFENPYVDAIKSLWN
 DPGIQECYDRRREYQLSDSTKY YLNDLDRVADPAYLPTQQDVLRVRVPTTGII EYPFDLQSVIFRM
 VDVGGRQSRERKWIHCFENVTSIMFLVALSEYDQVLVESDNENRMEESKALFRTIITYPWFQNSS

VILFLNKKDLLEEKIMYSHLVDYFPEYDGPQRDAQAAREFILKMFVDLNPDSDKIIYSHFTCATDTEN
IRFVFAAVKDTILQLNLKEYNLV (SEQ ID NO:54)

Human GαS subunit with an RlucII inserted at position 67:

MGCLGNSKTEDQRNEEKAQREANKKIEKQLQKDKQVYRATHRLLLLGAGESGKSTIVKQMRILHV
5 NGSGGGGSMSTSKVYDPEQRKRMITGPQWWARCKQMNVLDSFINYYDSEKHAENAVIFLHGNAT
SSYLWRHVPHIEPVARCIIPDLIGMGKSGKSGNGSYRLLDHYKYLTAWFELLNLPKKIIFVGHWDW
GAALAFHYSYEHQDKIKAIVHAESVVDVIESWDEWPDIEEDIALIKSEEKEMVLENNFFVETVLP
KIMRKLEPEEFAAYLEPFKEKGEVRRPTLSWPREIPLVKGGKPDVVQIVRNYNAYLRASDDLPKMF
IESDPGFFSNAIVEGAKKFPNTEFVKVKGLHFSQEDAPDEMCKYIKSFVERVLKNEQSGGGGGSFN
10 GEGGEEDPQAARSNSDGEKATKVQDIKNNLKEAETIVAAMSNLVPPVELANPENQFRVDYILSVM
NVPDFDFPPEFYEHAKALWEDEGVRACYERSNEYQLIDCAQYFLDKIDVIKQADYVPSDQDLLRC
RVLTS GIFETKFQVDKVNFMFDVGGQDERRKWIQCNDVTAIIFVVASSSYNMVIREDNQTNRL
QEALNLFKSIWNNRWLRTISVILFLNKQDLLAEKVLGKSKIEDYFPEFARYTTPEDATPEPGEDPR
VTRAKYFIRDEFLRISTASGDGRHYCYPHFTCAVDTENIRRVFNDCRDIIQRMHLRQYELL (SEQ ID
15 NO:55)

human Gβ1 subunit

MSELDQLRQEAQLKNQIRDARKACADATLSQITNNIDPVGRIQMRTRRTLGRHLAKIYAMHWGT
DSRLLVSASQDGKLIWDSYTTNKVHAIPLRSSWVMTCAAYAPSGNYVACGGLDNICSIYNLKTREG
NVRVSRELIGHTGYLSCCRFLDDNQIVTSSGDTTCALWDIETGQQTFTTFTGHTGDVMSLSLAPDT
20 RLFVSGACDASAKLWDVREGMCRQTFTGHESDINAICFFPNGNAFATGSDDATCRLFDLRADQE
LMTYSHDNIICGITSVSFSKSGRLLLAGYDDFNCNVWDALKADRAGVLGHNDNRVSCLGVTDDGM
AVATGSWDSFLKIWN (SEQ ID NO:56)

human Gγ1 subunit

MPVINIEDLTEKDKLKMEVDQLKKEVTLERMLVSKCCEEVRDYVEERSGEDPLVKGIPEDKNPFKE
25 LKGCCVIS (SEQ ID NO:57)

human Gγ2 subunit

MASNNTASIAQARKLVEQLKMEANIDRIKVSAAAADLMAYCEAHAKEDPLLTPVPASENPFREKKF
FCAIL (SEQ ID NO:58)

human Gγ3 subunit

30 MKGETPVNSTMSIGQARKMVEQLKIEASLCRIKVSAAAADLMTYCDAHACEDPLITPVPTSENPF
EKKFFCALL (SEQ ID NO:59)

human Gγ4 subunit

MKEGMSNNSTTSISQARKAVEQLKMEACMDRVKVSQAAADLLAYCEAHVREDPLIIPVPASENPF
REKKFFCTIL (SEQ ID NO:60)

35 human Gγ5 subunit

MSGSSSVAAMKKVVQQLRLEAGLNRVKVSQAAADLKQFCLQNAQHDPLLTVSSSTNPFRPQKV
CSFL (SEQ ID NO:61)

human G γ 7 subunit

MSATNNIAQARKLVEQLRIEAGIERIKVSKAASDLMSYCEQHARNDPLLVGVPASENPFKDKKPCII
L (SEQ ID NO:62)

human G γ 8 subunit

- 5 MSNNMAKIAEARKTVEQLKLEVNIDRMKVSQAAAELLAFCETHAKDDPLVTPVPAENPFRDKRLF
CVLL (SEQ ID NO:63)

human G γ 9 subunit

MAQDLSEKDLLKMEVEQLKKEVKNTTRIPISKAGKEIKEYVEAQAGNDPFLKGIPEDKNPFKEKGGC
LIS (SEQ ID NO:64)

- 10 human G γ 10 subunit

MSSGASASALQRLVEQLKLEAGVERIKVSQAAAEQQYCMQNACKDALLVGVPAGSNPFREPRS
CALL (SEQ ID NO:65)

human G γ 11 subunit

- MPALHIEDLPEKEKLKMEVEQLRKEVKLQRQQVSKCSEEIKNYIEERSGEDPLVKGIPEDKNPFKE
15 KGSCVIS (SEQ ID NO:66)

human G γ 12 subunit

MSSKTASTNNIAQARRTVQQLRLEASIERIKVSKASADLMSYCEEHARSDPLLIGIPTSENPFKDKK
TCIIL (SEQ ID NO:67)

human G γ 13 subunit

- 20 MEEWDVPQMKKEVESLKYQLAFQREMASKTIPELLKWIEDGIPKDPFLNPDLMKNNPWVEKGKC
TIL (SEQ ID NO:68)

human G α 15 subunit

- MARSLTWRCCPWCLTEDEKAAARVDQEINRILLEQKKQDRGELKLLLLGPGESGKSTFIKQMRIIH
GAGYSEEERKGFRLVYQNIFVSMRAMIEAMERLQIPFSRPESKHASLVMSQDPYKVTTFEKRY
25 AAAMQWLWRDAGIRACYERRREFHLLDSAVYYLSHLERITEEGYVPTAQDVLRSRMPTTGINEYC
FSVQKTNLRIVDVGGQKSERKKWIHCFENVIALIYLASLSEYDQCLEENNQENRMKESLALFGTILE
LPWFKSTSVILFLNKTDILEEKIPTSHLATYFSPFQGPQDAEAAKRFILDMYTRMYTGCVDGPEGS
KKGARSRRFLFSHYTCATDTQNIRKVFKDVRDSVLARYLDEINLL (SEQ ID NO:69)

Rho-binding domain (CRIB) of the human Protein kinase 1 (PKN)

- 30 VQSEPRSWSLLEQLGLAGADLAAPGVQQQLELERERLRREIRKELKLKEGAENLRRATTDLGRSL
GPVELLLRGSSRRLDLLHQQQLQE (SEQ ID NO:70)

Linker between RlucII and the Rho binding domain of PKN1

GSASAGTATMASDA (SEQ ID NO:71)

DAG binding domain C1b from the human PKC δ

- 35 FNIDMPHRFKVHNYMSPTFCDHCGSLLWGLVKQGLKCEDCGMNVHHKCREKVANLCG (SEQ ID
NO:72)

Human β 1 adrenergic receptor (β 1AR)

MGAGVLVLGASEPGNLSSAAPLPDGAATAARLLVPASPPASLLPPASESPEPLSQQWTAGMGLL
MALIVLLIVAGNVLVIVAIKTPRLQTLTNLFIMSLASADLVMGLLVVPGATIVVWGRWEYGSFFCE
LWTSVDVLCVTASIELCVIALDRYLAI TSPFRYQSLLTRARARGLVCTVWAISALVSFLPILMHWW
RAESDEARRCYNDPKCCDFVTNRAYAIASSVVSYFVPLCIMA FVYLRVFREAQKQVKKIDSCERRF
5 LGGPARPPSPSPSPVPAPAPPPGPPRPA AAAATAPLANGRAGKRRPSRLVALREQKALKTLGIIM
GVFTLCWLPFFLANVVKAFHRELVPDRLFVFFNWLG YANS AFNPIIYCRSPDFRKA FQGLLCCARR
AARRRHATHGDRPRASGCLARPGPPSPGAASDDDDDDVVGATPPARLLEPWAGCNGGAAAD
SDSSLDEPCRPGFASESKV (SEQ ID NO:73)

Human $\beta 2$ adrenergic receptor ($\beta 2AR$)

10 MGQPGNGSAFLLAPNRSHAPDHDVTQQRDEVWVVG MGIVMSLIVLAIVFGNVLVITAIKFERLQT
VTNYFITS LACADLVMGLAVVPFGAAHILMKMWTFGNFWCEFWTSIDVLCVTASIELCVIAVDRYF
AITSPFKYQSLLTKNKARVIILMWIVSGLTSFLPIQMHWYRATHQEAINCYANETCCDFFTNQAYAI
ASSIVSYFVPLVIMVFVYSRVFQEAQRQLQKIDKSEGRFHVQNLSQVEQDGRGTGHGLRRSSKFCL
KEHKALKTLGIIMGTFTLCWLPFFIVNIVHVIQDNLRKEVYILLNWIGYVNSGFNPLIYCRSPDFRIAF
15 QELLCLRRSSLKAYGNGYSSNGNTGEQSGYHVEQEKENKLLCEDLPGTEDFVGHQGTVPDNIID
SQGRNCSTNDSLL (SEQ ID NO:74)

Human prostaglandin 2α receptor isoform a (FP)

MSMNNSKQLVSPA AALLSNTTCQTENRLSVFFSVIFMTVGILSN SLAIAILMKAYQRFQKSKASFL
LLASGLVITDFFGHLINGAIAVFVYASDK EWIRFDQSNVLC SIFGICMVFSGLCPLLLGSVMAIERCIG
20 VTKPIFHSTKITSKHVKMMLSGVCLFAVFIALLPILGHRDYKIQASRTWCFYNTEDIKDWEDRFYLLL
FSFLGLLALGVSLLCNAITGITLLRVKFKSQQHRQGRSHHLEMVIQLLAIMCVSCICWSPFLVTMANI
GINGNHSLET CETTLFALRMATWNQILD PWVYILLRKAVLKNLYKLASQCCGVHVISLHIWELSSIK
NSLKVA AISESPVAEKSAST (SEQ ID NO:75)

Human Thromboxane A_2 receptor isoform α ($TP\alpha R$)

25 MWPNGSSSLGPCFRPTNITLEERRLIASPWFAASFCVVGLASNLLALS VLAGARQGGSHTRSSFLT
FLCGLVLTD FLGLLVTGTIVVSQHAALFEWHAVDPGCRLCRFMGVVMIFFGLSPLLLGAAMASERY
LGITRPF SRPAVASQRRAWATVGLVWAAALALGLLPLLGVGRYTVQYPGSWCFLT LGAESGDVA
FGLLFSMLGGLSVGLSFLNNTVSVATLCHVYHGQEAAQQRPRDSEVEMMAQLLGIMVVASVCWL
PLLVFIAQTVLRNPPAMSPAGQLSRTTEKELLYLRVATWNQILD PWVYILFRRAVLRRLQPRLSTR
30 PRSLSLQPQLTQRSGLQ (SEQ ID NO:76)

Human Urotensin II receptor (GPR14)

MALTPESPSSFPGLAATGSSVPEPPGGPNATLNSSWASPT EPSSLEDLVATGTIGTLLS AMGVVG
VVGNAVYTLVVTCSRSLRAVASMYYVYNLALADLLYLLSIPFIVATYVTKEWHFGDVGCRVLFGLDFL
TMHASIFTLTMSSERYAAVLRPLDTVQRPKG YRKLLALGTWLLALLLTLPVMLAMRLVRRGPKSL
35 CLPAWGPRAH RAYLTLLFATS IAGPGLLIGLLYARLARAYRRSQRASFKRARRPGARALRLVLGIVL
LFWACFLPFWLWQLLAQYHQAPLAPRTARIVNYLTTCLTYGNSCANPFLYTLLTRNYRDHLRGRV

RGPGSGGGRGPVPSLQPRARFQRCSGRSLSSCSPQPTDSLVLAPAAPARPAPEGPRAPA (SEQ ID NO:77)

Human histamine type 1 receptor (H1R)

MSLPNSSCLEDKMCEGNKTTMASPQLMPLVVVLSTICLVTVGLNLLVLYAVRSEKLTHTVGNLYI
 5 VSLSVADLIVGAVVMPMNILYLLMSKWSLGRPLCLFWLSMDYVASTASIFSVFILCIDRYRSVQQPL
 RYLKYRTKTRASATILGAWFLSFLWVIPILGWNHFMQQTSVRREDKCETDFYDVTWFKVMTAINF
 YLPTLLMLWFYAKIYKAVRQHCQHRELINRSLPSFSEIKLRPENPKGDAKKPGKESPWVFLKRKPK
 DAGGGSVLKSPSQTPKEMKSPVFSQEDDREVDKLYCFPLDIVHMQAAAEGSSRDYVAVNRSHG
 QLKTDQGLNTHGASEISEDQMLGDSQSFSRTSDSTTTTETAPGKGKLRSGSNTGLDYIKFTWKRL
 10 RSHSRQYVSGLHMNRERKAAKQLGFIMAAFILCWIPYFIFFMVIAFCKNCCNEHLHMFTIWLGYINS
 TLNPLIYPLCNENFKKTFKRILHIRS (SEQ ID NO:78)

human Bradykinin type 2 receptor (BKR2)

MFSPWKISMFLSVREDSVPTTASFADMLNVTLQGPTLNGTFAQSKCPQVEWLGLNLTQPPFL
 WVLFVLATLENIFVLSVFCLHKSSCTVAEIYLGNLAAADLILACGLPFWAITISNNFDWLFGETLCRV
 15 VNAIISMNLYSSICFLMLVSDRYLALVKTMSMGRMRGVRWAKLYSLVIWGCTLLLSSPMLVFRTM
 KEYSDEGHNVTA CVISYPSLIWEVFTNMLLN VVGFLPLSVITFCTMQIMQVLRNNEMQKFKEIQTE
 RRATVLVLVLLLFICWLFPFQISTFLDTLHRLGILSSCQDERIIDVITQIASFMAYSNSCLNPLVYVIV
 GKRFRKKSWEVYQGVQCQKGGCRSEPIQMENSMGTLRTSISVERQIHKLQDWAGSRQ (SEQ ID
 NO:79)

20 human dopamine type 2 receptor isoform 1(D2R)

MDPLNLSWYDDDRLERQNWSPFNGSDGKADRPHYNYATLLTLLIAVIVFGNVLCMAVSREKAL
 QTTTNYLIVSLAVADLLVATLVMPWVVYLEVVGWKFSSRIHCDIFVTLDVMMCTASILNLCAISIDRY
 TAVAMPMLYNTRYSSKRRVTVMISIVWVLSFTISCPLLFGLNNADQNECIANPAFVYSSIVSFYVP
 FIVTLLVYIKIYIVLRRRRKRVTNKRSSRAFRAHLRAPLKGNCTHPEDMKLCTVIMKSNGSFPVNRR
 25 RVEAARRAQELEMELSSSTSPPERTRYSPIPPSHHQLTLPDPSHHGLHSTPDSPAKPEKNGHAK
 DHPKIAKIFEIQTMPNGKTRTSLKTMSRRKLSQQKEKKATQMLAIVLGVFIICWLPFFITHILNIHCDC
 NIPPVLYSAFTWLGYNVSAVNPIIYTTFNIEFRKAFLKILHC (SEQ ID NO:80)

GFP2-RlucII fusion

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTCLKFICTTGKLPVPWPPTLVTTLS
 30 YGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDF
 KEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLP
 DNHYLSTQSALS KDPNEKRDHMLLEFVTAAGITLGMDELYKGGGGGDIEFLQPGGSGGGGMS
 KVDPEQRKRMITGPQWWARCKQMNVLDSFINYYDSEKHAENAVIFLHGNAASSYLWRHVPHI
 EPVARCIIPDLIGMGKSGKSGNGSYRLLDHYKYLTAWFELLNLPKKIIFVGHWDGAALAFHYSYEH
 35 QDKIKAIVHAESVVDVIESWDEWPDIEEDIALIKSEEKGMVLENNFFVETVLP SKIMRKLEPEEFAA
 YLEPFKEKGEVRRPTLSWPREIPLVKGGKPDVVQIVRNYNAYLRASDDL PKMFIESDPGFFSNAIV
 EGAKKFPNTEFVKVKGHLHFSQEDAPDEM GKYIKSFVERVLKNEQ (SEQ ID NO:81)

rGFP-RlucII fusion

MDLAKLGLKEVMPTKINLEGLVGDHAFSMEGVGEGNILEGTQEVKISVTKGAPLPFAFDIVSVAFS
 YGNRAYTGYPEEISDYFLQSFPEGFTYERNIRYQDGGTAIVKSDISLEDGKFIVNVDFKAKDLRRM
 GPVMQQDIVGMQPSYESMYTNVTSVIGECIIAFKLQTGKHFTYHMRTVYKSKKPVETMPPLYHFIQH
 5 RLVKTNVDTASGYVVQHETAIAAHSTIKKIEGSLPGGGGGDIEFLQPGGSGGGGMTSKVYDPEQR
 KRMITGPQWWARCKQMNVLDSFINYYDSEKHAENAVIFLHGNAASSYLWRHVPHIEPVARCIIPD
 LIGMGKSGKSGNGSYRLLDHYKYLTAWFELLNLPKKIIFVGHWDGAALAFHYSYEHQDKIKAIVHA
 ESVVDVIESWDEWPDIEEDIALIKSEEGERKMLENNFFVETVLP SKIMRKLEPEEFAAYLEPFKEKG
 EVRRPTLSWP REIPLVKGGKPDVVQIVRNYNAYLRASDDL PKMFIESDPGFFSNAIVEGAKKFPNT
 10 EFVKVKGLHFSQEDAPDEM GKYIKSFVERVLKNEQ (SEQ ID NO:82)

Venus-RlucII fusion

MVSKGEELFTGVVPIVELDGDVNGHKFSVSGEGEGDATYGKLTCLKICTTGKLPVPWPTLVTTLG
 YGLQCFARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDF
 KEDGNILGHKLEYNYN SHNVYITADKQKNGIKANFKIRHNIEDGGVQLADHYQQNTPIGDGPVLLP
 15 DNHYLSYQSALSKDPNEKRDHMLLEFVTAAGITLGMDELYKGGGGGDIEFLQPGGSGGGGMTS
 KVYDPEQRKRMITGPQWWARCKQMNVLDSFINYYDSEKHAENAVIFLHGNAASSYLWRHVPHI
 EPVARCIIPDLIGMGKSGKSGNGSYRLLDHYKYLTAWFELLNLPKKIIFVGHWDGAALAFHYSYEH
 QDKIKAIVHAESVVDVIESWDEWPDIEEDIALIKSEEGERKMLENNFFVETVLP SKIMRKLEPEEFAA
 YLEPFKEKG EVRRPTLSWP REIPLVKGGKPDVVQIVRNYNAYLRASDDL PKMFIESDPGFFSNAIV
 20 EGAKKFPNTEFVKVKGLHFSQEDAPDEM GKYIKSFVERVLKNEQ (SEQ ID NO:83)

Linker between β arrestin (1&2) and RlucII

KLPAT(SEQ ID NO:84)

Human β arrestin1

MGDKGTRVFKKASPNGKLTVYLGKRDFVDHIDLVDVPDGVVLVDPEYLKERRVYVTLTCAFRYGR
 25 EDLDVLGLTFRKDLFVANVQSFPPAPEDKKPLTRLQERLIKKLGEHAYPFTFEIPP NLPCSVTLQPG
 PEDTGKACGV DYE VKAFCAENLEEKIHKRNSVRLVIRKVQYAPERPGPQPTAETTRQFLMSDKPL
 HLEASLDKEIYYHGEPISVNVHVTNNTNKT VKKIKISVRQYADICLFNTAQYKCPVAMEEADDTVAP
 SSTFCKVYTLTPFLANNREKRGLALD GKLKHEDTNLASSTLLREGANREILGIIVSYKVVKLVVSR
 GGLGLD LASSDVAVELPFTLMHPKPKEPPHREVPENETPVDTNLIELDTNDDDIFEDFARQRLK
 30 GMKDDKEEEEEDGTGSPQLNNR (SEQ ID NO:85)

Human β arrestin2

MGEKPGTRVFKKSSPNCKLTVYLGKRDFVDHLDKVDVPDGVVLVDPDY LKDRKVFVTLTCAFRY
 GREDLDVLGLSFRKDLFIATYQAFPPVPNPPRPPTRLQDRLLRKL GQHAHPFFFTIPQNLPCSVTL
 QPGPEDTGKACGVDFEIRAFCAKSLEEKSHKRNSVRLVIRKVQFAPEKPGPQPSAETTRHFLMSD
 35 RSLHLEASLDKELYHGEPLNVNVHVTNNTSKTVKKIKVSVRQYADICLFSTAQYKCPVAQLEQDD
 QVSPSSTFCKVYTITPLSDNREKRGLALD GKLKHEDTNLASSTIVKEGANKEVLGILVSYRVKVKL

VVSRGGDVSVLPFVLMHPKPHDHIPLPRPQSAAPETDVPVDTNLIEFDTNATDDDIVFEDFARL
RLKGMKDDDYDDQLC (SEQ ID NO:86)

Example 2: Generation and validation of new BRET sensors for GPCR trafficking

New BRET acceptors based on *Renilla reniformis* GFP (rGFP) were generated for assessing receptor internalization and their targeting with β -arrestins to endosomes. These BRET acceptors were engineered for their specific expression either at the plasma membrane or in the endosomes, and for being used with the RET donors: RLucII-tagged GPCRs and β -arrestins (**Figures 1A-C**). The BRET assay disclosed herein is based on changes in the local concentration of the donor relative to the acceptor rather than a specific protein-protein interaction; hence not limited by the requirement for protein interaction and the avidity of one's complex. For its plasma membrane localization, rGFP was first tagged with a lyn domain. Lyn-rGFP localized mainly at the plasma membrane when expressed in HEK293 cells (**Figure 1D and F**). Moreover, adding the endofin FYVE domain to rGFP (rGFP-endofinFYVE) showed clear and exclusive endosomal localization (**Figure 1E, left panel**). A mCherry-labeled variant of the endofin FYVE (mCherry-FYVE) sensor also co-localized with the small G protein Rab5, which populates EE (**Figure 1G**). Notably, blocking PI3K using wortmannin delocalized the rGFP-endofinFYVE into the cytosol and enlarged endosomal vesicles (**Figure 1E, right panel**), consistent with its tethering to endosomes through PI3P binding. To visualize GPCR trafficking, the bradykinin B2 receptor tagged with CFP (B2R-CFP) was used, and lyn-rGFP and mCherry-endofin FYVE were expressed simultaneously (**Figure 1F**). At basal state, B2R-CFP localized at the plasma membrane with lyn-rGFP (*top panel*). Upon agonist stimulation, B2R-CFP separated from the lyn-rGFP, and moved into the endosomes where it colocalized with the mCherry-endofinFYVE (*bottom panel*). Only the receptor redistributed from one cellular compartment to another upon agonist, as both the plasma membrane and endosomal markers (*i.e.* lyn-rGFP and GFP-endofinFYVE, respectively) remained in their respective compartments, making this system suitable to dynamically track receptor trafficking using BRET.

Example 3: Assessing AT1R internalization and its trafficking to endosomes with β -arrestin

BRET experiments were performed to monitor receptor endocytosis. The RLucII was fused onto the C-terminal domain of the angiotensin type 1 receptor (AT1R-RLucII), another GPCR, which traffic with β -arrestin through the clathrin pathway and is targeted to endosomes (Hein, Meinel et al. 1997; Zhang, Barak et al. 1999; Anborgh, Seachrist et al. 2000; Oakley, Laporte et al. 2000; Gaborik, Szaszak et al. 2001). Using radio-ligand binding, it was first validated that the engineered AT1R-RLucII internalized to the same extent as the untagged receptor (**Figure 7A**). Co-expression of AT1R-RLucII and lyn-rGFP did not prevent efficient agonist-mediated removal of receptors from the plasma membrane (**Figure 7A**), which internalization increased furthermore by

the expression of β -arrestin2 or was inhibited with the dominant negative Dynamin K44A (DynK44A; **Figure 7B**). Consistent with their co-localization at the plasma membrane, expressing AT1R-RlucII and lyn-rGFP revealed a high BRET ratio at basal state (**Figure 2A**). The signal rapidly decreased in a concentration-dependent manner following challenge of live cells with AngII and the removal of receptor from the plasma membrane. Expressing lyn-GFP10, on the other hand, generated both a lower basal and AngII-induced BRET ratio changes. Remarkably, expressing AT1R-RlucII with rGFP-endofinFYVE rather than GFP10-endofinFYVE resulted in a 7.7-fold increase in AngII-mediated BRET changes (Δ BRET) (**Figure 2B**). Similarly, expressing AT1R with β -arrestin2-RlucII and rGFP-endofinFYVE instead of GFP10-endofinFYVE resulted in an increase of 4.5-fold in Δ BRET (**Figure 2C**). The temporal process of receptor endocytosis from the plasma membrane and its targeting to endosomes was next resolved using AT1R-RlucII with either the plasma membrane or endosome BRET acceptor sensors (e.g. lyn-rGFP and rGFP-endofinFYVE, respectively). AT1R disappearance from the plasma membrane was faster ($t_{1/2} \approx 3$ min) than its accumulation in endosomes ($t_{1/2} \approx 9$ min) (**Figures 2D-F**).

The extent to which AT1R internalization and its targeting to endosomes with β -arrestin could be regulated was next investigated. Dynamin K44A (DynK44A), a dominant negative of Dynamin, which is key for clathrin-coated pit invagination, and sucrose have both been used as endocytosis blockers (Zhang, Ferguson *et al.* 1996). AngII-mediated BRET responses at the plasma membrane and in the endosomes (AT1R-RlucII/lyn-rGFP and AT1R-RlucII/rGFP-endofinFYVE, respectively) were efficiently inhibited by the expression of DynK44A (**Figures 3A and B**). Consistent with the lack of accumulation of AT1R/ β -arrestin complexes in endosomes in presence of DynK44A, very little AngII-mediated BRET ratio changes were observed between β -arrestin2-RlucII and rGFP-endofinFYVE (**Figure 3C**). Similarly, sucrose efficiently blocked the AngII-induced BRET responses between AT1R-RlucII and lyn-rGFP at the plasma membrane and in the endosomes between AT1R-RlucII and either β -arrestin2-RlucII or rGFP-endofinFYVE (**Figures 3A-C**). Surprisingly, over-expression of DynK44A or sucrose treatment decreased the basal BRET ratio at the plasma membrane (with Lyn-rGFP) (**Figure 3A**), but not in the endosome (with rGFP-endofinFYVE). β -arrestin expression facilitates AT1R endocytosis (Gaborik, Szaszak *et al.* 2001). The vesicle acidification inhibitors bafilomycin A (Baf) and Chloroquine (CQ), which prevent receptor degradation and AT1R recycling (Heinz *et al.*, *Mol Endocrinol.* 1997 Aug;11(9):1266-77), both increased the agonist-mediated accumulation of AT1R in endosomes (**Figure 3F**). Consistent with its important role in agonist-mediated GPCRs endocytosis, over-expression of β arrestin2 enhanced AngII-mediated Δ BRET by more than 50% for both receptor internalization (AT1R-RlucII and lyn-rGFP) and its targeting to endosomes (AT1R-RlucII and rGFP-endofinFYVE) (**Figures 3D and E**). The effects of DynK44A or β -arrestin2 on AngII-mediated AT1R endocytosis were also validated by ligand binding experiments, and found consistent with what observed in the BRET assay (**Figure 7B**). Together, these results highlight the utility of the

BRET-based assays to monitor, in a dose- and time-dependent fashion, AT1R endocytosis and its trafficking with β -arrestin into endosomes.

Example 4: Measurement of the endocytosis and trafficking of various receptors to endosomes

Different GPCRs were tagged with RlucII in order to examine their trafficking. When the vasopressin V2 receptor-RlucII (V2R-RlucII) was expressed with lyn-rGFP, AVP-dose dependently decreased the BRET ratio response. Similarly to AngII-stimulated AT1R, AVP promoted the internalization of V2R with an EC_{50} in the nM range (1.1 nM and 7.8 nM, respectively) (**Figure 4A**). On the other hand, oxytocin, which has low affinity for V2R (Barberis, Audigier et al. 1992), promoted the internalization of the receptor with lower potency (**Figure 4A**, $EC_{50}=2.2\mu M$). B2R-RlucII and β 2-adrenergic receptor (β 2AR)-RlucII also respectively showed dose-dependent decrease in the BRET ratio by their cognate ligands, bradykinin and isoproterenol. However, we observed differences in the basal BRET ratio between receptors. When receptor trafficking into the endosome was vetted, B2R-RlucII and β 2AR-RlucII also showed different potencies in agonist-mediated increasing in BRET ratio (**Figure 4B**). Notably, V2R-RlucII and rGFPendofinFYVE showed high basal BRET ratio (**Figure 4B**), as compared to B2R and the other receptors, though we could still detect robust increased in the BRET ratio upon AVP stimulation. The higher basal signal was likely caused by high basal endosomal localization of V2R as suggested by microscopy (**Figure 8**). Indeed, contrarily to B2R, colocalization of V2R with mCherry-endofinFYVE was more present in absence of agonist stimulus (**Figures 1 and 8**). Next, the agonist-mediated receptor/ β -arrestin targeting to endosomes was examined using β arr2-RlucII and rGFPendofinFYVE with different GPCRs. AT1R, V2R and B2R promoted a 6-8-fold increase over basal in BRET ratios upon agonist stimulation (**Figure 4C**), which is consistent with the trafficking of class B GPCR (Oakley, Laporte et al. 2000), which traffic to endosomes with β -arrestins. However, isoproterenol stimulation of β 2AR failed to generate a BRET signal, consistent with the internalization of a Class A GPCR in endosomes without β -arrestins (Oakley, Laporte et al. 2000). Oxytocin showed very marginal trafficking of the V2R/ β -arrestin complex to endosome, while for the PGF2 α receptor (FP), which does not internalize (Goupil, Wisheart et al. 2012), no increase over basal in the BRET ratio was detected upon agonist stimulation (**Figure 4C**). These results support the use of these plasma membrane and endosome BRET sensors for studying the ligand-mediated trafficking of different classes of GPCRs. **Figure 4D** shows the plasma membrane and endosome BRET sensors may also be used to study the endocytosis and trafficking of other types of receptors (i.e., non-GPCRs), such as the epidermal growth factor receptor (EGFR), a receptor tyrosine kinase (RTK). **Figure 4E** shows that the Z' factor of over 0.73 for the AT1R-RlucII/rGFP-endofinFYVE biosensors following AngII stimulation, which indicates a robust and HTS compliant assay for receptor internalization in endosomes.

Example 5: Studying receptor recycling using BRET

Following GPCR internalization, many receptors have been shown to recycle back to the plasma membrane or to traffic to other intracellular compartments (Tsao, Cao et al. 2001). The dynamics of receptor trafficking following agonist removal was assessed using the different rGFP/RlucII BRET sensor pairs. For receptor recycling at the plasma membrane, cells expressing AT1R-RlucII and lyn-rGFP that have been challenged with AngII for 30 min, were washed to remove the agonist, and left to recover for another 45 min, before BRET measurements. Results show that in cells pre-treated with AngII, BRET ratio decreased by around 50% compared to control, and the signal recovered to about ~90% of control after 45 min of agonist removal (*i.e.* ~80% of receptor recycling to the plasma membrane; **Figure 5A**). The same paradigm was applied to the RlucII-tagged B2R, β 2AR, and V2R. These findings revealed that more than 80% of the endocytosed B2R and β 2AR recycled to the cell surface, while only about 30% of the endocytosed V2R recycled back to the plasma membrane (**Figure 5B**). These results are in good agreement with previous studies (Innamorati, Sadeghi *et al.* 1998; Tsao and von Zastrow 2000; Zimmerman, Simaan *et al.* 2011). The disappearance of receptor for the early endosomes was next monitored using the AT1R-RlucII and rGFP-endofinFYVE. AngII treatment for 30 min increased the BRET ratio by 3-fold compared to unstimulated cells (control, **Figure 5C**). 45 min after AngII removal, BRET ratio was 1.5-fold of basal, implying that 75% of AT1R disappeared from early endosomes, either by recycling back to the cell surface or other endocytotic sorting. These results provide evidence that the endocytosis BRET assays can be applied to monitor receptor recycling to the plasma membrane and the dynamics of endosomal sorting.

Figures 26A to 26C show that the changes in BRET signal resulting from β arrestin translocation/recruitment to different compartments may be measured by BRET microscopy, and that the use of rGFP as the BRET acceptor results in a stronger BRET signal as compared to other BRET acceptors such as Venus and GFP2. BRET-based microscopy imaging opens up the possibility of image-based multiplexing for monitoring the translocation of an Rluc-tagged protein to distinct subcellular compartments in response to diverse stimuli.

Example 6: Differential sorting of AT1R by angiotensin analogs

The ligand-mediated receptor endocytosis was next examined using different AT1R ligands: AngII, SI, and DVG, which were previously shown to have distinct biased signalling properties (Zimmerman, Beautrait et al. 2012). Their ability to temporally regulate the trafficking of AT1R-RlucII to endosomes was first evaluated using rGFP-endofinFYVE. Results revealed that the initial rates of AT1R trafficking to endosomes were similar upon ligand incubation (*e.g.* 0-5 min; **Figure 6A**). However, DVG-bound AT1R reached maximal internalization at 10 min, while SI produced its maximal effect only after 20 min, and both ligands were respectively 40% and 20%

less efficacious than AngII at promoting AT1R concentration in early endosomes. Taking the time of maximal internalization of 40 min as reference, the potency and efficacy of the different AngII ligands to promote AT1R internalization was compared. As shown in **Figure 6B**, AngII, SI, and DVG decreased the BRET ratio between AT1R-RlucII and lyn-rGFP to the same extent at maximal concentrations of ligand. While AngII and SI had the same propensity to promote AT1R internalization (1.3 nM and 1.5 nM, respectively), DVG was less potent (75 nM). Both SI and DVG promoted less receptor accumulation in endosomes, as compared to AngII (**Figure 6C**), although their relative order of potency remained the same as for promoting AT1R internalization. Interestingly, SI and DVG weakly promoted β -arrestin2 trafficking to endosomes with receptors as compared to AngII (**Figure 6D**). Together, these findings provide evidence that the different AngII ligands cause distinct AT1R/ β -arrestin2 complex sorting.

To test the potential differential intracellular trafficking of AT1R, other BRET-based sensors of endosomes were generated by tagging Rab proteins (Rabs) with rGFP. Rabs coordinate vesicle transport between a number of intracellular compartments and have been used to identify the pathways followed by GPCR trafficking (Seachrist and Ferguson 2003). Rab5 is found on both endocytosed and recycling vesicles of the short cycle, while rab4 is on recycling vesicles of short and long cycles, and rab11 on recycling vesicles of the long cycle and vesicles directed to lysosomes (Seachrist and Ferguson 2003). rGFP-tagged Rab4 (rGFP-rab4) and rab11 (rGFP-Rab11) were generated, since the rGFP-endofinFYVE labelled endosomes are mainly Rab5-positive. Both rGFP-rab4 and rGFP-rab11 showed good vesicular localizations when expressed in HEK293 cells (**Figure 9A**). When AT1R-RlucII/rGFP-rab4 expressing cells were incubated with AngII, SI, or DVG, the BRET ratios were increased over time (**Figure 6E**). Interestingly, SI and DVG stimulation generated a significantly higher BRET signal than AngII (5 and 10 min, **Figure 6E**). At 10 min, the BRET ratio increased by SI and DVG were more than 2-fold of that of AngII. The signals plateaued after 10 min with SI and DVG, and at 30 min with AngII. Similarly to rGFP-rab4, rGFP-rab11 also revealed overall higher BRET signals with SI and DVG, than with AngII (**Figure 6F**). Signals from AT1R-RlucII/rGFP-rab11 slowly increased over time as compared to rGFP-rab4, in good agreement with previous findings involving rab4 and rab11 in fast and slow recycling, respectively (Hunyady, Baukal et al. 2002; Li, Li et al. 2008). In both rGFP-rab4 and rGFP-rab11 BRET assays, SI and DVG showed no significant difference. These results provide evidence that SI and DVG drive AT1R into rab4- or rab11-positive vesicles and less into rab5-positive vesicles, relative to AngII.

Recent evidence suggests that G proteins play some functions in membrane trafficking, but the role of G α_q in AT1R trafficking is ill studied. The BRET-based sensor was used to assess how inhibiting G α_q affected receptor internalization. Treating cells with Ubo-QIC, an inhibitor that locks specifically G α_q in its inactive state, did not prevent the AngII-dependent AT1R internalization as assessed by the PM EsBRET assay (**Figure 9B**). Interestingly however,

inhibiting Gαq reduced by more than 25% the targeting of AngII-bound AT1R to Rab5 containing endosomes (**Figure 9C**). Consistent with the lack of DVG and SI in activating Gαq, Ubo-QIC had no effect on the ligand-mediated accumulation of receptors in these endosomes. Inhibiting Gαq, increased AngII-bound AT1R in Rab 4 and Rab11 vesicles, whereas it had little effects on the sorting of the receptor to endosomes promoted by either DVG or SI (**Figure 9D**). These findings suggest that AT1R sorting can be biased by different ligands.

Example 7: BRET-based pharmacological chaperone (PC) assay and sequestration assay to assess functional rescue

In order to measure cell surface expression, an assay was developed based on plasma density of an RlucII-tagged protein (in **Figure 10A**, a receptor) as detected in BRET between the RlucII-tagged protein and an energy acceptor (rGFP) located at the plasma-membrane by a subcellular localization tag. In **Figure 10A**, examples of tags used for rGFP localization: the polybasic sequence and prenylation signal sequence from KRAS splice variant b (CAAX), the palmitoylation and myristoylation signal sequence from the Lyn kinase (Lyn) and plasma-membrane targeting polybasic sequence from the human GRK5 (PB). A schematic representation of an assay for evaluation of cell surface expression PC-rescue of otherwise ER-retained proteins tagged with RlucII is presented and described in **Figure 10A**. A BRET-based assay to evaluate cell surface expression can also be used to evaluate agonist-induced sequestration of receptors as depicted and described in **Figure 10B**. Most of GPCRs and other receptors internalize or are sequestered to sub-domains of the plasma-membrane upon agonist stimulation. A sequestration assay post PC-rescue of cell surface expression of receptors can be used to evaluate receptor activation, which reflects agonist binding and thus functionality. The different constructs used in this study are described in **Figure 11**; **Figure 11A**: description of MC4R-RlucII; **Figure 11B**: description of V2R-RlucII, **Figure 11C**: description of a voltage-gated Potassium channel (hERG) that was used as an example of a non-GPCR. Three different rGFP constructs with distinct plasma-membrane targeting sequences were tested as described in **Figure 11D**. For most of the assays presented, the rGFP-CAAX and MC4R-RlucII constructs were used to illustrate the robustness of the assay, resistance to DMSO and functional rescue, as evaluated by using a MC4R agonist (α-MSH) to induce agonist-promoted sequestration.

For optimization of the cell surface expression assay, two different plasma-membrane targeting sequences were tested for rGFP (constructs and tags described in **Figure 11D**); the KRAS fragment-tag is targeted to the plasma-membrane by a combination of lipidation (rGFP-CAAX; prenylation) and a polybasic domain and the GRK5 fragment-tag is targeted to the plasma membrane by a polybasic domain (rGFP-PB), not requiring a lipidation of the rGFP fusion protein. In **Figure 12**, titrations of the two rGFP constructs (**Figure 12A** = rGFP-CAAX and **Figure 12B** = rGFP-PB) were obtained from cells transiently expressing the mutant hMC4R (R165Q)-RlucII. As

shown in **Figure 12A**, a DCPMP-treatment leads to saturable BRET response for rGFP-CAAX, and this construct was selected for the subsequent experiments.

For optimization and validation of PC-mediated rescue of the cell surface expression and functionality of MC4R, cells transiently expressing rGFP-CAAX and 3 forms of the hMC4R, the wt receptor (hMC4R wt-RlucII), a PC rescuable mutant MC4R (hMC4R-R165Q-rlucII) and a mutant MC4R known as resistant to DCPMP-treatment (non PC-rescuable), were tested for cell surface expression following PC treatment and a 1h-agonist treatment to induce agonist-mediated sequestration. Three different ratios receptor to rGFP-CAAX were tested. As shown, DCPMP-treatment led to an increase in BRET signal for the WT and R165Q MC4R but not for the P299H-mutant MC4R. Both wt and R165Q mutant MC4R expressed at the cell surface post-DCPMP treatment showed agonist-induced sequestration as described in **Figures 13A to C**. The condition equivalent to 24ng of plasmid DNA per 10 wells of a 96-well plate was selected for the subsequent assays. **Figure 13D** shows that the different components of the biosensors may be encoded and co-expressed from the same mRNA (polycistronic construct). Polycistronic constructs encoding rGFP-CAAX(Kras) and either a WT or mutant hMC4R was used to show the PC rescue of cell surface expression. Polycistronic constructs offer the advantage of a fixed ratio of donor to acceptor and the possibility of using only one construct for viral infection or for establishing stable cell lines. **Figure 13E** shows the PC-mediated rescue of V2R mutants known to be intracellularly retained (Y128S and W164S) by the chaperone SR121463.

Example 8: BRET-based cell surface expression assay can be used for pharmacological evaluation of chaperone potency and efficacy

In order to verify whether this assay could be used to characterize drugs with PC properties, dose-response curves were obtained with 2 different PC (DCPMP and Compound 1) treatment of cells coexpressing rGFP-CAAX and either the hMC4R wt-RlucII construct or the hMC4R (R165Q)-rlucII construct (**Figure 14**). Characteristics of the dose-response curves were compatible with data obtained with a previously described FACS-based assay (P. René *et al. J Pharmacol Exp Ther.* 2010 Dec;335(3):520-32), indicating that the BRET-based assay can be used to characterize ligands.

Z'-factors were determined for the PC-mediated rescue of MC4R cell surface expression, to evaluate the robustness of the developed BRET-based assay. Z' factors were obtained for both hMC4R wt-RlucII (**Figures 15A and 15B**) and hMC4R (R165Q)-RlucII (**Figures 15C and 15D**). Cell surface expression was evaluated in BRET2 in **Figures 15A and 15C** using coelenterazine 400a, and in BRET1 using coelenterazine H (**Figures 15B and 15D**) following a 16h-treatment with 10 μ M DCPMP vs. vehicle. Z' factor were evaluated to over 0.63 with the hMC4R wt receptor and over 0.82 with the mutant R165Q mutant hMC4R. The results show that the robustness of this assay is compatible with the requirements of screening applications, even with the WT MC4R.

Example 9: Evaluation of resistance to DMSO.

Libraries of ligands and compounds are often dissolved in DMSO. To evaluate whether the BRET-based assay for cell surface evaluation is sensitive to concentrations of DMSO usually reached with dose-response curves of ligands selected from library of compounds, hMC4R wt-RlucII and hMC4R (R165Q)-RlucII expressing cells were DCPMP-treated at 10uM or with vehicle (DMSO) in well containing different concentrations of DMSO. As indicated in **Figure 16**, this assay is resistant to at least 3% DMSO, which is compatible with high throughput screening (HTS) applications and characterization of compounds in dose-response curves.

Example 10: Generation of stable cell lines.

Cells stably expressing biosensors are usually preferred for screening purposes. PC-mediated rescue of MC4R and V2R expression was then evaluated in cells transiently expressing rGFP-CAAX and in stable rGFP-CAAX cell lines, in order to determine if the level of rGFP-CAAX reached in stable cell lines is compatible with a robust assay for screening applications. 3 different amounts (as indicated on the graphs: 6, 12 and 24 ng for 10 wells) of hMC4R (R165Q)-RlucII (**Figure 16A**) or in hV2R (Y128S)-RlucII (**Figure 16B**) were transfected in stable lines expressing different levels of rGFP-CAAX (low, med, high) or co-transfected in HEK293 cells along with the rGFP-CAAX construct in order to test different ratios of BRET donor to acceptor. The PC-mediated rescue of cell surface expression for MC4R was evaluated in BRET2, as indicated in **Figure 17**. The data presented indicates that better responses can be obtained with stable cell lines expressing higher levels of rGFP. These stable cell lines could be used to establish cell lines expressing both receptor-RlucII and rGFP-CAAX, which would be useful for screening applications.

Example 11: Biosensors to detect the PC-mediated cell surface rescue of an ion channel.

In order to verify whether a BRET-based PC-mediated cell surface expression assay could be used to identify and characterize drugs that would bind hERG, RlucII-tagged constructs were created using the WT sequence of hERG and a known intracellularly retained mutant (G601S) and tested for Astemizole-mediated rescue of cell surface expression (**Figures 18A and B**). Dose-response curves were obtained with the wt-hERG (**Figure 18C**) and mutant (**Figure 18D**) constructs, for drugs that are known to act as ligands and pharmacological chaperones on hERG with different efficacy and potency. Characteristics of the dose-response curves were compatible with data obtained with an ELISA-based assay (HERG-Lite: Wible BA *et al.* J Pharmacol Toxicol Methods. 2005, 52(1):136-45). The Z'factor obtained using the hERG-(G601S) (**Figure 18E**) indicates that this BRET-based assay is robust and could be used for high throughput application such as HTS to identify hERG chaperones capable of rescuing cell surface expression of different

naturally occurring mutant hERG or to identify drugs that could have off-target effects mediated through hERG binding.

Example 12: Biosensors to monitor β -arrestin recruitment to GPCRs.

It was next tested whether it is possible to monitor β -arrestin (β -arr) recruitment to GPCRs (i.e. to the plasma membrane where GPCRs are localized) using a BRET biosensor that rely on changes in the concentration/density of the donor relative to the acceptor at the plasma membrane. As shown in **Figure 19A**, a BRET acceptor (e.g., GFP) is tagged with a PM targeting moiety (thus tethering the BRET acceptor at the PM), and a β -arrestin is tagged with a BRET donor (e.g., RlucII). In the presence of a GPCR agonist, β -arr is recruited to the GPCR, thus increasing the concentration of RlucII- β -arr at the plasma membrane, which in turn results in an increase in energy transfer (BRET) between RlucII and the PM-tagged GFP.

Figures 19B and 19C show the increase in the BRET ratio for β -arrestin₁ (**Figures 19B and 19D**) and β -arrestin₂ (**Figures 19C and 19E**) with two different GPCRs, a class A receptor that has lower affinity for β -arrestin: β_2 AR (**Figures 19B and 19C**) and a class B receptor that has higher affinity for β -arrestin: V₂R (**Figures 19D and 19E**), following stimulation with increasing doses of isoproterenol (iso) and AVP, respectively. The results show that a suitable BRET signal is obtained using different PM targeting moieties (including a non-lipid based targeting moiety such as the polybasic domain of GRK5) and different BRET acceptors, and the best signal being obtained using the CAAX (Kras) PM targeting moiety and rGFP as the BRET acceptor (triangles). **Figure 19F** shows that using β -arr-RlucII with rGFP-CAAX, lyn or PB, all generated greater BRET responses than the traditional RlucII:GFP10 BRET pair such as with GFP10-CAAX. This assay to monitor beta-arrestin recruitment advantageously does not require modification of the receptor and also offers a robust assay (Z'factor of at least 0.74; **Figures 19G and H**) amenable to screening applications (including HTS) for both class A and B receptors. **Figures 21A to 21E** show the assessment of β -arrestin translocation using a unimolecular biosensor, which allows performing the experiments in membrane extracts, for example. A flexible polypeptide linker of 300 amino acids provided a better BRET signal relative to shorter polypeptide linkers (**Figure 21C**).

Example 13: Biosensors to monitor PI(4,5)P₂ amount at the plasma membrane.

The biosensor was applied to detect membrane PI(4,5)P₂ generation using PLC δ 1-PH domain. In the basal state, PLC δ 1-PH-RlucII and PLC δ 1-PH-rGFP (or lyn-rGFP or rGFP-CAAX) are localized at the PM where PI(4,5)P₂ is located, so their local concentration/density is high enough to generate a detectable BRET signal. When the phospholipase C (PLC) was activated through activation of AT1R by its ligand AngII (thus inducing PI(4,5)P₂ hydrolysis), the PLC δ 1-PH domain tagged with RlucII and rGFP diffused into the cytosol, thereby reducing the local

concentration of rGFP and RlucII and consequently the BRET signal in a dose-dependent manner (**Figures 20A and B**).

Example 14: Biosensors to monitor diacylglycerol (DAG) at the plasma membrane

5 Upon activation of PLC, membrane PIP_2 is hydrolysed into IP_3 and DAG. Although inositol trisphosphate diffuses into the cytosol, DAG remains within the plasma membrane, due to its hydrophobic properties. **Figures 22A and 23A** show schematic representations of biosensors for measuring the translocation of the diacylglycerol- (DAG-) binding domain of PKCdelta (C1b) to the plasma membrane. The biosensors comprise a PM-targeting domain/moiety attached to a
10 BRET acceptor (e.g., rGFP, GFP10) and a BRET donor (e.g., RLucII) linked to the DAG-binding domain of PKC δ , C1b. The DAG enrichment at the membrane following PIP_2 causes the C1b domain to bind to the membrane, bringing the BRET acceptor (e.g., rGFP) and BRET donor (e.g., RLucII) closer to each other, inducing a higher BRET signal. In the biosensor depicted in **Figure 22A**, the BRET acceptor and BRET donor components are linked together (unimolecular
15 biosensor), which allows performing the experiments in membrane extracts, for example, whereas these components are expressed separately in the biosensor depicted in **Figure 23A**. The results depicted in **Figures 22B to 22H** and **Figures 23B to 23E** show that DAG accumulation at the plasma membrane may be monitored using both biosensors. Figure 22F shows that direct activation of PLC using N-(3-Trifluoromethylphenyl)-2,4,6-trimethylbenzenesulfonamide (*m*-
20 3M3FBS), which induces the hydrolysis of membrane PIP_2 into IP_3 and DAG (thus increasing the amount of DAG at the membrane), led to an increase of the BRET signal detected using the unimolecular biosensor.

Example 15: Biosensors to monitor G protein subunit sequestration

25 In the absence of agonist, the G protein subunits are localized at the plasma membrane. Upon GPCR activation using an agonist (A), the G protein subunits are released from the plasma membrane. Using a PM-targeting domain/moiety attached to a BRET acceptor (e.g., rGFP, GFP10) and a BRET donor linked to a G protein subunit, it is possible to monitor GPCR activation by measuring the decrease in the BRET signal that results from the release of the G protein
30 subunits from the PM (**Figure 24A**). **Figures 24B and 24C** show the changes in BRET ratio following activation of $\beta 1\text{AR}$ and $\beta 2\text{AR}$, respectively, with isoproterenol using different RLucII-tagged $\text{G}\gamma$ subunits, which provides evidence that $\text{G}\gamma 1$ and $\text{G}\gamma 11$ are mainly involved in signaling of $\beta 1\text{AR}$, and $\text{G}\gamma 1$ is mainly involved in signaling of $\beta 2\text{AR}$. **Figures 24D to 24H** show that the sequestration/translocation of different G protein subunits to different cellular compartments
35 following agonist stimulation of GPCRs may be monitored using the biosensors.

Example 16: Biosensors to monitor RhoA activation

A biosensor of Rho activation was designed by monitoring the recruitment of PKN's CRIB domain, which binds the active form of Rho (Rho-GTP) that localizes at the plasma membrane, to the plasma membrane using BRET. The BRET pair is the RlucII-tagged CRIB domain of PKN as a BRET donor and the plasma membrane bound rGFP (rGFP-CAAX) as an acceptor (**Figure 25A**).

Figures 25B to 25G show that PKN CRIB domain is translocated to the plasma membrane upon agonist stimulation of GPCRs coupled to Gq/12/13, and that the translocation is decreased in the presence of specific Gq inhibitors. **Figures 25H to 25J** show the effect of Rho modulators on the BRET ratio measured using the Rho biosensor. The BRET ratio is increased in the presence of Rho activators, and decreased in the presence of Rho inhibitors, confirming the specificity of the assay.

Example 17: Identification of regulators of AT1R by high-throughput screening using a localization/trafficking biosensor

Using the AT1R with β arr2-RLucII and rGFP-FYVE, 115,000 were screened to identify by a BRET assay compounds that either potentiated or inhibited AngII-mediated internalization of AT1R in endosomes. 30 potentiators and 42 inhibitors were identified (**Figure 27A**). **Figure 27B** shows that compound #21 (Traf 21) identified in the screen blocks the targeting of B2R-YFP or β arr2-YFP to endosomes, as compare to untreated, agonist-stimulated cells. **Figure 27C** shows that compounds #10 (Traf 10) and #29 (Traf 29) identified in the screen which enhanced the targeting of B2R-YFP or β arr2-YFP to endosomes, as compare to untreated, agonist-stimulated cells. These results show that the biosensors described herein may be used to identify regulators (e.g., agonists, antagonists) of protein localization/trafficking by high-throughput screening.

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WHAT IS CLAIMED IS:

1. A biosensor for assessing the trafficking and/or localization of a protein of interest comprising;
 - a first component comprising said protein of interest tagged with a *Renilla* green fluorescent protein (*Renilla* GFP) or a *Renilla* luciferase protein (*Renilla* Luc);
 - a second component comprising a cellular compartment targeting moiety tagged with a *Renilla* GFP or a *Renilla* Luc;
 wherein if said first protein is tagged with said *Renilla* GFP, said cellular compartment targeting moiety is tagged with said *Renilla* Luc, and if said first protein is tagged with said *Renilla* Luc, said cellular compartment targeting moiety is tagged with said *Renilla* GFP.
2. The biosensor of claim 1, wherein said protein of interest is tagged with said *Renilla* Luc and said cellular compartment targeting moiety is tagged with said *Renilla* GFP.
3. The biosensor of claim 1 or 2, wherein said protein of interest is i) a protein that binds to a small GTPase, for example a Rho-binding polypeptide, ii) a β -arrestin polypeptide, iii) a cell surface receptor protein, iv) a G protein subunit polypeptide, v) a signalling protein, vi) an adaptor protein, vii) a protein recruited to the plasma membrane upon stimulation of a receptor, viii) a protein sequestered away from the plasma membrane upon stimulation of a receptor, or ix) a fragment thereof.
4. The biosensor of claim 3, wherein said protein of interest is a cell surface receptor, for example a G protein-coupled receptor (GPCR) or a Receptor Tyrosine Kinase (RTK).
5. The biosensor of claim 3, wherein said protein of interest comprises a Pleckstrin homology (PH) domain, for example the PH domain of PLC δ 1.
6. The biosensor of claim 3, wherein said protein of interest comprises a phorbol esters/diacylglycerol binding domain, for example the phorbol esters/diacylglycerol binding domain of PKC (C1b).
7. The biosensor of claim 3, wherein said protein of interest is an adaptor protein or a fragment thereof comprising at least one SH2 and/or SH3 domains.
8. The biosensor of any one of claims 1 to 7, wherein said cellular compartment targeting moiety is a plasma membrane (PM) targeting moiety, an endosomal targeting moiety, a Golgi targeting moiety, a lysosomal targeting moiety, a peroxisomal targeting moiety, an autophagosomal targeting moiety, a ribosome targeting moiety, a mitochondria targeting moiety, a cytoskeleton targeting moiety or a nuclear targeting moiety.

9. The biosensor of claim 8, wherein said cellular compartment targeting moiety is a plasma membrane (PM) targeting moiety or an endosomal targeting moiety.
10. The biosensor of claim 9, wherein said PM targeting moiety comprises (a) a palmitoylation, myristoylation, and/or prenylation signal sequence and/or (b) a polybasic sequence, for example the amino acid sequence MGCIKSKGKDS (SEQ ID NO:1); GKKKKKKSKTKCVIM (SEQ ID NO:7), CMSCKCVLS (SEQ ID NO:47), CMSCKCCIL (SEQ ID NO:43), or SPKKGLLQRLFKRQHQNNSKS (SEQ ID NO:8).
11. The biosensor of claim 9, wherein said endosomal targeting moiety comprises a FYVE domain, and preferably comprises residues 739 to 806 of human endofin (SEQ ID NO:20).
12. The biosensor of claim 10 or 11, wherein said endosomal targeting moiety is fused to the C-terminal end of said *Renilla* Luc or said *Renilla* GFP; and/or said protein of interest is fused to the N-terminal end of said *Renilla* Luc or said *Renilla* GFP.
13. The biosensor of any one of claims 1 to 12, wherein said first and second components are covalently linked through a flexible linker, preferably a polypeptide of about 50 to about 500 amino acids, and more preferably a polypeptide of about 300 amino acids.
14. A host cell expressing the biosensor of any one of claims 1 to 13.
15. A method for determining whether an agent modulates the trafficking of a protein of interest in a cell, said method comprising:
 - measuring the Bioluminescence Resonance Energy Transfer (BRET) signal in the biosensor of any one of claims 1 to 13 in the presence and absence of said agent; wherein a difference in said BRET signal in the presence of said agent relative to the absence thereof is indicative that said agent modulates the trafficking of said protein of interest in said cell.
16. A method for assessing a modulation in the amount of a biomolecule at a cellular compartment between a first and a second condition, said method comprising:
 - providing a biosensor according to any one of claims 1-13 comprising:
 - a first component comprising a *Renilla* green fluorescent protein (*Renilla* GFP) tagged with a protein marker that binds to said biomolecule; and
 - a second component comprising a *Renilla* luciferase protein (*Renilla* Luc) tagged with said protein marker;
 - measuring the Bioluminescence Resonance Energy Transfer (BRET) acceptor signal in said first and second conditions;
 - wherein a difference in the BRET signal between said first and second conditions is indicative of a modulation in the amount of said biomolecule at said cellular compartment between said first and second conditions.

17. The method of claim 16, wherein said first condition is the presence of an agent and said second condition is the absence of said agent.

18. The method of claim 16 or 17, wherein said biomolecule is a phospholipid, preferably phosphatidylinositol 4,5-bisphosphate (PIP₂), or a second messenger, preferably diacylglycerol (DAG).

19. The method of claim 16 or 17, wherein if said biomolecule is a phospholipid said protein marker comprises a Pleckstrin homology (PH) domain, and if said biomolecule is a second messenger, said protein marker comprises a phorbol esters/diacylglycerol binding domain.