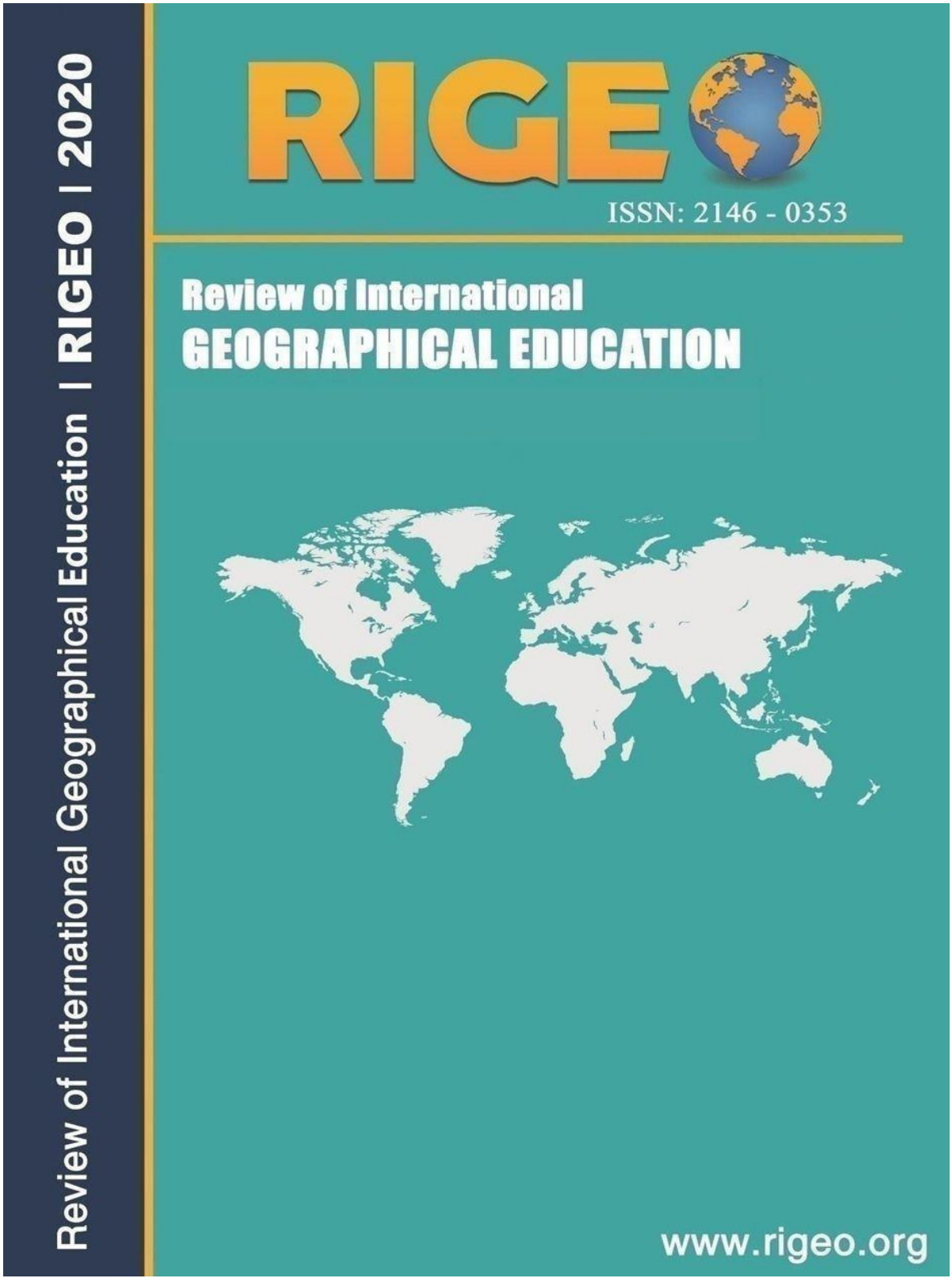
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Utilizing the innovative HiBiT tag to mark relaxin receptors on cell surfaces for BRET proximity experiments

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Abstract

Because its corresponding pep-tide ligand, relaxin, has therapeutic promise, the Relaxin family peptide 1 (RXFP1) receptor, a Class A G protein-coupled receptor (GPCR), is highly sought after as a potential pharmacological target. 1, 2 This Class A GPCR, RXFP1, stands out due to its unusually large extracellular domain, which includes an N-terminal low-density lipoprotein class A (LDLa) module before the so-called "linker" that connects the LDLa module to a domain that contains 10 leucine-rich repeats (LRR) (Figure 1A). 3 There are two locations where high-affinity relaxin binding may take place: one in the LRR4,5 and one in the linker. 6 The LDLa module is crucial for receptor activation in both RXFP1 and RXFP2, the only mammalian GPCRs that possess it (the receptor for insu- lin-like peptide 3). 7 The transmembrane domain of RXFP1,8,9 may be activated by the LDLa module, which is a tethered agonist, and RXFP1 can form dimers or oligomers in the cell membrane, according to other findings. Hence, it has been previously hypothesized that RXFP1 may activate as a homodimer, with one receptor subunit's LDLa module activating the other subunit's transmembrane domain via a trans-activation mechanism (Figure 1B). 12 Weak evidence suggests that RXFP1 produces persistent homodimers on the cell surface; however, a process whereby relaxin activates an RXFP1 homodimer needs more research.

Bioluminescence Resonance Energy Transfer (BRET)13,14 is a commonly used technique for detecting GPCR dimers. In this method, various receptors are transiently expressed in a model cell line like HEK293T after being tagged with either a luminescent "donor" protein or a fluorescent "acceptor" protein. In most cases, the distance at which resonance energy transmission occurs is often described as being less than 10 nm.

bandpass filter-based light emission measurements make it easy to quantify the closeness of a population of labeled receptors (thanks to donor/acceptor pairs). Saturation BRET assays maintain a constant quantity of donor (related to GPCR) and increasing quantities of acceptor (connected to the same or a different GPCR), while varying the stoichiometry of acceptor:donor expression (A:D). 15 to 17 Encouraging evidence of a specific connection between the two partners is often a nonlinear or hyperbolic relationship between the BRET signal and A:D. 13, 16, 18, 19, 20 Applying this sort of experiment to RXFP1 shows that the receptor is homodimerized in a "constitutive" way that is unaffected by relaxin activation. 10,11 That being said, this is the primary evidence for RXFP1 homodimer formation at the moment.

Consistent with earlier findings, this work used standard saturation BRET studies to demonstrate that RXFP1 receptors were located close together across the whole cell, but did not find any indication of relaxin-induced alterations in BRET. Nevertheless, it was shown that overexpressed RXFP1 did not adhere well to the cell surface, suggesting that the BRET signal could be due to receptor buildup in intracellular compartments instead of being directly implicated in the activation process at the surface of the cell. We were able to get around this issue by using the HiBiT split Nanoluc luciferase system (Promega), which allows us to label cell surface RXFP1 receptors with a Nanoluc donor and mCitrine-tagged RXFP1. This allows us to assess the receptor proximity on the cell surface using the BRET method. While the HiBiT tag provided a uniform way to quantify receptor expression, BRET tests showing co-expression of HiBiT-RXFP1 and mCitrine-RXFP1 suggested that RXFP1 may not mainly reside as a homodimer on the surface of the cell. Therefore, receptor homodimerization is not necessary for relaxin-mediated activation of RXFP1. Utilizing the simple and consistent HiBiT labeling method, this study lays the groundwork for the investigation of cell surface-expressed GPCR expression and proximity alone.

1 | INTRODUCTION

Because its corresponding pep-tide ligand, relaxin, has therapeutic promise, the Relaxin family peptide 1 (RXFP1) receptor, a Class A G protein-coupled receptor (GPCR), is highly sought after as a potential pharmacological target. 1, 2 This Class A GPCR, RXFP1, stands out due to its unusually large extracellular domain, which includes an N-terminal low-density lipoprotein class A (LDLa) module before the so-called "linker" that connects the LDLa module to a domain that contains 10 leucine-rich repeats (LRR) (Figure 1A). 3 There are two locations where high-affinity relaxin binding may take place: one in the LRR4,5 and one in the linker. 6 The LDLa module is crucial for receptor activation in both RXFP1 and RXFP2, the only mammalian GPCRs that possess it (the receptor for insu- lin-like peptide 3). 7 The transmembrane domain of RXFP1,8,9 may be activated by the LDLa module, which is a tethered agonist, and RXFP1 can form dimers or oligomers in the cell membrane, according to other findings. Hence, it has been previously hypothesized that RXFP1 may activate as a homodimer, with one receptor subunit's LDLa module activating the other subunit's transmembrane domain via a trans-activation mechanism (Figure 1B). 12 Weak evidence suggests that RXFP1 produces persistent homodimers on the cell surface; however, a process whereby relaxin activates an RXFP1 homodimer needs more research.

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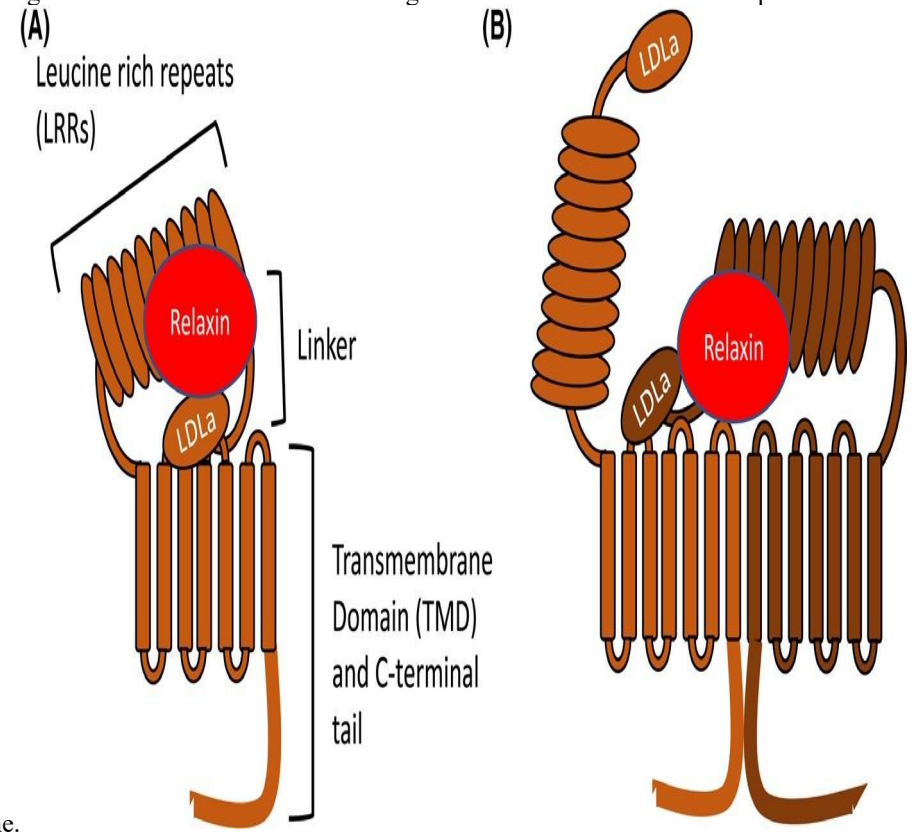


FIGURE 1 Theoretical models for RXFP1 activation by relaxin. Relaxin binds to the extracellular domain of RXFP1, but activation of the receptor requires interactions between the LDLa module and N-terminal residues on the linker and the transmembrane domain. Interactions of the LDLa module with the transmembrane domain may be occurring within a monomeric receptor (A) or could possibly involve a receptor homodimer where the LDLa-linker of one receptor subunit activates the transmembrane domain of the second receptor subunit via a *trans*-activation mechanism (B)

2 MATERIALS AND METHODS

2.1 | Cell culture

Human embryonic kidney (HEK) 293T cells used to express recep- tors were maintained in DMEM (Life Technology) supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, and 500 U/mL penicillin/streptomycin. Cells were cultured in 175 cm^2 flasks in incu- bators maintained at 37°C, with 5% CO_2 and 85% humidity.

2.2 | Receptor constructs

All RXFP1 receptor constructs were based on the previously pub- lished RXFP1 mammalian expression vector[3] which was cloned into pcDNA3.1/Zeo, containing an initial bovine prolactin signal peptide (BPLSP) followed by a FLAG epitope tag and then the RXFP1 re- ceptor sequence (with the exception of the HiBiT tagged receptor which did not contain a FLAG tag). N-terminal Nanoluc and mCitrine fusions were added via the insertion of an EcoRI site between FLAG and LDLa module. RXFP1-Rluc8 and RXFP1-Venus constructs were constructed by insertion of Rluc8/Venus fragments to the C-ter- minal end of the receptor between XhoI/NotI sites. For the N-ter- minal HiBiT tagged receptor, a pcDNA3.1/Zeo vector containing BPLSP-HiBiT was synthesized (Genscript) such that RXFP1 could be inserted C-terminally to the HiBiT tag via BamHI/NheI sites, and including a 12 amino acid linker (GGGSGGGSGGSG) between HiBiT tag and the start of RXFP1. The pcDNA3.1/Zeo BPLSP-HiBiT vector was also used for insertion of $GABA_{B1}$ (Genscript ORF clone OHu03752C)

between BamHI/NheI sites. The $GABA_{B2}$ (Genscript ORF clone OHu26227C) construct was synthesized and inserted into a custom-made pcDNA3.1 BPLSP-HA vector, which was then further modified by insertion of an mCitrine fusion tag between an EcoRI site. All plasmids were sequenced through the entirety of the ORF to ensure correct sequences, and full amino acid sequences of the ORF for all receptor constructs used are presented in the sup- plementary information.

2.3 | Venus/Rluc8 Saturation BRET experiments

For saturation BRET style experiments using RLuc8/Venus-tagged receptors, HEK 293T cells (15 000 cells/well) were seeded into poly-L-lysine coated white, opaque 96-well microplates (Perkin Elmer). Transient transfections using a constant amount of donor (RXFP1-Rluc8; 5 ng/well) and increasing amount of acceptor (RXFP1-Venus; 0-245 ng/well) were performed the following day using LipofectAMINE 2000 (Invitrogen). Forty-eight hours after transfection, BRET measurements were performed. In brief, cells were treated with 5 μmol/L coelenterazine *h* (Promega) in phenol red-free DMEM containing 10% FBS and 25 mmol/L HEPES buffer (henceforth referred to as PRF-DMEM). The BRET ratio was defined as the emission intensity at 520-550 nm divided by the emission in- tensity at 460-490 nm. BRET unit was defined as the BRET ratio minus that obtained in cells expressing only RXFP1-Rluc8. Following BRET measurements, cells were washed with PBS and Venus fluo- rescence emission was measured at 520-550 nm after excitation at 479-491 nm. Background fluorescence from cells expressing only RXFP1-Rluc8 was subtracted. All measurements were performed using a Polarstar Omega platereader (BMG Labtech) at 37°C. The Venus/Rluc8 expression ratio for each well was defined as RXFP1- Venus fluorescence signal (a.u.) divided by RXFP1-Rluc8 lumines- cence signal (a.u.). All saturation experiments were plotted using GraphPad PRISM and curves fitted using a nonlinear regression one-site binding curve.

2.4 | cAMP activity assays

All RXFP1 receptor constructs used here were tested for their ability to signal in response to relaxin stimulation using a cAMP reporter gene assay[21] as previously described.[7] Briefly, HEK 293T cells (5×10^5 cells/well) were seeded into six-well plates followed by transfection the following day with 50 ng receptor DNA and 2 μg of β-galactosidase reporter gene DNA using LipofectAMINE 2000 (Invitrogen). Twenty-four hours after transfection, cells were lifted and seeded into CELLBIND 96-well plates (Corning) at a density of 5×10^4 cells/well. The following day, cells were stimulated for 6 hours at 37°C with varying concentrations of relaxin or 5 μmol/L forskolin and subsequently frozen at −80°C overnight. Cells were then lysed and the amount of cAMP driven β-galactosidase reporter expression was determined. Experiments were performed in triplicate and were normalized to the cAMP response induced by 5 μmol/L Forskolin. A nonlinear regression sigmoidal dose-response curve was fit using GraphPad PRISM to obtain pEC_{50} and E_{max} values.

2.5 | FLAG receptor expression assays

Cell surface and total cellular expression of FLAG-tagged RXFP1 re- ceptors were measured using a method described previously.[22] HEK 293T cells were seeded into poly-L-lysine coated clear 96-well plates (for Figure 2D) or 24-well plates (for Figure 3). Twenty-four hours later, cells were transfected with increasing amounts of FLAG-tagged RXFP1 receptor DNA using LipofectAMINE 2000. For all transfec- tions performed, the amount of transfected DNA was kept constant (250 ng/well for 96-well plate format and 1000 ng/well for 24-well plate format) between conditions using empty pcDNA3.1 vector DNA. Twenty-four hours after transfection, cells were washed once in assay buffer (TBS pH 7.4, 2 mmol/L $CaCl_2$) and fixed for 15 min- utes by addition of assay buffer containing 3.7% formaldehyde (for cell surface) or 3.7% formaldehyde/0.25% Triton-X (for total expres- sion). Cells were then washed twice with assay buffer, blocked for 45 minutes in assay buffer containing 1% BSA, incubated at room temperature with mouse anti-FLAG M1 monoclonal antibody (Sigma Aldrich), washed once in assay buffer, incubated at RT in goat anti- mouse Alexa 488 conjugated antibody

(Invitrogen), and washed twice in assay buffer. Finally, cells were lysed and transferred to black walled 96-well optiplates for fluorescence measurement at

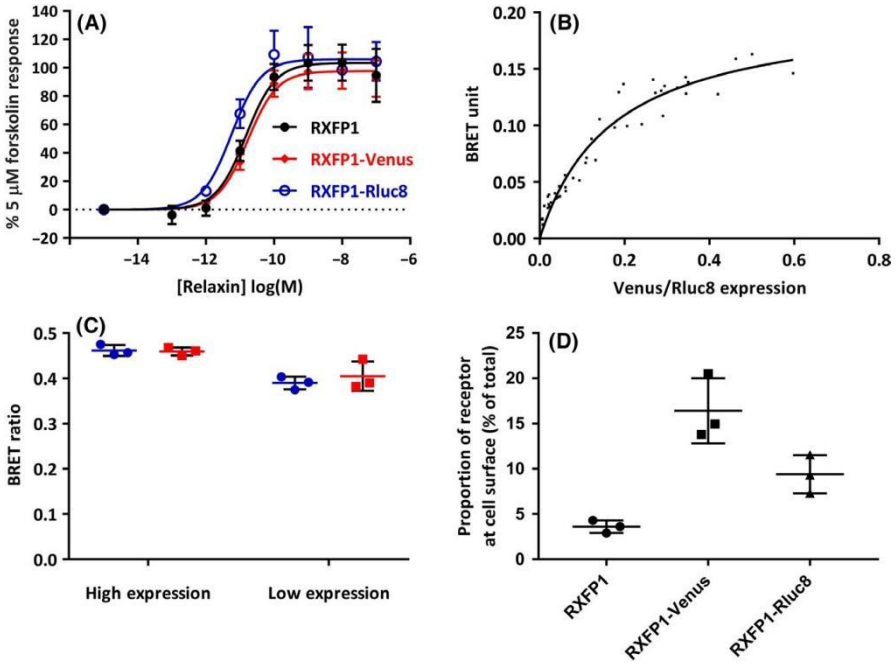


FIGURE 2 BRET experiments, low cell surface expression, and normal signalling from C-terminally tagged RXFP1 receptors (A) Dose- response curves showing relaxin-mediated cAMP responses in HEK293T cells transfected with C-terminally tagged RXFP1 receptors.

Experiments performed at least 3 times in triplicate, shown as mean ± SD (B) Saturation BRET curve using RXFP1-Rluc8 and RXFP1-Venus. Data are representative of an individual experiment which was performed three times. Data points represent single wells in which filtered luminescence was measured, followed by measurement of Venus fluorescence (C) HEK293T cells co-transfected with a 1:2 ratio of RXFP1- Rluc8 and RXFP1-Venus DNA at "High" (250 ng DNA transfected per well) or "Low" (25 ng DNA transfected per well) expression levels.

BRET ratios determined after 20 minutes incubation with vehicle (blue circles) or 100 nmol/L relaxin (red squares). Data represent the

mean ± SD of a single experiment performed 3 times. (D) Expression of RXFP1, RXFP1-Venus, or RXFP1-Rluc8 in HEK293T cells by virtue or N-terminal FLAG tags. The percentage of receptor trafficked to the cell surface was determined as a ratio of cell surface expression (intact cells) vs total expression (Triton-X permeabilized). Cells were transfected with 250 ng/well receptor DNA in a 96-well plate, and the total expression of each receptor construct was the same. Data points are pooled from three independent experiments performed in triplicate and error bars represent SD

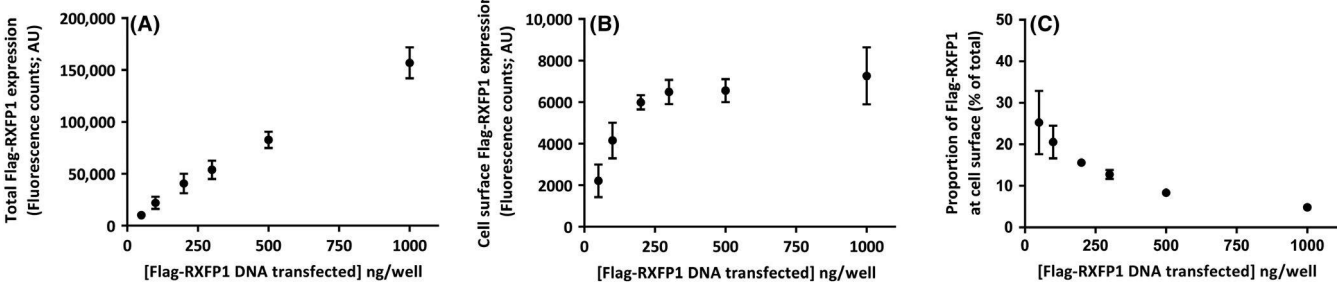


FIGURE 3 Cell surface and total expression of FLAG-RXFP1 in HEK293T cells by detection of the FLAG epitope tag.

HEK293T cells transfected with increasing amounts of FLAG-RXFP1 in a 24-well plate. (A) Total expression in fixed cells permeablized with 0.05% Triton-X.
(B) Cell surface expression in fixed intact cells. (C) Relative proportion of FLAG-RXFP1 detected at the cell surface. Pooled results from three independent experiments performed in triplicate, presented as mean ± SD520 nm after excitation at 479-491 nm. Measurements were per- formed using a Polarstar Omega platereader (BMG Labtech).

2.6 | HiBiT receptor expression assays

HEK293T cells were seeded into clear 24-well plates (Costar) at a density of 200 000 cells/well and the following day were transfected with receptor DNA using LipofectAMINE 2000 (Invitrogen). Twenty- four hours after transfection, cells were resuspended and seeded into white, opaque 96-well microplates (Perkin Elmer) at a density of 5×10^4 cells/well for assay the following day. On the day of the ex- periment, cell culture media was aspirated from all wells and replaced with 100 µL of PRF-DMEM and placed back in the 37°C incubator for 1 hour before further addition of 100 µL HiBiT complementation reagent and subsequent luminescence measurements in a Polarstar Omega platereader (BMG Labtech). HiBiT complementation reagent was prepared according to the manufacturer's instructions using the Nano-Glo ® HiBiT Extracellular Detection system kit (Promega) and contained a final concentration of 200 nmol/L LgBiT protein. To per- meablize cells and label intracellular HiBiT tags for measurement of total expression, digitonin (Sigma) was added to the HiBiT comple- mentation reagent to achieve a final concentration of 0.01%.

2.7 | Nanoluc/HiBiT BRET assays

HEK293T cells were prepared for assay the same as above (HiBiT receptor expression assays), but co-transfected with HiBiT/Nanoluc- and mCitrine-tagged receptor DNA. For saturation-style BRET as- says 10 ng per well of HiBit/Nanoluc-labeled RXFP1, or 5 ng per well of HiBit-labeled $GABA_{B1}$, and increasing amounts (0-4000 ng) mCitrine-labeled receptor per well were transfected, with empty pcDNA3.1 vector included to make up equal total DNA amounts. BRET measurements were taken immediately after addition of HiBiT complementation reagent. For experiments where Nanoluc was used, PRF-DMEM containing a 1:250 dilution of Nano-Glo® luciferase substrate (Promega) was used instead of HiBiT comple- mentation reagent. The BRET ratio was defined as the filtered light emission intensity at 520-620 nm divided by the emission intensity at 410-490 nm and measured on a Polarstar Omega platereader (BMG Labtech) at 37°C. BRET unit was defined as the BRET ratio of wells transfected with both donor and acceptor tagged receptor, minus the BRET ratio obtained in cells transfected with Nanoluc/ HiBiT-tagged receptors and the appropriate untagged receptor.

3 | RESULTS

Previous studies investigating RXFP1 homodimerization with sat- uration BRET used RXFP1-Rluc/ RXFP1-Venus[10] or RXFP1-Rluc/ RXFP1-GFP[2] [11] pairings for their receptor constructs. We chose to use a Rluc8/Venus pairing as it has been shown to give improved sensitivity in BRET measurements.[23,24] The functionality of newly cloned RXFP1-Rluc8 and RXFP1-Venus receptor constructs were tested using a cAMP reporter gene assay (Table 1 and Figure 2A), confirming that the C-terminal fusions did not adversely affect re- ceptor signaling as compared to the untagged receptor. Saturation BRET experiments yielded a hyperbolic curve, indicative of prox- imity between RXFP1-Venus and RXFP1-Rluc8 (Figure 2B), con- cordant with that previously published. We also co-expressed RXFP1-Rluc8 and RXFP1-Venus at different levels and treated cells with vehicle or 100 nmol/L relaxin before measuring BRET (Figure 2C) which showed no changed in BRET ratio due to relaxin activation of receptors, also as previously published. Notably, we have not performed control saturation BRET experiments with RXFP1-Rluc8 and another Venus-tagged GPCR as these have been performed previously[10,11] and this study

is focussed on measuring cell surface proximity. These experiments report on the proximity

TABLE 1 Relaxin-mediated cAMP activity of tagged RXFP1 constructs used in this study

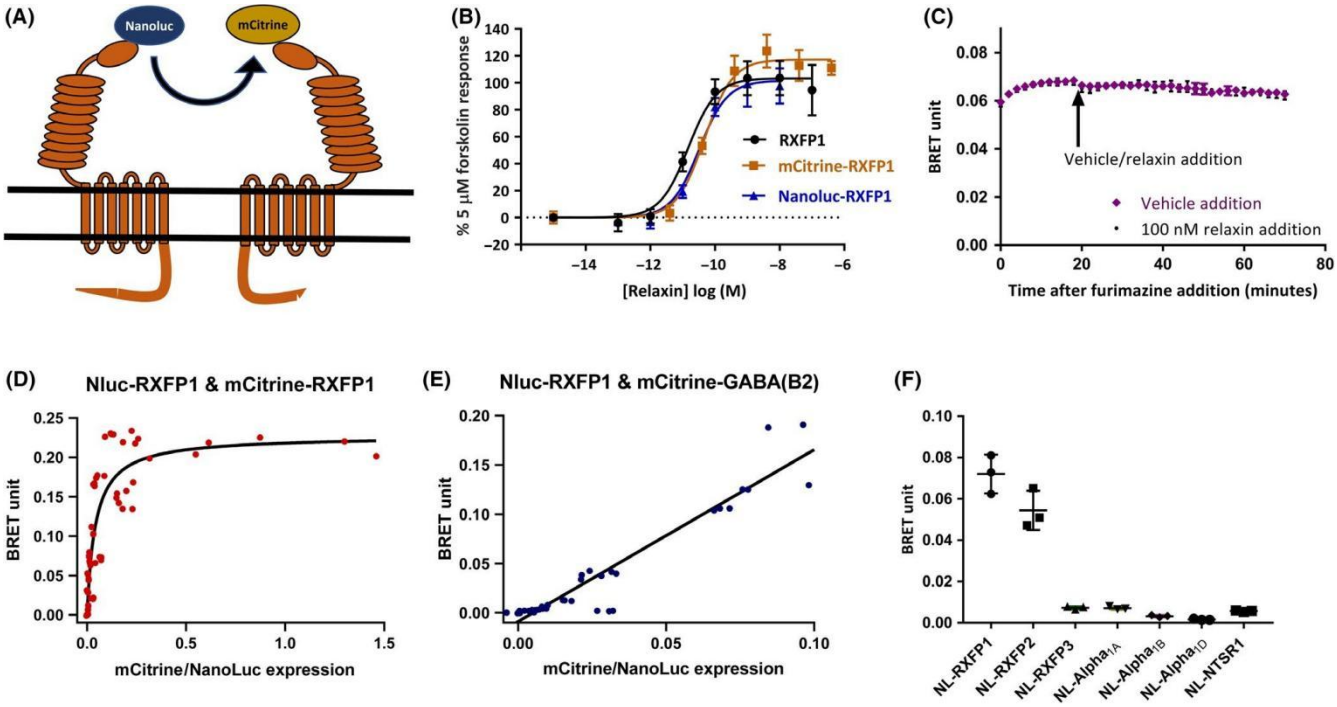
	pEC_{50}	E_{max} (% of 5 µmol/L forskolin response)	n
[a]RXFP1	10.80 ± 0.10	114 ± 17	9
[a]RXFP1-Venus	10.77 ± 0.10	98 ± 1.9	3
[a]RXFP1-Rluc8	11.23 ± 0.20	106 ± 4.9	3
[a]mCitrine-RXFP1	10.32 ± 0.10	117 ± 3.6	3
[a]Nanoluc-RXFP1	10.53 ± 0.03	101 ± 7.6	3
HiBiT-RXFP1	10.43 ± 0.04	113 ± 12	3

Values represent the mean ± SD of n experiments performed in triplicate. [a]Receptor also contain a FLAG epitope tag at the N-terminus.

of tagged receptors in all compartments of the cell, not just on those at the cell surface. It was therefore important to investi- gate the cell localization of RXFP1 receptors to allow interpreta- tion of BRET data. Receptor expression was quantified by virtue of an N-terminal FLAG tag. It was found that, when overexpressed in HEK 293T cells, only about 5% of RXFP1 was trafficked to the cell surface (Figure 2D). C-terminal Venus- and Rluc8-tags ap- peared to improve the trafficking of RXFP1 to the cell surface when expressed at similar levels; however, cell surface expression remained only around 15% and 10% of the total receptor pool, respectively (Figure 2D).

Further analysis of RXFP1 expression in HEK 293T cells using increasing DNA transfection levels revealed that, while there was a good linear relationship between the DNA transfection amount and total protein expression (Figure 3A), there was a saturable limit to the amount of receptor that could be trafficked to the cell surface (Figure 3B) hence the relative percentage of the total receptor pool which is actually trafficked to the cell surface is highly dependent on the total expression of the receptor (Figure 3C).

As the majority of RXFP1 appears to be located intracellularly when overexpressed in HEK293T cells, it could be possible that sat- uration BRET experiments (using Rluc8/Venus fusions) are predomi- nantly reporting interactions occurring in intracellular compartments (especially at the highest expression levels), which could explain the lack of relaxin-mediated BRET change that may hypothetically be occurring. We therefore tagged RXFP1 at the extracellular N-ter- minus, which we reasoned offered the possibility that if RXFP1 is indeed activated as a homodimer, conformational changes in the extracellular domain due to relaxin binding to both receptors might be more readily detectable since the efficiency of resonance energy transfer is influenced by both distance and angular orientation of the donor/acceptor species.[25] Tagging of GPCRs for BRET analysis often uses a variant of *Renilla* luciferase; however, this has been noted to adversely affect cell surface trafficking when tagged to the N-ter- minus.[26] To assess the possibility of BRET transfer between RXFP1 receptors suitably tagged at their N-terminus, we used RXFP1 with Nanoluc at the N-terminus[27] and paired that with N-terminally mCi- trine tagged RXFP1 as the acceptor (Figure 4A). Importantly, both fusions (Nanoluc and mCitrine) were well tolerated and did not



F I G U R E 4 Specific BRET between Nanoluc- and mCitrine-tagged RXFP1 is not affected by relaxin stimulation (A) Cartoon representation of BRET between RXFP1 containing N-terminal Nanoluc- and mCitrine-fusion tags. (B) Dose-response curves showing relaxin-mediated cAMP responses in HEK293T cells transfected with a 1:1 ratio of Nanoluc-RXFP1 or mCitrine-RXFP1 compared to RXFP1. Experiments performed 3 times in triplicate, shown as mean ± SD. (C) HEK293T cells co-transfected with Nanoluc-RXFP1 mCitrine- RXFP1. BRET signal determined over a 70 minute timecourse after addition of furimazine, with vehicle or 100 nmol/L relaxin added after 20 minutes. Data representative of a single experiment performed three times in duplicate, presented as mean ± SD. (D) and (E) Saturation BRET curve using Nluc-RXFP1 and mCitrine-RXFP1 (D) or mCitrine-$GABA_{B2}$ (E). Data points represent single wells in which filtered luminescence was measured, followed by measurement of mCitrine fluorescence, and is pooled from three independent experiments. (F) BRET signal in HEK293T cells co-transfected with a 1:1 ratio (200 ng total DNA per well) of mCitrine-RXFP1 and other Nanoluc (NL) tagged related and unrelated GPCRs, showing the specificity of Nanoluc/mCitrine BRET signal for RXFP1. Pooled data from three independent experiments performed in triplicate presented as mean ± SD

perturb relaxin-mediated signaling from these receptors (Table 1 and Figure 4B).

Co-expression of Nanoluc-RXFP1 and mCitrine-RXFP1 pro- duced a BRET signal which was stable for at least 20 minutes after addition of furimazine (the coelenterazine analogue developed spe- cifically for Nanoluc),[28] indicating close proximity of Nanoluc- and mCitrine-tagged RXFP1 receptors across the whole cell, and again there was no effect of relaxin treatment (Figure 4C). Additionally, the specificity of the BRET signal between Nanoluc-RXFP1 and mCi- trine-RXFP1 was tested (Figure 4F and Figure S1) by co-expressing mCitrine-RXFP1 with other related and unrelated Nanoluc-tagged GPCRs – RXFP2, RXFP3, α_1-adrenoceptors (α_{1A}, α_{1B}, α_{1D}), and the neurotensin receptor 1 (NTS_1). Notably only Nanoluc-RXFP2 demonstrated a specific BRET signal with mCitrine-RXFP1 to a similar level (~75% of the RXFP1 BRET signal) that has previously been shown using C-terminally tagged receptors.[29] Importantly, all Nanoluc-la- beled receptors were expressed as demonstrated by luminescent measurements and none of the paired receptors disrupted mCitrine- RXFP1 expression measured as fluorescence (Figure S1).

Furthermore, saturation BRET style analyses demonstrated a hyperbolic curve for Nluc-RXFP1/mCitrine-RXFP1 co-transfections (Figure 4E) and a linear relationship for Nluc-RXFP1/mCitrine- $GABA_{B2}$ co-transfections (Figure 4F) which were used as a negative control.

To separate BRET signal originating from intracellular compart- ments from that at the cell surface, we next took advantage of the recently described split Nanoluc complementation system called HiBiT[30] both as a homogeneous assay of receptor expression and as a labeling technique for cell surface receptors. The FLAG tag present in our RXFP1 expression construct was replaced with the 11 amino acid HiBiT tag (VSGWRLFKKIS) which did not adversely affect re- laxin-mediated signaling (Table 1: Figure 5A). Addition of the HiBiT complementation reagent (containing furimazine and LgBiT, the pro- tein which binds the HiBiT tag to form the active Nanoluc luciferase) to HEK293T cells transiently expressing HiBiT-RXFP1 resulted in a lu- minescence signal indicative of RXFP1 expression at the cell surface, with negligible luminescence from untransfected cells (Figure 5B). The luminescence signal from complemented HiBiT-RXFP1 rose slowly after addition of the HiBiT complementation reagent and generally reached a maximum after 20 minutes (Figure 5B), hence we used the luminescence values at this timepoint to indicate the re- ceptor cell-surface expression level. To further demonstrate that cell

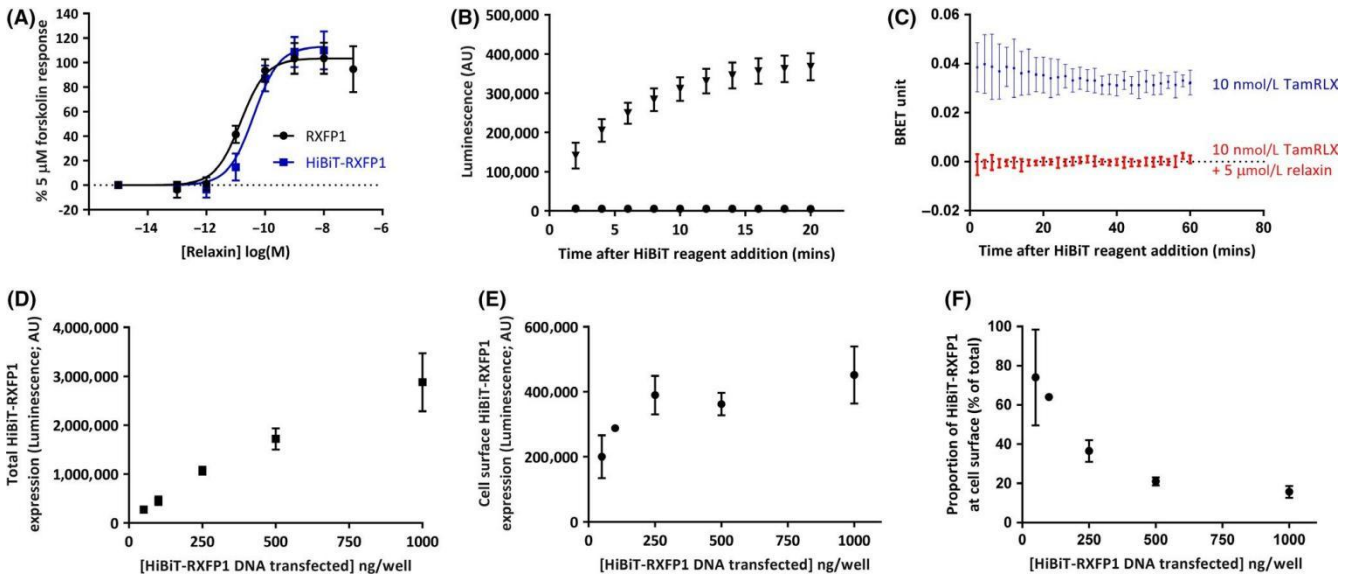


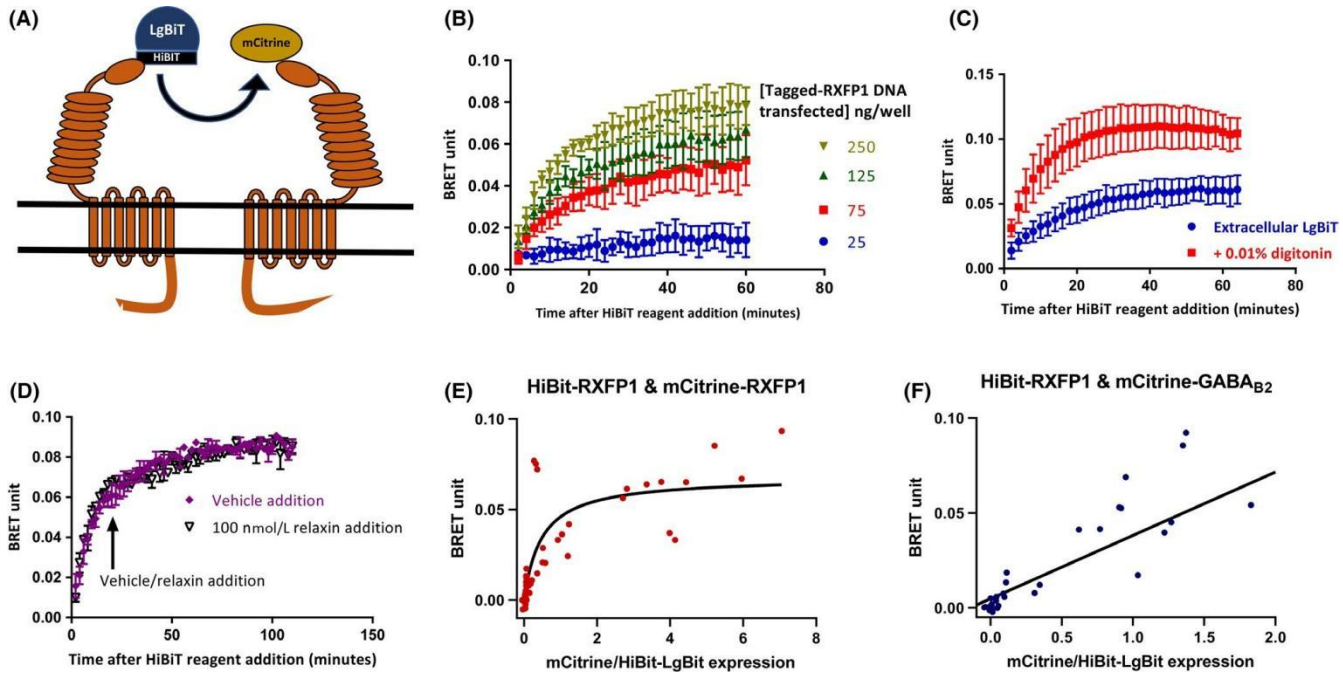
FIGURE 5 Cell surface and total expression of HiBiT-RXFP1 in HEK293T cells using HiBiT complementation assay.

(A) Dose-response curves showing relaxin-mediated cAMP responses in HEK293T cells transfected with HiBiT-RXFP1 compared to RXFP1. (B) HiBiT complementation progress over time. HEK293T cells transfected with 500 ng/well of either empty vector (circles) or HiBiT-RXFP1 DNA (triangles). Luminescence measured every 2 minutes after addition of the HiBiT complementation reagent to live cells. (C) HiBiT-RXFP1 expressing HEK293T cells were incubated with 10 nmol/L TamRLX (±5 μmol/L unlabeled relaxin) for 30 minutes at 37°C before addition of HiBiT complementation reagent. (D) and (E) HEK293T cells transfected with increasing amounts of HiBiT-RXFP1, with luminescence measured after 20 minutes of incubation with the HiBiT complementation reagent. For (D), 0.01% digitonin was added with the HiBiT complementation reagent to permeabilize cells and give a measurement of the total HiBiT-RXFP1 expression. (F) Relative proportion of HiBiT-RXFP1 detected at the cell surface. All data are pooled from three independent experiments performed in triplicate (A) or duplicate (B-F), shown as mean ± SDsurface localized HiBiT-RXFP1 receptors were successfully being la- beled, we also used fluorescently labeled relaxin (TamRLX); used as a fluorescent BRET acceptor with Nanoluc-RXFP1 in recent studies concerning relaxin binding kinetics.[27] Preincubation of HiBiT-RXFP1 expressing cells with 10 nmol/L of TamRLX for 30 minutes before addition of the HiBiT complementation reagent produced a BRET signal between bound TamRLX and labeled HiBiT-RXFP1 which was stable for 60 minutes and the signal was fully attenuated by co-incu- bation with a large excess of non-fluorescent relaxin as a competitor (Figure 5C).

Our previous experiments using a FLAG tag to determine total receptor expression used 0.25% Triton-X (to permeabilize cell mem- branes and allow labeling of the whole receptor pool); however, we found that Triton-X strongly inhibited Nanoluc luminescence (Figure S2). Instead, by co-addition of 0.01% digitonin with the HiBiT com- plementation reagent we were able to obtain an estimate of the total expression of HiBiT-RXFP1 (Figure 5D). Similar to our experiments using a FLAG-tagged receptor, there was a saturable limit to the amount of HiBiT-RXFP1 receptor that could be trafficked to the cell surface (Figure 5E) and a good linear relationship between the DNA transfection amount and total protein expression (Figure 5D). Again, it was clear that there was an appreciable intracellular pool of HiBiT- RXFP1 (Figure 5F).

Having developed a suitable system to assess BRET between tagged RXFP1 receptors at the cell surface, we then co-expressed a 1:1 ratio of HiBiT- and mCitrine-tagged RXFP1 in live HEK293T cells (Figure 6A), at a range of transfection levels which should correlate to a varied cell surface expression based on the results from Figure 5E. This 1:1 DNA ratio was chosen in an attempt to express equimolar amounts of HiBiT/mCitrine-tagged receptors in cells, which seems likely given that all FLAG-tagged RXFP1 con- structs used in this study demonstrated similar molar expression levels (Figure S5).

We observed a slow increase in the BRET signal over time, with a magnitude correlating to the amount of receptor cell surface expres- sion (Figure 6B). This is partly unexpected since BRET is a ratiometric measurement and thus should produce a relatively stable BRET sig- nal over time even as the luminescence signal changes. The increase in overall BRET signal as a result of increased surface expression suggests that the BRET signal results from proximity (either random collisions from high receptor density or due to proximity “bystander BRET”) of receptors rather than strictly a specific dimeric physical interaction. Co-addition of digitonin with the HiBiT complementa- tion reagent, to label intracellularly expressed receptors, showed a sharper increase in the BRET signal, which plateaued at a higher level consistent with additional BRET signal from the now exposed intra- cellular receptors (Figure 6C). We then treated non-permeabilized cells with relaxin and saw no changes in BRET signal (Figure 6D), clearly demonstrating that relaxin binding does not influence the apparent proximity of cell surface RXFP1 protomers. These experi- ments thus confirm that there is no relaxin-mediated change in prox- imity of RXFP1 at the cell surface.



F I G U R E 6 BRET between HiBiT- and mCitrine-tagged RXFP1 is not affected by relaxin addition (A) Cartoon representation of BRET between RXFP1 containing N-terminal HiBiT- and mCitrine fusion tags, with HiBiT tag complemented to the LgBiT protein in the HiBiT complementation reagent. (B) HEK293T cells co-transfected with increasing amounts of a 1:1 ratio of HiBiT- and mCitrine tagged RXFP1, with BRET units measured over a timecourse of 60 minutes after addition of HibiT complementation reagent. Pooled data from three independent experiments performed in duplicate, shown as mean ± SD. (C) Comparison of tagged RXFP1 BRET signal increase over a 60 minute timecourse, with and without 0.01% digitonin to permeabilize cells and allow intracellular HiBiT labeling. Pooled data from three independent experiments performed in duplicate, shown as mean ± SD (D) BRET signal between HiBiT-RXFP1 and mCitrine-RXFP1 determined after over a 70 minute timecourse after addition of furimazine, with vehicle or 100 nmol/L relaxin added after 20 minutes. Data representative of a single experiment performed 3 times in duplicate, presented as mean ± SD. (E) and (F) Saturation BRET curve using HiBiT-RXFP1 and mCitrine-RXFP1 (E) or mCitrine-GABA$_{B2}$ (F). Data points represent single wells in which filtered luminescence was measured, followed by measurement of mCitrine fluorescence, and is pooled from three independent experiments Additionally, saturation BRET style analyses were at- tempted using HiBiT-RXFP1/mCitrine-RXFP1 co-transfections (Figure 6E), or HiBiT-RXFP1/mCitrine-GABA$_{B2}$ as a negative control (Figure 6F). The BRET signal for HiBiT-RXFP1/mCitrine-RXFP1 ap- peared pseudo-hyperbolic which may indicate RXFP1 homodimers at the cell surface. However, there were clear qualitative differences to that obtained using Nanoluc-RXFP1/mCitrine-RXFP1 (Figure 4D) suggesting that these interactions are different at the cell surface compared to the whole cell context.

Given these results for RXFP1, we applied this HiBiT/mCitrine BRET approach to the GABA$_B$ receptor, a *bona fide* stable GPCR het- eromer composed of GABA$_{B1}$ and a GABA$_{B2}$ subunits, as a positive control (Figure 7A). The GABA$_{B1}$ subunit contains an ER retention motif in the C-terminal tail that inhibits its trafficking to the cell sur- face when expressed alone. Co-expression of GABA$_{B1}$ with GABA$_{B2}$ masks the ER retention motif of GABA$_{B1}$, allowing both to traffic to the cell surface where they exist as a stable di-sulphide linked heteromer.[31-33] As expected, HiBiT-GABA$_{B1}$ was poorly expressed alone (though intracellular expression could be detected with the ad- dition of digitonin) and co-expression of GABA$_{B2}$ greatly enhanced the expression of HiBiT-GABA$_{B1}$ at the cell surface as measured by HiBit luminescence (Figure 7B). Co-expression of a 1:1 ratio of HiBiT-GABA$_{B1}$/mCitrine-GABA$_{B2}$ in HEK293T yielded results that were consistent with a specific, stable heterodimeric interaction. The BRET signal from labeled GABA$_B$ heteromers was stable over time and was not influenced by different expression levels of the receptor at the cell surface (Figure 7D-E). Additionally, a saturation BRET style analysis using a titration of mCitrine-GABA$_{B2}$ with a con- stant amount of HiBiT-GABA$_{B1}$ showed a clear saturable curve in- dicative of heterodimerization (Figure 7F). Comparison of the HiBiT/ mCitrine BRET results for the

GABA$_B$ heteromer with that of RXFP1 suggested that RXFP1 does not exist as a stable homodimer at the cell surface.

4 | DISCUSSION AND CONCLUSIONS

Hetero- and homodimerization of GPCRs has been a topic of great in- terest in the drug discovery field for several decades now. However, the functional implications of such interactions are difficult to re- solve. The receptor for relaxin, RXFP1, has a unique mode of acti- vation which lends itself to the possibility that the functional unit may be a homodimer (Figure 1). However, there were unresolved questions about whether RXFP1 is necessarily a homodimer at the

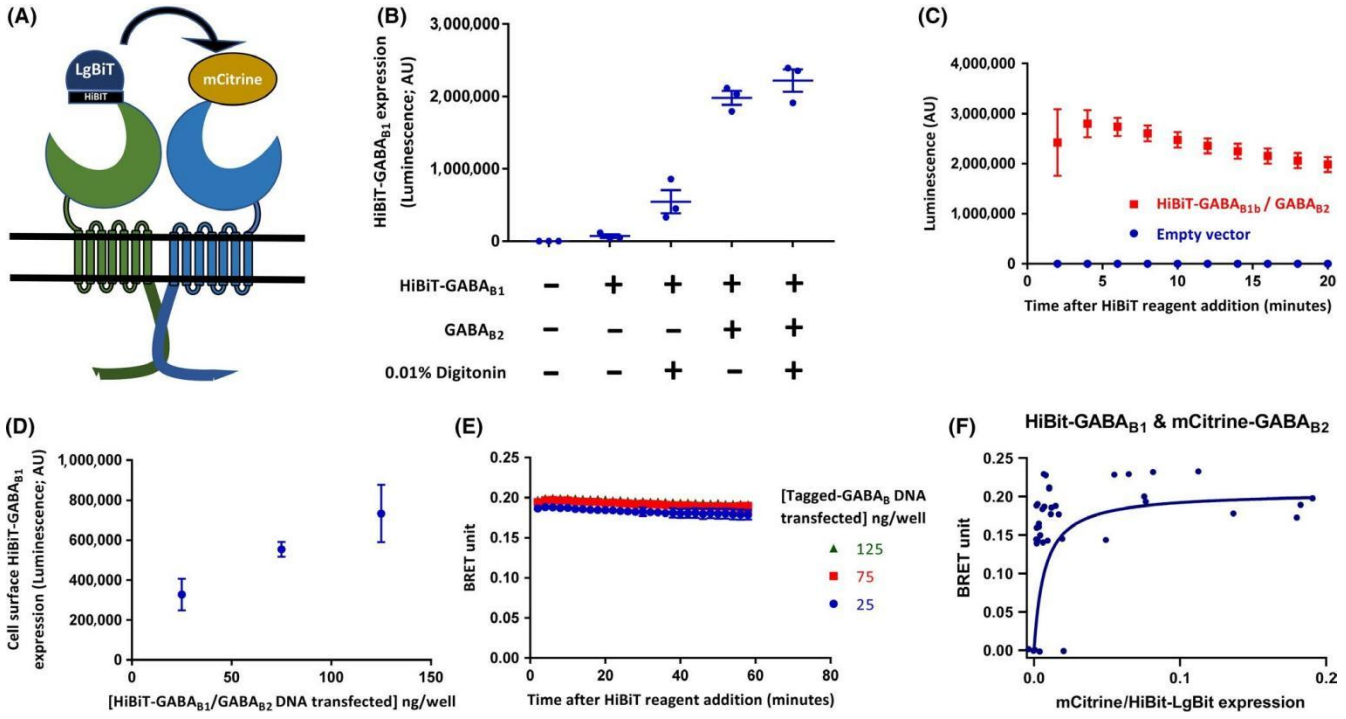


FIGURE 7 BRET between HiBiT- and mCitrine-tagged GABA$_B$ heteromer is consistent with a stable dimer (A) Cartoon representation of BRET between HiBiT-GABA$_{B1}$ (green) and mCitrine-GABA$_{B2}$ (blue), with HiBiT tag complemented to the LgBiT protein in the HiBiT complementation reagent. (B) HiBiT detected expression of HiBiT-GABA$_{B1}$with and without co-expression of GABA$_{B2}$ and/or co-addition of digitonin. (C) Timecourse of luminescence after adding HiBiT complementation reagent to HEK293T cells transiently expressing HiBiT-GABA$_{B1}$ and untagged GABA$_{B2}$. (D) Expression of HiBiT-GABA$_{B1}$ at different transfection levels (E), A 1:1 ratio of HiBiT-GABA$_{B1}$ and mCitrine-GABA$_{B2}$ were co-transfected, and BRET measured over a 60 minute timecourse after addition of HiBiT complementation
reagent. Data (A-E) are pooled from three independent experiments performed in duplicate, shown as mean ± SD. (F) Saturation BRET curve using HiBiT-GABA$_{B1}$ and mCitrine-GABA$_{B2}$. Data points represent single wells in which filtered luminescence was measured, followed by measurement of mCitrine fluorescence, and is pooled from three independent experiments cell surface and what role, if any, homodimerization of RXFP1 plays
on its mechanism of activation.

A common method for determining GPCR proximity is the use of saturation BRET experiments, and these have been published for RXFP1 showing "constitutive" homodimerization, which appear to be unaffected by relaxin binding.[11] Our primary aim was to further investigate RXFP1 homodimerization in order to assess whether it is indeed a necessary requirement for relaxin-mediated activation of RXFP1, thus we sought appropriate tools to investigate RXFP1 proximity in live cells. Saturation BRET experiments inherently give a readout of the proximity of receptors across the whole cell (not simply at the cell surface) and involve a titration of receptor expression by increasing DNA transfection amounts. We produced

our own saturation BRET experiments, using slightly different fusion pro- teins (Rluc8/Venus) to previously published reports.[10,11] This yielded a BRET saturation curve indicative of a close proximity between RXFP1-Venus and RXFP1-Rluc8 receptors across the whole cell. We could not, however, detect any change in the BRET ratio upon stim- ulation with relaxin using this technique. Only a small proportion of RXFP1 (with or without BRET tags) was reaching the cell surface, thus indicating that a large proportion of the BRET signal was com- ing from receptors expressed in intracellular compartments, which

may obscure any potential relaxin-induced change in BRET signal at the cell surface. As our main goal was to understand what is happen- ing at the cell surface, we looked at the localization of FLAG-tagged RXFP1 when overexpressed in HEK 293T cells over a range of DNA transfection amounts. While receptor DNA transfection amounts corresponded linearly with the total amount of receptor expressed in the cell, the level of receptor expressed at the cell surface had a saturable limit. Thus, increasing the amount of RXFP1 DNA trans- fected (as in saturation BRET experiments) leads to accumulation of receptor within intracellular compartments. It has previously been published that RXFP1 targets poorly to the cell surface when tran- siently expressed in HEK 293 cells,[10] however, this involved confocal imaging of permeabilized vs non-permeabilized transfected cells, and no quantification of the percentage of receptor at the cell sur- face was presented. Kern et al[10] also neatly showed co-localization of RXFP1 receptor with RXFP1 splice variants that were retained in the endoplasmic reticulum. Co-expression of these splice vari- ants with full length RXFP1 resulted in a decrease in the cell sur- face targeting of the receptor which, taken together, supports the view that RXFP1 homodimerization in the endoplasmic reticulum is involved in receptor maturation and subsequent targeting to the cell surface. This is not unique to RXFP1, however, as homo- and

heterodimerization of receptors in the endoplasmic reticulum is be- lieved to be common across the GPCR family to allow appropriate trafficking to the cell surface.[34,35]

Due to the significant intracellular accumulation of RXFP1 re- ceptor, we therefore aimed to develop a system where the BRET signal from intracellular compartments could be excluded, by only detecting signal from cell surface receptors. This is not a new idea – previous successful approaches to investigate GPCR oligomeriza- tion have used fluorescent antibodies directed against N-terminal epitope tags[36,37] or used specific labeling proteins such as the SNAP tag[38] to perform time resolved FRET experiments. More recently, surface labeled SNAP tag fused receptors were used in combination with Nanoluc-tagged VEGR2 (a receptor tyrosine kinase) to investi-

gate the possibility of interactions between VEGR2 and the β_2-ad-
renergic receptor.[39]

We chose a new approach in using the Nanoluc split luciferase system, HiBiT, which was developed by Promega and has recently entered use by academic labs.[40-43] This involved fusion of the HiBiT tag to the N-terminus of RXFP1, such that cell surface expression could be measured by exogenous addition of the complementary Nanoluc fragment LgBiT (an 18 kDa protein which does not cross the cell membrane). This has many advantages – no requirement for removal of unbound labeling reagent, and no requirement for laser excitation of the donor as is necessary for FRET approaches. Indeed, the HiBiT tag proved to be an excellent method for detection of RXFP1 cell-surface and total expression (as well as for the $GABA_B$ heteromer), comparable to using a FLAG epitope tag but far less labor-intensive and applicable for use on live cells at 37°C. In future, HiBiT labeling of receptors could be combined with previous strate- gies (ie, SNAP surface labeling) to further refine BRET experiments investigating cell surface receptor interactions.

In order to quickly and fully label all cell surface HiBiT-tagged RXFP1 receptors, the concentration of LgBiT used in our assay con- ditions was around 100 nmol/L – well above the reported dissocia- tion constant (K_D) for the LgBiT:HiBiT interaction (K_D = 700 pM).[30] It is generally found that the on-rate (k_{on}) for purely diffusion limited protein association is in the 10^5-10^6 M/sec range[44] which, through some basic simulations (Figure S3) shows it reasonable that equi- librium should be attained within a few minutes at most, on the assumption of a simple reversible one-step interaction occurring ac- cording to the law of mass action.

The ability to detect the real-time presentation of membrane- bound, extracellularly expressed HiBiT tag in live cells at

37°C is unique and may potentially provide information about the dynam- ics of trafficking of membrane receptors to and from the cell sur- face. We note that the increase in luminescence upon addition of HiBiT complementation reagent to HiBiT-RXFP1 expressing cells was unusually slow (Figure 5B), contrasting with the profile of HiBiT- $GABA_{B1}$ labeling which showed the expected rapid rise in lumines- cence followed by the expected slow signal decay (Figure 7C). In our early experiments using HiBiT for labeling of RXFP1, we used a short four residue glycine/serine linking sequence between HiBiT and RXFP1 and considered that this slow increase in luminescence may be due to poor accessibility of the HiBiT tag due to being too close to the N-terminus of RXFP1. However, the slow rise in luminescence remained even in the 12 residue linker which we subsequently used for these studies (Figure S4), indicating that steric inaccessibility of the HiBiT tag is not an issue. An alternative explanation for the slow rise in luminescence for HiBiT-RXFP1 complementation is that the receptor is constitutively being recycled between the cell surface and endosomal compartments during the experimental time course. RXFP1 has been demonstrated to undergo constitutive endocytosis in HEK293T cells,[45] therefore it follows that there must also be a constitutive recycling of receptors back to the cell surface in order to maintain a dynamic equilibrium of cell surface expression. Hence, the luminescence signal of labeled HiBiT-RXFP1 is likely to be a summation of all receptors which have resided at the cell membrane over the experimental time course, including those that have subse- quently been endocytosed but continue to emit luminescence. This explanation would also resolve the slow rise in BRET between HiBiT/ mCitrine tagged RXFP1 receptors (Figure 6B), which contrasts with the temporal stability of the BRET signal for the obligate $GABA_B$ het- eromer (Figure 7E), meaning that RXFP1 receptors may only come into proximity upon constitutive internalization into endosomes. These possibilities can easily be investigated in future using pharma- cological inhibitors of endocytic machinery, such as pitstop 1/2[46] or dynasore.[47] Additionally, live cell imaging techniques could also be applied to investigate the dynamics of RXFP1 subcellular localization and trafficking in future.

Plate-based assays which use BRET to investigate GPCR:GPCR interactions provide only limited evidence concerning GPCR di- merization. Indeed, the great difficulty and caveat of resonance energy transfer methods to investigate protein-protein interac- tions is that it is inherently a readout of proximity only, rather than black-and-white evidence of a molecular interaction. On the other hand, single-molecule microscopy methods have previously been employed to determine the 2D interaction kinetics of a few Class A GPCRs, demonstrating that these interactions are quite tran- sient.[48-51] Such methods are far more labor intensive; however, and so higher throughput plate-based methodologies such as saturation BRET and the experiments described here are still important tools when weighed against other available data.

As previously mentioned, the evidence that RXFP1 is indeed a functional homodimer at the cell surface is weak. Alongside pre- viously published saturation BRET experiments, relaxin has been reported to bind RXFP1 with negative cooperativity,[11] which seemingly strengthens a theory RXFP1 may be a functional ho- modimer. A co-operative binding interaction implies the existence of more than one binding site, in which the occupancy of one site allosterically decreases (negative cooperativity) or increases (pos- itive cooperativity) the affinity of a second binding site. An RXFP1 homodimer provides a structural basis by which two allosterically coupled relaxin binding sites might exist. Svendsen et al[11] re- ported negative cooperativity of relaxin binding using a method originally developed for the insulin receptor,[52] in which an unde- fined concentration of radio-labeled relaxin was incubated with

high expressing HEK293T-RXFP1 cells, and the amount of remain- ing bound radio-labeled relaxin was later quantified after removal of unbound radio-labeled relaxin (to allow dissociation of bound radio-labeled relaxin) with or without varying concentrations of unlabeled relaxin. The observation that increasing concentrations of unlabeled relaxin led to a concentration dependent decrease in the amount of remaining bound radio-labeled relaxin indicated a competitor induced "acceleration" of radiolabeled relaxin dis- sociation which was taken as evidence of negative cooperativity (even though dissociation rates were not quantified). However, this methodology does not take into account the general phe- nomena that even strictly isolated single-site binding processes will necessarily show an apparent competitor-induced accelera- tion of dissociation due to the competitors ability to occlude the rapid rebinding of the initially bound ligand.[53,54] Additionally, the complex mechanism by which relaxin is now understood to bind RXFP1 (co-ordinated by multiple distinct sites within the ECD) could also explain an apparently cooperative binding interaction when a receptor monomer is assumed. Furthermore, the most recent investigations into the kinetics of relaxin:RXFP1 binding found no evidence of negative binding cooperativity

when relaxin dissociation rates were quantified in the presence of varying con- centrations of competitor relaxin.[27] Therefore, the most current evidence concerning the mode of relaxin binding to RXFP1 does not support the idea that it is activated as homodimer.

Given the results of these studies, a non-homodimer mechanism of RXFP1 activation by relaxin currently appears more likely. These studies have shown that the localization of receptors is an important consideration when interpreting the results of plate-based assays using BRET methodologies. We have demonstrated that the HiBiT tag is an excellent tool for cell labeling both Class A (RXFP1) and Class C ($GABA_B$) GPCRs with a luminescent tag which can partici- pate in BRET transfer, and that it may even be useful to study the real-time dynamics of receptor trafficking in future. Importantly, by isolating the BRET signal occurring at the cell surface, we can say with more certainty that activation of RXFP1 by relaxin does not induce homodimerization at the cell surface. Indeed, the outcomes of these studies suggest that further investigations should focus on the dynamics of RXFP1 trafficking to and from the cell surface for which the use of a real-time labeling strategy such a HiBiT may be informative.

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