

## Quantification of the Surface Expression of G Protein-coupled Receptors Using Intact Live-cell Radioligand Binding Assays

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**[Abstract]** G protein-coupled receptors (GPCRs) are the most structurally diverse family of signaling proteins and regulate a variety of cell function. For most GPCRs, the cell surface is their functional destination where they are able to respond a wide range of extracellular stimuli, leading to the activation of intracellular signal transduction cascades. Thus, the quantity of receptor expression at the cell surface is a crucial factor regulating the functionality of the receptors. Over the past decades, many methods have been developed to measure the cell surface expression of GPCRs. Here, we describe an intact live-cell radioligand binding assay to quantify the surface expression of GPCRs at the endogenous levels or after overexpression. In this assay, cell cultures will be incubated with specific cell-nonpermeable radioligands which selectively and stoichiometrically bind to individual GPCRs and the receptor numbers at the cell surface are quantified by the radioactivity of receptor-bound ligands. This method is highly specific for measuring the functional GPCRs at the surface of intact live cells and is particularly useful for endogenous, low-abundant GPCRs.

**Keywords:** G protein-coupled receptor, Adrenergic receptor, Angiotensin II receptor, Muscarinic receptor, Cell surface, Trafficking, Radioligand, Live cell

**[Background]** G protein-coupled receptors (GPCRs) constitute the largest superfamily of cell surface receptors and modulate a variety of cell functions under physiological and pathological conditions (Hauser *et al.*, 2017; Hilger *et al.*, 2018; Weinberg and Puthenveedu, 2019). The precise functions of individual GPCRs at the cell surface are initiated by their binding to specific ligands which in turn activates cognate heterotrimeric G proteins or other signaling molecules, leading to the activation of intracellular signal transduction cascades. Although GPCR-mediated signaling and functioning are sophisticated processes which are coordinated by many factors, the net amount of receptor surface expression is undoubtedly a crucial element regulating the magnitude and duration (Zhang and Wu, 2019).

The quantity of GPCR expression at the cell surface is a balance of highly regulated, dynamic intracellular trafficking, including maturation, internalization, recycling, and degradation. Over the past decades, numerous methods have been well established to quantify the surface GPCRs, such as ELISA, flow cytometry, biotinylation, radioligand binding and imaging (Dunham *et al.*, 2009; Qin *et al.*, 2011; Zhu *et al.*, 2015; Shiowski *et al.*, 2017 and 2019). Here, we describe an intact live-cell ligand binding assay to quantify their surface expression at steady state by using cell membrane-nonpermeable radioligands.

In this assay, live cells will be incubated with individual receptor-specific antagonists or agonists labelled with radioactive isotopes at a saturating concentration. Since these radioligands are not able to penetrate the plasma membrane, the radioactivity of ligands bound to the cells will reflect the quantity of receptor expression at the cell surface. As the ligands used are radiolabeled and stoichiometrically bind to specific GPCRs, this method provides a very sensitive and highly specific approach to accurately quantify the surface functional GPCRs which are able to bind to their ligands in intact live cells and is particularly useful for endogenous, low-abundant GPCRs whose quantification by other methods is extremely difficult.

We have used this method to measure the surface expression of a number of family A GPCRs in different cell types at the endogenous levels or after overexpression (Filipeanu *et al.*, 2004; Dong and Wu, 2006 and 2007; Dong *et al.*, 2008; Duvernay *et al.*, 2009a and 2009b; Dong *et al.*, 2010a and 2010b; Duvernay *et al.*, 2011; Zhang *et al.*, 2011; Dong *et al.*, 2012; Fan *et al.*, 2012; Li *et al.*, 2012; Zhang *et al.*, 2016a and 2016b; Li *et al.*, 2017; Wei *et al.*, 2019; Zhang *et al.*, 2019). Here, we first describe the procedures to measure the surface expression of  $\alpha_{2B}$ -adrenergic receptor (AR), which has long been used as a model GPCR in our studies, in NG108-15 neuroblastoma-glioma and MCF-7 breast cancer cells which express  $\alpha_{2B}$ -AR endogenously, and in human embryonic kidney 293 (HEK293) cells in which  $\alpha_{2B}$ -AR was overexpressed by transient transfection or inducible systems. We will then briefly discuss the quantification of surface expression of other GPCRs by using radioligand binding of intact live cells.

## **Materials and Reagents**

1. 6-well plates (Thermo Fisher Scientific, catalog number: 130184)
2. 12-well plates (Thermo Fisher Scientific, catalog number: 130185)
3. HEK293 cells (ATCC, catalog number: CRL-1573)
4. NG108-15 cells (ATCC, catalog number: HB-12317)
5. MCF-7 cells (ATCC, catalog number: HTB-22)
6. [6,7- $^3\text{H}$ (N)]-RX821002 (PerkinElmer, catalog number: NET1153250UC)
7. [7-methoxy- $^3\text{H}$ ]-Prazosin (PerkinElmer, catalog number: NET823025UC)
8. [5,7- $^3\text{H}$ ]-CGP12177 (PerkinElmer, catalog number: NET1061250UC)
9. [ $^{125}\text{I}$ ]-Angiotensin II, [ $^{125}\text{I}$ ]-Ang II (PerkinElmer, catalog number: NEX105050UC)
10. [N-methyl- $^3\text{H}$ ]-Scopolamine methyl chloride, [ $^3\text{H}$ ]-NMS (PerkinElmer, catalog number: NET636250UC)
11. Phentolamine hydrochloride (Sigma, catalog number: P7547)
12. Rauwolscine hydrochloride (Tocris, catalog number: 0891)
13. Alprenolol hydrochloride (Sigma, catalog number: A8676)
14. Angiotensin II, Ang II (Calbiochem, catalog number: 05-23-0101)
15. Atropine (Sigma, catalog number: A0132)
16. Doxycycline hyclate (Sigma, catalog number: D9891)
17. Dulbecco's modified Eagle's medium, DMEM (Sigma, catalog number: D6429-500ML)

18. Fetal bovine serum, FBS (HyClone, catalog number: SH30396.03)
19. Penicillin-streptomycin solution (HyClone, catalog number: SH40003.01)
20. Hypoxanthine (Sigma, catalog number: H9377)
21. Aminopterin (Sigma, catalog number: A3411)
22. Thymidine (Sigma, catalog number: T9250)
23. Poly-L-lysine hydrobromide (Sigma, catalog number: P1274)
24. Trypsin-EDTA (0.25%) (Sigma, catalog number: T4049-100ML)
25. Opti-MEM (Gibco, catalog number: 31985-070)
26. Lipofectamine 2000 (Invitrogen, catalog number: 11668-019)
27. 10× Phosphate buffered saline, 10× PBS (Sigma, catalog number: P5493-4L)
28. NaOH (Sigma, catalog number: S5881-500G)
29. Scintillation solution, ScintiVerse™ BD cocktail (Fisher Chemical, catalog number: SX18-4)
30. High density polyethylene vials, 20 ml capacity (RPI, catalog number: 121043)
31. Poly-L-lysine (see Recipes)
32. NG108-15 growth medium (see Recipes)
33. DMEM complete medium (see Recipes)
34. <sup>3</sup>H-RX821002 binding solution (see Recipes)
35. <sup>3</sup>H-RX821002 binding solution containing 10 μM rauwolscine (see Recipes)

## **Equipment**

1. Low speed orbital shaker (Southwest Science, catalog number: SBT30)
2. Hemocytometer (Hausser Scientific, catalog number: 1483)
3. Cell culture incubator (Thermo Scientific, Forma series II water jacketed CO<sub>2</sub> incubator)
4. Tissue culture microscope (Nikon, model: Eclipse TS100)
5. Scintillation counter (Hitachi, model: AccuFLEX LSC-8000c)

## **Software**

1. Microsoft Excel

## **Procedure**

### **A. Quantification of endogenous α<sub>2B</sub>-AR at the cell surface**

There are three different α<sub>2</sub>-AR subtypes: α<sub>2A</sub>-AR, α<sub>2B</sub>-AR and α<sub>2C</sub>-AR. NG108-15 neuroblastoma-glioma cells express only α<sub>2B</sub>-AR but not α<sub>2A</sub>-AR and α<sub>2C</sub>-AR, whereas MCF-7 breast cancer cells express both α<sub>2B</sub>-AR and α<sub>2C</sub>-AR but not α<sub>2A</sub>-AR.

1. Cell culture

- a. To pre-coat 6-well plates, add 2 ml of poly-L-lysine (Recipe 1) each well and incubate at 37 °C for 15 min. Wash the plate twice, each with 1 ml of sterilized MilliQ-water.
  - b. Seed NG108-15 or MCF-7 cells on 6-well plates at a total of  $5 \times 10^5$  cells per well. For each experiment, a total of 6 wells are needed; 3 wells are used to measure total radioligand binding and other 3 wells used to measure non-specific radioligand binding.
  - c. Culture NG108-15 cells in NG108-15 growth medium (Recipe 2) under 95% air and 5% CO<sub>2</sub> at 37 °C.
  - d. Culture MCF-7 cells in DMEM complete medium (Recipe 3) under 95% air and 5% CO<sub>2</sub> at 37 °C.
  - e. When cells become 70-90% confluent, conduct radioligand binding.
  2. Radioligand binding
    - a. Aspirate the growth medium completely.
    - b. To measure total radioligand binding, add 500 µl of <sup>3</sup>H-RX821002 at a concentration 2 nM diluted in DMEM (Recipe 4).
    - c. To measure non-specific radioligand binding, add 500 µl of <sup>3</sup>H-RX821002 at 2 nM containing nonradioactive rauwolsine at 10 µM (Recipe 5).
    - d. Incubate cells for 90 min at room temperature (RT) on a low speed orbital shaker with constant shaking at 20-40 rpm.
    - e. Aspirate radioligand binding solution and wash cells twice, each with 1 ml of 1× PBS (10× PBS diluted with MilliQ-water) for 5 min at RT with shaking to remove the excess radioligand.
    - f. Remove PBS and add 500 µl of 1 M NaOH to digest cells for 2 h at RT with shaking.
    - g. Add 4 ml of scintillation solution to each high density polyethylene vial, transfer cell lysates to vials, and mix vigorously by inverting the tubes or using a vortex.
    - h. Count radioactivity in a scintillation counter.
    - i. In a separate vial, add 4 ml of scintillation solution and 5 µl of radioligand binding solution, mix and count radioactivity. This number will be used as input to calculate receptor numbers.
- B. Quantification of transiently expressed α<sub>2B</sub>-AR at the cell surface
1. Cell culture
    - a. Seed HEK293 cells into 6-well plates pre-coated with poly-L-lysine at a total of  $5 \times 10^5$  cells per well and culture cells in DMEM complete medium at 37 °C overnight.
    - b. Check the cell density under a tissue culture microscope. When cells become 70-90% confluent, aspirate culture medium and add 1.8 ml of DMEM.
  2. Transient transfection
    - a. α<sub>2B</sub>-AR plasmids in any mammalian expression vectors can be used. For example, we have used the pEGFP-N1 vector to generate α<sub>2B</sub>-AR tagged with GFP at the C-terminus (Dong *et al.*, 2010a and 2010b). Based on our experience, transfection of 1 µg of α<sub>2B</sub>-AR plasmids in each well of 6-well plates is sufficient to achieve the maximal receptor expression.

- b. In an Eppendorf tube, add 125  $\mu$ l of serum-free Opti-MEM and 1  $\mu$ g of  $\alpha_{2B}$ -AR plasmids, mix gently, and incubate for 5 min at RT.
    - c. In another tube, add 125  $\mu$ l of Opti-MEM and 2.5  $\mu$ l of Lipofectamine 2000, mix and incubate for 5 min at RT.
    - d. Combine two solutions, mix gently by pipetting 3-4 times, and incubate for additional 20 min at RT.
    - e. Add the mixture slowly into cells cultured in 1.8 ml of DMEM, mix gently, and culture cells for 6 h at 37 °C.
    - f. Remove cell culture medium and wash cells once with 1 ml of sterilized 1 $\times$  PBS.
    - g. Treat cells with 100  $\mu$ l of trypsin-EDTA for 2 min at 37 °C and add 1 ml of DMEM complete medium.
    - h. Split cells from each well of 6-well plates into four wells of 12-well plates pre-coated with poly-L-lysine (0.27 ml each well). Two wells are used to measure total radioligand binding and other two wells used to measure non-specific radioligand binding.
    - i. Culture cells in DMEM complete medium for 24-36 h at 37 °C.
  3. Radioligand binding
    - a. Aspirate the growth medium completely.
    - b. For measurement of total radioligand binding, add 200  $\mu$ l of  $^3$ H-RX821002 at 20 nM diluted in DMEM (Recipe 4) into cells
    - c. For measurement of non-specific radioligand binding, add 200  $\mu$ l of  $^3$ H-RX821002 at 20 nM plus rauwolscline at 10  $\mu$ M (Recipe 5) into cells.
    - d. Incubate cells for 90 min at RT with constant shaking at 20-40 rpm.
    - e. Aspirate radioligand binding solution and wash cells twice, each with 0.5 ml of 1 $\times$  PBS for 5 min at RT with shaking.
    - f. Remove PBS and add 500  $\mu$ l of 1 M NaOH to digest cells for 2 h at RT with shaking.
    - g. Transfer cell lysates to a vial containing 4 ml of scintillation solution, mix well and count radioactivity in a scintillation counter.
    - h. Add 5  $\mu$ l of radioligand binding solution and 4 ml of scintillation solution to a vial, mix and count radioactivity. This number will be used as input to calculate receptor numbers.
- C. Quantification of inducibly expressed  $\alpha_{2B}$ -AR at the cell surface
  1. Stable HEK293 cells inducibly expressing  $\alpha_{2B}$ -AR were generated by using Tet-On 3G tetracycline-inducible gene expression system as described previously (Zhang *et al.*, 2016a and 2016b).
  2. Seed cells into 12-well plates pre-coated with poly-L-lysine at a density of  $2 \times 10^5$  cells per well and culture cells in DMEM complete medium at 37 °C overnight.
  3. To measure doxycycline dose-dependent induction of  $\alpha_{2B}$ -AR expression, add doxycycline to the final concentrations of 0, 2.5, 5, 7.5, 10, 20, 40, 60, 80 and 100 ng/ml and incubate for 24 h at 37 °C.

4. To measure time-dependent induction of  $\alpha_{2B}$ -AR expression, add doxycycline at the final concentration of 40 ng/ml and incubate cells for 2, 4, 6, 8, 10, 12, 14, 16, 20, 24, and 36 h.
5. Conduct radioligand binding as described in Procedure B.

#### D. Measurement of the cell surface expression of other GPCRs

Over the past years, we have used intact live-cell ligand binding assays to quantify the cell surface expression of several family A GPCRs, including  $\alpha_{1A}$ -AR,  $\alpha_{1B}$ -AR,  $\alpha_{2A}$ -AR,  $\alpha_{2B}$ -AR,  $\alpha_{2C}$ -AR,  $\beta_1$ -AR,  $\beta_2$ -AR, Ang II type 1 (AT1R) and type 2 (AT2R) receptors, and M3-muscarinic receptor (M3-MR) using different radioligands (Table 1). One issue associated with intact cell radioligand binding assays is that radiolabeled ligands may be able to induce receptor internalization. For example, Ang II is an agonist of AT1R, thus incubation with  $^{125}$ I-Ang II may induce the internalization of AT1R from the cell surface to the endosomal compartment. One strategy to limit receptor internalization during incubation with radiolabeled agonists is to carry out ligand binding assays at low temperature (Filipeanu *et al.*, 2004).

**Table 1. Measurement of the surface expression of GPCRs by intact cell ligand binding assays**

Receptors	Radioligands	References
$\alpha_1$ -AR ( $\alpha_{1A}$ - and $\alpha_{1B}$ -AR)	[ $^3$ H]-Prazosin (Phentolamine)	Duvernay, 2009a and 2009b; Zhang <i>et al.</i> , 2011; Li <i>et al.</i> , 2017
$\alpha_2$ -AR ( $\alpha_{2A}$ -, $\alpha_{2B}$ - and $\alpha_{2C}$ -AR)	[ $^3$ H]-RX821002 (Rauwolscine)	Dong and Wu, 2007; Dong <i>et al.</i> , 2008; Duvernay 2009a and 2009b; Dong <i>et al.</i> , 2010a and 2010b; Zhang <i>et al.</i> , 2011; Filipeanu <i>et al.</i> , 2015; Li <i>et al.</i> , 2017; Wei <i>et al.</i> , 2019
$\beta$ -AR ( $\beta_1$ - and $\beta_2$ -AR)	[ $^3$ H]-CGP12177 (Alprenolol)	Dong and Wu, 2007; Dong <i>et al.</i> , 2008; Duvernay 2009a and 2009b; Dong <i>et al.</i> , 2010a and 2010b; Zhang <i>et al.</i> , 2011; Filipeanu <i>et al.</i> , 2015; Li <i>et al.</i> , 2017; Wei <i>et al.</i> , 2019
AT1R and AT2R	[ $^{125}$ I]-Ang II (Ang II)	Filipeanu <i>et al.</i> , 2004; Dong and Wu, 2007; Dong <i>et al.</i> , 2008; Duvernay <i>et al.</i> , 2009a and 2009b; Dong <i>et al.</i> , 2010b; Zhang <i>et al.</i> , 2011
M3-MR	[ $^3$ H]-NMS (Atropine)	Dong <i>et al.</i> , 2010b

Drugs indicated in ( ) are used for measurement of non-specific radioligand binding.

#### E. Data analysis

1. Calculate specific radioligand binding to receptors using the values of disintegrations per minute (DPM):

$$\text{Specific binding (DPM)} = \text{total binding (DPM)} - \text{non-specific binding (DPM)}$$

2. Calculate surface receptor numbers per cell

In order to calculate the receptor numbers at the surface in each cell, the total cell numbers used in ligand binding assays need to be determined. This can be done by preparing cell

cultures in exactly the same way as for ligand binding and count the cell numbers in each well by using a hemocytometer.

a. Endogenous receptors

$$\text{Surface receptor numbers per cell} = \frac{\text{Specific binding (DPM)} \times 6.02 \times 10^9}{\text{Input (DPM)} \times \text{Cell number}}$$

Detailed calculation:

$$\begin{aligned} \text{Input ligand number (moles)} &= \text{Input ligand concentration} \times \text{Input ligand volume} \\ &= 2 \text{ nM} \times 5 \text{ }\mu\text{l} = 2 \times 10^{-9} \text{ M} \times 5 \times 10^{-6} \text{ L} = 10^{-14} \end{aligned}$$

$$\begin{aligned} \text{Ligand number per DPM (moles)} &= \text{Input ligand (moles)} \div \text{Input DPM} \\ &= 10^{-14} \text{ moles} \div \text{Input DPM} \end{aligned}$$

$$\begin{aligned} \text{Sample ligand number per well (moles)} &= \text{Sample DPM} \times \text{Ligand number per DPM} \\ &= \text{Sample DPM} \times 10^{-14} \text{ moles} \div \text{Input DPM} \end{aligned}$$

As 1 mole =  $6.02 \times 10^{23}$  molecules,

$$\begin{aligned} \text{Sample ligand number per well (molecules)} &= \text{Sample DPM} \times 10^{-14} \text{ moles} \times 6.02 \times 10^{23} \div \text{Input DPM} \\ &= \text{Sample DPM} \times 6.02 \times 10^9 \div \text{total input DPM} \\ \text{Sample ligand number per cell (molecules)} &= \text{Sample DPM} \times 6.02 \times 10^9 \div \text{total input DPM} \div \text{cell number} \end{aligned}$$

b. Overexpressed receptors

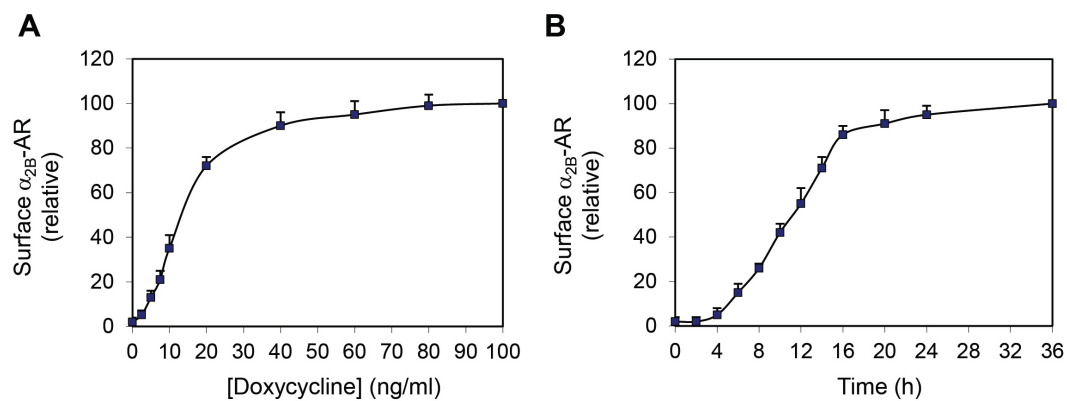
$$\text{Surface receptor numbers per cell} = \frac{\text{Specific binding (DPM)} \times 6.02 \times 10^{10}}{\text{Input (DPM)} \times \text{Cell number}}$$

## F. Experimental results

### 1. Inducible expression of the surface expression of $\alpha_{2B}$ -AR

By using intact live-cell radioligand binding assays as described in the section C, we have measured the surface expression of  $\alpha_{2B}$ -AR in a stable HEK293 cell line which inducibly expresses  $\alpha_{2B}$ -AR. Our data have shown that doxycycline incubation induces the expression of  $\alpha_{2B}$ -AR at the cell surface in a dose- (Figure 1A) and time-dependent (Figure 1B) fashion. Doxycycline-induced  $\alpha_{2B}$ -AR expression reaches a plateau at a concentration of about 40 ng/ml (Figure 1A) and after 20 h of induction (Figure 1B), resulting in a total of  $8.5 \times 10^5$   $\alpha_{2B}$ -AR at the surface per cell (Zhang *et al.*, 2016a and 2016b).



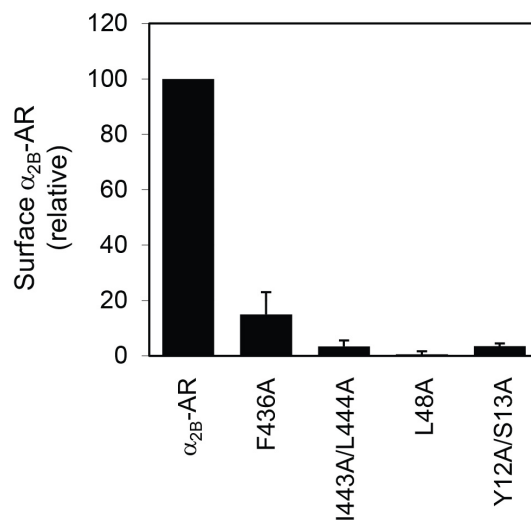


**Figure 1. Dose- (A) and time-dependent (B) induction of  $\alpha_{2B}$ -AR expression at the cell surface by doxycycline in HEK293 cells.** The data shown are percentages of specific binding obtained from cells treated with doxycycline at 100 ng/ml (A) or from cells after induction for 36 h (B) (Zhang *et al.*, 2016a and 2016b).

## 2. Structural determinants of $\alpha_{2B}$ -AR transport to the cell surface

Over the past decades, most studies on GPCR trafficking have focused on the events involved in receptor internalization from the cell surface to the endosomal compartments and recycling of internalized receptors back to the cell surface (Tan *et al.*, 2004; Hanyaloglu and von Zastrow, 2008; Marchese *et al.*, 2008; Kang *et al.*, 2014). In contrast, the molecular mechanisms underlying the cell surface transport of newly synthesized receptors along the biosynthesis pathways are much less well understood. By using intact live cell radioligand binding assays as described above, we have successfully identified a number of motifs or specific residues which are essential for receptor transport to the cell surface either from the endoplasmic reticulum (ER) or from the Golgi apparatus. For example, we have demonstrated that mutation of the residues Y12/S13 in the N-terminus, L48 in the first intracellular loop, and I443/F444 and F436 in the C-terminus markedly reduces the cell surface expression of  $\alpha_{2B}$ -AR (Figure 2) (Dong and Wu, 2006; Duvernay, Dong *et al.*, 2009a and 2009b), indicating important roles of these residues in  $\alpha_{2B}$ -AR biosynthesis.

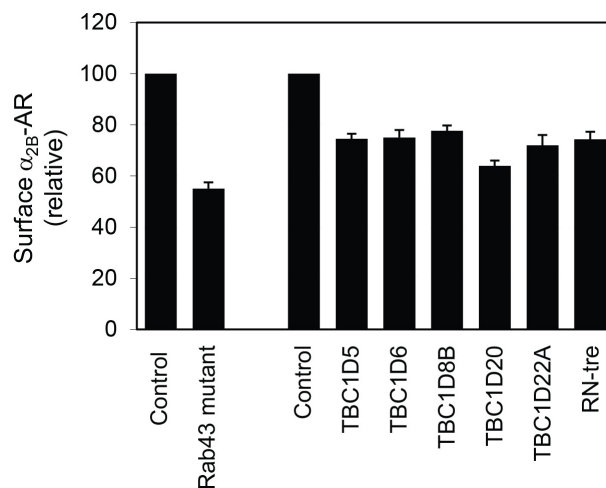




**Figure 2. Inhibition of the cell surface transport of  $\alpha_{2B}$ -AR by mutating residues Y12/S13, L48, F436 and I443/L444.** Wild-type and mutated  $\alpha_{2B}$ -AR were transiently expressed in HEK293 cells and their surface expression was measured by radioligand binding of intact live cells using [ $^3$ H]-RX821002.

### 3. Identification of $\alpha_{2B}$ -AR surface transport regulators

We have also employed intact live cell radioligand binding assays in searching for proteins that regulate  $\alpha_{2B}$ -AR cell surface transport. For instance, in recent studies we have investigated the function of 48 Rab GTPases (Li *et al.*, 2017) and 44 putative Rab GTPase-activating proteins (GAPs) (Wei *et al.*, 2019) in the cell surface transport of  $\alpha_{2B}$ -AR. In these experiments, individual Rab mutants or Rab GAPs were transiently expressed in stable HEK293 cells expressing  $\alpha_{2B}$ -AR and their effects on the cell surface expression of  $\alpha_{2B}$ -AR were measured by radioligand binding of intact live cells as described above. Our results have shown that the expression of Rab43 mutants and six TBC domain-containing proteins, namely TBC1D5, TBC1D6, TBC1D8B, TBC1D20, TBC1D22A and RN-tre significantly reduces the cell surface numbers of  $\alpha_{2B}$ -AR (Figure 3) (Li *et al.*, 2017; Wei *et al.*, 2019). These results demonstrate important functions of Rab43 and the six TBC proteins in the forward trafficking of nascent GPCRs and reveal novel regulatory mechanisms underlying GPCR targeting to the functional destination.



**Figure 3. Inhibition of the cell surface transport of  $\alpha_{2B}$ -AR by mutated Rab43 and TBC domain-containing proteins.** The Rab43 mutant or individual TBC proteins were transiently expressed in HEK293 cells stably expressing  $\alpha_{2B}$ -AR and  $\alpha_{2B}$ -AR expression at the surface was measured by intact cell ligand binding using [ $^3$ H]-RX821002.

## Recipes

### 1. Poly-L-lysine

Dissolve poly-L-lysine hydrobromide in sterilized Milli-Q water to a final concentration of 25  $\mu$ g/ml

### 2. NG108-15 growth medium

DMDM

10% FBS

100 units/ml penicillin

100  $\mu$ g/ml streptomycin

100  $\mu$ M hypoxanthine

0.4  $\mu$ M aminopterin

16  $\mu$ M thymidine

### 3. DMEM complete medium

DMEM

10% FBS

100 units/ml penicillin

100  $\mu$ g/ml streptomycin

### 4. $^3$ H-RX821002 binding solution

As the concentration of  $^3$ H-RX821002 varies in different batches, the following is just an example of concentration calculation of the radioligand in a vial containing 250  $\mu$ Ci in a total volume of 250  $\mu$ l with specific activity of 63.9 Ci/mmol.

$$\text{The concentration of RX821002 (nM)} = \frac{250 \times 10^6}{250 \times 63.9} = 15,600$$

Dilute <sup>3</sup>H-RX821002 in DMEM to 2 nM for measurement of endogenous receptors or to 20 nM for measurement of overexpressed receptors.

5. <sup>3</sup>H-RX821002 binding solution containing 10 μM rauwolscine
  - a. To prepare 5 mM rauwolscine stock solution, dissolve 19 mg of rauwolscine hydrochloride in 10 ml of Milli-Q water
  - b. Dilute rauwolscine stock solution to 10 μM in radioligand binding solution prepared as Recipe 4

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## **Competing interests**

The authors declare no conflict of interests.

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