A Naturally Occurring Splice Variant of GGA1 Inhibits the Anterograde Post-Golgi Traffic of $\alpha_{2B}$-Adrenergic Receptor

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The regulatory mechanisms of cell surface targeting of nascent G protein-coupled receptors (GPCRs) en route from the endoplasmic reticulum through the Golgi remain poorly understood. We have recently demonstrated that three Golgi-localized, $\gamma$-adaptin ear domain homology, ADP ribosylation factor-binding proteins (GGAs) mediate the post-Golgi export of $\alpha_{2B}$-adrenergic receptor ($\alpha_{2B}$-AR), a prototypic GPCR, and directly interact with the receptor. In particular, GGA1 interaction with $\alpha_{2B}$-AR is mediated via its hinge domain. Here we determined the role of a naturally occurring truncated form of GGA1 (GGA1t) which lacks the N-terminal portion of the hinge domain in $\alpha_{2B}$-AR trafficking and elucidated the underlying mechanisms. We demonstrated that both GGA1 and GGA1t were colocalized and mainly expressed at the Golgi. In marked contrast to GGA1, the expression of GGA1t significantly attenuated the cell surface export of newly synthesized $\alpha_{2B}$-AR from the Golgi and in parallel receptor-mediated signaling. Furthermore, we found that GGA1t formed homodimers and heterodimers with GGA1. More interestingly, GGA1t was unable to bind the cargo $\alpha_{2B}$-AR and to recruit clathrin onto the trans-Golgi network. These data provide evidence implicating that the truncated form of GGA1 behaves as a dominant-negative regulator for the cell surface export of $\alpha_{2B}$-AR and this function of GGA1t is attributed to its abilities to dimerize with its wide type counterpart and to inhibit cargo interaction and clathrin recruitment to form specialized transport vesicles.

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 the functional destination where they are able to bind to ligands. Agonist binding to GPCRs induces a conformational change of the receptors which in turn activate cognate heterotrimeric G proteins or other signaling molecules, such as arrestins, leading to the activation of a diverse array of downstream effectors. It is apparent that the magnitude and duration of receptor-mediated signaling is, at least in part, controlled by the amount of receptor expression at the cell surface. However, compared to the well-characterized mechanisms underlying the internalization, recycling and degradation pathways$^{1-3}$, how GPCRs transport the cell surface remains poorly defined$^4$.

$\alpha_{2}$-Adrenergic receptors ($\alpha_{2}$-ARs) are prototypic GPCRs which play an important role in regulating sympathetic nervous system, both centrally and peripherally. There are three different $\alpha_{2}$-AR subtypes: $\alpha_{2A}$-AR, $\alpha_{2B}$-AR and $\alpha_{2C}$-AR. All three $\alpha_{2}$-ARs have similar structural features, including a relatively large third intracellular loop (ICL3) and a short C-terminus (CT). Numerous studies have demonstrated that, by virtue of their ability to directly interact with many other proteins, both the ICL3 and the CT are the most important domains in the receptors that control almost every function the receptors perform, including G protein-coupling, phosphorylation, signal termination and trafficking$^{5-7}$. Our studies have focused on the elucidation of the molecular mechanisms underlying the intracellular trafficking of $\alpha_{2}$-ARs, particularly their anterograde transport en route from the endoplasmic reticulum (ER) through the Golgi to the cell surface$^{8-17}$.

The family of Golgi-localized, $\gamma$-adaptin ear domain homology, ADP ribosylation factor (ARF)-binding proteins (GGAs) includes GGA1, GGA2 and GGA3, all of which contain the VHS, GAT, hinge and GAE domains.
It has been well defined that all three GGAs function as adaptor proteins for clathrin-coated vesicles derived from trans-Golgi network (TGN) which specifically mediate protein traffic from the TGN to endosomes. One of the important findings in the studies of GGA-mediated trafficking over the past decades is the demonstration that each domain of GGAs is able to interact with other proteins to regulate the trafficking process. In particular, cargo proteins use specific motifs to interact with the VHS domain of GGAs to dictate their sorting to the endosomal transport pathway, whereas specific interaction of GGAs, via the hinge domain, with clathrin is an crucial event required for the recruitment of clathrin onto the TGN, as well as the formation of clathrin-coated vesicles. In addition, it has been shown that GGAs may be involved in regulation of the expression of epidermal growth factor receptor and the localization of phosphatidylinositol 4-kinase at the TGN. More recently, we have demonstrated that three GGAs regulate α2B-AR transport specifically from the Golgi to the cell surface.

Each GGA isoform has different spliced variants. However, the precise function of these variants remains unknown. As we have demonstrated that three GGAs use different domains to interact with α2B-AR, among them the GGA1 hinge domain is responsible for the interaction with α2B-AR. In this study, we have determined the role of a spliced variant of GGA1 lacking the N-terminal portion of the hinge domain (GGA1t) in the cell surface trafficking of α2B-AR. We have demonstrated that this truncated GGA1 variant plays a dominant negative role in the export of α2B-AR and its inhibitory function is likely mediated through multiple mechanisms, including dimerization with wild-type counterpart and loss of the abilities to interact with the receptor and to recruit clathrin onto the membrane.

Results
Subcellular distribution of GGA1 and GGA1t. GGA1 is composed of 639 amino acid residues. Based on the unigene and uniprot databases, there are multiple spliced variants of GGA1. Among these spliced variants, the second form is truncated from L277 to S363 in the GAT and hinge domains and therefore, was designated GGA1t (Fig. 1A). As an initial approach to study the function of GGA1t, we compared the subcellular localization of GGA1 and GGA1t in HeLa cells. Myc-tagged GGA1t and GGA1 were transiently expressed together with venus-tagged giantin, a Golgi marker. Confocal microscopy revealed that both GGA1 and GGA1t were strongly co-localized with giantin (Fig. 1B,C), suggesting their expression on the Golgi apparatus.

We next studied the colocalization of GGA1 and GGA1t with other GGAs. For this purpose, myc-tagged GGA1 or GGA1t was transiently expressed together with green fluorescent protein (GFP)-tagged GGA3 in HeLa cells and their colocalization was visualized by confocal microscopy. Both GGA1 and GGA1t were well colocalized with GFP-GGA3 in HeLa cells (Fig. 1D,E). These data demonstrate that the truncation of the N-terminal portion of the hinge domain does not significantly impact the subcellular distribution of GGA1.

GGA1t inhibits the cell surface transport from the Golgi and signaling of α2B-AR. We then determined the effect of overexpression of GGA1t on the cell surface expression of α2B-AR. In this experiment, α2B-AR was tagged with HA at its N-terminus and stably expressed in HEK293 cells using an inducible system. After the cells were transfected with GGA1 or GGA1t and incubated with doxycyclin to induce receptor expression, the numbers of α2B-AR expression at the cell surface was quantified by ligand binding of live cells using [3H]-RX821002 and the total receptor expression was measured by flow cytometry following staining with high affinity anti-HA antibodies in permeabilized cells. The expression of GGA1t significantly inhibited the cell surface expression of α2B-AR as compared with cells transfected with control vector or GGA1 (Fig. 2A,B). In contrast, the expression of GGA1t did not influence overall receptor expression as measured by flow cytometry (Fig. 2B). These data suggest that GGA1t may function as a dominant-negative mutant for the transport of α2B-AR to the cell surface.

To determine if GGA1t could influence other trafficking processes of α2B-AR, we determined its effect on the internalization of α2B-AR. In this experiment, arrestin-3, which was shown to enhance the internalization of α2B-AR, was transiently expressed in HEK293 cells that inducibly express HEK293 cells using an inducible system. The internalization of α2B-AR in response to stimulation of epinephrine at each time point was almost identical in control and GGA1t-expressing cells (Fig. 2C), indicating that GGA1t does not affect agonist-induced α2B-AR internalization.

α2B-AR has been well demonstrated to activate the mitogen-activated protein kinases (MAPK), inhibit adenyl cyclases and suppress voltage-gated calcium channels. To define if GGA1t overexpression could inhibit the function of α2B-AR, the activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) was used as a functional readout. Consistent with its ability to inhibit α2B-AR cell surface transport, transient expression of GGA1t significantly reduced ERK1/2 activation in response to UK14,304 stimulation in cells inducibly expressing α2B-AR, as compared to cells transfected with control vector or GGA1 (Fig. 2D,E). These data suggest that GGA1t modulates not only α2B-AR cell surface transport but also receptor-mediated signal transduction.

To further confirm the inhibitory effect of GGA1t on the cell surface transport of α2B-AR, GGA1t and GGA1t were tagged with GFP at their N-termini and transiently expressed in HEK293 cells stably expressing HA-α2B-AR. The cell surface expression of α2B-AR in cells transfected with GGA1 and GGA1t was visualized by confocal microscopy following staining with HA antibodies in permeabilized cells. As expected, α2B-AR was robustly expressed at the cell surface in cells expressing GFP alone (Fig. 3A). The expression of GFP-GGA1 did not clearly alter the cell surface expression of α2B-AR (Fig. 3A). In contrast, GFP-GGA1t markedly attenuated α2B-AR expression at the cell surface and the receptors were partially co-localized with GGA1t (Fig. 3A,B). These data demonstrate that, consistent with the ligand binding data, expression of GGA1t blocks the cell surface export of α2B-AR. As GGA1t is mainly expressed at the Golgi (Fig. 1C), these data suggest that it regulates the Golgi-to-cell surface transport of α2B-AR.

Possible homo- and hetero-dimerization of GGA1 and GGA1t. We then sought to elucidate the molecular mechanisms underlying the function of GGA1t in regulating α2B-AR trafficking. First, we determined if GGA1 and GGA1t were able to form homodimers and heterodimers using co-immunoprecipitation (IP) and...
bioluminescence resonance energy transfer (BRET) assays. In co-IP assays, HEK293 cells were transfected with myc-tagged GGA1 (B) or GGA1t (C) together with venus-tagged giantin and then stained with anti-myc antibodies. The subcellular distribution of GGA1 and GGA1t and their colocalization with giantin were revealed by confocal microscopy. Red, myc-GGA1 (B) or myc-GGA1t (C); Green, giantin-venus; blue, DNA staining by DAPI. Similar results were obtained in four individual experiments. (D,E), Colocalization of GGA1 (D) and GGA1t (E) with GGA3. HeLa cells were transfected with myc-tagged GGA1 (D) or GGA1t (E) together with GFP-tagged GGA3 and then stained with anti-myc antibodies. Red, myc-GGA1 (D) or myc-GGA1t (E); Green, GFP-GGA3. Similar results were obtained in three experiments. Scale bars, 10µm.

Figure 1. Subcellular localization of GGA1 and GGA1t. (A) A diagram showing the domain arrangement of GGA1 and its truncated mutant GGA1t. (B,C) Colocalization of GGA1 (B) and GGA1t (C) with the Golgi marker giantin. HeLa cells were transfected with myc-tagged GGA1 (B) or GGA1t (C) together with venus-tagged giantin and then stained with anti-myc antibodies. The subcellular distribution of GGA1 and GGA1t and their colocalization with giantin were revealed by confocal microscopy. Red, myc-GGA1 (B) or myc-GGA1t (C); Green, giantin-venus; blue, DNA staining by DAPI. Similar results were obtained in four individual experiments. (D,E), Colocalization of GGA1 (D) and GGA1t (E) with GGA3. HeLa cells were transfected with myc-tagged GGA1 (D) or GGA1t (E) together with GFP-tagged GGA3 and then stained with anti-myc antibodies. Red, myc-GGA1 (D) or myc-GGA1t (E); Green, GFP-GGA3. Similar results were obtained in three experiments. Scale bars, 10µm.
GGA1t is unable to interact with the cargo α2B-AR. We have recently demonstrated that, although full length α2B-AR and GGA1 are not able to form a complex in co-IP assays, the ICL3 of α2B-AR and the hinge domain of GGA1 strongly interacted. As the hinge domain is partially deleted in GGA1t, we compared the...
interaction of α2B-AR with GGA1 and GGA1t. In the first experiment, the hinge domains of GGA1 and GGA1t were tagged with GFP (Fig. 5A). Confocal microscopy revealed that both hinge domains were largely expressed in the cytoplasm (Fig. 5B). To measure the interaction of GGA1 and GGA1t hinge domains with the ICL3 of α2B-AR, the ICL3 was generated as glutathione S-transferase (GST) fusion proteins (Fig. 5C) and incubated with total cell lysates expressing individual hinge domains. GST-ICL3 fusion proteins, but not GST, strongly bound to the GGA1 hinge domain. In contrast, the GGA1t hinge domain did not bind to the ICL3 (Fig. 5D,E).

In the second experiment, we measured the interactions of full length GGA1 and GGA1t with the ICL3 in GST fusion protein pulldown assays. Consistent with the data observed using the hinge domains, full length GGA1, but not full length GGA1t, interacted with GST-ICL3 (Fig. 5F,G). These data suggest that GGA1t is unable to associate with α2B-AR.

**GGA1t is unable to interact with clathrin and recruit clathrin onto the TGN.** GGA1 has been shown to enhance the recruitment of clathrin on the TGN and two clathrin-binding sites have been identified in GGA1 hinge region, As both clathrin-binding sites are deleted in GGA1t (Fig. 6A), we compared the interaction of GGA1 and GGA1t with clathrin in co-IP assays. GGA1 clearly interacted with clathrin, whereas GGA1t did not (Fig. 6B).
We then determined the effect of GGA1 and GGA1t on the recruitment of clathrin on the TGN. Consistent with previous reports, transient expression of GGA1-GFP markedly induced the concentration of clathrin in the perinuclear regions, presumably at the TGN, as compared to cells without transfection of GGA1-GFP (Fig. 6C,E). However, expression of GGA1t-GFP did not affect subcellular localization of clathrin (Fig. 6D,E). These data suggest that, in addition to the loss of its ability to interact with the cargo α2B-AR, GGA1t is unable to recruit clathrin onto the Golgi body.

**Discussion**

The idea to study the function of truncated GGA1t lacking the N-terminal portion of the hinge domain stems from our recent publications showing that, unlike the interactions between GGAs and other cargoes that bear DxxLL-type motifs and transport to endosomes, three GGAs use different domains to interact with different regions of α2B-AR and, in particular, GGA1 interacts with α2B-AR via its hinge domain.

The most important finding presented here is that GGA1t functions as a dominant-negative regulator in the cell surface export of newly synthesized α2B-AR. We have demonstrated that expression of GGA1t markedly inhibited the cell surface expression of α2B-AR without altering overall receptor expression. As the experiments were carried out in cells which inducibly express α2B-AR, the inhibitory effect is likely due to the reduced export trafficking of newly synthesized receptors. Consistent with the intact cell ligand binding
Figure 5. Interaction of GGA1 and GGA1t with the ICL3 of α2B-AR in GST fusion protein pulldown assays. (A) A diagram showing the generation of GFP-tagged hinge domains of GGA1 and GGA1t. (B) Subcellular distribution of GFP-tagged GGA1 and GGA1t hinge domains in HeLa cells. Similar results were obtained in at least three separate experiments. Scale bar, 10 μm. (C) The amino acid sequences of the ICL3 of α2B-AR (left panel) and Coomassie Brilliant Blue staining of purified GST fusion proteins (right panel). (D) Interaction of the α2B-AR ICL3 with the hinge domains of GGA1 and GGA1t. The α2B-AR ICL3 was generated as GST fusion proteins. The hinge domains of GGA1 and GGA1t were expressed in HEK293 cells and total cell homogenates were incubated with GST-ICL3 fusion proteins. Bound GGA1 and GGA1t were revealed by immunoblotting using anti-GFP antibodies. (E) Quantitative data shown in D (n = 3). (F) Interaction of the α2B-AR ICL3 with full length GGA1 and GGA1t. Myc-tagged GGA1 and GGA1t were expressed in HEK293 cells and total cell homogenates were incubated with GST-ICL3 fusion proteins. Bound GGA1 and GGA1t were revealed by immunoblotting using anti-myc antibodies. (G) Quantitative data shown in F (n = 3). Input −5% of total input.
data, subcellular localization analysis by confocal microscopy revealed that α₂B-AR was indeed expressed in the perinuclear region, presumably the Golgi and TGN compartments, which was in marked contrast to robust cell surface expression in GGA1-expressing cells. Furthermore, expression of GGA1t attenuated α₂-AR-mediated signaling, which was presumably caused by an attenuation of receptor transport to the cell surface. Altogether, these data strongly demonstrate a dominant negative function of GGA1t in the cell.
surface transport of $\alpha_{2B}$-AR. However, whether or not GGA1t is a dominant negative regulator in other transport pathways, such as the TGN-endosomes pathway, remains unknown.

There are several possible mechanisms responsible for the dominant-negative effect of GGA1t on the transport of $\alpha_{2B}$-AR. First, co-IP and strong BRET between GGA1 and GGA1t suggest that GGA1t is likely able to form heterodimers with GGA1 in cells. This dimerization may inhibit the normal function of endogenous GGA1 to mediate receptor export to the cell surface.

Second, GGA1t is unable to interact with the cargo $\alpha_{2B}$-AR. We have shown that GGA1t and its hinge domain were unable to interact with the ICL3 of $\alpha_{2B}$-AR in GST fusion protein pulldown assays. These data identify the $\alpha_{2B}$-AR binding site in the N-terminal portion of the hinge domain. These data further support a crucial role of GGA1-$\alpha_{2B}$-AR interaction in receptor forward trafficking (Fig. 7).

Third, GGA1t is unable to recruit clathrin on the Golgi membrane. The most important function of GGAs is to recruit clathrin on the TGN which is crucial for the formation of clathrin-coated vesicles. There are two clathrin-binding sites identified in the hinge domain which are lost in GGA1t. Consistently, GGA1t did not interact with clathrin and was unable to recruit clathrin on the TGN. Therefore, expression of GGA1t may disrupt the formation of clathrin-coated vesicles (Fig. 7).

Fourth, GGAs are well defined Golgi-localized proteins and their Golgi localization is, at least in part, attributed to the interaction with activated ARF1. The ARF1-binding site has been mapped to the N-terminal portion of the GAT domain which remains intact in GGA1t. Consistently, we have found that GGA1t and GGA1 have very much similar Golgi localization and both are colocalized with GGA3 as revealed by confocal microscopy. It is most likely that GGA1t is able to interact with ARF1, which enhances its localization to the Golgi. In addition, we have previously shown that expression of ARF1 mutants markedly inhibits the cell surface transport of $\alpha_{2B}$-AR and that ARF1 directly interacts with the C-terminus of the receptor which may form a unique transport machinery for the forward trafficking of the receptor (Fig. 7). Although GGA1t interacts with ARF1 and ARF1 interacts with the receptor, these interactions may not be able to form the clathrin-coated vesicles due to the lack of clathrin binding as discussed above. It is reasonable to speculate that GGA1t expression will block the function of endogenous ARF1 which is essential for the transport of the receptor at many different steps, including ER-Golgi and post-Golgi transport13.

Physiological significance of spliced GGA variants is not clear at this point. It has been shown that a spliced GGA2 variant containing the N-terminal VHS domain is able to bind to the cytoplasmic domain of sortilin23 and that the short isoform of GGA3 is predominantly expressed in different cell lines and tissues38. Our data suggest that GGA1t functions as a dominant negative regulator for the cell surface transport of a GPCR. One can imagine that expression of these dominant negative GGA isoforms will maintain the transport of the receptors at a relatively low level, which can be easily modulated in response to environmental insults by either enhancing the expression of functional GGAs or reducing the expression of their dominant-negative isoforms.

GPCRs represent therapeutic targets of approximately one-third of the drugs on the market to treat human diseases39. However, the regulatory mechanisms underlying their targeting to the cell surface, the functional
destination for most GPCRs, are largely unexplored. Recent studies have identified a number of specific structural determinants, highly conserved motifs and regulatory proteins (such as Rab GTPases, TBC proteins, ER chaperones, receptor interacting proteins and kinases) to coordinate GPCR export from the ER and the Golgi and transport to the cell surface.\(^{11,17,40–50}\) Identification of GGAs, a family of proteins involved in the transport from the TGN to the endosomal compartment, as well as their naturally occurring mutants to be involved in regulation of the cell surface transport of GPCRs, suggests a novel trafficking function for the GGA family proteins, which may provide an important foundation for the development of novel therapeutic strategies by targeting GPCR anterograde trafficking processes.

**Materials and Methods**

**Materials.** Antibodies against GFP, myc, clathrin and phospho-specific ERK1/2 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-Erk antibodies detecting total ERK1/2 expression were from Cell Signaling Technology, Inc. (Beverly, MA). \(^{[3H]}\)-RX821002 (63 Ci/mmol) was purchased from Perkin Elmer Life Sciences (Waltham, MA). All other materials were obtained as described elsewhere.\(^{35,36}\)

**Plasmid constructions.** \(^{3}α_{2B}\)-AR tagged with three HA (YPYDVPDYA) at its N-terminus in the pCDNA3.1(+) vector was generated as described previously.\(^{3}GGA1\) cDNA was cloned from HEK293 cells into the pCMV-Myc vector at the restriction sites of EcoRI and Xhol (forward primer, 5′-GGGAATCCGGGAGCCCGCGATGGAGCCGGAG-3′ and reverse primer, 5′-CCGCTGAGCTAGAGGCTACCCAGGTTTC-3′). \(^{3}\) GGA1 tagged with Venus and Rluc8 were generated in the pEFGP-C1 vector as described previously.\(^{35,36}\) A similar strategy was used to generate GFP-GGA1t. To generate GFP-tagged GGA1 hinge domain (303–513 residues) and GGA1t hinge domain, each domain was generated by PCR and then cloned into the pEFGP-C1 vector. To generate GGA1 and GGA1t tagged with Venus and Rluc8, each was amplified by PCR and then cloned into the Venus-C1 and Rluc8-C1 vectors at the restriction sites of Xhol and EcoRI. The GST fusion protein construct of the third intracellular loop (ICL3, 205–369 residues) of \(^{3}α_{2B}\)-AR was generated using the pGEX-4T-1 vector as described previously.\(^{3,14}\) The sequence of each construct used in this study was verified by restriction mapping and nucleotide sequence analysis.

**Cell culture and transient transfection.** HEK293 and HeLa cells were cultured in Dulbecco’s Modified Eagle’s medium (DMEM) with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Transient transfection of cells was carried out using Lipofectamine 2000 reagent (Invitrogen) as described previously.\(^{32}\)

**Generation of inducible cell lines expressing \(^{3}α_{2B}\)-AR.** The Tet-On 3G Tetracycline Inducible Gene Expression System (Clontech Laboratories, Inc.) was utilized to generate stable cell lines inducibly expressing HA-\(^{3}α_{2B}\)-AR in HEK293 cells as described previously.\(^{17,35}\) Briefly, HA-\(^{3}α_{2B}\)-AR was cloned into the pTRE3G-TRES vector at the BglII and ClaI restriction sites and co-transfected with the PLKO.1 vector. HEK293 and HeLa cells were transfected with \(^{3}α_{2B}\)-AR in HEK293 cells as described previously.\(^{17,35}\) Briefly, HA-\(^{3}α_{2B}\)-AR was cloned into the pTRE3G-TRES vector at the BglII and ClaI restriction sites and co-transfected with the PLKO.1 vector. HEK293 cells using Lipofectamine 2000 reagent (Invitrogen) as described previously.\(^{32}\)

**Fluorescence microscopy.** The subcellular localization of \(^{3}α_{2B}\)-AR, GGAs and clathrin was visualized by fluorescence microscopy as described previously.\(^{32}\) Briefly, HEK293 cells inducibly expressing HA-\(^{3}α_{2B}\)-AR were transfected with 2 µg of GGA-GFP for 24 h. After induction with doxycycline at 40 µg/ml for 5 min and blocked with 5% normal donkey serum for 1 h. For other co-localization studies, HeLa cells were transiently transfected for 36 h, permeabilized and blocked as described above. The cells were then incubated with antibodies against HA (1:500 dilution), Myc (1:500 dilution) or clathrin (1:100 dilution) for 1 h. After washing, the cells were incubated with Alexa Fluor 546-labeled secondary antibody (1:2000 dilution) for 1 h. The images were captured using a confocal microscope (Zeiss LSM780) and a 63× objective.

**Intact live cell ligand binding.** The cell surface expression of \(^{3}α_{2B}\)-AR was measured by ligand binding of intact live cells using \(^{[3H]}\)-RX821002 as described.\(^{14}\) Briefly, HEK293 cells expressing HA-\(^{3}α_{2B}\)-AR were transiently transfected with control vector, myc-GGA1 or myc-GGA1t for 24 h. The cells were split into 12-well plates and incubated with doxycycline (40 ng/ml) for additional 24 h. The cells were then incubated with DMEM plus \(^{[3H]}\)-RX821002 (20 nM) in a total volume of 400 µl for 90 min and then washed with ice-cold DMEM to remove excess radioligands. The retained radioligands were extracted by digestion in 1 M NaOH for 2 h. The radioactivity was counted by liquid scintillation spectrometry. For measurement of \(^{3}α_{2B}\)-AR internalization, HEK293 cells expressing \(^{3}α_{2B}\)-AR were cultured on 6-well dishes and transfected with control or GGA1t with 1 µg of arrestin-3 for 24 h. After induction with doxycycline, the cells were starved for 3 h and then stimulated with epinephrine (100 µM) for different time periods. The cells were washed 3 times and the cell surface expression of \(^{3}α_{2B}\)-AR was measured by intact cell ligand binding.

**Flow cytometry.** Total \(^{3}α_{2B}\)-AR expression was measured by flow cytometry as described previously.\(^{9,51}\) Briefly, HEK293 cells expressing HA-\(^{3}α_{2B}\)-AR were suspended in PBS containing 1% fetal calf serum at a density of 4 × 10⁶ cells/ml and permeabilized with 0.2% Triton X-100 in PBS for 5 min on ice. The cells were then incubated with high affinity anti-HA-fluorescein (3F10) at a final concentration of 2 µg/ml at 4°C for 30 min. After washing with 0.5 ml of PBS twice, the cells were re-suspended and the fluorescence was analyzed on a flow cytometer (Dickinson FACSCalibur).
**Measurement of ERK1/2 activation.** Inducible HEK293 cells expressing α2B-AR were cultured in 6-well dishes and transfected for 24 h and incubated with tetracycline at 40 ng/ml for 24 h. After starvation for at least 3 h, the cells were then stimulated with UK14304 for 5 min. ERK1/2 activation was determined by immunoblotting using phospho-specific ERK1/2 antibodies as described previously23.

**Co-IP assays.** HEK293 cells were cultured on 100-mm dishes and transfected with 5 μg of Myc-GGA1 together with 5 μg of pEGFP-C1, GFP-GGA1 or GFP-GGA1t for 48 h. The cells were lysed with 500 μl of lysis buffer (50 mM Tris–HCl, pH 7.4, 140 mM NaCl, 1% Nonidet P-40, and complete Mini protease inhibitor mixture). After rotation for 1 h at 4 °C, the samples were centrifuged at 12,000 g for 15 min. To remove non-specific binding proteins, the cell lysates were incubated with 2 μg of normal mouse IgG and Dynabeads protein G (Life Technologies, Oslo, AS). The supernatants were then incubated with 2 μg of anti-Myc antibodies (Santa Cruz, CA) overnight at 4 °C followed by incubation with 30 μl of Dynabeads protein G for 1 h. The resin was collected and washed four times each with 1 ml of lysis buffer. Immunoprecipitated proteins were eluted with 30 μl of 1x SDS-gel loading buffer and separated by SDS-PAGE. Myc- and GFP-tagged GGA in the immunoprecipitate were detected by immunoblotting using anti-Myc and anti-GFP antibodies, respectively. To study the interaction of GGA1 and GGA1t with endogenous clathrin, HEK293 cells were transfected GFP-GGA1 or GFP-GGA1t and immunoprecipitated with anti-GFP antibodies. GGA and clathrin in the immunoprecipitate were detected by immunoblotting using anti-GFP and anti-clathrin antibodies, respectively.

**BRET assays.** Live cell-based BRET assays were carried out as described previously14,53,54. HEK293 cells were cultured on 6-well dishes and transfected with 0.1 μg of GGA-Rluc8 and 1.5 μg of GGA-Venus for 24 h. The cells were transferred to black 96-well plates. Coelenterazine h (5 mM) was added to all wells immediately prior to making measurements. Raw BRET signals were calculated by dividing the emission intensity at 520–545 nm by the emission intensity at 475–495 nm. Net BRET was this ratio minus the same ratio measured from cells expressing only the BRET donor (Rluc8).

**GST fusion protein pulldown assays.** GST fusion proteins were purified as described previously8,14. Purified GST fusion proteins were used immediately or stored at 4 °C for no longer than 3 days. GST fusion proteins tethered to the glutathione resin were incubated with total cell lysates in 500 μl of buffer (20 mM Tris–HCl, pH 7.5, 1% NP-40, 140 mM NaCl and 1 mM MgCl2) at 4 °C for 4–6 h. The resin was washed 4 times with 0.5 ml of binding buffer and the retained proteins were solubilized in SDS-gel loading buffer and separated by SDS-PAGE. Proteins bound to GST fusion proteins were detected by immunoblotting.

**Statistical analysis.** Differences were evaluated using Student’s t-test, and p < 0.05 was considered as statistically significant. Data are expressed as the mean ± S.E.

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Author Contributions
M.Z., C.L. and G.W. conceived and designed the experiments. M.Z., X.X., C.L., W.H., N.X. and G.W. performed the experiments and analyzed the results. M.Z. and G.W. wrote the manuscript with the input of all authors.

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