Regulation of $\alpha_{2B}$-Adrenergic Receptor Cell Surface Transport by GGA1 and GGA2

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The molecular mechanisms that control the targeting of newly synthesized G protein-coupled receptors (GPCRs) to the functional destinations remain poorly elucidated. Here, we have determined the role of Golgi-localized, $\gamma$-adaptin ear domain homology, ADP ribosylation factor-binding proteins 1 and 2 (GGA1 and GGA2) in the cell surface transport of $\alpha_{2B}$-adrenergic receptor ($\alpha_{2B}$-AR), a prototypic GPCR, and studied the underlying mechanisms. We demonstrated that knockdown of GGA1 and GGA2 by shRNA and siRNA significantly reduced the cell surface expression of inducibly expressed $\alpha_{2B}$-AR and arrested the receptor in the perinuclear region. Knockdown of each GGA markedly inhibited the dendritic expression of $\alpha_{2B}$-AR in primary cortical neurons. Consistently, depleting GGA1 and GGA2 attenuated receptor-mediated signal transduction measured as ERK1/2 activation and cAMP inhibition. Although full length $\alpha_{2B}$-AR associated with GGA2 but not GGA1, its third intracellular loop was found to directly interact with both GGA1 and GGA2. More interestingly, further mapping of interaction domains showed that the GGA1 hinge region and the GGA2 GAE domain bound to multiple subdomains of the loop. These studies have identified an important function and revealed novel mechanisms of the GGA family proteins in the forward trafficking of a cell surface GPCR.
of clathrin-coated vesicles. The C-terminal GAE domain interacts with a number of accessory proteins regulating GGA-mediated TGN-to-endosome transport37–46.

Our laboratory is interested in dissecting the mechanisms of anterograde trafficking of GPCRs. We have recently demonstrated that GGA3 is required for the TGN-to-cell surface transport of α2B-adrenergic receptor (α2B-AR), a prototypic member of the GPCR superfamily, and that the function of GGA3 in modulating α2B-AR export is mediated through its VHS domain interaction with the receptor, providing the first evidence implicating a role of the GGA family proteins in GPCR trafficking47. Here we have expanded these studies to define the role of GGA1 and GGA2 in α2B-AR cell surface export and elucidate the underlying mechanisms. We have found that all three GGAs are equally important in regulating the cell surface export of α2B-AR and more interestingly, three GGAs physically associate with the receptor via distinct domains. These studies have revealed novel mechanisms of the GGA-mediated cell surface GPCR trafficking.

Results
Depletion of GGA1 and GGA2 by shRNA and siRNA attenuates the cell surface transport of inducibly expressed α2B-AR. We have generated stable cell lines by using the Tet-On 3G inducible expression system to drive the expression of HA-α2B-AR in HEK293 cells and utilized these inducible cells to define the function of GGA3 in the cell surface transport of newly synthesized α2B-AR47. In the current study, we determined the role of GGA1 and GGA2. HEK293 cells were transfected with previously characterized shRNAs targeting GGA1 and GGA2 (Fig. 1A) and the effect of depleting individual GGAs on the numbers of α2B-AR at the cell surface was quantified by intact cell ligand binding assays using the cell nonpermeable radioligand [3H]-RX821002 after doxycycline induction for different time periods. shRNA-mediated knockdown of GGA1 and
2B-AR in the primary cultures of neurons. For this purpose, α2B-AR ICL3 interaction with GGA1 and GGA2 is direct. These data also suggest that both GGA1 and GGA2 interact the α2B-AR ICL3 with comparable efficiencies.

Depletion of GGA1 and GGA2 inhibits α2B-AR expression in the dendrites of cortical neurons. As α2B-AR plays an important role in regulating the sympathetic nervous system, we addressed the question if GGA1 and GGA2 could modulate the transport of α2B-AR in the primary cultures of neurons. For this purpose, the cortical neurons were prepared from embryonic rat pups and transfected with α2B-AR-GFP together with siRNA targeting individual GGAs. The effect of siRNA-mediated knockdown of GGAs on the expression of α2B-AR in the cortical neurons was measured by confocal microscopy. α2B-AR-GFP was expressed in the cell body of cortical neurons with or without GGA knockdown. However, its expression in the dendrites was markedly reduced by GGA1 siRNA with an average reduction of 76% as compared with neurons with normal expression of GGA1 (Fig. 3A and C). Knockdown of GGA2 also dramatically reduced the dendritic expression of α2B-AR in primary neuronal cultures (Fig. 3B and C).

Knockdown of GGA1 and GGA2 inhibits α2B-AR-mediated signaling. To determine if GGA-mediated α2B-AR trafficking could modulate the function of the receptor, we measured the effect of GGA1 and GGA2 knockdown on the activation of mitogen-activated protein kinases (MAPK) ERK1/2 and the reduction of cAMP production in HEK293 cells. Consistent with the reduction of the cell surface expression of α2B-AR, ERK1/2 activation in response to UK14304 stimulation was significantly inhibited by approximately 50% by shRNA targeting GGA1 and GGA2 as compared to cells transfected with control shRNA (Fig. 4A and B). Consistently, shRNA-mediated knockdown of GGA1 and GGA2 reduced α2B-AR-mediated inhibition of cAMP production in response to forskolin stimulation (Fig. 4C).

Differential interaction of GGA1 and GGA2 with α2B-AR. We have previously shown that GGA3 interacts with α2B-AR and the interaction is mediated through the VHS domain of GGA3 and the third intracellular loop (ICL3) of the receptor21. To elucidate the possible molecular mechanisms underlying the function of GGA1 and GGA2 in α2B-AR export, we determined if GGA1 and GGA2 could interact with the receptor in co-immunoprecipitation and GST fusion protein pulldown assays. HEK293 cells stably expressing α2B-AR were transiently transfected with myc-GGA1 or myc-GGA2 followed by immunoprecipitation using α2B-AR antibodies. GGA2 was clearly detected in the immunoprecipitates of α2B-AR antibodies, whereas GGA1 was undetectable in the immunoprecipitates (Fig. 5A).

In GST fusion protein pulldown assays, the ICL1, ICL2, ICL3 and the C-termius of α2B-AR were generated as GST fusion proteins (Fig. 5B) and incubated with cell lysates expressing myc-GGA1 or myc-GGA2. The GST-ICL3 strongly interacted with GGA2, whereas the ICL1, ICL2 and C-termius GST fusion proteins did not (Fig. 5C). Interestingly, although GGA1 did not associate with α2B-AR in co-immunoprecipitation assays, it interacted with the ICL3, but not with the ICL1, ICL2 and the C-termius in GST fusion protein pulldown assays (Fig. 5C).

We next determined if GGA1 and GGA2 were able to directly interact with the α2B-AR ICL3. In this experiment, GGA1 and GGA2 were tagged with the epitope His and purified (Fig. 5D). GST-ICL3 fusion proteins, but not GST alone, bound to His-tagged GGA1 and GGA2 in GST fusion protein pulldown assays (Fig. 5E). These data indicate that the α2B-AR ICL3 interaction with GGA1 and GGA2 is direct. These data also suggest that both GGA1 and GGA2 interact the α2B-AR ICL3 with comparable efficiencies.

Identification of interaction domains of GGAs and α2B-AR. To define the domains of GGA1 and GGA2 interacting with α2B-AR, their VHS, GAT, hinge and GAE domains were tagged with GFP. Confocal microscopy showed that full length GGA1 and GGA2 and their GAT domains were mainly localized to the Golgi whereas the hinge, the VHS and GAE domains were largely expressed in the cytoplasm. In addition, the VHS and GAE domains were also found to be partially expressed in the nuclear compartment (Fig. 6A). The GGA hinge domain, but not the VHS, GAT and GAE domains, strongly bound to GST-ICL3 fusion proteins, whereas
Figure 2. Inhibition of cell surface expression of $\alpha_{2B}$-AR by siRNA-mediated depletion of GGA1 and GGA2. (A) siRNA-mediated depletion of GGA1 and GGA2 in HEK293 cells. (B) Effect of siRNA-mediated knockdown of GGA1 and GGA2 on the cell surface expression of $\alpha_{2B}$-AR. HEK293 cells inducibly expressing $\alpha_{2B}$-AR were transfected with control siRNA or siRNA targeting GGA1 and GGA2 and incubated with doxycycline as described in legends of Fig. 1B. The average specific binding of [3H]-RX821002 from cells without siRNA transfection and treated with doxycycline for 28 h was 34,423 $\pm$ 563 cpm per well. (C) Effect of combination knockdown of GGA1, GGA2 and GGA3 on the cell surface expression of $\alpha_{2B}$-AR in HEK293 cells. (D) Effect of knockdown of GGA1, GGA2 and GGA3 on the Golgi structure. HEK293 cells were transfected with control or GGA siRNA for 48 h and then stained with antibodies against GM130 (1:200 dilution) and p230 (1:100 dilution) overnight. Scale bar, 10 $\mu$m. (E) Effect of GGA1 and GGA2 knockdown on total $\alpha_{2B}$-AR expression. HEK293 cells inducibly expressing $\alpha_{2B}$-AR were transfected with control or GGA shRNA or siRNA for 24 h and incubated with doxycycline (40 ng/ml) for another 24 h. The overall $\alpha_{2B}$-AR expression was measured by flow cytometry following staining with HA antibodies in permeabilized cells (n = 3). (F) Effect of GGA1 and GGA2 knockdown on the internalization of $\alpha_{2B}$-AR. HEK293 cells stably expressing $\alpha_{2B}$-AR were transfected with arrestin-3 and control or GGA shRNA and incubated with doxycycline as described above. The cells were then stimulated with epinephrine (100 $\mu$M) for 10, 20 and 30 min (n = 3). The cell surface expression of $\alpha_{2B}$-AR was determined by intact cell ligand binding using [3H]-RX821002. The data are presented as the mean ± S.E. of at least three individual experiments in (B,C,E,F). *p < 0.05 versus respective control.
Figure 3. Effect of GGA1 and GGA2 depletion on the dendritic expression of α2B-AR in primary cortical neurons. (A) Effect of GGA1 knockdown on α2B-AR expression in the dendrites of primary cortical neurons. The cortical neurons were transfected with α2B-AR-GFP together with GGA1 siRNA at DIV 5. Two days after transfection, the neurons were stained with antibodies against GGA1. The distribution of α2B-AR was visualized by confocal microscopy. (B) Effect of GGA2 knockdown on the dendritic expression of α2B-AR. The data shown are representative images in at least 4 individual experiments. Arrows indicate the expression of GGA1 or GGA2. Scale bars, 20 μm. (C) Quantitative data shown in (A,B) (n = 17). α2B-AR expression in the dendrites was determined by measuring the GFP signal. *p < 0.05 versus control.
the GGA2 GAE domain, but not the VHS, the GAT and the hinge domains, was found to interact with the ICL3 of α₂B-AR (Fig. 6B). These data demonstrate that the α₂B-AR ICL3 specifically interacts with the hinge domain of GGA1 and the GAE domain of GGA2.

To further map the GGA1 and GGA2-binding sites in the α₂B-AR ICL3, the ICL3 was progressively deleted and generated as GST fusion proteins. The C-terminal portion R285-E369 strongly interacted with the GGA1 hinge and the GGA2 GAE whereas the N-terminal portion K205-P284 did not (Fig. 7A). Furthermore, the GST fusion proteins encoding R285-C326, N327-E369 and L339-Q358 interacted with the GGA1 hinge domain and the GGA2 GAE domain whereas the fragments N327-L348 and G349-369 did not (Fig. 7A). These data demonstrate that there are two binding sites for GGA1 and GGA2, one located in the region R285-C326 and the other in the region L339-Q358 (Fig. 7B).

Discussion

Three GGA family proteins are well-characterized adaptor proteins for clathrin-coated vesicles that transport cargo proteins specifically from the TGN to the endosomal compartment. We have recently demonstrated that GGA3 depletion attenuated the cell surface transport of α₂B-AR and arrested the receptor in the Golgi/TGN compartment47. These data provide the first direct evidence implicating a role for the GGA family proteins in controlling the cell surface GPCR transport. Our current studies have shown that, similar to GGA3, knockdown of GGA1 and GGA2 significantly reduced the cell surface expression of α₂B-AR in cells and primary neurons as quantified by intact live cell ligand binding and direct visualization of receptor subcellular localization. These data indicate that all three GGAs are involved in the anterograde cell surface traffic of α₂B-AR.

There are several interesting points regarding the regulation of cell surface transport of α₂B-AR by the GGA family proteins. First, as depleting GGA1, GGA2, and GGA3 individually or in combination similarly inhibited the cell surface transport of α₂B-AR, three GGAs are equally important in mediating the export of newly synthesized receptor. These data also suggest that the anterograde transport of α₂B-AR requires all three GGAs and
the lack of any one GGA will disrupt the transport. Second, inhibitory effects on the cell surface \(\alpha_{2B}\)-AR export caused by depleting individual GGAs were moderate (less than 40%). One possible explanation for this phenomenon is that there are multiple pathways to direct \(\alpha_{2B}\)-AR export trafficking and GGAs mediate only one of these pathways. It should be pointed out that the data described here do not provide sufficient evidence indicating that

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**Figure 5. Interaction of \(\alpha_{2B}\)-AR with GGA1 and GGA2.** (A) Interaction of \(\alpha_{2B}\)-AR with GGA1 and GGA2 in co-immunoprecipitation assays. HEK293 cells stably expressing HA- \(\alpha_{2B}\)-AR were transfected with control vector or myc-tagged GGA1 and GGA2. The receptors were immunoprecipitated with \(\alpha_{2B}\)-AR antibodies. The amounts of GGA1 and GGA2 (upper panel) and \(\alpha_{2B}\)-AR (lower panel) were determined by immunoblotting using myc and \(\alpha_{2B}\)-AR antibodies, respectively. Lysate - 1% of total input. Similar results were obtained in three experiments. (B) Sequences of the ICL1, ICL2, ICL3 and C-terminus (CT) of \(\alpha_{2B}\)-AR (upper panel) and Coomassie blue staining of purified GST fusion proteins (low panel). The calculated molecular weights of GST and the ICL1, ICL2, ICL3, and CT GST fusion proteins are 27,898, 27,422, 28,070, 43,779 and 29,348 daltons, respectively. (C) Interaction of different intracellular domains of \(\alpha_{2B}\)-AR with GGA1 and GGA2. Myc-tagged GGA1 and GGA2 were expressed in HEK293 cells and total cell homogenates were incubated with GST fusion proteins. Bound GGAs were revealed by immunoblotting using anti-myc antibodies. (D) Purified His-tagged GGA1 and GGA2. The molecular weight (MW) markers (KDa) are indicated on the left. (E) Direct interaction of the \(\alpha_{2B}\)-AR ICL3 with GGA1 and GGA2. Purified His-tagged GGA1 and GGA2 were incubated with GST-ICL3 fusion proteins and bound GGAs were detected by immunoblotting using anti-His antibodies. Similar results were obtained in at least three separate experiments. Lysate – 5% of total input. Similar results were obtained in at least 3 experiments.
α2B-AR transport from the Golgi/TGN to the cell surface is direct. As proteins destined for the plasma membrane can be transported either directly from the Golgi to the plasma membrane or from the Golgi through the recycling endosomal compartment to the plasma membrane, it is possible that α2B-AR targeting to the cell surface passes through the recycling endosomal compartment. In support of this possibility, GGA3 was shown to modulate the transport of internalized Met receptor tyrosine kinase from the recycling endosomes.

Third, GGA knockdown markedly inhibited the expression of α2B-AR in the dendrites of primary cortical neurons, implicating that GGAs may play a more important role in the dendritic transport of α2B-AR in the native neurons. Fourth, consistent with the reduction of cell surface receptor expression, knockdown of individual GGAs suppressed α2B-AR-mediated signaling measured as ERK1/2 activation and cAMP reduction, suggesting that GGAs modulate not only the cell surface trafficking but also the function of the receptor.

Another important finding presented here is that we have elucidated novel mechanisms underlying the function of the GGA family proteins in the cell surface transport of α2B-AR. It has been well described that the function of GGAs in sorting proteins into the TGN-to-endosome pathway is tightly controlled by their VHS domain interaction with the DxxLL-type motifs of cargo proteins. These proteins include cation-dependent and cation-independent mannose 6-phosphate receptors, sortilin, sorting-protein-related receptor, low-density lipoprotein receptor-related proteins and β-secretase.

We previously showed that the GGA3 VHS domain interacted with the ICL3 of α2B-AR. Here we found that GGA1 and GGA2 also interacted with the ICL3 of α2B-AR in GST fusion protein pulldown assays. Although the VHS domains are highly conserved amongst three GGAs, the VHS domain of GGA1 and GGA2 did not interact with α2B-AR. Interestingly, the α2B-AR-binding domains were identified as the hinge domain of GGA1 and the GAE domain of GGA2. Although our data have clearly shown that GGA2 and GGA3 were able to form complexes with α2B-AR in co-immunoprecipitation assays, GGA1 did not under the same experimental condition, suggesting that interactions of three GGAs with α2B-AR have different regulatory properties.

Nevertheless, our previous and current studies indicate that the GGA1 hinge domain, the GGA2 GAE domain and the GGA3 VHS domain are responsible for the interaction with α2B-AR (Fig. 7C). These data suggest that the interaction of α2B-AR with each GGA is highly specific. To the best of our knowledge, α2B-AR is the only cargo molecule identified thus far which is able to interact with distinct domains of three GGAs (Fig. 7C).
Consistent with the identification of different domains in three GGAs responsible for interaction with \( \alpha_{2B} \text{-AR} \), different GGAs may have different binding sites in the ICL3 of \( \alpha_{2B} \text{-AR} \). We have previously shown that the 3 R motif in the ICL3 of the receptor and the acidic motif EDWE located in the VHS domain of GGA3 are responsible for the interaction between the receptor and GGA3. \(^{47}\) Consistent with the fact that the VHS domains of GGA1 and GGA2 did not bind to \( \alpha_{2B} \text{-AR} \), or the \( \alpha_{2B} \text{-AR} \) ICL3, an alignment of three GGA VHS domains showed that the GGA1 and GGA2 do not have the GGA3-binding motif EDWE, but instead have the sequences LDWA and QDWS, respectively (data not shown). We have used the progressive deletion strategy to successfully map the binding sites of both GGA1 and GGA2 to the regions R285-C326 and L339-Q358. As the GGA-binding subdomains R285-C326 and L339-Q358 do not possess the D/EFGXØ and DxxLL-type motifs and thus, they may contain novel, yet unidentified, GGA-binding signals. Our unpublished data showed that deletion of the fragment R285-Q358 significantly reduced the interaction of \( \alpha_{2B} \text{-AR} \) with GGA2 in coimmunoprecipitation assays. However, the truncated receptor was unable to export to the cell surface. These data suggest that, in addition to binding to GGAs, the ICL3 fragment R285-Q358 may contain other signals important for receptor transport to the cell surface. Nevertheless, these data strongly demonstrate differential interactions of three GGAs with \( \alpha_{2B} \text{-AR} \) which are mediated through different domains/motifs in individual GGAs and the receptor (Fig. 7C).
Similar to $\alpha_{2B}$-AR, many GPCRs possess a large ICL3. In addition to heterotrimeric G proteins coupled to the receptors, a number of ICL3-interacting proteins have been identified and described to play a crucial role in regulating the phosphorylation, trafficking and signal initiation, propagation and termination of the receptors. $\alpha_{2B}$-AR ICL3 interacts with, among others, kinases, 14-3-3, spinophilin, the ubiquitin carboxy terminal esterase L1, ADP ribosylation factor 1 and Sec24C/D. Our previous reports and current studies have demonstrated that $\alpha_{2B}$-AR uses its ICL3 as a docking site for multiple GGAs. It is interesting to note that different proteins may bind to different ICL3 regions. For example, spinophilin binding sites were mapped to the extreme N-terminal and C-terminal ends of the ICL3 of $\alpha_{2B}$-AR, whereas the C-terminal portions of the loop is important for arrestin interaction. The identification of two subdomains in the ICL3 which are capable of binding to GGA1 and GGA2 suggests multiple contacting points between $\alpha_{2B}$-AR and GGA1 or GGA2. These data also imply that GGAs may interact with a specific three dimensional surface of the loop and these specific interactions form a unique transport machinery that drives forward trafficking of the receptor from the Golgi/TGN to the cell surface.

It has become increasingly apparent that mistrafficking of GPCRs which leads to the dysfunction of the receptors directly links to pathogenesis of human diseases, such as nephrogenic diabetes insipidus, retinitis pigmentosa and male pseudohermaphroditism. However, the molecular mechanisms of anterograde transport of the GPCR superfamily to their functional destinations are poorly understood. Our previous studies have identified several highly conserved motifs and regulatory proteins that are required for the cell surface export of $\alpha_{2B}$-AR, as well as other GPCRs, en route from the ER and the Golgi. Our previous and current studies have also clearly demonstrated an important role of the GGA family proteins in the cell surface targeting of nascent $\alpha_{2B}$-AR which is likely mediated through physical interactions. In addition to $\alpha_{2B}$-AR, GGA3 was shown to regulate the transport of $\alpha_{2A}$-AR, but not $\alpha_{1D}$-AR, suggesting that there is specificity of GGA3 for different GPCRs. As the GGA3-binding motif of $\alpha_{2B}$-AR is highly conserved in many GPCRs, such as some muscarinic and serotonin receptor types. GGA3 may regulate the cell surface transport of a group of GPCRs. However, it still remains unknown if GGA1 and GGA2 are involved in the cell surface export of other GPCRs. To further elucidate the function of the GGA family proteins in the trafficking of the GPCR superfamily will enhance our understanding of GPCR targeting process and may be used to design novel therapeutics for effective therapy of human diseases, involving abnormal trafficking and signaling of GPCRs.

Materials and Methods

Materials. Full length GGA1 and GGA2 tagged with myc at their N-termini were generously provided by Dr. Juan S. Bonifacino (Eunice Kennedy Shriver National Institute of Child Health and Human Development, NIH). Arrestin-3 was obtained from Dr. Jeffrey L. Benovic (Thomas Jefferson University). Antibodies against GGA1 were purchased from Abcam Inc. (Cambridge, MA). Antibodies against GGA2, GM130 and p230 were from BD Transduction Laboratories (San Diego, CA). Antibodies against GFP, myc and phospho-ERK were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-ERK antibodies detecting total ERK1/2 expression were purchased from Transduction Laboratories (San Diego, CA). Antibodies against GGA1, GGA2, GM130, p230, and arrestin-3 were obtained from Dr. Jeffrey L. Benovic (Thomas Jefferson University). Antibodies against GGA1 and GGA2, GM130 and p230 were from BD Transduction Laboratories (San Diego, CA). Antibodies against GFP, myc and phospho-ERK were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-ERK antibodies detecting total ERK1/2 expression were purchased from Transduction Laboratories (San Diego, CA). Antibodies against GGA1, GGA2, GM130, p230, and arrestin-3 were obtained from Dr. Jeffrey L. Benovic (Thomas Jefferson University).

Plasmid constructions. $\alpha_{2B}$-AR tagged with either GFP at its C-terminus in the pEGFP-N1 vector or three HA (YPYDVPDYA) at its N-terminus in the pcDNA3.1 (-) vector were generated as described previously. The GST fusion protein constructs coding the first (ICL1, 44–53 residues), the second (ICL2, 117–131 residues), and the third intracellular loops (ICL3, 205–369 residues), different lengths of the ICL3 (K205–P284, R285–C326, N327–E369, N327–L348, L339–Q359 and G349–E369), and the C-terminus (430–453 residues) of $\alpha_{2B}$-AR were cloned into the BamH1 and XhoI restriction sites of the pEGFP-N1 vector. Then, the XhoI restriction site was truncated by PCR and then cloned into the pEGFP-N1 vector. The sequence of each construct used in this study was verified by restriction mapping and nucleotide sequence analysis.

Cell culture, primary neuronal preparation 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. The transfection efficiency was estimated to be greater than 70% based on the GFP fluorescence. Neuronal cultures were prepared from the cortex of embryonic day 18 rat pups and grown on glass coverslips precoated with poly-L-lysine. After 4–5 days in vitro (DIV), the neurons were transfected with $\alpha_{2B}$-AR-GFP with or without co-transfection with GGA siRNA by Lipofectamine 2000. The use of the Tet-On 3 G Tetracycline Inducible Gene Expression System (Clontech Laboratories, Inc.) was utilized to generate stable cells inducibly expressing HA--$\alpha_{2B}$-AR in HEK293 cells as described previously. Intact cell ligand binding assays, immunoblotting and confocal microscopy were used to characterize inducible expression of $\alpha_{2B}$-AR at the cell surface. A cell line expressing 8.5 × 10^5 $\alpha_{2B}$-AR per cell after incubation with doxycycline at a concentration of 40 ng/ml for 24 h was utilized in the current study.
shRNA- and siRNA-mediated depletion of GGAs. shRNA targeting GGA1 (463AAGCTTCCAGATGCACTACC483) and GGA2 (1428ATACACCTCTGCTAAGTG1448) were kindly provided by Dr. Stuart Kornfeld (Washington University School of Medicine) as described25. For shRNA-mediated knockdown of GGA1 and GGA2, cells cultured on 6-well plates were transiently transfected with 2 μg of control shRNA or shRNA targeting individual GGAs for 24 h. The cells were split into 12 wells at a density of 5 × 10^5 cells per well and cultured for additional 24 h before measuring the cell surface expression of α₂B-AR in intact cell ligand binding assays. For siRNA-mediated knockdown of GGAs, siRNAs targeting GGA1 (173CACAGAGGAGGCGAT191), GGA2 (1291TGATATTGTTTCCGAGAA1310) and GGA3 (1703TTGACAGCTACGATAA1721) and a negative control med GC duplex were purchased from Invitrogen. HEK293 cells were cultured in 6-well dishes at a density of 1 × 10^6 cells/well for 24 h and transfected with control or GGA siRNA. The expression of GGA1 and GGA2 was measured by GGA isoform-specific antibodies.

Measurement of the cell surface and total expression of α₂B-AR. The cell surface expression of α₂B-AR was measured by ligand binding of intact live cells using [³H]-RX821002 as described25. Briefly, inducible HEK293 cells expressing α₂B-AR were cultured on 6-well dishes and transiently transfected as described above for 12 h. The cells were split into 12-plates and cultured for an additional 24 h. After induction with doxycycline at a concentration of 40 ng/ml for different time periods, the cells were incubated with DMEM plus [³H]-RX821002 at a concentration of 20 nM in a total volume of 400 μl for 90 min at room temperature. The non-specific binding of α₂B-AR was determined in the presence of rauwolscine (10 μM). The binding was terminated and excess radioligand eliminated by washing the cells with ice-cold DMEM. The retained radioligands were then extracted by digesting the cells in 1 M NaOH for 2h. The radioactivity was counted by liquid scintillation spectrometry. All radioligand binding assays were performed in triplicate. For measurement of α₂B-AR internalization, HEK293 cells stably expressing α₂B-AR were cultured on 6-well dishes and transfected with control or GGA shRNA together with 1 μg of arrestin-3 for 24 h. After starvation for 3h, the cells were stimulated with epinephrine at a concentration of 100 μM for different time periods. The cells were washed three times with cold phosphate buffered saline (PBS) and α₂B-AR expression at the cell surface was measured by intact cell ligand binding at 4°C as described above.

Total α₂-AR expression was measured by flow cytometry as described previously27. Briefly, HEK293 cells expressing HA-α₂B-AR were suspended in PBS containing 1% fetal calf serum at a density of 4 × 10^5 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 resuspended and the fluorescence was analyzed on a flow cytometer (Dickinson FACS Calibur).

Fluorescence microscopy. Fluorescence microscopic analysis of the subcellular localization of α₂B-AR was carried out as described previously27. Briefly, cells were grown on coverslips precoated with poly-L-lysine in 6-well plates and transfected with 50 ng of α₂B-AR-GFP together with 400 ng of GGA shRNA. The coverslips were mounted with prolong antifade reagent (Invitrogen) and images were captured using a LSM510 Zeiss confocal microscope equipped with a 63 × objective. To visualize the localization of α₂B-AR in primary neurons, the neuronal cultures were transfected with α₂B-AR-GFP with or without co-transfection with GGA siRNA for 2 days. The neurons were fixed, permeabilized and stained with GGA isoform specific antibodies at a dilution of 1:500. The amounts of α₂B-AR-GFP signal pixels in the dendrites were determined by using NIH Image J software as described previously28.

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Total α₂-AR expression was measured by flow cytometry as described previously27. Briefly, HEK293 cells expressing HA-α₂B-AR were suspended in PBS containing 1% fetal calf serum at a density of 4 × 10^5 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 resuspended and the fluorescence was analyzed on a flow cytometer (Dickinson FACS Calibur).

Fluorescence microscopy. Fluorescence microscopic analysis of the subcellular localization of α₂B-AR was carried out as described previously27. Briefly, cells were grown on coverslips precoated with poly-L-lysine in 6-well plates and transfected with 50 ng of α₂B-AR-GFP together with 400 ng of GGA shRNA. The coverslips were mounted with prolong antifade reagent (Invitrogen) and images were captured using a LSM510 Zeiss confocal microscope equipped with a 63 × objective. To visualize the localization of α₂B-AR in primary neurons, the neuronal cultures were transfected with α₂B-AR-GFP with or without co-transfection with GGA siRNA for 2 days. The neurons were fixed, permeabilized and stained with GGA isoform specific antibodies at a dilution of 1:500. The amounts of α₂B-AR-GFP signal pixels in the dendrites were determined by using NIH Image J software as described previously28.

Measurement of the cell surface and total expression of α₂B-AR. The cell surface expression of α₂B-AR was measured by ligand binding of intact live cells using [³H]-RX821002 as described25. Briefly, inducible HEK293 cells expressing α₂B-AR were cultured on 6-well dishes and transiently transfected as described above for 12 h. The cells were split into 12-plates and cultured for an additional 24 h. After induction with doxycycline at a concentration of 40 ng/ml for different time periods, the cells were incubated with DMEM plus [³H]-RX821002 at a concentration of 20 nM in a total volume of 400 μl for 90 min at room temperature. The non-specific binding of α₂B-AR was determined in the presence of rauwolscine (10 μM). The binding was terminated and excess radioligand eliminated by washing the cells with ice-cold DMEM. The retained radioligands were then extracted by digesting the cells in 1 M NaOH for 2h. The radioactivity was counted by liquid scintillation spectrometry. All radioligand binding assays were performed in triplicate. For measurement of α₂B-AR internalization, HEK293 cells stably expressing α₂B-AR were cultured on 6-well dishes and transfected with control or GGA shRNA together with 1 μg of arrestin-3 for 24 h. After starvation for 3h, the cells were stimulated with epinephrine at a concentration of 100 μM for different time periods. The cells were washed three times with cold phosphate buffered saline (PBS) and α₂B-AR expression at the cell surface was measured by intact cell ligand binding at 4°C as described above.

Total α₂-AR expression was measured by flow cytometry as described previously27. Briefly, HEK293 cells expressing HA-α₂B-AR were suspended in PBS containing 1% fetal calf serum at a density of 4 × 10^5 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 resuspended and the fluorescence was analyzed on a flow cytometer (Dickinson FACS Calibur).

Co-immunoprecipitation. HEK293 cells inducibly expressing HA-α₂B-AR were cultured on 100-mm dishes and transfected with 2 μg of GGA shRNA as described above. The cells were starved for at least 3 h before stimulation with 1 μM UK14304 for 5 min. Stimulation was terminated by addition of 1 × SDS-loading buffer. After solubilizing the cells, 20 μl of total cell lysates were separated by 12% SDS-PAGE. ERK1/2 activation was determined by measuring the levels of phosphorylation of ERK1/2 by phospho-specific ERK1/2 antibodies by immunoblotting29.
GST fusion protein pulldown assays. The GST fusion proteins were expressed in bacteria and purified using a glutathione affinity matrix as described previously. GST fusion proteins immobilized on the glutathione resin were either used immediately or stored at 4 °C for no longer than 3 days. Each batch of fusion proteins used in experiments was first analyzed by Coomassie Brilliant Blue R250 staining following SDS-PAGE. GST fusion proteins tethered to the glutathione resin were incubated with total cell lysates in 500 μl of binding buffer containing 20 mM Tris-HCl, pH 7.5, 1% NP-40, 140 mM NaCl, 1 mM MgCl₂ and 0.5% bovine serum albumin at 4 °C for 4–6 h. The resin was washed four 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.

To determine if GGAs could directly interact with α₂B-AR, GGAs and GGA2 were tagged with the epitope His at their N-termini in the pET-28 vector and purified by using His SpinTrap kit (GE Healthcare) as described previously. About 1 μg of purified Hi-GGAs was incubated with GST-ICl3 fusion proteins in 600 μl of binding buffer at 4 °C for 4 h and the retained His-GGAs were measured by immunoblotting using anti-His antibodies.

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**

G.W. conceived the study. M.Z., J.G., W.H., A.V.T. and G.W. performed experiments. M.Z. and G.W. wrote the manuscript.

**Additional Information**

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