Molecular determinants of the human $\alpha_{2C}$-adrenergic receptor temperature-sensitive intracellular traffic.

Catalin M. Filipeanu, Ashok K. Pullikuth, Jessie J. Guidry.

Affiliations:

Department of Pharmacology, College of Medicine, Howard University, Washington, DC 20059, USA: CMF

Department of Pharmacology and Experimental Therapeutics, Louisiana State University Health Sciences Center, New Orleans, Louisiana, 70112, USA: AKP, JJG

Department of Cancer Biology, Wake Forest University School of Medicine, Winston Salem, NC 27157: AKP

Louisiana State University Health Sciences Center Proteomics Core Facility, New Orleans, LA 70112, USA: JJG
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Corresponding author: Catalin M. Filipeanu, Department of Pharmacology, Howard University, tel: 202-806-6311, fax: 202-806-4453, email: catalin.filipeanu@howard.edu

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Abbreviations:

Adrenergic receptor – AR; GPCR – G protein-coupled receptors; VSMC – vascular smooth muscle cells; COPI - Coat protein complex I; AAA+ ATP-ases adenosine triphosphases associated with diverse cellular activities; Epac - the exchange protein directly activated by cyclic AMP;
Abstract.
Human $\alpha_{2C}$-AR is localized intracellularly at physiological temperature. Decreasing the environmental temperature strongly stimulates the receptor transport to the cell surface. In contrast, rat and mouse $\alpha_{2C}$-AR plasma membrane levels are less sensitive to decrease in temperature, whereas the opossum $\alpha_{2C}$-AR cell surface levels are not changed in these conditions. Structural analysis demonstrated that human $\alpha_{2C}$-AR has a high number of arginine residues in the third intracellular loop and in the C-terminus, organized as putative RXR motifs. Although these motifs do not affect the receptor subcellular localization at 37°C, deletion of the arginine clusters significantly enhanced receptor plasma membrane levels at reduced temperature. We found that this exaggerated transport of the human receptor is mediated by two functional arginine clusters, one in the third intracellular loop and one in the C-terminus. This effect is mediated by interactions with COPI vesicles, but not by 14-3-3 proteins. In rat $\alpha_{2C}$-AR, the arginine cluster from the third intracellular loop is shifted to the left, due to three missing residues. Reinsertion of these residues in the rat $\alpha_{2C}$-AR restored the same temperature-sensitivity as in the human receptor. Proteomic and co-immunoprecipitation experiments identified pontin as a molecule having stronger interactions with human $\alpha_{2C}$-AR compared to rat $\alpha_{2C}$-AR. Inhibition of pontin activity enhanced human receptor plasma membrane levels and signaling. Our results demonstrate that human $\alpha_{2C}$-AR has a unique temperature-sensitive traffic pattern within the GPCR class due to interactions with different molecular chaperones, mediated in part by strict spatial localization of specific arginine residues.
Introduction.

Thermoregulation is a highly regulated process in the homoeothermic mammals required to maintain the body temperature independent of ambient temperature (Morrison 2011; Romanovsky, 2014). The cutaneous circulation plays a major role in this process, functioning as a heat insulator below the epidermal surface (Johnson and Kellogg, 2010; Morrison 2011). Exposure to cold in the homoeothermic mammals leads to local vasoconstriction, a physiological response limiting the heat loss (Flavahan, 2008). As in all vascular beds, the cutaneous circulation is controlled by autonomic nervous system and local endogenous mediators (Morrison, 2011). However, in certain individuals the local vasoconstriction in response to cold is exaggerated and accompanied by triphasic color changes of the affected skin region: pallor, cyanosis and red flushing (Pope, 2007; Prete et al., 2014). These features define Raynaud Phenomenon, a disease described first by Maurice Raynaud in 1862 (Raynaud, 1862). Although Raynaud Phenomenon is regarded as a rare disease, in fact is a common clinical encounter affecting between 3-10% of the general population with higher incidence in women (Brand et al., 1997; Prete et al., 2014). Even if Raynaud Phenomenon was described 150 years ago, a specific treatment is still missing today. The therapy consists in palliative measures, associated with non-specific smooth muscle relaxants (calcium channel blockers, nitrates) and no specific biomarkers for early identification of the individuals susceptible to Raynaud Phenomenon were identified.

Several mechanisms have been proposed to mediate the exaggerated vasoconstriction in Raynaud Phenomenon, like enhanced endothelin levels, augmented serotonin release, or increased activation of the renin-angiotensin system, but the experimental findings do not support the involvement of these factors (Pope et al., 2000; Wood et al., 2006; Nguyen et al., 2010). In contrast, the role of α2-adrenergic receptors (α2-AR) in cold-induced vasoconstriction
was demonstrated in the mid-nineties, by results showing that vasospastic attacks in Raynaud Phenomenon patients were prevented by the $\alpha_2$-AR antagonist yohimbine, but not by the $\alpha_1$-AR blocker prazosin (Freedman et al., 1995). These studies in vivo were complemented by findings in isolated human skin arterioles and mouse or rat tail arteries, showing that the contractile response to moderate cooling is reduced by $\alpha_2C$-AR antagonists (Chotani et al., 2000; Bailey et al., 2004, Filipeanu et al., 2011a). Evaluation of the subcellular localization of $\alpha_2C$-AR in VSMC and fibroblasts confirmed that at 37°C this receptor accumulates in the endoplasmic reticulum (Daunt et al., 1997; Angelotti et al., 2010; Filipeanu et al., 2011). Interestingly, in cells with a neuronal phenotype, $\alpha_2C$-AR displayed strong plasma membrane localization, indicating that its intracellular trafficking is cell-dependent (Hurt et al., 2000). We have demonstrated that intracellular accumulation of $\alpha_2C$-AR at 37°C is not related to aberrant receptor internalization/recycling, but to defects in the export trafficking, which can be corrected by non-specific pharmacological chaperones like DMSO or glycerol (Filipeanu et al., 2011a). We also identified HSP90 as an interacting-partner of $\alpha_2C$-AR and we showed that inhibition of HSP90 activity increases the $\alpha_2C$-AR traffic to the cell surface at 37°C, but has no effect at 30°C. Further, we found that in VSMC from rat tail artery, exposure to 30°C decreased HSP90 (but not HSP70) cellular levels (Filipeanu et al., 2011a). These results indicate that fine interactions with specific molecular chaperones can be responsible for the temperature-dependent $\alpha_2C$-AR traffic.

$\alpha_2C$-AR belongs to the seven GPCR superfamily, the largest family of the human genome and the target for about 30-40 % of the current therapeutic drugs (Duvernay et al., 2005; Dong et al., 2007). In this class, certain GPCR were shown to be exported in an inefficient manner and only a fraction of the synthesized receptors reach the plasma membrane, like the $\delta$-opioid receptors and the human gonadotropin releasing hormone receptor (Petaja-Repo et al., 2000; Re et al., 2010).
The transport of GPCR to the plasma membrane is critically dependent by interactions at different steps with various accessory proteins, which are also called molecular chaperones (Dong et al., 2007). These accessory proteins are heterogeneous and can interact in a highly specific manner with individual GPCR, like AHA1 (a HSP90 co-chaperone) for CB1R (Filipeanu et al., 2011b). Also, the cellular levels of these accessory proteins can be cell-type specific and could be altered in the diseased state, therefore changing the expression, subcellular localization and the functional responses of different GPCR. Based on this concept, in the present work we further focused on the elucidation of the mechanisms modulating \( \alpha_{2C}-\text{AR} \) intracellular traffic and to identify interacting-partners which modulate the receptor temperature-dependent traffic.
Materials and Methods.

Cell Culture and transfection. HEK293T cells were from ATCC (Manassas, VA) and the experiments were performed in passages 7 to 20. The cells were cultured in Dulbecco’s modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (all the cell culture reagents being from Life Technologies, Carlsbad, CA). OK (opossum kidney cortex proximal tubule epithelial cells) were a gift from Dr. Guangyu Wu (Medical College of Georgia) and were grown in similar conditions as HEK293T cells. Human coronary vascular smooth muscle cells were a gift from Dr. T. Cooper Woods (Tulane University, New Orleans) and were routinely cultured in full SmGM-2 Growth Medium (Lonza, Walkersville, MD). Transient transfection of the HEK293T cells (70-80% confluent) was carried out in 6-well plates using LipofectAMINE 2000 (Life Technologies, Carlsbad, CA) for 6 h with a cDNA:liposomes ratio of 2.5:1 as described (Filipeanu et al., 2011a; Deshotels et al., 2014). Six hours later the cells were trypsinized and plated at a density of 10^6 cells/well in 6-well plates for western blot experiments, or 4 × 10^5 cells/well in 12-well plates for radioligand binding experiments and cAMP determination. Based on the examination under the fluorescent microscope about 85-90% of the cells were transfected in these conditions. Human coronary VSMC were transfected using Neon Electroporation System (Life Technologies Carlsbad, CA) with two pulses of 1475 V and 20 ms duration. The transfection efficacy in these conditions was found to be about 50%. The experiments were performed 72 h after electroporation with the cells being kept in SmGM-2 medium without growth supplements for the last 6 h.

Protein Interaction with α2C-AR Identification by LC-MS analysis. The experiments were carried as previously described (Filipeanu et al., 2011b). Cell lysates from human and rat α2C-AR transfected HEK293T cells were incubated overnight with 20 μl/mg anti-GFP Agarose (MBL
International Woburn, MA), and subsequently were harvested into 7 M urea, 2 M thiourea, 4%
3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 20% glycerol (Sigma,
St. Louis, MO). The resulting mixture was subjected to a brief centrifugal step before
determining protein concentration using Bradford (Bio-Rad, Hercules, CA) method. DIGE 2-
Dimensional gel electrophoresis labeling and analysis was performed as previously described
(Filipeanu et al., 2011b). Fluorophore-labeled protein gels were scanned using a Typhoon 9400
Variable Mode Imager (GE Healthcare, Piscataway, NJ) at 100 um resolution. DIGE CyDyes
(GE Healthcare, Piscataway, NJ) are detected using the following wavelength settings: Cy2,
excitation 488 nm, emission 520 nm; Cy3, excitation 532 nm, emission 580 nm; Cy5, excitation
633 nm, emission 670 nm. Spot detection and quantification were performed using DeCyder
differential analysis software DIA, Version 5.0 (GE Healthcare, Piscataway, NJ) and individual
gels were imported into DeCyder biological variation analysis BVA for t-test analysis. Spots of
interest were determined to be any difference of greater than 50% or a t-test p-value of less than
0.05. Gels were then post-stained with Sypro Ruby (Bio-Rad, Hercules, CA), and images were
captured again using a Typhoon 9400 Imager. Spots of interest were excised and trypsin
(Promega, Madison, WI) digested using the automated Spot Handling Workstation (GE
Healthcare, Piscataway, NJ).

Mass spectrometry data was acquired using a Thermo LTQ-XL linear ion trap mass spectrometer
(Thermo, Waltham, MA) coupled to an Eksigent nanoLC (Dublin, CA). Peptide samples were
loaded onto a Dionex C18 PepMap 100 trap column with dimensions of 300 μm (inside
diameter) × 5 mm (Dionex, Sunnyvale, CA) and were separated by a New Objective reversed-
phase C18 PicoFrit emitter with dimensions of 75 μm (inside diameter) × 10 cm (bed length)
with 15 μm tip size (part number PF7515-100-H002) (Woburn, MA). Peptides were loaded at
500 nL/min using a mobile phase of 2% acetonitrile and 0.1% formic acid and then eluted using a gradient of 5–40% acetonitrile and 0.1% formic acid over 16 min, with a ramp to 60% acetonitrile and 0.1% formic acid for 10 min, and finally a ramp to 95% acetonitrile and 0.1% formic acid for 10 min. A top-five data-dependent scan strategy was utilized. The MS1 scan range is between m/z 300 and 2000. The top five most abundant peptides in this MS1 scan were chosen for MS/MS. The MS/MS parameters are the following; isolation window is set to 2 Da, 35% relative collision energy (CID), dynamic exclusion enabled with repeat count set to 1, repeat duration of 30 s, and an exclusion size of 100 with an exclusion duration of 20 s.

Mass Spectrometry data was then analyzed by Proteome Discoverer 1.4 (Thermo, Waltham, MA) against the Human SwissProt database. Database searching was performed for b and y ion series and up to 1 missed trypsin cleavage. Precursor Mass Tolerance was set to 1.2 Daltons and Fragment Mass Tolerance set to 0.8 Daltons. Methionine Oxidation and Cysteine Carbamidomethylation were considered as possible modifications.

Antibodies and chemicals. The antibodies used in the present study were from the following sources: anti-α2C-AR antibody (ab46536) was from Abcam (Cambridge, MA), anti-GFP Agarose (D153-8) was from MBL International (Woburn, MA), anti-GFP (sc9996) anti-14-3-3ζ (sc-1019), anti-β-COP (sc-368581), anti-pontin (sc-393854), anti-β-actin (sc-69879), anti-phospho-ERK 1/2 (sc-7976) and anti-total ERK 1/2 (sc-5626) were from Santa Cruz (Dallas, TX), anti-mouse and anti-rabbit HRP-secondary antibodies were from Perkin-Elmer (Waltham, MA). Pontin shRNA lentiviral particles (sc-43543-V) and puromycin were obtained from Santa Cruz, whereas β-COP shRNA was from Life Technologies (Carlsbad, CA). Rottlerin was obtained from Sigma-Aldrich (St. Louis, MO) and forskolin and UK14304 were from Tocris (Minneapolis, MN).
Plasmid Constructions and generation of the α2C-AR mutants. GFP-tagged human α2C-AR and α2B-AR at their C-termini were generated by PCR after the stop codon was mutated, and the sequences restricted with HindIII/SalI in frame with GFP were ligated into the pEGFP-N1 vector (Clontech, Mountain View, CA), as described (Filipeanu et al., 2011a). Flag-tagged rat α2C-AR, a kind gift from Dr. Randy Hall (Emory University), was used to generate GFP-tagged rat α2C-AR by a similar procedure. As demonstrated previously (Filipeanu et al., 2011a) these GFP-tagged constructs have similar binding properties as not tagged receptors.

All receptor mutants were obtained using Quick Change site-directed mutagenesis kit (Agilent, Santa Clara, CA). The following primers were used to obtain the following mutants (mutation is shown in bold italics characters): human α2C-AR 240ATR242: 5’ gtg gcc aag ctg cgc acg ctc 3’; 3’ gtg gcc aag ctg gec acg ctc aag cgc acg ctc 5’. human α2C-AR 294RRAAR298: 5’ gca gcg ggc gag agg cgg cgc cgc acg cgc ttg cgg cgg 5’. human α2C-AR 306RRA 308 5’ cgg ggc ggg cgg cgg cgg cgg ggc ggc gag agg cgc acg cgc acg ctc 3’; 3’ cgg ggc ggc gag agg cgc acg cgc acg ctc 5’. human α2C-AR 359RRAAR364: 5’ tc ttc ctg tgc gcg gcc ggc ggc ggc gag agg cgg cgc acg cgc acg ctc 3’; 3’ tc ttc ctg tgc gcg gcc ggc ggc gag agg cgg cgc acg cgc acg ctc 5’.

rat α2C-AR 269GA 270: 5’ gtt cgg cgg cgg ggc ggc ggc ggc ggc ggc gag agg gag cgc acg cgc acg ctc ttc ctg cgg cgc cgc ggc ggc gag agg cgc acg cgc acg ctc 3’; 3’ cgg cgc acg cgc acg ctc ttc ctg cgg cgc cgc ggc ggc gag agg cgc acg cgc acg ctc 5’; rat α2C-AR 290A: 5’ gtg gag cgc gag cgc ggc ggc ggc ggc gag agg cgc acg cgc acg ctc ttc ctg cgg cgc cgc ggc ggc gag agg cgc acg cgc acg ctc 3’; 3’ cgg cgc acg cgc acg ctc ttc ctg cgg cgc cgc ggc ggc gag agg cgc acg cgc acg ctc 5’; rat α2C-AR 326G: 5’ cgg cgc acg cgc acg ctc ttc ctg cgg cgc cgc ggc ggc gag agg cgc acg cgc acg ctc ttc ctg cgg cgc cgc ggc ggc gag agg cgc acg cgc acg ctc 3’; 3’ cgg cgc acg cgc acg ctc ttc ctg cgg cgc cgc ggc ggc gag agg cgc acg cgc acg ctc 5’.
α<sub>2C</sub>-AR/α<sub>2B</sub>-AR chimeric construct was generated by inserting the selected sequence using BamH1 restriction sites introduced by PCR. The sequence of each construct was confirmed by restriction mapping and nucleotide sequence analysis (Integrated DNA Technologies, Coralville, IA).

Co-immunoprecipitation. Immuno-precipitation of the receptors was performed in similar manner as described (Filipeanu et al., 2011a; Deshotels et al., 2014). In brief, HEK293T cells were cultured on 10 cm<sup>2</sup> dishes and transfected at ~80% confluency with 10 μg of GFP-tagged human or rat α<sub>2C</sub>-AR for 48 h, FBS being omitted during the last 18 h. The cells were washed twice with PBS and lysed in 300 μl of lysis buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and Complete Mini protease inhibitor cocktail (Roche, Indianapolis, IN). After gentle rotation for 1 h, samples were centrifuged for 15 min at 14,000 × g and the supernatant was incubated with 50 μl of protein G Sepharose for 1 h at 4 °C to remove non-specific bound proteins. Samples were then incubated with 5 μg of anti-GFP antibodies overnight at 4 °C with gentle rotation followed by incubation with 50 μl of protein G sepharose beads for 5 h. Resin was collected by centrifugation and washed four times with 500 μl of lysis buffer. Immunoprecipitated receptors were eluted with 100 μl of 1× SDS-PAGE loading buffer, separated by 10% SDS-PAGE and visualized by immunoblotting using specific antibodies.

Western blotting. The cell lysates were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The signal was detected using ECL Plus (BioRad Laboratories, Hercules, CA) and a Fuji Film luminescent image analyzer (LAS-1000 Plus) and quantitated using the Image Gauge program (version 3.4, GE Healthcare, Piscataway, NJ).
Radioligand binding. The experiments were carried out as described (Filipeanu et al., 2006; Filipeanu et al., 2011a) on intact cells plated on 12-well plates at a density of 5 x 10^4 cells/well. The cells were serum starved after 24 h to prevent differential proliferation at different temperatures. Some cells were incubated at 30°C for 18 h prior the measurements. The determinations were performed at 4°C in 400 μl of DMEM containing 20 nM [3H]-RX821002. Then the medium was aspirated and the cells were washed three times with DMEM, digested with 1 M NaOH, and the bound radioactivity was determined in a β-scintillation counter. The non-specific binding determined in parallel experiments in the presence of non-radioactive rauwolscine (10 μM) represented less than 10% of the total radioactivity and it was subtracted from the presented results. In these experimental conditions, the internalization of the ligand is minimal (~5%) as shown by our previous experiments (Filipeanu et al., 2011a).

cAMP determinations. cAMP cellular levels were determined using cAMP enzyme immunoassay system (Cayman Chemical Company, Ann Arbor, MI) as described (Filipeanu et al., 2011a, b). HEK293T cells on 10 cm² plates at ~80% confluency were transfected with 3 μg α2C-AR receptor constructs. After six hours the cells were plated on 12-well plates at a density of 5 x 10^4 cells/well. Subsequently the cells were serum starved for 24 h and then incubated at 37°C or at 30°C for 18 h. One hour before stimulation the medium was changed to PBS supplemented with isobutylmethylxanthine (100 μM). Then the cells were incubated with 10^-8 M UK14304 for 5 min, followed by stimulation with forskolin (3 μM) for 15 min. The reactions were stopped by aspirating the medium and addition of 200 μl of acetic acid (4%). Twenty microliters of cell lysate was then transferred into microtitre plate and the cAMP levels were determined by ELISA according to the manufacturer protocol.
Statistical analysis. The experiments were repeated at least in three different cell passages. The statistical differences were evaluated using Student's t test with \( p < 0.05 \) was considered as statistically significant. Data are expressed as the mean ± S.D.
Results

Previous studies demonstrated that $\alpha_{2C}$-AR accumulates in the endoplasmic reticulum at 37°C and displays little localization at the plasma membrane, the common functional site of GPCR (Daunt et al., 1997 Angelotti et al., 2010; Filipeanu et al., 2011a). Reducing the environmental temperature to 28°C-30°C greatly enhanced the plasma membrane receptor levels. This particular receptor localization pattern was observed for both heterogeneously human $\alpha_{2C}$-AR transfected cell lines and in vascular smooth muscle cells from rodent tail artery or human cutaneous arteries which endogenously express this receptor subtype (Chotani et al., 2000; Bailey et al., 2004; Filipeanu et al., 2011a; Jeyaraj et al., 2012). However, during our previous investigation (Filipeanu et al., 2011a) we observed that the effects of low-temperature are higher on the translocation of human $\alpha_{2C}$-AR transfected HEK293T compared to endogenous rat $\alpha_{2C}$-AR in VSMC from rat tail artery. We analyzed in detail the plasma membrane receptor levels in human $\alpha_{2C}$-AR transfected HEK293T cells, in mouse and rat VSMC and in OK cell line by radioligand binding. The latter is an opossum kidney cell line and it was included because $\alpha_{2C}$-AR is expressed endogenously and it was the historically typical choice to study this receptor subtype before heterogenous transfection was available (Bylund et al., 1992). The incubation at 30°C was performed for 18 h, the time previously demonstrated by us to induce maximal translocation of $\alpha_{2C}$-AR to the cell surface in cell lines in these experimental conditions (Filipeanu et al., 2011a). This apparent time required for the development of the full response may appear slow in comparison with the fast reactions observed in Raynaud Phenomenon, probably because the experiments were performed in transfected cell lines which may mask the contribution of different multiple mechanism involved in the temperature-sensitive $\alpha_{2C}$-AR intracellular traffic. In these experimental conditions, the human $\alpha_{2C}$-AR plasma membrane levels were enhanced by
73 ± 9% after exposure to 30°C, whereas mouse and rat receptor cell surface levels were increased only 33 ± 6% and 29 ± 4%, respectively (Fig 1A). In stark contrast, exposure to low-temperature did not change α2C-AR plasma membrane levels in OK cell line (Fig 1A).

To test if the observed differences were caused by the expression in different cell lines or by intrinsic species differences in the receptor intracellular traffic, we determined the effects of low-temperature on the transfected human and rat α2C-AR in HEK293T cells and human coronary VSMC, which both do not express the endogenous receptor. In these cell lines we found that at 37°C, plasma membrane levels of human receptor were much lower compared to rat receptor levels, despite comparable total amount of expressed receptors (Fig 1B and 1C). Exposure to low-temperature had a much greater effect in enhancing human α2C-AR plasma membrane levels in both cell lines (73 ± 9% and 86 ± 11% in HEK293T and coronary VSMC) compared to rat α2C-AR (36 ± 6% and 31 ± 8%, respectively, Fig 1B). In fact, the effects of low-temperature on the rat α2C-AR plasma membrane levels were similar in rat tail VSMC artery, human coronary VSMC and HEK293T cells (compare Fig 1A and 1B). Also, confocal microscopy examination of subcellular localization demonstrated that rat α2C-AR has a more prominent plasma membrane localization compared to human receptor at 37°C in human coronary VSMC (Fig 1D). Thus, our data suggest that the temperature-sensitive α2C-AR transport to the cell surface is regulated in a specie-dependent manner.

In an attempt to determine the receptor characteristics underlying these traffic patterns we analyzed the amino acid sequence of α2C-AR in several species. No major differences were observed in the receptor structure in the extracellular loops and transmembrane domains between human, rat and mouse α2C-AR. (Fig 2A). Also, the amino acid composition of first and second intracellular loop were very similar in human, rat, mouse and opossum receptor (Fig 2A).
However, in the third intracellular loop and in the C-terminus, regions which are known to contain amino acid motifs essential for GPCR trafficking (Dong et al., 2007; Wu et al., 2012), several differences were clearly noticeable. First, many arginine rich clusters are present in the third intracellular loop and C-terminus of $\alpha_2$C-AR from higher mammals (Fig 2A). In contrast, the opossum receptor is missing the cluster from the C-terminus (Fig 2A) and its third intracellular loop has a higher percentage of alanine and histidine residues (Fig 2A). The long arginine clusters from rodent and human $\alpha_2$C-AR are organized as putative RXR motifs which when present in other proteins such motifs act as endoplasmic reticulum retention motif, preventing the transport of the proteins bearing these motifs to the cell surface. Indeed these motifs are largely absent in the $\alpha_2$B-AR third intracellular loop and C-terminus (Fig 2B), which despite sharing over 80% homology to $\alpha_2$C-AR, does not change its plasma membrane levels after exposure to low-temperature (Filipeanu et al., 2011a).

We hypothesize that these RXR motifs may be responsible for the temperature-dependent transport of $\alpha_2$C-AR to the plasma membrane. To test this hypothesis, we initially generated two human $\alpha_2$C-AR deletion mutants. In the first mutant ($\alpha_2$C-3iC), we deleted 125 amino acids from the third intracellular loop of human $\alpha_2$C-AR, removing the putative eight RXR motifs located at this level. In the second mutant ($\alpha_2$C-R9) we removed the last nine amino acids from the C-terminus, including the $^{454}$RRRR$^{458}$ sequence which contains three putative RXR motifs (Fig 2A, B). Surprisingly, in transfected HEK293T cells, at 37°C these deletion mutants have similar cell surface levels as the wild-type human $\alpha_2$C-AR (Fig 2B). However, at 30°C, both mutants displayed statistically significant higher plasma membrane levels (Fig 2B). Further, a mutant carrying both deletions has higher plasma membrane levels compared with single deletion mutants at 30°C, but the same levels as $\alpha_2$C-AR at 37°C (Fig 2B). These results suggest that at
37°C these RXR motifs are silent, but become functional at 30°C, preventing exaggerated receptor transport to the cell surface. Since we have previously shown that the α2B-AR traffic is not temperature-sensitive (Filipeanu et al., 2011a), we constructed a chimeric α2C-AR which contained the third intracellular loop and the C-terminus of α2B-AR (Fig 2B). The corresponding chimera further substantiated our conclusion, in that this construct had slightly elevated cell surface levels at 37°C, but exposure to low-temperature (30°C) had no effect on its plasma membrane levels (Fig 2B). These results taken together clearly implicate the RXR motifs in the third intracellular loop and the C-terminus of α2C-AR as being responsible for the temperature sensitive trafficking.

To precisely identify which arginine clusters modulate the α2C-AR temperature-dependent trafficking we performed progressive alanine mutagenesis. Surprisingly, individual disruption of any single RXR motif did not change the cell surface levels at 37°C or at 30°C (Table 1). These data suggest that these RXR motifs are redundant and multiple motifs are involved in the temperature-sensitive receptor trafficking. We further generated α2C-AR mutants having multiple RXR motifs disrupted throughout the sequence and found that only mutants with disrupted arginine clusters in the positions 293RRRRR298 and 453RRRRR458 have significantly enhanced plasma membrane receptor levels after exposure to low-temperature, without significant changes at 37°C (Table 1). In fact this pattern fully resembles the results obtained with α2C-3ic and α2C-R9 deletion individual and double mutants. Any additional mutation of other RXR motifs did not significantly change the cell surface levels of human α2C-AR at 37°C or at 30°C in HEK293T cells (Table 1). The plasma membrane α2C-AR levels are directly correlated with the effects of receptor stimulation on cAMP levels (Filipeanu et al., 2011a; Jevaraj et al., 2012). Accordingly, the effects of UK14304, an α2-AR agonist on forskolin-stimulated cAMP increases in α2C-AR
wild-type or $^{294}\text{RRRR}^{298}/^{454}\text{RRRR}^{458}$- $\alpha_{2C}$-AR transfected HEK293T cells were minimal at 37°C (Table 2). In contrast, the effects of $^{294}\text{RRRR}^{298}/^{454}\text{RRRR}^{458}$- $\alpha_{2C}$-AR stimulation in cells exposed to low temperature were significantly greater (Table 2). The observed differences in the plasma membrane receptor levels and in signaling abilities are not related to different total cellular expression levels of the receptor constructs as demonstrated by western blot (Supplementary Figure 1A). These data clearly demonstrate that the RXR motifs embedded in the arginine clusters from positions $^{294}\text{RRRR}^{298}$ and $^{454}\text{RRRR}^{458}$ clusters of human $\alpha_{2C}$-AR prevent the receptor transport at physiological temperature and this inhibitory effect is released at low-temperature resulting in enhanced traffic to the plasma membrane and augmented functional responses.

Two major mechanisms were proposed to underlie the endoplasmic reticulum retention by the RXR motifs, namely retrograde transport through COPI vesicles mediated by interactions with $\beta$-COP subunit and binding to 14-3-3 proteins (Okamoto and Shikano, 2011; Cunningham et al., 2012). However, 14-3-3ζ overexpression did not change the human $\alpha_{2C}$-AR plasma membrane levels at 37°C or at 30°C in transfected HEK293T cells (Fig 3A). In contrast, down-regulation of $\beta$-COP slightly reduced the $\alpha_{2C}$-AR cell surface localization at 37°C, but greatly stimulated the effects of low-temperature on the receptor trafficking (Fig 3B). In agreement, we found that the $^{294}\text{RRRR}^{298}/^{454}\text{RRRR}^{458}$- $\alpha_{2C}$-AR mutant has diminished interactions with $\beta$-COP at 30°C compared to $\alpha_{2C}$-AR wild-type as demonstrated by co-immunoprecipitation (Fig 3C), indicating that the retrograde traffic through COPI vesicles plays a major role in preventing exaggerated $\alpha_{2C}$-AR to plasma membrane at low-temperature.

Still, rat $\alpha_{2C}$-AR had larger plasma membrane levels at 37°C and displayed a reduced sensitivity after exposure to low-temperature, although it contains the same arginine clusters in its structure.
The \(^{450}RRRRR^{454}\) cluster from the C-terminus is located in the same position in human and in rat receptor, and thus is probably not responsible for the observed differences (Fig 2A). In contrast, in rat \(\alpha_2C-AR\) the \(^{291}RRRRR^{296}\) cluster from the third intracellular loop is shifted three amino acids to the left due to the absence in the rat receptor of three residues, \(^{268}AG^{269}\) and \(^{291}A\) (Fig 2A). To determine if strict spatial positioning of this arginine cluster is essential for the temperature-sensitive intracellular trafficking, we generated mutants of the rat \(\alpha_2C-AR\), in which the missing amino acids were inserted at the same positions as in the human receptor. Both \(^{268}AG^{269}\) - and \(^{291}A\) - rat \(\alpha_2C-AR\) mutants had significant lower plasma membrane levels at 37°C in HEK293T cells (Fig 4A), despite similar total receptor levels as detected by western blot (Supplementary Figure 1B). The cell surface levels of these mutants were increased by exposure to low temperature with significantly greater extent compared to wild-type rat receptor (Fig 4A). Further, the mutant carrying the insertion of all of these three amino acids located proximal to \(^{291}RRRRR^{296}\) had similar plasma membrane levels at 37°C and displayed the same temperature sensitivity as the human receptor (Fig 4A). Interestingly, the fourth amino acid missing in the rat \(\alpha_2C-AR\) third intracellular loop compared to the human receptor is located distal to \(^{291}RRRRR^{296}\) cluster in the position \(^{325}G\) (Fig 2A). Supporting the idea that differences in the temperature sensitivity of the human and rat \(\alpha_2C-AR\) are due to strict spatial requirements of this arginine cluster, the cell surface levels of rat \(^{325}G\) \(\alpha_2C-AR\) mutant are not different compared to the rat wild-type receptor (Fig 4A). In agreement with the radio-ligand binding determinations, in transfected HEK293T cells at 37°C, stimulation of the rat \(\alpha_2C-AR\) reduced the cAMP levels with a greater extent compared to human receptors (Fig 4B). In contrast, stimulation of \(^{268}AG^{269}/^{291}A\) rat \(\alpha_2C-AR\) had minimal effects on cAMP levels, as in the case of human \(\alpha_2C-AR\) (Fig 4B). Thus,
the insertion of these three amino acids into the rat $\alpha_{2C}$-AR appears to have restored temperature sensitive trafficking to the levels seen in the human $\alpha_{2C}$-AR.

Regulation of GPCR intracellular trafficking involves interactions with accessory proteins along the biosynthetic pathway. To determine the regulatory proteins responsible for the trafficking differences observed between human and rat $\alpha_{2C}$-AR, we performed a proteomic analysis in rat and human $\alpha_{2C}$-AR transfected HEK293T cells. From this analysis three proteins were identified to have differences in the interactions with the two receptors, namely pontin (also known as RuvB-like 1 or Tip49), p97 (also named VCP, for valosin-containing protein) and sorting nexin 9 (Table 3 and Fig 5A). The first two, pontin and p97, belong to the group of AAA+ ATPases (ATPase associated diverse cellular activities), a class of proteins associated with a variety of cellular processes ranging from DNA replication, transcription regulation to protein degradation. The interactions of human $\alpha_{2C}$-AR with pontin were confirmed by co-immunoprecipitation (Fig 5B) and we confirmed that these interactions were decreased at low-temperature (Fig 5B). Rat $\alpha_{2C}$-AR displayed lower interactions with pontin which were not temperature-sensitive (Fig 5B).

Also, supporting the role of pontin in the regulation of $\alpha_{2C}$-AR intracellular traffic, we found that $^{268}$AG$^{269}$/291 A rat $\alpha_{2C}$-AR mutant interacts with this protein in the temperature-sensitive manner in similar manner as human receptor (Fig 5B), indicating once again that the three residues are responsible for the differences in the temperature-sensitivity between human and rat receptor.

Further, HEK293T cells with genetically reduced pontin levels and HEK293T cells treated with the recently described pontin inhibitor rottlerin (5 $\mu$M, Elkaim et al., 2012), show significantly enhanced human $\alpha_{2C}$-AR plasma membrane levels at 37°C. However, in HEK293T cells with genetically reduced levels of pontin the cell surface levels of rat $\alpha_{2C}$-AR were not changed (Fig 6A), and neither pharmacological inhibition of pontin activity using rottlerin did not change the
plasma membrane levels of rat α2C-AR (Fig 6A). The effects of reduced pontin activity were not observed at 30°C (Fig 6A). Genetic or pharmacological inhibition of pontin activity, also specifically enhanced human α2C-AR signaling, by potentiating the effects of receptor stimulation on p-ERK 1/2 (Fig 6B) and on inhibition of cAMP levels (Fig 6C) at 37°C.
Discussion

GPCR intracellular traffic plays fundamental roles in targeting the receptors to the functional site and modulates the amplitude of cellular signaling to receptor stimulation. The temperature-sensitive $\alpha_{2C}$-AR export to plasma membrane in VSMC is responsible for the exaggerated vasoconstriction in response to cold and emotional stress observed in Raynaud Phenomenon. The present results unveil several novel features of this receptor’s distinct intracellular trafficking. Most important, we found that the effects of low-temperature on $\alpha_{2C}$-AR are specie-dependent, being absent in lower mammals as opossum. This differential temperature-sensitivity is mediated by specific arginine clusters organized as RXR motifs in the third intracellular loop and the C-terminus of the receptor. Surprisingly, we found that disruption of these RXR motifs does not change the plasma membrane receptor levels at 37°C, suggesting that multiple mechanisms may contribute to $\alpha_{2C}$-AR intracellular retention at physiological temperature. However, the roles of specific RXR motifs became evident at reduced temperature, as the mutation of motifs in the positions $^{294}$RRRR$^{298}$ and $^{458}$RRRR$^{462}$ prevent the exaggerated transport of human $\alpha_{2C}$-AR to cell surface. These two clusters operate in a redundant manner and disruption of both is required for maximal transport to the plasma membrane in response to cold.

In contrast to the well-characterized mechanisms involved in GPCR internalization, the motifs involved in GPCR traffic from the endoplasmic reticulum to plasma membrane remain poorly characterized (Wu, 2012). A highly conserved F(x)6LL motif located in proximal C-terminus of many GPCR is a common motif mediating the transport from the endoplasmic reticulum to plasma membrane (Duvernay et al., 2004). Another motif present in most GPCR is a cluster of three to five basic residues (arginine or lysine) in the third intracellular loop which interacts with
Sec24 subunit of the cargo complex to stimulate the receptor export (Dong et al., 2012). Both these motifs are embedded in α2C-AR structure at positions FRRSFKHL and RRK. However, these motifs are involved in the general GPCR transport to the plasma membrane, as indicated by the common presence in many GPCR, including α2B-AR. This is the closest homologue of α2C-AR, but it does not display temperature-sensitive trafficking. In addition to export motifs from intracellular domains, the YS motif located in the N-terminus was demonstrated to mediate the traffic of all three α2-AR from the Golgi to the plasma membrane (Dong and Wu, 2006). Regarding α2C-AR, a hydrophobic motif, ALAAALAAAAA, from N-terminus of receptor has been shown to contribute to its endoplasmic reticulum retention (Angelotti et al., 2010). However, this motif appears to not mediate the temperature-sensitive receptor traffic because it is present also in the mouse and rat α2C-AR. In contrast to other export motifs, our results demonstrate that RRRR and RRRR are a unique human α2C-AR feature, preventing the exaggerated receptor transport to cell surface at low-temperature.

Two major mechanisms were demonstrated to modulate in opposite ways the ability of RXR motifs to mediate the endoplasmic reticulum retention. β-COP subunit of COPI complex recognizes RXR motifs stimulating the retrograde traffic from Golgi to endoplasmic reticulum, whereas 14-3-3 proteins assist motif masking and promote export trafficking (Dong et al., 2007). Both these mechanisms were shown to modulate subcellular localization of different members of GPCR family, like PAR2 and GPR15 (Okamoto and Shikano, 2011; Cunningham et al., 2012). However, our results indicate that 14-3-3ζ is not involved in the temperature-sensitive α2C-AR transport. This finding can be explained by the absence of the distal phosphorylation sites adjacent to RXR motifs required for interactions with 14-3-3 proteins in α2C-AR. In contrast, reducing β-COP cellular levels enhanced the effects of low-temperature on the α2C-AR transport.
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whereas it had a minimal effect at 37°C. Disruption of the 294RRRRR298 and 458RRRRR462 regulating the temperature-sensitive α2C-AR traffic attenuated the interactions between receptor and β-COP only at 30°C, explaining the enhanced plasma membrane localization of this mutant at reduced temperature.

However, unlike the opossum receptor, rat and mouse α2C-AR have equivalent arginine clusters, although the temperature-sensitivity of these receptors is reduced. This pattern might be due to enhanced plasma membrane localization of rat and mouse receptor at 37°C. As the C-terminus arginine cluster 458RRRRR462 is similar in all three species, we focused on 294RRRRR298 cluster from the third intracellular loop. Our insertion experiments demonstrate that indeed the absence in the rat receptor of the three amino acids located at 269GA270/291A proximal to this cluster are responsible for enhanced rat α2C-AR plasma membrane localization at 37°C. Noteworthy, insertion of these residues in rat α2C-AR did not enhance the cell surface receptor levels at low-temperature, resembling the traffic pattern of human receptor. In contrast, insertion of the fourth missing amino acid in the rat α2C-AR third intracellular loop, located distal to this cluster, 356G, has no effect on receptor traffic, suggesting that strict spatial positioning of 294RRRRR298 cluster is required to limit human α2C-AR transport to the cell surface. These results indicate that a rodent model is not suitable to study the pathology of Raynaud Phenomenon. Further, only a few studies have analyzed the fine differences between GPCR from different species, focusing mostly on the different receptor affinities generated by evolutionary mutations in the transmembrane domains (Schattauer et al., 2012; Scott et al., 2013; Strasser et al., 2013). To our best knowledge, this is the first study demonstrating major specie-dependent intracellular traffic differences in the GPCR family and this finding should be considered in the future studies on receptor traffic, subcellular localization and signaling.
A major mechanism involved in the GPCR localization is represented by receptor interactions with specialized intracellular accessory proteins generically named molecular chaperones (Dong et al., 2007). In the last few years, specific molecular chaperones for different GPCR were identified and we and others have demonstrated that the cellular levels of these accessory proteins can affect the subcellular localization and the signaling of the ‘chaperoned’ receptor (Williams and Devi 2010; Filipeanu et al., 2011b). Based on the observed differences between human and rat α2C-AR plasma membrane levels at 37ºC, we performed a proteomic analysis to identify the different protein interactions of these receptors. We found that pontin, p97 and nexin as accessory proteins interacting differentially with the two receptors. Nexin has been previously shown to regulate the intracellular traffic of P2Y1 and PAR1 in a retromer-independent manner (Nisar et al., 2010; Gullapalli et al., 2006). In contrast, the involvement of pontin and p97 in the modulation of GPCR traffic has not been reported before. Both pontin and p97 are members of AAA+ ATP-ases, a large and diverse superfamily, characterized by the presence of a highly conserved ATPase module of 200-250 amino acids (Grigoletto et al., 2011). The exact roles of each member of this family are diverse and not fully characterized, but include chromatin remodeling, transcription regulation, protein unfolding and degradation, and many of its members, including p97 and pontin, are overexpressed in cancer or neurodegenerative disorders (Jha and Dutta, 2009). Out of these three proteins we selected pontin to further study its role in modulation of α2C-AR temperature-sensitive traffic, because recently auto-antibodies against pontin were detected in systemic sclerosis, a disease that may be associated with Raynaud Phenomenon (Kaji et al., 2014). We found that the direct interactions with human α2C-AR in a temperature-sensitive manner were confirmed by co-immunoprecipitation, and pontin did not change the total cellular receptor levels. These results indicate that pontin effects on human α2C-
AR are not related to changes in transcription or degradation. However, reducing pontin activity by genetic or pharmacological approaches strongly enhanced human receptor plasma membrane levels and signaling at physiological temperature, indicating that these interactions limit the $\alpha_{2C}$-AR transport to the cell surface at $37^\circ C$. Our data are in agreement with recent findings in patients with systemic sclerosis and suggest that decreased pontin activity may represent a diagnostic biomarker for Raynaud Phenomenon (Kaji et al., 2014). Interestingly, pontin together with its partner reptin, has been shown to interact with HSP90 (Izumi et al., 2012). We previously demonstrated that inhibition of HSP90 enhanced the $\alpha_{2C}$-AR plasma membrane levels at $37^\circ C$ (Filipeanu et al., 2011a). Together with the present demonstration that pontin limits receptor transport at physiological temperature, we propose that multiple interactions with various chaperones along the biosynthetic pathway are responsible for the unique temperature-sensitive pattern of human $\alpha_{2C}$-AR. Recently, cAMP -mediated Epac activation was proposed to mediate $\alpha_{2C}$-AR plasma membrane delivery through a Rap1-Rho-actin-dependent mechanism (Jeyaraj et al., 2012, Michel and Insel, 2012). However, these effects were also observed at $37^\circ C$ and with both human and murine receptor and thus seem to mediate receptor traffic from Golgi apparatus to cell surface. In contrast, HSP90 and pontin appear to be specific mediators of temperature-sensitive $\alpha_{2C}$-AR intracellular traffic and may constitute valuable biomarkers to identify people predisposed to Raynaud Phenomenon.

Overall, our data represent the first demonstration of unique mechanisms underlying the temperature-sensitivity of human $\alpha_{2C}$-AR trafficking, particularly the prevention of the exaggerated traffic to plasma membrane at reduced temperature by specific arginine clusters from third intracellular loop and C-terminus through retrograde traffic mediated by COPI vesicles and receptor interactions in temperature-sensitive manner with pontin.
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Authorship Contributions.

Participated in research design: Filipeanu and Guidry.

Conducted experiments: Filipeanu, Pullikuth and Guidry

Contributed new reagents or analytic tools: Filipeanu and Guidry

Performed data analysis: Filipeanu and Guidry

Wrote or contributed to the writing of the manuscript: Filipeanu and Guidry
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*Leclerc*


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Footnotes.

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Address for reprints requests: Catalin M. Filipeanu, Associate Professor, Department of Pharmacology, Howard University, tel: 202-806-6311, fax: 202-806-4453, email: catalin.filipeanu@howard.edu
Figure legends.

**Figure 1.** A. The effects of temperature on the subcellular localization of α2C-AR from different species. The receptor plasma membrane levels were determined in HEK293T cells transfected with human α2C-AR, mouse and rat VSMC from tail artery, and OK cells at 37°C (black columns) and at 30°C (white columns) using [3H]-RX821002 as described (Filipeanu et al., 2011a). n=8-12 in each case from at least three different passages. **- indicate p<0.01 and * - indicate p<0.05 at 30°C compared to 37°C. B. The cell surface levels of human (left columns) and rat α2C-AR (right columns) in HEK293T (top panel) and human coronary VSMC cells (bottom panel) at different 37°C and 30°C. n=6-12 in each case, * - indicates significant differences between 37°C and at 30°C. C. The total α2C-AR cellular levels at 37°C and at 30°C in HEK293T cells (upper panel) and in human coronary VSMC (bottom panel) detected by Western blot using a receptor raised against receptor (Abcam ab46536). Similar results were obtained in two different experiments. D. Subcellular localization of human (left) and rat (right) α2C-AR in human coronary VSMC at 37°C. Blue – nucleus staining using DAPI. The images are representative from three independent electroporation experiments.

**Figure 2.** A. Alignment of α2C-AR from different species. The amino acids shown in blue are forming the extracellular domains, in brown are shown the amino acids forming the transmembrane domains, and in black are shown the amino acids constituting the intracellular loops. The amino acids differing from human sequence are italicized. The putative RXR motifs are shown in red bold characters. In green are indicated the four amino acids missing in the rat and mouse α2C-AR third intracellular loop. The alignment was performed using ClustalW2
program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). **B.** The effects of deletion of selected sequences on the human \(\alpha_2C\)-AR temperature-dependent traffic. The receptor regions deleted are shown in green. In the \(\alpha_2C\)-AR/\(\alpha_2B\)-AR chimeric construct, the domains shown in red from \(\alpha_2B\)-AR were incorporated in \(\alpha_2C\)-AR structure between the using BamH1 restriction sites (residues shown in green in the bottom panel). The plasma membrane \(\alpha_2C\)-AR levels were determined as in Fig 1 and are expressed as 30\(^\circ\)C/37\(^\circ\)C levels ratio. n=6-10 in each case. *- indicate p<0.05 compared to wild-type \(\alpha_2C\)-AR human receptor.

**Figure 3. A.** Top: the cellular levels of 14-3-3\(\zeta\) in pcDNA3.1 and 14-3-3\(\zeta\) transfected cells. Bottom: the effects of 14-3-3\(\zeta\) overexpression on the human \(\alpha_2C\)-AR traffic in HEK293T cells, as determined by specific \[^3\text{H}\]RX821002 binding. n=6 from three independent transfections. **B.** The cellular levels of \(\beta\)-COP in cells transfected with scrambled shRNA and \(\beta\)-COP shRNA. Bottom: plasma membrane levels of \(\alpha_2C\)-AR in control cells and in cells with reduced \(\beta\)-COP levels. The experiments were performed in similar manner as in A. n=6 in each case from three independent transfections. * - indicate p<0.05 compared to scrambled shRNA. **C.** Interactions between human wild-type \(\alpha_2C\)-AR or \(^{294}\text{RRRAARR}^{298}/^{454}\text{RRRAARR}^{458}\)-\(\alpha_2C\)-AR mutant with \(\beta\)-COP at 37\(^\circ\)C and at 30\(^\circ\)C in HEK293T cells detected by co-immunoprecipitation. HEK293T cells were transfected with the indicated receptors and three days later the cells were solubilized and immunoprecipitated with GFP antibody as described (Filipeanu et al., 2011a). The immunoprecipitates (20 \(\mu\)g/lane) were separated by 10% SDS-Page and the \(\beta\)-COP levels were revealed by western-blotting. The experiment shown is representative from three independent transfections. In the left, the quantification of these three independent experiments is shown, n = 3, * - p<0.05.
Figure 4. A. The four amino acids missing in the rat \( \alpha_{2C} \)-AR third intracellular loop were inserted by site-mutagenesis and the cell surface levels of these mutants were determined at 37°C and at 30°C by \[^3H\]RX821002 binding. \( ^{269} \)GA\(^{270} \) and \( ^{291} \)A mutants displayed significantly lower plasma membrane levels at 37°C, and greater enhancement after exposure to low-temperature. Triple \( ^{269} \)GA\(^{270}/^{291} \)A mutant behaved similarly to human \( \alpha_{2C} \)-AR. The insertion of \( ^{326} \)G amino acid has no effect on the receptor plasma membrane levels. n=6-8 in each case, *- p<0.05 compared to wild-type \( \alpha_{2C} \)-AR rat receptor. B. The effects of human, rat and \( ^{269} \)GA\(^{270}/^{291} \)A rat \( \alpha_{2C} \)-AR at 37°C on forskolin induced cAMP increases at 37°C. The experiments were performed as described, n=6 in each case from two different transfections. *- p<0.05

Figure 5. A. The histograms representing the volumes of specific spot interactions with human and rat \( \alpha_{2C} \)-AR in HEK293T cells determined with the DeCyder software. Subsequently the spot was picked, subjected to trypsin digestion and the peptides were subjected to tandem mass spectrometry analysis. LC-MS data was analyzed by Proteome Discoverer 1.4 using the Human SwissProt database. On the right, in red are shown the peptides obtained during analysis, identifying pontin (also named RuvBL-1 or TIP49) as an interacting partner of human \( \alpha_{2C} \)-AR. The experiments were repeated three times and the statistics are shown in the bar graph. *- p<0.05. B. Validation of proteomics data by co-immunoprecipitation. GFP-tagged human, rat and \( ^{269} \)GA\(^{270}/^{291} \)A rat mutant \( \alpha_{2C} \)-AR were transfected in HEK293T cells. Two days later the cells were serum starved and exposed to 37°C or 30°C for 18 h. and then were processed as described in Figure 3. Pontin levels in immunoprecipitates and in inputs were determined by western blot. Similar results were obtained in two other experiments.
Figure 6. A. The effects of down-regulation of pontin on the human and rat α2C-AR plasma membrane levels. A clone of HEK293T cells with reduced pontin levels (23 ± 12 % from control, n=4) was generated using a shRNA lentiviral construct (sc-43543) and puromycin selection. The receptors were transfected for 72 h in control or pontin depleted cells. When present, Rottlerin (pontin inhibitor, 5 µM) was incubated for 18 h. n=9 from three independent cell passages. * - indicates p<0.05 compared to control cells. B. The effects of pontin depletion on α2C-AR stimulation of P-ERK 1/2 phosphorylation at 37°C. The cells were processed as in A., and 72 h post-transfection were stimulated for 5 min with indicated concentrations of specific α2-AR agonist UK14304. Black circles indicate the effects obtained in control cells and open circles indicate the effects obtained in HEK293T cells with reduced pontin levels. n=3 from three independent experiments, * - indicate p<0.05 compared to control cells. C. The effects of reducing pontin cellular levels on α2C-AR mediated decrease in levels cAMP levels at 37°C. The cells were pretreated with UK14304 (10⁻⁸M) for 5 min and then stimulated with forskolin (3 µM) for 15 min and cAMP determined as described (Filipeanu et al., 2011 a,b). n=9 from three independent experiments, *- indicate p<0.05 compared to control cells.
Table 1. Identification of RXR motifs preventing exaggerated transport of α2C-AR to the plasma membrane at 30°C.

<table>
<thead>
<tr>
<th>Mutants with single cluster disrupted</th>
<th>Wild-type</th>
<th>100 %</th>
<th>169 ± 6.2 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td></td>
<td>100 %</td>
<td>169 ± 6.2 %</td>
</tr>
<tr>
<td>240RTA242</td>
<td>96 ± 4.2 %</td>
<td>168 ± 8.4 %</td>
<td></td>
</tr>
<tr>
<td>294RRARR298</td>
<td>93 ± 7.3 %</td>
<td>175 ± 10.1 %</td>
<td></td>
</tr>
<tr>
<td>306RRA308</td>
<td>102 ± 6.7 %</td>
<td>166 ± 7.3 %</td>
<td></td>
</tr>
<tr>
<td>358RRARR364</td>
<td>107 ± 9.5 %</td>
<td>164 ± 8.5 %</td>
<td></td>
</tr>
<tr>
<td>454RRARR458</td>
<td>95 ± 5.5 %</td>
<td>176 ± 6.6 %</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mutants with two clusters disrupted</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>240RTA242/293RRAAR297</td>
<td>92 ± 6.8 %</td>
<td>173 ± 8.0 %</td>
<td></td>
</tr>
<tr>
<td>240RTA242/306RRA308</td>
<td>101 ± 3.1 %</td>
<td>168 ± 6.5 %</td>
<td></td>
</tr>
<tr>
<td>240RTA242/358RRARR364</td>
<td>97 ± 5.5 %</td>
<td>177 ± 7.5 %</td>
<td></td>
</tr>
<tr>
<td>240RTA242/454RRARR458</td>
<td>94 ± 6.8 %</td>
<td>173 ± 6.6 %</td>
<td></td>
</tr>
<tr>
<td>294RRARR298/306RRA308</td>
<td>104 ± 7.7 %</td>
<td>165 ± 8.2 %</td>
<td></td>
</tr>
<tr>
<td>294RRARR298/358RRARR364</td>
<td>101 ± 3.1 %</td>
<td>177 ± 6.2 %</td>
<td></td>
</tr>
<tr>
<td>294RRARR298/454RRARR458</td>
<td>103 ± 6.2 %</td>
<td>215 ± 7.4 %</td>
<td></td>
</tr>
</tbody>
</table>

*
The residues shown in bold and italicized in human α<sub>2C</sub>-AR were mutated from arginine to alanine using the specific primers indicated in Material and Methods. Subsequently, the mutants with single cluster disruption served as a template for generating double or triple mutants. The expected mutation was confirmed in each case by sequence analysis. The plasma membrane levels were determined by [3H]RX821002 binding and the results are expressed as a percentage of α<sub>2C</sub>-AR wild-type levels determined in the same experiment. n=8-12 in each case, from three independent transfections. * indicate p<0.05 compared to wild-type human α<sub>2C</sub>-AR receptor.

<table>
<thead>
<tr>
<th>Mutants with three clusters disrupted</th>
<th>104 ± 7.8 %</th>
<th>221 ± 8.3 % *</th>
</tr>
</thead>
<tbody>
<tr>
<td>294RRAAR298/358RRAAR364/454RRAAR458</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. The effects of human α2C-AR wild type and α2C-ARmutant stimulation on cAMP levels.

<table>
<thead>
<tr>
<th></th>
<th>α2C-AR wild-type</th>
<th>294RRRRR298/454RRRRR458 - α2C-AR</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C</td>
<td>84.89 ± 13.5 %</td>
<td>81.24 ± 12.3 %</td>
</tr>
<tr>
<td>30°C</td>
<td>57.26 ± 8.6 %</td>
<td>24.62 ± 10.1 %*</td>
</tr>
</tbody>
</table>

HEK293T cells transiently transfected with 3 µg/plate of each receptor construct for 48 h and subsequently starved for 24 h. The cells were then treated with 10^{-8} M UK14304 for 5 min, followed by incubation with forskolin (3 µM) for 15 min. cAMP levels were determined as described (Filipeanu et al., 2011a,b) using cAMP enzyme immunoassay system (Cayman Chemical Company). n=6 in each case from three independent transfections. * - indicate p<0.05 compared to wild-type human α2C-AR receptor.
Table 3. Proteins identified to interact differentially with human and rat $\alpha_2$C-AR in HEK293T cells.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular mass</th>
<th>Score</th>
<th>Peptides matched</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pontin (RUVBL1, TIP49)</td>
<td>50524</td>
<td>762</td>
<td>40</td>
</tr>
<tr>
<td>P97 (PCP)</td>
<td>89950</td>
<td>2180</td>
<td>145</td>
</tr>
<tr>
<td>SNX9 (SH3PX1)</td>
<td>66903</td>
<td>100</td>
<td>4</td>
</tr>
</tbody>
</table>

Human and rat GFP-tagged $\alpha_2$C-AR were transiently transfected in HEK293T cells (8 $\mu$g/plate) and the cells were starved 24 h before the experiment. Three days after transfection the cells were lysed and incubated with Anti-GFP mAb-Agarose (MBL) overnight. Dimensional gel electrophoresis labeling and analysis was performed as previously described (Filipeanu et al., 2011b). Fluorophore-labeled protein gels were scanned, detected and analyzed as described in Materials and Methods. Implicated spots were subjected to LC-MS and analysis as described in Material and Methods and spot verification was performed by western blot.
Figure 2

B. Ratio 30°C/37°C binding

3ic Human α<sub>2c</sub>-AR:

232<br>RI YVAKL <RTRGFTL SKEKRAPVGPDASPPTTENGLGAAAGAGHCEPPADVEPDESAAAEERRRRRGRALLRRGRRR141 <AGAEAGGAGGADGQQAGGPAAESGALTSRSPGGRSLRSRSSRSEFLLSRRRRARRSSVCRRGF141 <KVQRAREKR 379

C-terminus human α<sub>2c</sub>-AR:

442<br>QDFRRSFKHILFGPRRRRGFRQ 462

3ic Human α<sub>2b</sub>-AR:

192<br>RI YLIAKRKNRRGPRAKGPGQEGKQGFRPDHGALASAKLPALASVASEVNGHSKSTKEEGETPDGTGALPPSWALPP<br>NSGQQOKEGVCKGASPDEAI</br>E<br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br><br>EEEEE</br<br>C-terminus human α<sub>2b</sub>-AR:

450<br>NQDFRRAFRRTLGCRPRWQTAW 450
Figure 3

A.  

<table>
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<th></th>
<th>pcDNA 3.1</th>
<th>14-3-3ζ</th>
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<tr>
<td>14-3-3ζ</td>
<td>![Image 1]</td>
<td>![Image 2]</td>
</tr>
<tr>
<td>β-actin</td>
<td>![Image 3]</td>
<td>![Image 4]</td>
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B.  

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<th>control shRNA</th>
<th>β-COP shRNA</th>
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<tr>
<td>β-COP</td>
<td>![Image 5]</td>
<td>![Image 6]</td>
</tr>
<tr>
<td>β-actin</td>
<td>![Image 7]</td>
<td>![Image 8]</td>
</tr>
</tbody>
</table>

C.  

IP: GFP  
IB: β-COP  

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<tr>
<th></th>
<th>NT</th>
<th>α₂C-AR</th>
<th>α₂C-AR&lt;sup&gt;294RRAAR&lt;sup&gt;298&lt;/sup&gt;/&lt;sub&gt;454RRAAR&lt;sup&gt;458&lt;/sup&gt;</th>
<th>α₂C-AR&lt;sup&gt;294RRAAR&lt;sup&gt;298&lt;/sup&gt;/&lt;sub&gt;454RRAAR&lt;sup&gt;458&lt;/sup&gt;</th>
<th>α₂C-AR&lt;sup&gt;294RRAAR&lt;sup&gt;298&lt;/sup&gt;/&lt;sub&gt;454RRAAR&lt;sup&gt;458&lt;/sup&gt;</th>
<th>α₂C-AR&lt;sup&gt;294RRAAR&lt;sup&gt;298&lt;/sup&gt;/&lt;sub&gt;454RRAAR&lt;sup&gt;458&lt;/sup&gt;</th>
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<th>control</th>
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<th>30°C</th>
<th>37°C</th>
<th>30°C</th>
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<th>30°C</th>
<th>37°C</th>
<th>30°C</th>
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<td>% of α₂C-AR wild-type interactions at 37°C</td>
<td>![Image 21]</td>
<td>![Image 22]</td>
<td>![Image 23]</td>
<td>![Image 24]</td>
<td>![Image 25]</td>
<td>![Image 26]</td>
<td></td>
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<td>20</td>
<td>10</td>
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</tbody>
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A. Bound[^3H]-RX821002 (cpm)

B. cAMP levels (pmol/well)
Figure 5

A. GFP-\(\alpha_{2C}\)-AR

human  rat

B. IP: GFP
IB: Pontin

GFP-\(\alpha_{2C}\)-AR

human  rat  rat \(^{269}\)GA\(^{270/291}\)A

NT  37\(^\circ\)C  30\(^\circ\)C  37\(^\circ\)C  30\(^\circ\)C

IP  inputs