# Insights into G Protein Coupled-Receptor Functional Regulation by Interacting Effectors: G Proteins and β-arrestins

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# DEDICATION

This dissertation is dedicated to my dear parents, Hazem Sedki and Mayada Al Azmeh. This work would never have been accomplished without their faith and unconditional support.

# ABSTRACT

G protein-coupled receptors (GPCRs) are cell surface proteins that play a fundamental role in mediating diverse physiological processes by converting extracellular stimuli into cellular signals. Upon ligand interaction, activated receptors propagate signals by engaging GPCR-interacting proteins, particularly heterotrimeric G proteins and β-arrestins. While more than 800 GPCR members exist, their signaling activity is governed by a limited number of interacting effectors. Moreover, many GPCRs interact with multiple G protein and/or β-arrestin subtypes, giving each receptor a potential distinctive signaling profile or signature. To achieve such accurate profiling, GPCRs and their signaling partners develop intricate coupling and signaling selectivity mechanisms for their communication. Understanding such mechanisms is critical for the proper exploitation of GPCRs as drug targets. Especially since these receptors allow for functional selective targeting by ligands, thus providing advantageous therapeutic approaches.

The first half of this thesis explores the G protein coupling selectivity for two promiscuous Gαq/11- and Gα12/13-coupled receptors: prostaglandin F2α (FP) and angiotensin II type 1 receptor (AT1R). These receptors are simultaneously expressed in vascular smooth muscle cells, and their signaling through Gαq/11 and Gα12/13 is tightly regulated to mediate vascular contraction regulation. Through altering the levels of Gαq and Gα13 accessibility for receptor binding, a unidirectional and receptor-specific competition of these G protein binding to FP and AT1R was observed. This alteration also affected the bias modulation of functionally selective ligands on both receptors. Thus, competitive G protein receptor binding not only highlights a novel phenomenon governing G proteins' selectivity, but also suggests a mechanism by which bias ligands exert their functional selectivity modulation.

The second half of this thesis explores the β-arrestin conformational arrangements in function of the receptor partner and cellular compartmentalization. The existence of differential conformational arrangements provides an important link for the numerous functional outcomes that transpire from β-arrestin activation. A set of photo-activable β-arrestin mutants was generated using site-directed bioorthogonal labeling with an unnatural amino acid. Complex formation between these mutants and one of the three activated GPCRs: AT1R, bradykinin receptor B2, and vasopressin receptor 2, was performed, and was followed by UV-mediated photolysis to induce complex cross-linking. This enabled the generation of receptor-specific imprints, reflective of the distinct β-arrestin conformations. Moreover, β-arrestin conformation when bound to the same GPCR partner was shown to vary depending on the cellular localization of the complex between the plasma membrane and endosomes.

Altogether, this thesis sheds light on mechanisms for regulating GPCR signaling through G proteins and β-arrestins. It also provides novel insights for GPCR–β-arrestin complex arrangement that could be further applied to understand β-arrestin-mediated signal transduction. Insights from this thesis are ultimately beneficial for the design of better GPCR targeted therapeutics.

# RÉSUMÉ

Les récepteurs couplés aux protéines G (RCPGs) sont des protéines de surface cellulaire jouant un rôle fondamental dans la communication de divers processus physiologiques en convertissant les stimuli extracellulaires en signaux cellulaires. Lors d'interaction avec leurs ligands, les récepteurs activés propagent les signaux en engageant des protéines intracellulaires interagissant avec eux, en particulier les protéines G hétérotrimériques et les β-arrestines. Alors que plus de 800 membres des RCPGs ont été identifiés, leur activité de signalisation est régulée par un nombre limité de partenaires interactifs. De plus, de nombreux RCPGs interagissent avec plusieurs sous-types de protéines G et/ou β-arrestines, permettant ainsi à chaque récepteur un profil ou une signature de signalisation distincte. Afin d'atteindre ce profil de signalisation, les RCPGs et leurs partenaires de signalisation ont développé des mécanismes complexes de couplage et de signalisation sélective. Une compréhension de ces mécanismes est donc essentielle pour exploiter adéquatement les RCPGs comme cibles médicamenteuses; d'autant plus que la nature dynamique de ces récepteurs permet un ciblage sélectif fonctionnel par des ligands, offrant ainsi des approches thérapeutiques avantageuses.

La première moitié de cette thèse explore la sélectivité de couplage de deux familles de protéines G, les protéines Gαq/11 et Gα12/13, aux récepteurs de la prostaglandine F2α (FP) et le récepteur de type 1 de l'angiotensine II (AGTR1). Ces récepteurs sont exprimés de manière ubiquitaire dans les cellules musculaires lisses vasculaires, et leur signalisation via Gαq/11 et Gα12/13 est étroitement régulée pour induire des contractions vasculaires. En modifiant les niveaux d'accessibilités cellulaires de Gαq et Gα13, une compétition diffèrent entre les protéines G pour la liaison à FP et AT1R, a été observé. Cette altération dans les niveaux d'accessibilités cellulaires de protéines G a également affecté la modulation sélective des ligands biaisés sur ces

deux récepteurs. Ainsi, la liaison compétitive de la protéine G à ces récepteurs met non seulement en lumière un nouveau mécanisme de liaison sélective des protéines G, mais suggèrent également un mécanisme par lequel les ligands biaisés exercent leur modulation de sélectivité fonctionnelle sur ces récepteurs.

La deuxième moitié de cette thèse explore les différents arrangements de conformations qui peuvent exister dans la β-arrestine en fonction de sa liaison avec différents récepteurs et du compartimentage cellulaire de ces complexes. L'existence de différents arrangements de conformations pourrait révéler un lien fonctionnel dans l'activation de la signalisation découlant des β-arrestines. Un ensemble de mutants de la β-arrestine ayant la propriété d'être photo-réticulés et de former des liens covalents avec les RCPGs a été généré en marquant la β-arrestine à différentes positions avec un acide aminé photo-réactif. La photolyse par UV des β-arrestines a été réalisé pour induire une liaison covalente entre β-arrestine et un des trois RCPGs: AT1R, le récepteur de la bradykinine B2 et le récepteur de la vasopressine 2. Cela a permis la génération d'empreintes spécifiques de liaisons aux récepteurs, révélant ainsi des conformations distinctes de la β-arrestine. De plus, la photoréticulation de complexes isolés à la membrane plasmique ou dans les endosomes a également révélé d'importantes divergences dans l'arrangement de la β-arrestine avec le même RCPG.

En somme, cette thèse met en lumière des mécanismes de régulation de la signalisation des RCPGs par les protéines G et les β-arrestines. Elle fournit également de nouvelles connaissances sur l'arrangement des complexes RCPG–β-arrestine et de la transduction de signaux émanant des β-arrestines. Ces nouvelles connaissances pourraient s'avérer ultimement bénéfiques pour la conception de meilleurs traitements ciblant les RCPGs.

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# CONTRIBUTION OF AUTHORS

This thesis is assembled and written in the manuscript-based format in compliance with the regulations of the McGill University Faculty of Graduate Studies. It is composed of two original manuscripts; one is submitted and is currently under peer revision (Chapter 3), and the second is in preparation and soon to be submitted (Chapter 4). Each of the chapters contains their own abstract, introduction, materials and methods, results, and discussion sections. A connecting text is added at the beginning of each chapter to provide the rationale of the study and a logical connection between chapters. The detailed contributions of contributing authors are listed below.

***Selectivity Regulation of Gq/11 and G12/13 at Promiscuous GPCRs.*** Submitted, June, *2022.*
Dana Sedki, Aaron Cho, Yubo Cao, Ljiljana Nikolajev, N. D, Prasad Atmuri, William D. Lubell, & Stéphane A. Laporte

Dana Sedki (candidate) designed and performed all the experiments excluding those listed below, analyzed and interpreted data, performed statistical analysis, generated figures, and wrote the manuscript. Aaron Cho generated the GαqNull FP mutant and did experiments related to its validation. Dr. Yubo Cao generated the schematic in Fig. 6, participated in discussions, and edited the manuscript. Ljiljana Nikolajev was involved in the initial conception of the idea and participated in the generation of data presented in Figures S4, S7, and S8. Dr. Yoon Namkung participated in the discussions and provided guidance on troubleshooting experimental protocols. N.D. Prasad Atmuri generated the az-PDC analogue. Dr. William D. Lubell supervised the work of N.D. Prasad and participated in discussions and manuscript editing. Dr. Stéphane A. Laporte conceived the study and wrote the manuscript.

***Probing Differential β-arrestin Active Conformations Through Genetically Engineered Crosslinking.*** *In preparation.*

Dana Sedki, Yubo Cao, Yoon Namkung, & Stéphane A. Laporte

Dana Sedki (candidate) designed and performed all the experiments excluding those listed below, analyzed and interpreted data, performed statistical analysis, generated figures, and wrote the manuscript. Dr. Yubo Cao conducted the microscopy experiments presented in Figure 3A and participated in discussions. Dr. Yoon Namkung assisted in the generation of HA-AT1R construct, participated in the discussions, and provided guidance on troubleshooting experimental protocols. Dr. Stéphane A. Laporte conceived the study and edited the manuscript.

**Other contributions during the course of my Ph.D. training at the Laporte lab (not presented in this thesis):**

- Gagnon L., Cao Y., Cho A., Sedki D., Huber T., Sakmar T.P., & Laporte S.A. (2019) Genetic code expansion and photocross-linking identify different beta-arrestin binding modes to the angiotensin II type 1 receptor. J Biol Chem. 2019;294(46):17409-20.

- Cao Y*., Nivedha AK*., Cho A., Sedki D., Holleran B., Leduc R, Vaidehi N., & Laporte S.A. (2022) Structural nanodomains regulating functional selectivity of angiotensin II type 1 receptor to Gq and β-arrestin. *Equal contribution. *Submitted to Nature Chemical Biology.*

- Cho A., Heydenreich F.M., van der Velden W.J.C., Cao Y., Sedki D., Veprintsev D.B., Vaidehi N., & Laporte S.A. (2022) High throughput mutagenesis of FP receptor reveals functional selectivity between Gαq/11 and Gα12/13 activation. *In preparation.*

# ABBREVIATIONS

| | |
|---|---|
| AA | Arachidonic acid |
| aaRS | Aminoacyl-tRNA synthetase |
| AC | Adenylyl cyclase |
| AngII | Angiotensin II |
| AP-2 | Adaptor protein 2 |
| AT1R | Angiotensin II type 1 receptor |
| azF | p-azido-l-phenylalanine |
| β2AR | β2 adrenergic receptor |
| B2R | Bradykinin B2 receptor |
| BK | Bradykinin |
| BRET | Bioluminescence resonance energy transfer |
| cAMP | Cyclic adenosine monophosphate |
| CCP | Clathrin-coated pit |
| CCV | Clathrin-coated vesicle |
| DAG | Diacyl glycerol |
| ECL | Extracellular loop |
| ERK1/2 | Extracellular signal-regulated kinases 1 and 2 |
| FP | Prostaglandin F2α receptor |
| FRET | Fluorescence (Förster) resonance energy transfer |
| G protein | Guanine nucleotide-binding proteins |
| GAP | GTPase-activating protein |
| GDP | Guanosine diphosphate |
| GEF | Guanine nucleotide exchange factor |
| GFP | Green fluorescent protein |
| GPCR | G protein-coupled receptor |
| GRK | G protein-coupled receptor kinase |
| GTP | Guanosine triphosphate |
| HEK293 | Human embryonic kidney cells |

| | |
|---|---|
| ICL | Intracellular loop |
| IP | Immunoprecipitation |
| IP3 | Inositol 1,4,5-triphosphate |
| Jak2 | Janus kinase 2 |
| MAPK | Mitogen-activated protein kinase |
| $MLC_{20}$ | Myosin light chain |
| MLCP | Myosin light chain phosphatase |
| MLCK | Myosin light chain kinase |
| mGluR | Metabotropic glutamate receptor |
| NAM | Negative allosteric modulator |
| PAM | Positive allosteric modulator |
| PIP2 | Phosphatidylinositol 4,5-biphosphate |
| PKA/PKC | Protein kinase A/C |
| PLC | Phospholipase C |
| PM | Plasma membrane |
| PMA | Phorbol 12-myristate 13-acetate |
| PG | Prostaglandin |
| PGF2α | Prostaglandin F2α |
| PTX | Pertussis toxin |
| RAAS | Renin-angiotensin-aldosterone system |
| RGS | Regulators of G protein signaling |
| ROCK | Rho-associated protein kinase |
| RhoGEF | Rho-family guanine nucleotide exchange factor |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| TM | Transmembrane |
| UAA | Unnatural amino acid |
| TPα | Thromboxane A2 receptor alpha |
| V2R | Vasopressin 2 receptor |
| VFT | Venus flytrap' domain |

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# CHAPTER 1: General Introduction and Literature Review

## 1.1 G Protein-Coupled Receptors

### *1.1.1 Overview*

Throughout the evolutionary history, the ability of living organisms to receive and transduce signals from their environment has been a key necessity for their survival. Information from the extracellular milieu thus needs to cross the cellular membrane barrier to be translated into intracellular responses. This is mainly realized by numerous proteins embedded within the cell membrane that are involved in regulating physiological processes such as cell adhesion, ion conduction, and signaling. From these proteins, eukaryotic signal transduction is principally mediated by cell-surface receptors, with the largest family of those being the G protein-coupled receptor (GPCR) family. Approximately 2% of the human genome encodes GPCRs (Lander et al., 2001), amounting for over 800 ubiquitously expressed receptors that influence virtually all cellular responses (Sommer et al., 2020). As such, it is no surprise that GPCRs are the main focus for drug development research. Today, more than 30% of all currently used drugs in clinical practice target GPCRs, with therapeutic actions spanning a wide range of pathologies, ranging from allergic rhinitis, to hypertension, cardiovascular diseases, pain management, and schizophrenia (Lagerstrom & Schioth, 2008).

The modern study of GPCRs was launched with the first cloning of the β2-adrenergic receptor (β2AR) in 1986 by Robert Lefkowitz, together with Brian Kobilka (Dixon et al., 1986). Lefkowitz made the crucial observation of the shared seven transmembrane structure (7TM) between β2ARs and rhodopsin light-sensing receptors, establishing the common 7TM GPCR structure. This knowledge led to a rapid expansion in the number of cloned GPCRs and revolutionized pharmacological studies. In the last two decades, remarkable research efforts

focused on exploring the mechanisms for GPCR functional regulation, identifying a wide signaling network associated with their activity. Our expanding knowledge of GPCRs' complex signaling and their communication with interacting protein partners is thus crucial for the development of novel therapeutic approaches.

### 1.1.2 GPCR Structure

From the structural standpoint, GPCRs share a similar core that comprises a transmembrane domain consisting of seven transmembrane (7TM) α helical segments of 25–30 hydrophobic amino acid residues embedded within the plasma membrane. These TMs are linked together with alternating intracellular and extracellular loops (ICLs and ECLs) and are flanked by an extracellular N-terminus and a C-terminus (Fig. 1). Extracellular domains, either alone or in addition to the TM domain, generally constitute the main site for ligand binding in GPCRs. This site is often referred to as the "ligand-binding pocket". On the other hand, the C-terminus extremity, together with the ICLs, are targeted for palmitoylation and/or phosphorylation in order to facilitate receptor interactions with interacting effectors such as G proteins and arrestins (Gurevich & Gurevich, 2019). Despite their structural similarities, GPCRs exert unique signaling profiles through the different G protein subtypes as well as other interacting effectors. Therefore, intensive academic research efforts are dedicated to extending our knowledge of the structural basis for GPCR function. A high-resolution 3D structure of GPCRs have only been feasible in the past two decades with the first resolved GPCR crystal structure of the bovine rhodopsin in the year 2000 (Palczewski et al., 2000). Advancements in the field allowed the identification of several other GPCR crystal structures, including but not limited to, the human β1- and β2-adrenergic receptors (Cherezov et al., 2007; Rasmussen et al., 2011; Rosenbaum et al., 2007), the dopamine D3 receptor (Chien et al., 2010), the oxytocin receptor (Waltenspuhl et al., 2020), the adenosine

A2 receptor (Jaakola et al., 2008; Xu et al., 2011), prostaglandin E2 receptor 3 (Audet et al., 2019), and the angiotensin II type 1 receptor (AT1R) (Zhang et al., 2015; Wingler, Elgeti, et al., 2019; Wingler, McMahon, Staus, Lefkowitz, & Kruse, 2019). These breakthroughs provided valuable insights for the understanding of the structure-function relationship for the complex signaling regulation in GPCRs.

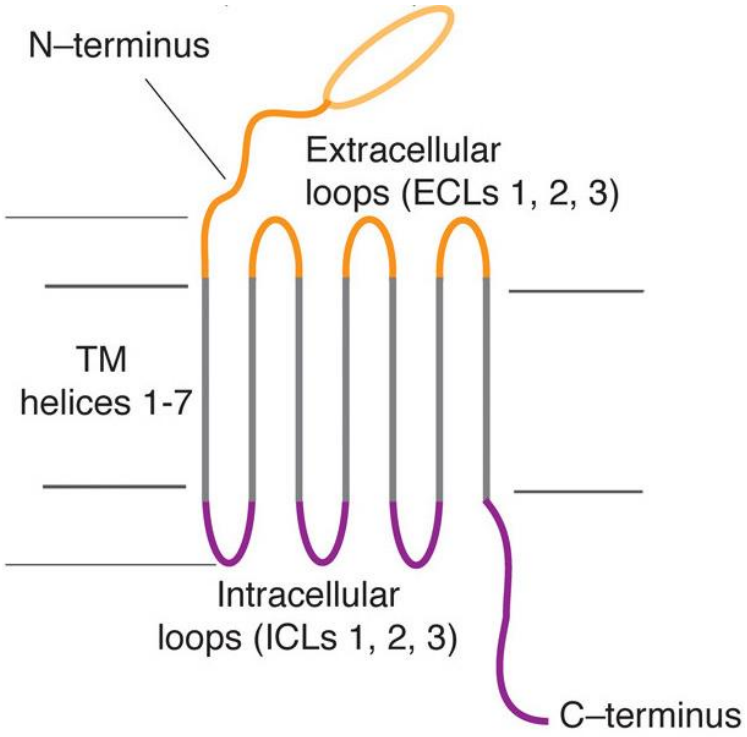


**Figure 1. Schematic representation of GPCR structure.**

Seven transmembrane spanning domains of GPCRs that form the transmembrane helices are shown in gray, the three alternating extracellular loops (ECLs) and the amino terminus are shown in orange, while the three intracellular loops (ICLs) and carboxy terminus are shown in purple. Adapted from (Latorraca, Venkatakrishnan, & Dror, 2017)

### 1.1.3 GPCR Classes

In order to better understand the biological and pharmacological applications of the ~800 GPCR members, GPCRs are classified based on their sequence homology and functional similarities into six major families (i.e., classes) according to the A–F classification system (structural features of the main 4 families are demonstrated in Fig. 2). The largest of those is the class A family (rhodopsin-like), accounting for 80% of receptor abundance in the human body and comprising around 701 GPCRs. Members of this family have a relatively simpler structural arrangement with short N-terminus. They also share two common ancestral fingerprint regions that are highly important for stabilizing and activating receptors: the DRY motif located between TM3 and ICL2, and the NPxxY motif in TM7 (Gacasan, Baker, & Parrill, 2017).

Class B family (secretin receptor family), named after the first discovered member, forms a small group comprising only 15 known receptors. Compared to class A GPCRs, class B receptors have a longer N-terminal domain. They also have conserved cystine residues forming a network of cysteine bridges that stabilize the N-terminus. This conserved N-terminus is critical for the binding of large peptides and stabilizing an active GPCR conformation. Owing to their involvements in important homeostatic processes, members of this family serve as great drug targets. Examples include: the glucagon, parathyroid hormone and calcitonin receptors, all of which are successfully used in the clinic for the management of hypoglycemia, osteoporosis, and hypercalcaemia, respectively (Hendy, D'Souza-Li, Yang, Canaff, & Cole, 2000).

Class C GPCRs (glutamate receptor family) predominantly include the metabotropic glutamate receptors (mGluRs) and the taste receptors. Members of this family are characterised by the largest extracellular domain with a long N-terminal sequence (500–600 amino acids), often referred to as the ‘Venus flytrap’ domain (VFT). Their activation distinctively requires the

formation of constitutive dimers; thus, they are viewed as noncanonical GPCRs. Ligand-binding to one protomer of the receptor dimer transcends, by allosteric communication, a conformational change in the second protomer, leading to receptor activation (Grushevskyi et al., 2019; Hlavackova et al., 2005). Moreover, class C GPCRs are distinct in that their orthosteric ligand-binding site is situated in the VFT domain and does not involve the TM region (Dore et al., 2014).

Other classes of GPCRs include: Class D (parasitic mating pheromone receptor family), Class E (cyclic AMP receptor family), and class F (frizzled and smoothened receptor family). The latter is known to be implicated in the Wnt signaling pathway involved in governing cell proliferation, embryonic development, and other physiological processes in developing and in adult organisms. Ever since their initial discovery in 1989 in drosophila (Vinson, Conover, & Adler, 1989), 10 mammalian receptor subtypes have been identified. Receptor from this family have been recently exploited as drug targets, and a monoclonal antibody is currently under development for cancer treatment (Diamond et al., 2020).

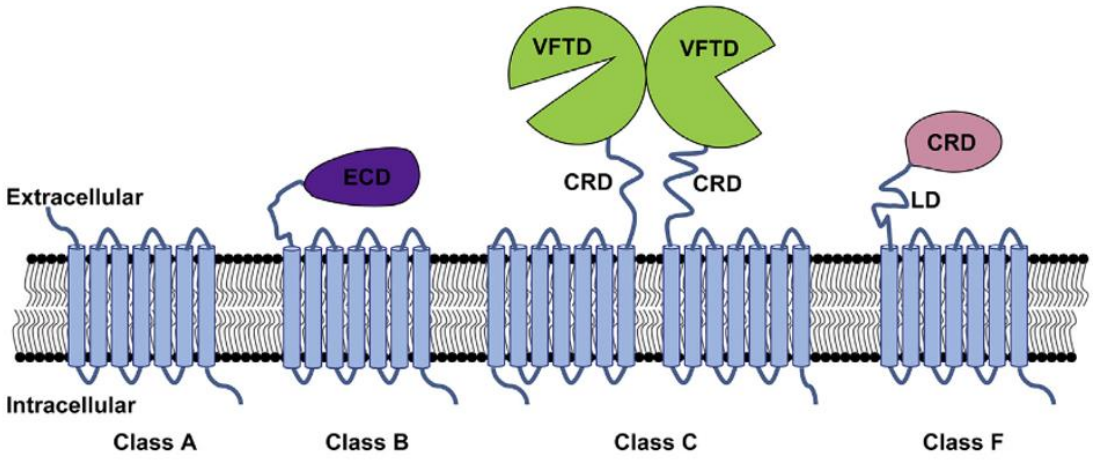


**Figure 2. Cartoon model displaying the structural features of the different classes of GPCRs.**

The canonical heptahelical transmembrane structure is shared by all GPCRs. Class A rhodopsin-like GPCRs have a relatively short N-terminus. Class B receptors have a longer N-terminal extracellular domain to accommodate for the large hormone peptide binding with the transmembrane domain. Class C receptors feature the VFT domain responsible for endogenous ligand-binding and for receptor dimerization. The VFT is connected with the TM region through a flexible cysteine-rich domain. Class F receptors feature a similar cysteine-rich domain at the N-terminus. Adapted from (Xiangli, Dejian, & Beili, 2020)

### 1.1.4 GPCR Activation

The localization of GPCRs on the plasma membrane and the exposition of their ligand-binding pocket on the extracellular side allows them to sense a wide diversity of extracellular signals. These signals include light photons, proteins, hormones, growth factors, small molecules, neurotransmitter, and ions. The classical model describing GPCR activation implies that agonist binding to receptors triggers a conformational change within the TM region that involves an outward movement of helices V and VI. As revealed by structural studies, TM movements creates a cavity on the cytoplasmic side that serves as a docking site for heterotrimeric guanine nucleotide-binding proteins (G proteins): αβγ (Carpenter, Nehme, Warne, Leslie, & Tate, 2016; Liang et al., 2017; Rasmussen et al., 2011). This interaction with G proteins represents the canonical view of GPCR activation and results in signal amplification through the different G protein-specific downstream effectors. Beyond G proteins, GPCRs interact with other effectors such as the G protein-coupled receptor kinases (GRKs) and arrestin proteins, leading to the desensitization of G protein-mediated signaling. Additionally, arrestins serve other functions such as receptor internalization and the activation of G protein-independent signaling (Marinissen & Gutkind, 2001). Elucidation of GPCR interactions with the varying interacting effectors is thus critical for the understanding of GPCR functional regulation.

## 1.2 Heterotrimeric G Proteins

### 1.2.1 Structure and Activation

The identification of heterotrimeric G proteins and their role in relaying information from plasma membrane receptors to intracellular effectors revolutionized our understanding of ligand-mediated functions. Indeed, Martin Rodbell and Alfred G. Gilman were awarded the Nobel Prize in Physiology or Medicine in 1994 for their discovery of G proteins and their role in in signal transduction in cells (Gilman, 1987; Rodbell, 1980). G proteins exist in a complex of three polypeptides (α, β, and γ), structured as two distinct units: the α subunit and the βγ dimer. The Gα and Gβγ subunits are post-translationally lipidated and confined at the inner leaflet of the cellular plasma membrane. Depending on the activation state, the Gα subunit binds guanosine diphosphate (GDP) and guanosine triphosphate (GTP) nucleotides. Typically, at the basal state, G proteins exist as heterotrimeric complexes with the Gα-GDP tightly associating with the Gβγ dimer. Agonist binding to GPCRs triggers a conformational rearrangement of receptors that allows for Gα association. Upon this association, active receptors act as guanine nucleotide exchange factors (GEFs), promoting GDP to GTP exchange on the Gα subunit. This in turn leads to the functional dissociation of the Gα-GTP from Gβγ, both becoming free to initiate downstream signaling (Alhosaini, Azhar, Alonazi, & Al-Zoghaibi, 2021). Two models of Gα and Gβγ dissociation after GDP/GTP exchange have been proposed. The first, representing the traditional view of G protein activation where there is a complete dissociation by physical distancing of the subunits "i.e., dissociation model". On the other hand, recent evidence points out another model of G protein activation where nucleotide exchange promotes a structural reorganization within the complex "i.e., conformational model" (Chung & Wong, 2021). Termination of effector signaling is

conveyed by GTP hydrolysis to GDP through the GTPase domain within the Gα subunit (Cabrera-Vera et al., 2003). Shortly after hydrolysis, reassociation of the Gα-GDP with Gβγ restores the basal inactive form of the G protein heterotrimer to initiate a new activation cycle (Fig. 3). It was initially thought that the duration of G protein signaling is determined solely by the rate of intrinsic GTP hydrolysis. However, a family of GTPase-activating proteins (GAPs), referred to as regulators of G protein signaling (RGS), were later revealed to expedite GTP hydrolysis on the Gα subunit, and hence desensitize heterotrimeric G protein signaling. (Pierce, Premont, & Lefkowitz, 2002).

While the precise timing for the functional and structural interaction between receptors and G proteins seems obscure, the classical signal transduction view suggests that such interaction occurs only after receptor activation. Nonetheless, several lines of evidence have questioned this view, suggesting that receptor activation might not be a prerequisite for G protein coupling and that G proteins may be pre-coupled to receptors at the basal state. This notion is supported by studies using fluorescence resonance energy transfer (FRET) which detected a low basal FRET signal between the α2-adrenergic receptor and Gαi (Gales et al., 2006). Another example of pre-coupling is the rhodopsin receptor, which exists at the basal state associated with a GDP-bound transducin (Gt). However, this pre-coupling renders rhodopsin in an intermediate state of weaker affinity than the full rhodopsin active state and is not sufficient to initiate GDP to GTP exchange (Alves et al., 2005; Morizumi, Imai, & Shichida, 2005).

An interrelationship between ligands, receptors, and G proteins was described in the ternary complex model. This mode distinguishes two interconvertible states of receptor agonist-binding affinities (low and high) coexisting in equilibrium at the basal level (De Lean, Stadel, & Lefkowitz, 1980). These affinity states are sensitive to G protein activity, where GTP

administration and G protein activation directly shifts the equilibrium towards the higher ligand affinity state. Thus, coupling of receptors to G proteins yields a more active state of the receptor that corresponds with higher ligand-binding affinity. Whereas the G protein-uncoupled receptor remains inactive and exhibits low ligand-binding affinity (Park, Lodowski, & Palczewski, 2008). According to this model, G proteins are suspected to behave as allosteric modulators (i.e., binding receptors at a site distinct from the orthosteric ligand binding pocket) that bind to receptors at the intracellular region and alter ligand-binding affinity.

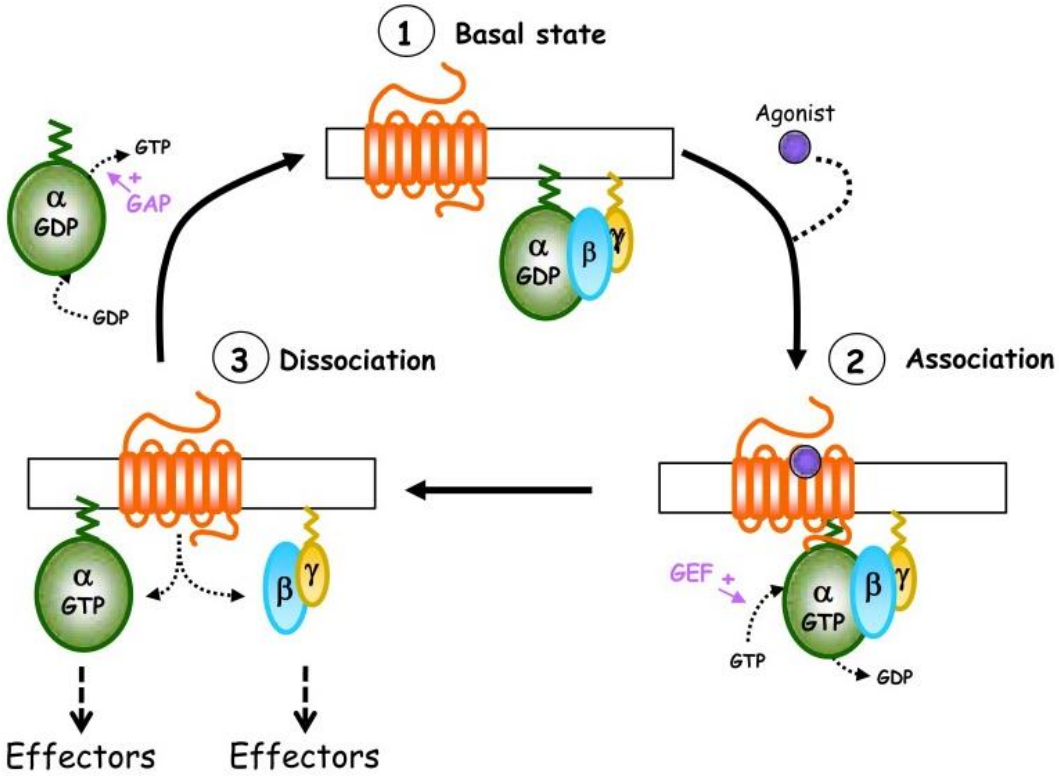


**Figure 3. GDP/GTP cycle of G protein activation.**

In the absence of ligand (basal state), the G protein heterotrimer exists as an inactive Gα-GDP/Gβγ complex. Ligand binding and GPCR activation allows the recruitment of the G protein heterotrimer and the subsequent GDP/GTP exchange on the Gα subunit (association), which in turn leads to Gα dissociation from the Gβγ subunits (dissociation). The activation cycle is then terminated by the intrinsic GTPase activity that mediates GTP hydrolysis and Gα-GDP reassociation with Gβγ subunits, restoring the initial inactive state (basal state). Adapted from (Denis, Sauliere, Galandrin, Senard, & Gales, 2012)

### 1.2.2 Gα Subunits

Gα subunits range in size between 39 and 45 kilodaltons (kDa) (Nurnberg, Gudermann, & Schultz, 1995) and are composed mainly of two domains. The RAS-like domain (αRas) shares a great structural homology to Ras-superfamily GTPases and is responsible for GTP hydrolysis; and the all-alpha-helical (αAH) domain that constitutes a long central helix and five shorter α-helices. Together with αRas, the αAH forms a deep guanine nucleotide binding pocket (McCudden et al., 2005). To date, 16 different Gα genes encoding 23 known Gα proteins have been identified. Based on their sequence homology and their effector selectivity, these proteins are classified into four main families: Gαs/olf, Gαi/o, Gαq/11, and Gα12/13 (Pfleger, Gresham, & Koch, 2019) (Fig. 4). Generally, GPCRs select for a specific Gα protein family, although some receptors may show more promiscuous coupling.

### 1.2.3 Gα Subtypes

#### *1.2.3.1 Gαs/olf*

GTP binding to Gαs/olf leads to the activation of adenylyl cyclase enzyme (AC). AC is a plasma-membrane bound protein that facilitates the synthesis of the cyclic adenosine monophosphate (cAMP) second messenger (Fig. 4). The rise in intracellular cAMP further activates protein kinase A (PKA), a serine/threonine kinase with diverse phosphorylation targets implicated in a number of cellular responses. For instance, PKA-mediated phosphorylation of myosin light-chain kinase (MLCK) regulates smooth muscle contraction. PKA may also phosphorylate GPCRs, leading to their G protein uncoupling and desensitization. Further PKA phosphorylation targets include transcription factors and other kinases such as members of the mitogen-activated protein kinase (MAPK) signaling cascade, among others (Oldham & Hamm,

2008). Prototypical Gαs/olf-coupled receptors include the isoproterenol-activated β-adrenergic receptor, the dopamine D1 receptor, and the vasopressin receptor 2 (V2R).

*1.2.3.2 Gαi/o*

Members of the Gαi/o protein family are encoded by 8 different genes. The three Gαi/o proteins (Gαi1, Gαi2, and Gαi3) inhibit AC activation and cAMP accumulation ("i" referring to the inhibitory effect), thereby counteracting Gαs activity (Fig. 4). Gαi can also activate the nonreceptor tyrosine kinase Src, leading to the activation of extracellular signal-regulated kinases (ERK) (Belcheva & Coscia, 2002). Other Gαi/o family members include: Gαt1 and Gαt2 (transducin), Gαgust (gustducin), Gαz and Gαo. Gαt1/2 and Gαgust are implicated in visual and taste regulation, respectively. Gαz also inhibits AC, in addition to its role in $K^+$ channel stimulation and RGS interaction. Finally, the Gαo ("o" standing for other) have an unclear effect on AC. It is presumed to have an effect only on some AC isoforms and not others. Gαo has also been reported to modulate other signaling pathways such as STAT3 and ERK (Birnbaumer, 2007). All members of the Gαi/o, except Gαz, are sensitive to pertussis toxin (PTX), which is often used to study Gαi/o functions. PTX is an endotoxin extracted from the bacterium Bordetella pertussis and catalyzes ADP-ribosylation on the Gαi subunit, locking it in its inactive GDP-bound form (Mangmool & Kurose, 2011). Interestingly, Gαi/o-coupled receptors are highly abundant in the brain and examples include the dopamine D2 receptor, acetylcholine receptor, opioid receptor, and cannabinoid receptors.

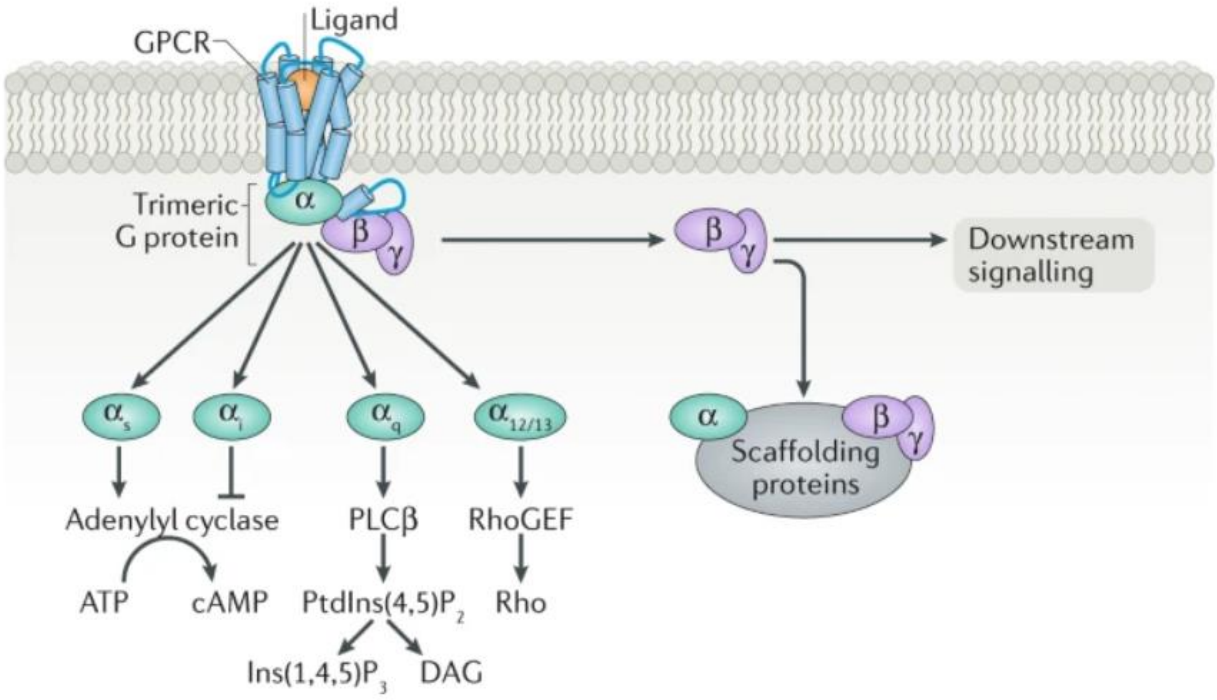


**Figure 4. The diversity of heterotrimeric G protein downstream signaling effectors.**

The four major Gα protein classes are shown. Each G protein subtype leads to the activation of specific downstream signaling effectors. Similarly, the Gβγ dimers initiate downstream signaling mainly by binding scaffolding proteins. From (Pfleger, Gresham, & Koch, 2019)

*1.2.3.3 Gαq/11*

The Gαq/11 family is composed of the ubiquitously expressed Gαq and Gα11 subtypes, and the less abundant Gα14 and Gα15 subtypes. Gαq/11 activation incites phosphoinositide turnover by the activation of phosphoinositide-specific phospholipase C-β (PLC-β) isozymes, which catalyzes the hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP2) to generate the two second messengers: inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) (Fig. 4). IP3 in the endoplasmic reticulum activates IP3 receptors responsible for controlling cytoplasmic $Ca^{2+}$ release. The increased $Ca^{2+}$ concentrations in the cytosol in turn initiates various cellular events such as cell proliferation, gene transcription, and muscle contraction. $Ca^{2+}$-mediated muscle contraction occurs through two mechanisms. The first involves MLCK activation, leading to the phosphorylation of myosin regulatory light-chain ($MLC_{20}$), and hence vasoconstriction. The second mechanism involves Janus kinase 2 (Jak2) and subsequent P115RhoGEF, RhoA, and Rho-associated protein kinase (ROCK) activation. ROCK phosphorylates and suppresses the myosin light chain phosphatase (MLCP) enzyme, leading to the same earlier result of increased $MLC_{20}$ phosphorylation and smooth muscle contraction (Momotani & Somlyo, 2012) (Fig. 5). The second released messenger, DAG, remains on the plasma membrane and activates the serine/threonine protein kinase C (PKC). PKC is involved in the activation of a wide range of signaling pathways including for the MAPK cascade that is responsible for regulating transcription factors that mediate cell growth, migration, and apoptosis. Interestingly, similar to PKA, PKC phosphorylation of GPCRs serves as a negative feedback mechanism and uncouples receptors from G proteins (Cordeaux & Hill, 2002).

In addition to this classical Gαq/11 signaling, studies identified p63RhoGEF as another effector which directly interacts with Gαq. Through its GEF function, p63RhoGEF may also

activate the RhoA pathway. The exact engagement mechanism between Gαq and p63RhoGEF have been identified and a Gαq-p63RhoGEF-RhoA crystal structure was resolved in 2007 (Lutz et al., 2007; Rojas et al., 2007). Such knowledge enabled further exploitation of this effector and the development of novel bioluminescence resonance energy transfer (BRET) biosensors to specifically monitor Gαq signaling (Namkung et al., 2018; Avet et al., 2022).

Another valuable approach commonly used for evaluating the coupling of receptors to Gαq/11 is using one of the two synthetic compounds that selectively inhibit Gαq/11 activity: FR900359 (UBO-QIC) and its closely related analogue, YM-254980 (YM). These compounds intercalate with the GDP binding domain of the Gαq/11 subunits and prevent GDP/GTP exchange, constraining the α subunit in its inactive form (Schrage et al., 2015). Examples of Gαq/11-coupled receptors mentioned in this study are the angiotensin II (AngII) type 1 receptor (AT1R), the prostaglandin F2α receptor (FP), the bradykinin type 2 receptor (B2R), and the thromboxane A2 receptor alpha (TPα).

*1.2.3.4 Gα12/13*

The Gα12/13 subfamily is the most recently discovered of the four G protein families and is composed of Gα12 and Gα13 proteins. To date, over 30 GPCRs coupling to Gα12/13 have been identified. Interestingly, these receptors invariably interact with other G proteins, particularly Gαq (Riobo & Manning, 2005). This made the dissection of signaling specificity downstream of Gα12 and Gα13 more complicated. Especially given that very few events are linked unambiguously to these G proteins. The most well-established Gα12/13-downstream effector is the monomeric GTPase RhoA, important for regulating cell contraction, gene transcription, and cytoskeletal remodeling (Gohla, Schultz, & Offermanns, 2000; Siehler, 2009). As mentioned earlier, RhoA activation is not exclusive to Gα12/13 and can also be mediated by Gαq/11. Thus, the absence of

specific inhibitors acting on these G proteins further limits the ability to develop a comprehensive analysis of Gα12/13 signaling.

Increased interest in studying Gα12/13-specific effectors led to the identification of specific Rho-family guanine nucleotide exchange factors (RhoGEFs) that directly link Gα12 and Gα13 with Rho. These RhoGEFs possess an amino-terminal RGS homology domain (RH) and central DH/PH (Dbl homology/pleckstrin homology) domain, which are characteristic features of GEFs for Rho family GTPases. Upon the activation of Gα12/13-coupled receptors, P115RhoGEF, leukemia-associated RhoGEF (LARG), and PDZRhoGEF are translocated to the plasma membrane to interact with activated Gα12 and Gα13 forms. This interaction triggers two functions associated within these RhoGEFs: a GEF activity that leads to RhoA activation, and a GAP activity inhibiting the Gα12/13 subunit (Momotani & Somlyo, 2012) (Fig. 5). Similar to p63RhoGEF for Gαq, a BRET-based biosensor for PDZ-RhoGEF activation relying on its plasma membrane translocation has been developed (Avet et al., 2022). Known GPCRs that couple Gα12/13 mentioned in this study are FP, AT1R, B2R, and TPα, all of which are also coupled to the Gαq/11 G protein family.

### 1.2.4 Gβγ

Similar to Gα, Gβγ subunits are also tethered to the plasma membrane by post-translational modifications. In the human genome, Gβ subunits are encoded by 5 different genes, while 12 different genes encode Gγ subunits. Although a large number of different Gβγ combinations can theoretically exist, only several are actually formed. This is partly explained by the tissue-specific expression of the Gβ and Gγ subtypes (Smrcka, 2008). To this day, the functional significance of individual Gβγ combinations is not completely understood. The role of Gβγ dimer in regulating the Gα subunit was the first to be described. Gβγ was shown to be important for properly orienting

Gα at the plasma membrane and exposing its amino terminus so that it may interact with activated GPCRs. Moreover, the Gβγ dimer catalyzes nucleotide exchange on Gα by virtue of its activity as a guanine nucleotide dissociation inhibitor. Newer lines of evidence revealed other functions of the free Gβγ released after Gα-GTP dissociation. Gβγ was reported to interact with phospholipases and ion channels, and the list of interacting partners is continuously growing (McCudden et al., 2005). An important function of free Gβγ is mediated by its association with GRK2 and GRK3 leading to GPCR desensitization (Daaka et al., 1997). The process of GPCR desensitization will be discussed in more details in section 1.3.3.1.

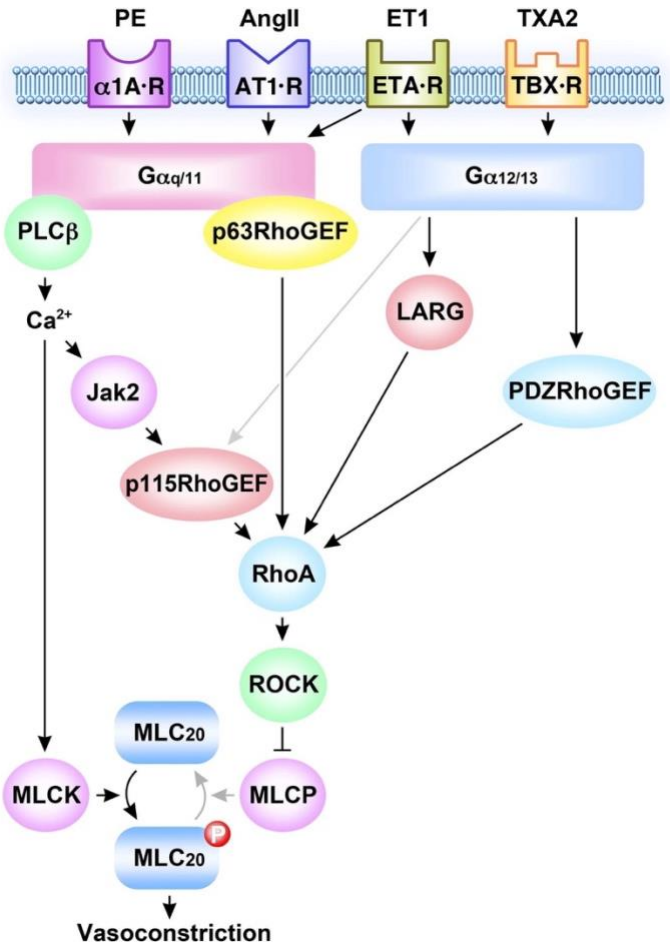


**Figure 5. Schematic illustration of RhoGEFs activated by Gαq/11 and Gα12/13.**

GPCRs coupled to Gαq/11 and/or Gα12/13 proteins activate a variety of RhoGEF. They activate p63RhoGEF through interacting with Gαq/11, and P115RhoGEF, LARG, and PDZRhoGEF through Gα12/13 interaction. Activated RhoGEFs catalyze GTP loading to activate RhoA. This leads to ROCK activation, MLCP inhibition, and an increase in phosphorylated $MLC_{20}$ and vasocontraction. Gαq-mediated increase in cytosolic $Ca^{2+}$ through PLCβ also leads to vasoconstriction, either by MLCK, or by Jak2 and P115RhoGEF activation. Adapted from (Momotani & Somlyo, 2012)

## 1.3 Arrestin Family Proteins

### 1.3.1 Overview

GPCR functional regulation is fine-tuned by the arrestin family of adaptor proteins. The original identification of arrestin proteins and their role in terminating G protein signaling was connected with the isolation of GRKs (Benovic, Strasser, Caron, & Lefkowitz, 1986). Further studies later established that both GRKs and arrestins are necessary to fully modulate GPCR's G protein decoupling (i.e., desensitization). GRKs serve to phosphorylate the C-tail of activated GPCRs leading to arrestins interaction and receptors' desensitization. Besides this classical function of arrestin proteins, these adaptor proteins mediate pleiotropic functions such as receptor internalization and trafficking, as well as the activation of G-protein independent signaling (Fig. 6). The arrestin protein family constitutes four members: the two visual arrestin proteins (arrestin 1 and 4, also called rod and cone arrestins, respectively) and the two non-visual arrestin proteins (arrestin2 and arrestin3, alternatively referred to as β-arrestin1 and β-arrestin2) (Caron & Barak, 2019). This thesis will focus on β-arrestins, which are more ubiquitous and interact with the majority of non-visual GPCRs, as opposed to the visual arrestins, expressed in the retina, and confined to photoreceptors.

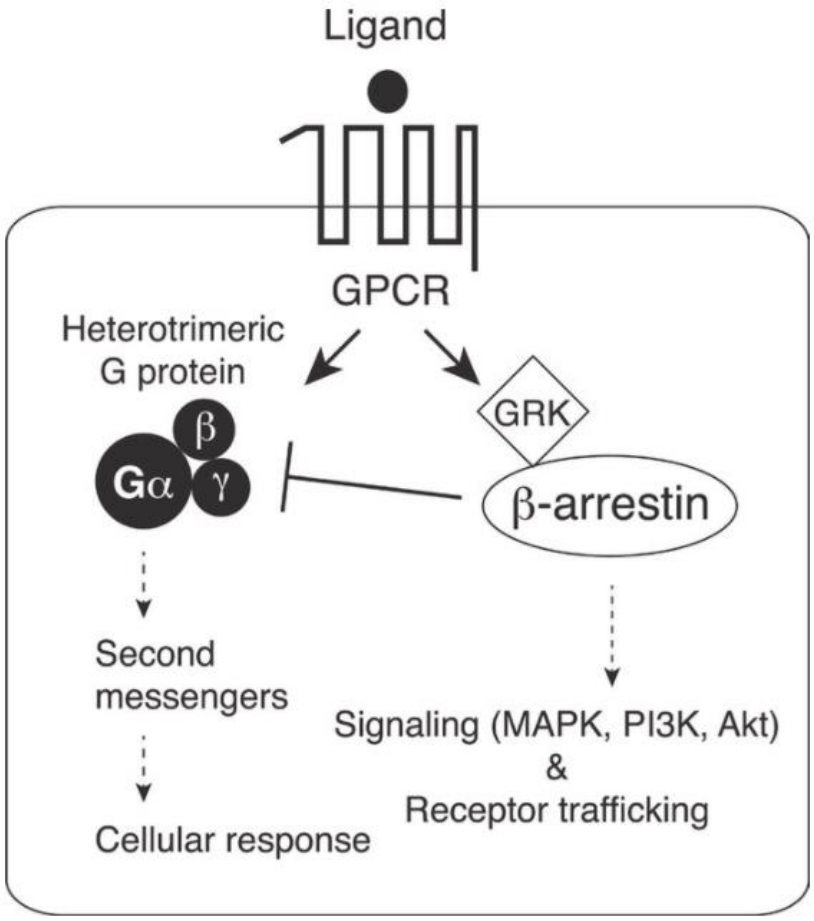


**Figure 6. Diversity of β-arrestin functions.**

Besides their classical role in G protein desensitization, β-arrestins regulate receptor trafficking and mediate G protein-independent downstream signaling. From (Ikeda, Kumagai, Motozawa, Suzuki, & Komuro, 2015)

### 1.3.2 Structure

The structure of arrestin proteins comprises two cup-like domains made up of β-sheets, termed as the N and C domains. These domains are capped with flexible loops composing the central crest (finger loop, middle loop and C-loop) and the C-edge loops (344-loop and 197-loop). The core domain interface is stabilized with hydrophobic interactions and a network of hydrogen bonds, making up the polar core important for maintaining the inactive "basal" arrestin state. Disruption of these interactions, by changing the charges of the five charged residues comprising the polar core yields an easily activable arrestin mutant (also referred to as "preactivated" arrestin). The inactive state of arrestin is also maintained by a hydrophobic "three-element interaction", involving two N domain elements (β-strand I and the α-helix) and the C-tail of arrestin. A natural splice variant lacking the C-tail, and hence the three-element interaction, produces a "preactivated" arrestin (Chen, Iverson, & Gurevich, 2018; Kim et al., 2013; Vishnivetskiy, Baameur, Findley, & Gurevich, 2013). All these interactions wind between the N and C domains and make multiple contacts within both. Receptor interaction is restricted by the non-flexible configuration of the central crest loops and the N and C domain imposed by the interdomain interface together with the C-tail (Sommer, 2017). Collectively these data infer that arrestin activation requires a conformational change that involves domains' movement relative to one another (Fig. 7).

The two crystal structures of the pre-activate arrestin, p44 (a naturally occurring arrestin-1 splice variant lacking the entire C-tail) (Kim et al., 2013) and active arrestin-2 bound to a phosphopeptide analogous to phosphorylated receptor C-terminus (Shukla et al., 2013), allowed researchers to pinpoint important conformational rearrangements that occur in arrestins upon receptor interaction and C-tail displacement. Arrestin's activation occurs in a multistep process. Initial engagement of arrestin with the phosphorylated C-tail of the active GPCRs leads to a

twisting displacement of the gate loop, which breaks the polar core and releases arrestin's C-tail, thereby relieving the restrictive conformation of the central crest loops. This in turn leads to major conformational changes within arrestin where the N and C domains exhibit a 21° rotation in respect to one another (Shukla et al., 2014) (Fig. 7). In addition to these interdomain rearrangements, other loops show mobility upon receptor interaction and arrestin activation. Namely, the finger loop (residues 64–72), the middle loop (residues 130–142), and the lariat loop (residues 275–315) which comprises the gate loop (residues 289–298) and the back loop (residues 311–315). The finger loop in particular shows significant flexibility and is highly conserved between all four arrestin subtypes. Moreover, the positioning of the finger loop in close proximity to the receptor's cytoplasmic interface allows it to insert within its binding cavity, and this engagement appears to be crucial in stabilizing the GPCR's active form (i.e., high affinity GPCR–arrestin interaction) (Sommer, 2017).

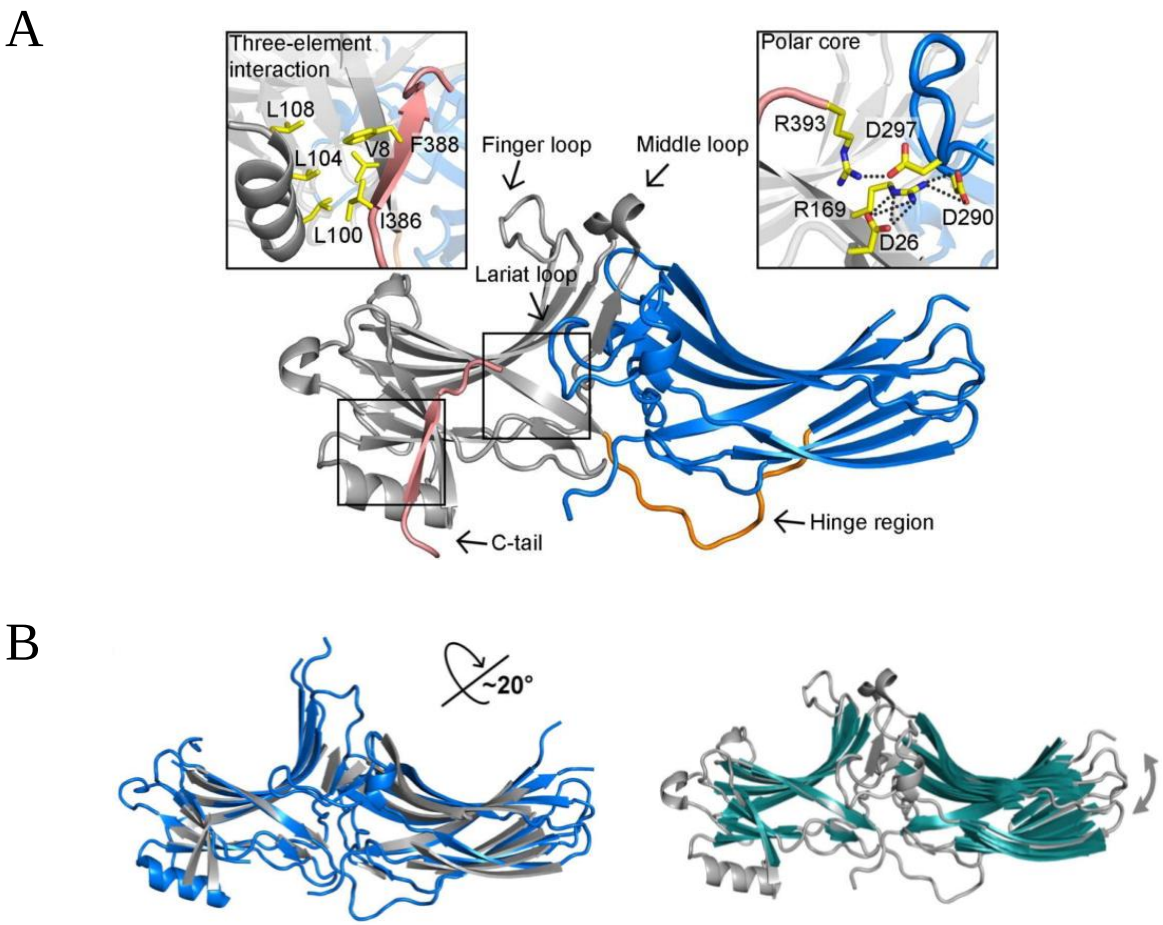


**Figure 7. Arrestin basal state structure and its conformational changes upon activation.**

A. Crystal structure of arrestin-2 in its basal state (PDB:1G4M). The N domain (grey) and C domain (blue) are linked by a hinge region (orange). Arrestin's C-tail (pink) is attached to the N domain. The upper left box shows the three-element interactions at the basal state. The upper right box shows the polar core interaction, involving five charged residues. B. Global conformational changes in arrestin upon its activation. The left panel is a superposition of arrestin's N domain in the active (blue, PDB:4JQI) and basal (grey, PDB:1G4M), highlighting the inter-domain rotation. The right panel is a superposition of arrestin's N domain for different arrestin-2 variants (grey and green), revealing a narrow range of interdomain rotational movements at the varying basal states. Adapted from (Chen, Iverson, & Gurevich, 2018)

### 1.3.3 Classical Functions

#### *1.3.3.1 Desensitization*

As its name implies, the initial identified function of β-arrestin was to "arrest" G protein-mediated signaling by desensitizing activated GPCRs. In conditions of prolonged agonist stimulation, receptor desensitization becomes important to prevent overstimulation. Principally, GPCR desensitization requires the phosphorylation of serine/threonine residues situated mainly in the receptor's ICL3 and C-terminal tail. While this phosphorylation is primarily achieved by GRKs, second messenger-activated kinases like PKA and PKC may also mediate receptor phosphorylation (Kelly, Bailey, & Henderson, 2008). Based on the kinase in play, two modes of receptor desensitization are distinguishable. The heterologous desensitization is driven by second messenger-activated kinases and does not necessarily target the active ligand-occupied receptors as their target (i.e., they phosphorylate active and inactive GPCRs indiscriminately) (Chuang, Iacovelli, Sallese, & De Blasi, 1996). As such, these second messenger kinases can mediate classical negative-feedback regulatory loops to regulate GPCR activity. On the other hand, homologous desensitization is governed by the specific GRK phosphorylation of active ligand occupied GPCRs (Freedman & Lefkowitz, 1996). To date, seven mammalian GRK subtypes, closely related to PKA and PKC, have been identified (GRK1–7). All GRK subtypes comprise distinct structural domains important for receptor specificity and portray increased activity towards the active GPCR conformation (Pitcher, Freedman, & Lefkowitz, 1998). The most important function of GRKs is to facilitate the recruitment of arrestin family proteins to the phosphorylated receptors, which in turn decouples GPCRs from their cognate G proteins in two manners: the first is by sterically occluding G proteins' binding site at the receptor interface, while the second is by

facilitating the association of elements of the endocytic machinery to the receptor and promoting GPCR internalization into the endosomes (further details about this process are in the next section).

*1.3.3.2 Internalization*

Receptor-bound β-arrestins act as scaffolding proteins to recruit elements of the endocytic machinery to the plasma membrane. Notably, desensitization and internalization processes are two distinct events; and efficient endosomal endocytosis is not always indispensable for receptor desensitization. For instance, some GPCRs, like the α2-adrenergic receptor, are desensitized following ligand stimulation without internalizing to intracellular endosomes (Daunt et al., 1997). For those receptors that do internalize, β-arrestins play a vital role in their sequestration from the plasma membrane to the endosomes through the process of clathrin-mediated endocytosis.

The interaction of β-arrestin with activated receptors and the ensuing conformational rearrangements in its structure not only enhance β-arrestin and GPCR interaction, but also results in the exposure of binding motifs within β-arrestin that can interact with endocytic proteins such as clathrin and the adaptor protein-2 (AP-2) complex (Tian, Kang, & Benovic, 2014). Clathrin is a well-studied 190 kDa protein that plays a central role in receptor endocytosis. As a monomer, clathrin forms a triskelion of three heavy chains and three light chains that oligomerize, to form the polyhedral lattice referred to as the clathrin-coated pit (CCP). AP-2 and other adaptor proteins are responsible for assembling clathrin monomers and the resulting formation of (CCPs), which envelope an invagination of the phospholipid bilayer that is later removed from the plasma membrane to form clathrin-coated vesicles (CCVs). β-arrestin acts as the link between receptors, AP2, and clathrin to co-localize receptor–β-arrestin complexes in the punctuated CCPs at the cell surface. Besides its role in mediating clathrin oligomerization, AP2 also recruit dynamin GTPase to pinch and separate the CCP from the plasma membrane, leading to CCV formation (Ferguson

& De Camilli, 2012; Pierce & Lefkowitz, 2001). Internalized GPCRs exhibit two behavioral patterns in term of their trafficking profile that depend on the strength of their β-arrestin interaction. Accordingly, GPCRs are classified into two classes: A and B (Fig. 8). Class A GPCRs, such as β2AR, are characterised by their poor and transient β-arrestin interaction. These receptors rapidly dissociate from β-arrestin at the plasma membrane and are rapidly recycled, leading to regain of signaling functions at the cell surface. On the other hand, class B receptors, such as the AT1R, V2R, and B2R, are characterized by their high avidity binding to β-arrestins, enabling them to form stable complexes that persist throughout the internalization process to accumulate in endosomes. Members of the class B family have a slower rate of recycling, ultimately leading to a decrease in receptor density and signaling from the plasma membrane (Oakley, Laporte, Holt, Barak, & Caron, 1999).

Although CCV-mediated endocytosis represents the classical mode of receptor internalization, some GPCRs can internalize via different mechanisms independent of clathrin and β-arrestin. The caveolae/lipid raft pathway is an alternative internalizing route utilized by some GPCRs such as the glucagon and chemokine receptors (CCR2 and CCR5) (Dzenko, Andjelkovic, Kuziel, & Pachter, 2001; Krilov, Nguyen, Miyazaki, Unson, & Bouscarel, 2008). Caveolins oligomerize in lipid-rich domain to form smooth muscle invaginations at the plasma membrane, which leads to receptors' internalization. Similar to CCV-mediated endocytosis, caveolae-dependent internalization also uses dynamin to remove vesicles from the plasma membrane. However, receptors are subsequently targeted to the endoplasmic reticulum rather than endosomes (Frank et al., 2006). Endocytic sorting of receptors varies based on the agonist type, receptor phosphorylation by GRKs, and the relative availability of the endocytic proteins.

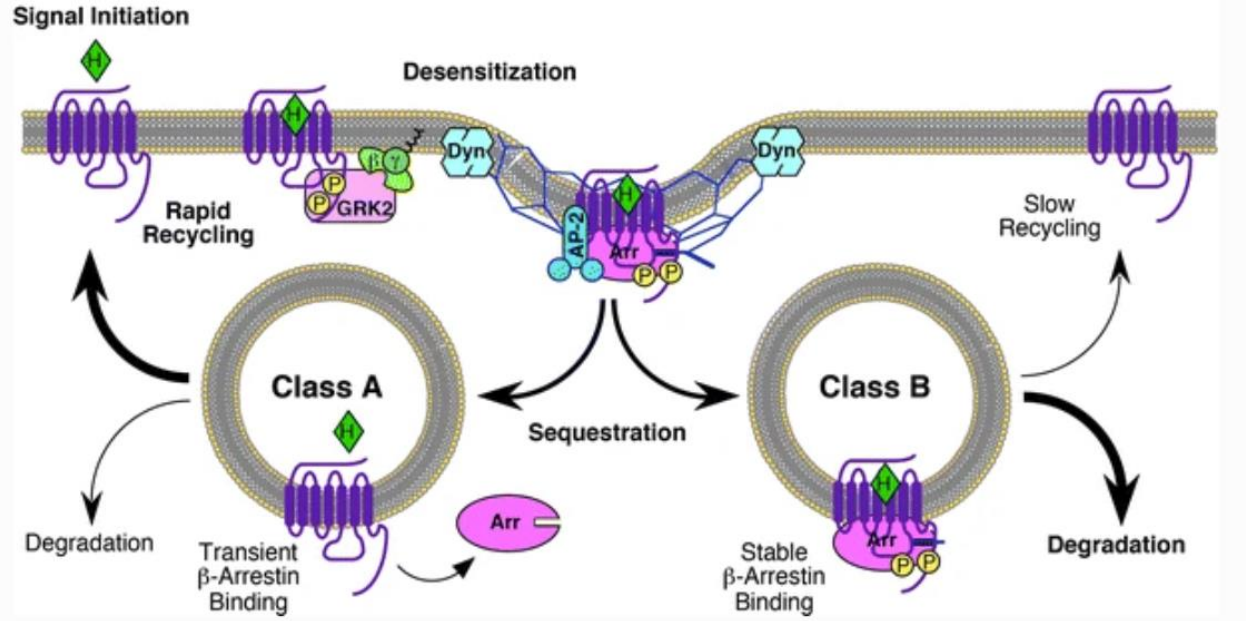


**Figure 8. β-arrestin-dependent internalization of class A and B GPCRs.**

Agonist-bound GPCRs are phosphorylated by GRKs at their ICL3 and C-terminal leading to β-arrestin recruitment. β-arrestin scaffolds clathrin and the AP-2 complex to form clathrin-coated pits, while dynamin pinches the formed vesicle and completes GPCR–β-arrestin endocytosis. The stability of GPCR–β-arrestin interaction distinguishes two classes of GPCRs. Weak interactors (i.e., class A), which dissociate from interacting β-arrestin and are thus rapidly recycled to the plasma membrane, and strong interactors (i.e., class B), which form stable GPCR–β-arrestin complexes that accumulate longer in the endocytic vesicle and exhibit much slower recycling. From (Kendall & Luttrell, 2009)

### 1.3.4 Signaling

In the last two decades, the list of arrestin functions expanded to include signal transduction. With their role as scaffolding proteins, arrestins recruit numerous signaling effectors to mediate their signaling activity. The first evidence of the involvement of β-arrestins in signaling was with the discovery that β-arrestin can interact with src to activate MAPK kinases (Luttrell, Daaka, & Lefkowitz, 1999). This finding directed scientists to a previously unappreciated mechanism for GPCR-dependant ERK1/2 activation. Later, the list of β-arrestin-interacting proteins kept continuously growing, today reaching more than a hundred interacting partners (Xiao et al., 2007). Of those, over 20 different kinases bind β-arrestin to support the "non canonical" G protein-independent GPCR signaling (Eichel & von Zastrow, 2018). Select examples of signaling proteins scaffolded by β-arrestins are the MAPK family members including Raf-1, MEK1, ERK and JNK, the ARF6-ARNO complex, the Src family of kinases, AKT, and phospholipase A2 (Beaulieu et al., 2005; Luttrell & Gesty-Palmer, 2010; Mukherjee et al., 2000). Some of β-arrestin interacting effectors, like ERK1/2, are jointly regulated by both G proteins and β-arrestins through differential mechanisms.

Investigation of β-arrestin signaling lead to the identification of the first evidence for GPCR-mediated signaling from the internal membrane. This was reported as a correlation between MAPK activation through β-arrestin and endocytosis (Luttrell, Daaka, Della Rocca, & Lefkowitz, 1997). GPCR signaling from the endosomes was further supported by the resulting MAPK inhibition upon blocking β2AR internalization (Daaka et al., 1998). Accumulating evidences of more GPCRs forming β-arrestin complexes with MAPK effectors in the endosomes led to the concept of endosomal β-arrestin signaling. The ability of β-arrestin to also signal from the plasma membrane was later suggested through studies showing that blocking endocytosis by dynamin

inhibition enhanced β-arrestin-mediated MAPK activation by some receptors (Eichel, Jullie, & von Zastrow, 2016; Weinberg, Zajac, Phan, Shiwarski, & Puthenveedu, 2017) (Fig. 9). Interestingly, the relative plasma membrane vs. endosomal ERK1/2 activation varies between receptors (Khoury, Nikolajev, Simaan, Namkung, & Laporte, 2014; Laporte & Scott, 2019; Peterson & Luttrell, 2017; Zimmerman et al., 2011). For example, ERK1/2 activation occurs both at the plasma membrane and in endosomes for B2R (Zimmerman et al., 2011). In the case of V2R, blocking AP2 interactions with β-arrestin, and hence the complex internalization, completely inhibited ERK1/2 signaling (Beautrait et al., 2017). For AT1R, while no clear distinction is established, studies suggest that AT1R's internalization is critical for proper β-arrestin-mediated ERK1/2 activation (Ahn, Wei, Garrison, & Lefkowitz, 2004; Cao et al., 2020). It remains not evident how the different receptor interactions and the subcellular localization affect β-arrestin-mediated signaling and function. In chapter 4 of this thesis, the binding conformation of β-arrestin was shown to be altered in the different compartments. Such alteration may directly associate with the different signaling outcomes of β-arrestin engagement.

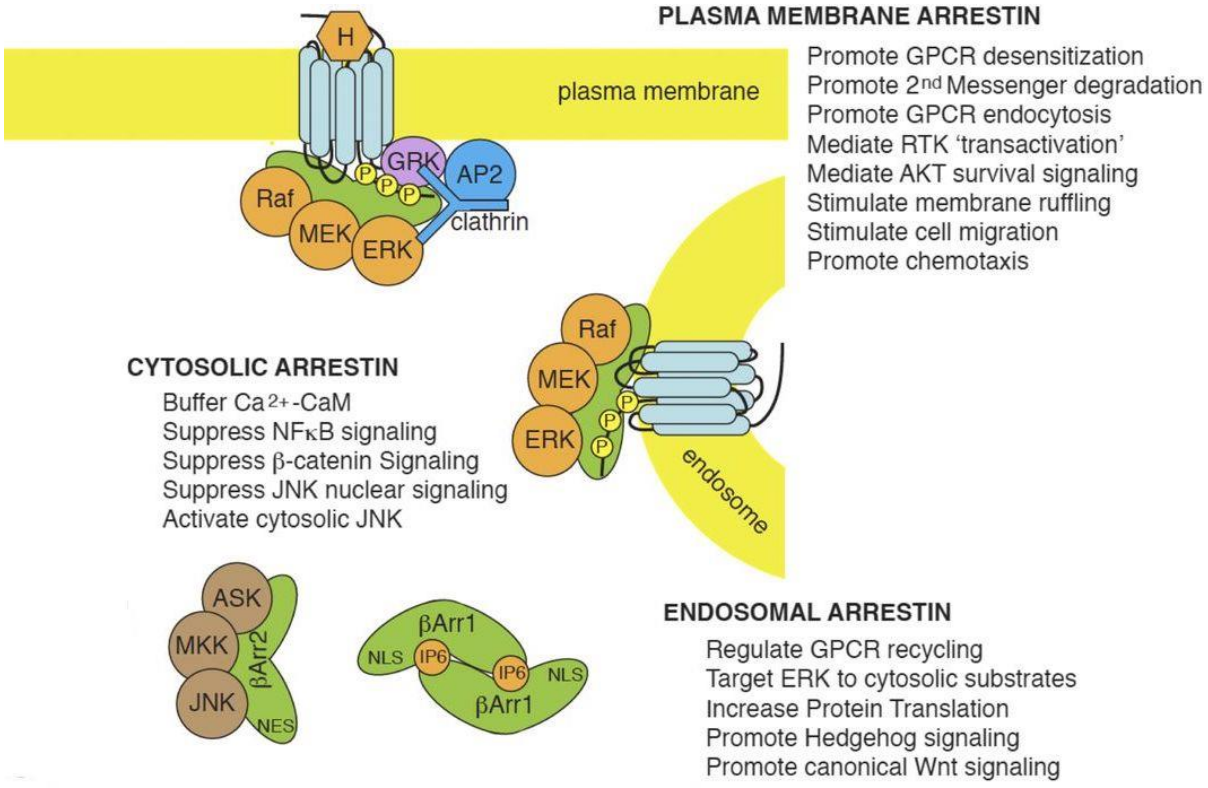


**Figure 9. β-Arrestin-signaling at different subcellular compartments.**

β-arrestins can associate with different cargos in the plasma membrane and in the endosomes. Free cytosolic β-arrestins can dampen basal signaling by sequestering signaling intermediates and prevent their interaction with other partners. Upon recruitment to GPCRs at the plasma membrane, β-arrestins desensitize receptors and initiate a number of signaling event. Interestingly, some β-arrestin cargos, such as ERK1/2, continue to signal from the GPCR-β-arrestins in the endosomes. Adapted from (Peterson & Luttrell, 2017)

## 1.4 Prostaglandin F2α Receptor, FP

### 1.4.1 Prostaglandins

Prostaglandins (PGs) are lipid derived compounds characterized by their 20-carbon chain. They contain the three subtypes: prostaglandins E (PGE2), F (PGF2α), and D (PGD2) that are ubiquitously expressed and have a hormone-like effect in mammals. PGs belong to the larger eicosanoid group which additionally includes prostacyclins (PGI2), thromboxanes (TXAs and TXBs), and leukotrienes (LTA, LTC, LTD, and LTE). PGs, PGIs, and TXAs form the "prostanoid" subgroup, and are all derived from the sequential enzymatic metabolism of the fatty acid, arachidonic acid (AA). Upon its plasma membrane release, AA is oxidized to PGH2 by the cyclooxygenase enzyme (COX). PGH2 is then converted by the prostaglandin synthase enzymes to the different prostanoid subtypes (Smith, DeWitt, & Garavito, 2000). PGs have potent pro-inflammatory effects, as implied by the pharmacological nonsteroidal anti-inflammatory agents (NSAIDs), which exert their functions through inhibiting PG biosynthesis by acting on one of the two COX isoforms. COX-1 is ubiquitously expressed in the different tissues, while COX-2 is inducible in response to inflammatory cytokines (Hata & Breyer, 2004). In addition to their implication in inflammatory regulation, PGs are also well known for their role in smooth muscle contraction, further broadening their pharmacological scope. Physiological effects of PGs are mediated through their interaction with prostanoid GPCRs that share 20–30% sequence homology.

### 1.4.2 PGF2α-Induced Signaling

The prostaglandin F2α receptor (FP) binds and mediates the functions of PGF2α by coupling to Gαq/11 and Gα12/13 families. Through its Gαq-mediated activity, FP promotes the canonical Gαq singling, leading to IP3 production, DAG generation, and intracellular $Ca^{2+}$ release.

Gαq activation also leads to PKC and MAPK activation. FP-mediated Gα12/13 leads to Rho GTPase/ROCK signaling cascade important for cytoskeletal remodeling (Chen, Fong, & Davis, 2001; Goupil et al., 2010). As described in sections 1.2.3.3 and 1.2.3.4, Gαq/11 and Gα12/13 are both involved in smooth muscle contraction; yet their relative contribution is not determined. Moreover, FP responses can be allosterically biased towards Gαq/11 activation while selectively inhibiting Gα12/13 pathway; this is discussed in further details in section 1.6.1.2. FP signaling is further complexed by its ability to bind other endogenous PGs, such as PGD2 and PGE2, although at a lower affinity (Abramovitz et al., 2000). Moreover, PGF2α binding to FP may induce signaling events through other receptors by transactivation or dimerization. A crosstalk between FP and the epidermal growth factor receptor (EGFR) signaling have been reported (Goupil et al., 2012). FP may also form heterodimers with AT1R, leading to altered receptors' signaling (Goupil et al., 2015). Sine FP and AT1R have mutual effects in regulating vessel contractility, such dimerization underscores an important means for these receptors' functional control. An example of FP and AT1R reciprocal regulation is the role of PGF2α in AngII release and renin production (Hayashi & Miyamoto, 1999; Yu et al., 2009). Similarly, AT1R activation can lead to an increase in AA production, and hence PGF2α synthesis (Zafari et al., 1999). With the limited knowledge of the interplay of these two GPCRs, it remains important to understand how these receptors exert their function through their coupled G proteins.

FP is one of the few receptors that do not internalize nor recruit β-arrestin following agonist stimulation (Goupil et al., 2012). Nonetheless, one of the ovine FP splice variants ($FP_A$) undergoes heterologous desensitization by PKC and is internalized in HEK 293 cells (Srinivasan, Fujino, & Regan, 2002). While the ovine and human FP share 90% sequence homology, the C-tail of human FP lacks threonine residues that are found in the ovine FP sequence. These represent important

phosphorylation sites for kinases that are involved in GPCR desensitization. Interestingly however, the heterodimerization of FP with AT1R was shown to facilitate its internalization (Goupil et al., 2015), highlighting other pathways that mediate FP and other receptors' internalization.

### 1.4.3 FP Physiology

The FP receptor is expressed in multiple tissue types and is involved in various physiological processes (Table. 1). Particularly, it is expressed in high abundance in the female reproductive system where it plays an important role in parturition, concomitant with an increase in PGF2α synthesis (Basu, 2007; Hao & Breyer, 2008). Studies on mice lacking FP or COX-2 expression showed disrupted parturition (Sugimoto et al., 1997). Accordingly, PGF2α replacements rescues a high number of neonatal mortalities. FP's activation in the eye vasculature and ciliary muscles results in the increased outflow of the aqueous humor and subsequent reduction in the intraocular pressure; this underlies the clinical implication of FP agonists, such as fluprostenol and latanoprost, in the treatment of glaucoma (Woodward, Jones, & Narumiya, 2011). FP is also abundantly expressed in the kidney's distal convoluted tubules and cortical collecting ducts to serve its role in maintaining water and electrolyte homeostasis (Hebert et al., 2005). Interestingly, the activation of FP was found to have a pathological consequence on the cardiac system relating to blood pressure, atherosclerosis, and cardiac function through its autocrine-mediated increase of renin release, and renin–angiotensin–aldosterone system (RAAS) activation. FP also promotes artery constriction in smooth muscle cells, which contributes to blood pressure increase and atherosclerosis (Zhang et al., 2010). Thus, PGF2α responses through FP are responsible for multiple aspects of cardiovascular disorders, and targeting FP provides a valuable strategy for controlling hypertension and the resultant vascular disorders.

| Tissue/cell distribution | Physiological/pathophysiological process |
|---|---|
| Ovary | Luteolysis, Parturition |
| Myometrium | Uterine contraction |
| Ocular vasculature, iris sphincter, ocular circular muscles | Aqueous humor homeostasis |
| Renal distal convoluted tubule, cortical collecting duct | Water and electrolyte reabsorption |
| Juxtaglomerular apparatus | Renin secretion, blood pressure regulation |
| Lung fibroblast | Pulmonary fibrosis |
| Cardiac fibroblast, cardiomyocyte | Myocardial fibrosis, arrythmias, myocyte hypertrophy |
| Vascular smooth muscle cell (VSMC) | VSMC hypertrophy, vasoconstriction |

**Table 1. FP expression and its physiological/pathological roles.**

This table is adapted from (Zhang et al., 2010)

## 1.5 Angiotensin II Type 1 Receptor, AT1R

### 1.5.1 Renin-Angiotensin-Aldosterone System

The renin-angiotensin-aldosterone system (RAAS) is the primary system in the human body that regulates blood pressure, electrolyte balance, and vascular resistance. It operates as a feedback mechanism to elevate blood pressure in response to a decrease in renal blood pressure or a decrease in salt delivery to the kidney's distal tubules by releasing the renin enzyme from Juxtaglomerular cells in the kidney. Renin in the bloodstream acts on its plasma circulating target, angiotensinogen, and cleaves it into angiotensin I. The inactive angiotensin I is converted to the active angiotensin II (AngII) by the angiotensin converting enzyme (ACE), which is primarily found in the vascular endothelium of kidneys and lungs. AngII activates AT1R to increases blood pressure by promoting arteriole constriction. AngII also triggers aldosterone and vasopressin release from the adrenal and pituitary glands, respectively. The effect of aldosterone and vasopressin on the kidney causes sodium retention, further increasing blood volume and blood pressure (Atlas, 2007). Accordingly, the RAAS system is often associated with chronic cardiovascular diseases such as hypertension and congestive heart failure; and drugs have been developed to target the RAAS system at varying steps to manage these diseases (Luther & Brown, 2011) (Fig. 10).

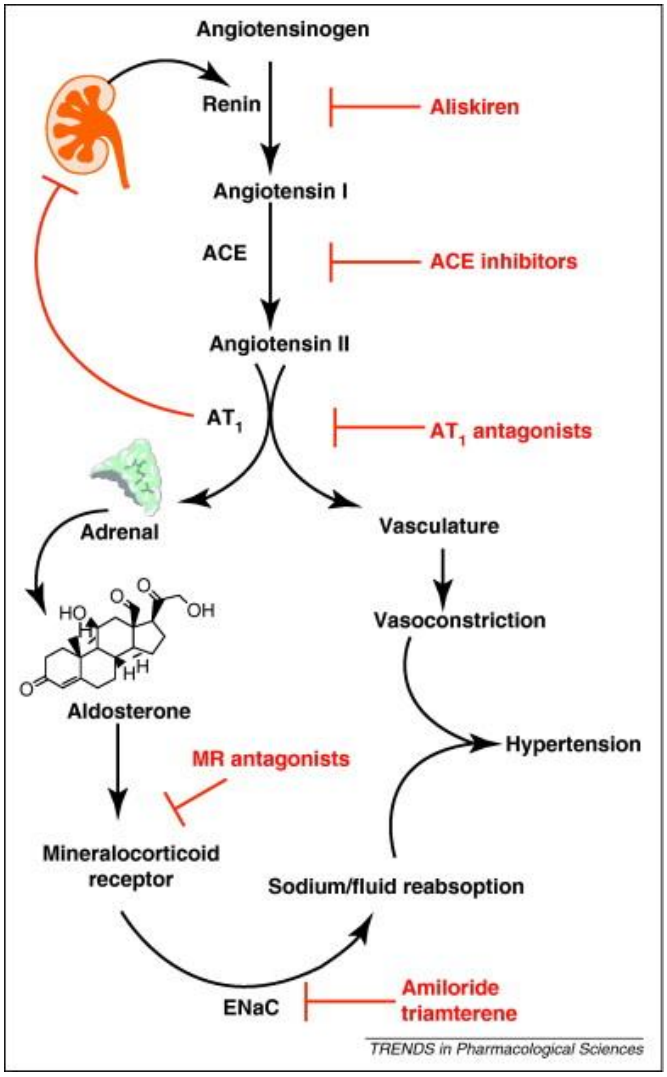


**Figure 10. The RAAS System.**

The RAAS system is initiated by renin secretion and the conversion of angiotensinogen to AngI. AngI is then converted to AngII, which activates AT1R to mediate vasoconstriction and adrenal aldosterone secretion. Pharmacological agents developed to block this pathway act on almost every step. Aliskiren is the only current inhibitor of renin. ACE inhibitors prevent AngII release. AT1R blocker prevent AngII-mediated signaling. MR antagonists block aldosterone's effects. From (Luther & Brown, 2011)

### 1.5.2 Angiotensin II-Induced Signaling

The octapeptide, AngII, exerts its functions in regulating vascular tone and blood pressure by binding AT1R and coupling it to Gαq/11. As described in section 1.2.3.3, Gαq/11 activation leads to PLC-β-mediated cleavage of PIP2 to DAG and IP3. $Ca^{2+}$ release by IP3 and the PKC/ERK activation by DAG, together regulate transcription factors implicated in cellular growth, migration, and apoptosis. AT1R is considered a promiscuous receptor, coupling to multiple other G protein families including Gα12/13, and Gαi/o (Touyz & Schiffrin, 2000). As detailed in sections 1.2.3.3 and 1.2.3.4, both Gαq/11 and Gα12/13 activate distinct RhoGEFs, leading to Rho/ROCK pathway activation responsible for cytoskeletal remodeling, migration, and contraction. Gαi activation leads to the inhibition of AC and the reduction in intracellular cAMP. Given that the physiological outcomes for AT1R's coupling to Gα12/13 and Gαi/o remain elusive and not clearly determined, the Gαq/11 pathways continues to be considered as AT1R's canonical pathway (Costa-Neto et al., 2014).

As a class B receptor, AT1R is a strong interacting partner of β-arrestin. The regulatory role of β-arrestin in AT1R signaling has been extensively studied and research efforts are focused on identifying the bias advantage and mechanisms for AT1R signaling between Gαq/11 and β-arrestin. Further details about AT1R bias modulation are in section 1.6.1.1. AngII-mediated β-arrestin recruitment leads to the activation of ERK, thereby dividing AT1R-mediated ERK1/2 activity to two different components depending on the time scale. The early phase, relating to G protein activation, is rapid and transient, and leads to nuclear ERK1/2 activation, while the late phase, relating to β-arrestin activation, is more sustained and leads to the phosphorylation of cytosolic substrates, thereby stimulating proliferation and cell motility (Ahn, Shenoy, Wei, & Lefkowitz, 2004; Wei et al., 2003; Zheng, Loh, & Law, 2010).

An important mechanism mediating AT1R signaling diversity is through interacting with other GPCRs to form homodimers or heterodimers. AT1R homodimers were shown to exist in high levels in the monocytes of hypertension patients (Hansen, Theilade, Haunso, & Sheikh, 2004). AT1R heterodimerization with other GPCRs has also been reported and is involved in regulating the RAAS system. For instance, AT1R and B2R heterotrimers were found to raise AngII hypersensitivity in Preeclampsia (AbdAlla, Lother, & Quitterer, 2000), a complication during pregnancy associated with a rise in blood pressure (Bokslag, van Weissenbruch, Mol, & de Groot, 2016). Heterodimerization of AT1R with the angiotensin II type 2 receptor (AT2R) was also reported and results in the inhibition of AT1R's IP3 production, thereby antagonizing AT1R-mediated signaling (AbdAlla, Lother, Abdel-tawab, & Quitterer, 2001; Inuzuka et al., 2016). AT1R is also reported to form heterodimers with the FP receptor (Goupil et al., 2015), the cannabinoid type 1 receptor (Rozenfeld et al., 2011), the dopamine type 2 receptor (Martinez-Pinilla et al., 2015). Such dimerization plays an important role not only in mediating AT1R signaling, but also its trafficking properties. While not focused on in this study, GPCR functional regulation by receptor interacting partners is significantly important.

## 1.6 Fine-Tuning of GPCR-mediated Signaling

### 1.6.1 Functional Selectivity of GPCR Signaling

GPCR activation was historically attributed to a two-state model which proposes that receptors toggle between two "states" or conformations: the inactive free state (R), and the active G protein-coupled state (R*). At the basal level, an equilibrium between the R and R* states persist, while ligand binding to receptors shifts this equilibrium towards the active R* state (Leff, 1995). At the time of this model development, the common assumption was that an active GPCR interacts with a single "cognate" G protein. Over the last two decades, it became evident that GPCR signaling is more complex and that a large number of GPCRs are pleiotropic receptors that may bind several G proteins and effector partners. With this realization, the simplified linear view of GPCR signaling was challenged. An alternative multi-state model is now considered, where GPCRs are more dynamic and assume multiple intermediate conformations, before and once occupied by ligand. As such, agonist binding to a GPCR may induce dynamic receptor conformational states, that allow the interaction with multiple different partners, such as different G protein and arrestin subtypes (Wang, Qiao, & Li, 2018). This model also infers that different ligands acting on the same receptor can have different propensities to stabilize unique receptor conformations. The different conformations allow for the engagement of specific signaling effectors to varying extents and result in varying functional outcomes; this phenomenon is commonly referred to as "functional selectivity" or "biased signaling" (Kenakin, 2011; Smith, Lefkowitz, & Rajagopal, 2018) (Fig. 11). Interestingly, not only bias ligands acting on the orthosteric ligand-binding site can induce functional bias, but also allosteric modulators (ligands and interacting partners) that bind topologically distant sites of the receptor and affect its

conformation, thereby altering the orthosteric site and/or effector binding sites (Foster, Roura, Molenaar, & Thomas, 2015) (Fig. 11). Moreover, numerous studies reveal that mutations in the receptor, whether lab-generated or naturally occurring, can modulate receptors' G protein activation and β-arrestin signaling (Yang, Hou, & Tao, 2021).

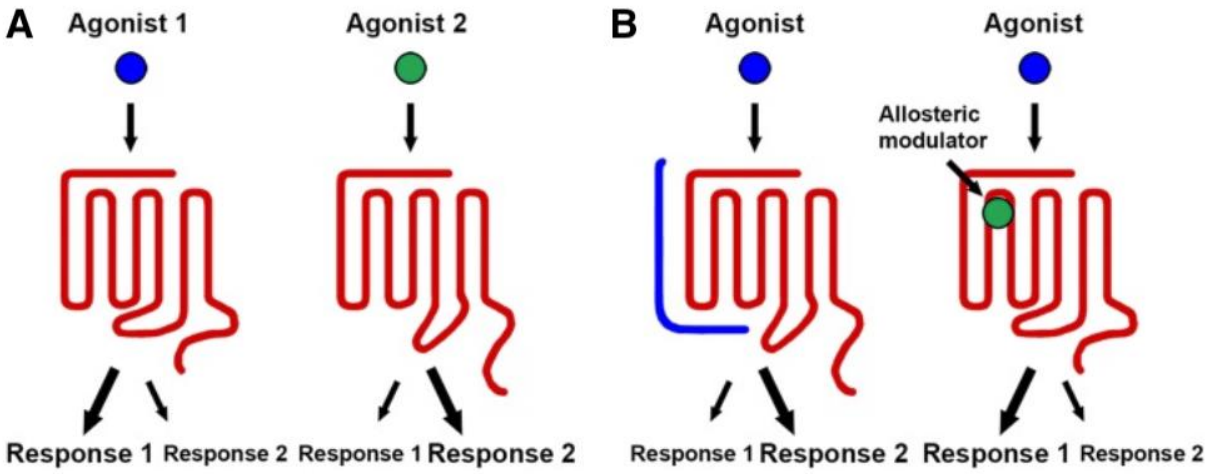


**Figure 11. Biased signaling at GPCRs.**

A) Bias signaling: two agonists binding the same receptor can elicit different receptor conformational states favoring different responses. B) Allosteric modulation: receptor signaling may be biased by allosteric interactions with an allosteric modulator. From (Foster, Roura, Molenaar, & Thomas, 2015)

*1.6.1.1 Biased Ligands*

During the last decade, numerous GPCR biased ligands which "select" for specific receptor conformational state that promote or inhibit select pathways were identified. Those ligands that preferentially activate signaling associated with advantageous responses represent a great potential for the development of new therapeutics with less side effects (for review, see (Kenakin, 2019; Kenakin & Christopoulos, 2013; Kenakin & Miller, 2010)). Biased ligands can preferentially activate G protein- or β-arrestin-mediated signaling; they can also distinguish between the different G protein subtypes. The effector protein associated with the advantageous effect readily varies depending on the receptor and pathophysiological condition. For example, while morphine produces a strong analgesic effect through its action on the μ-Opioid receptor, its clinical utility is significantly limited owing to the undesirable side effects associated with its use, such as morphine tolerance, nausea, vomiting, constipation, and respiratory depression. Studies using the β-arrestin knockout mice linked Gαi signaling to the analgesic effect, while β-arrestin signaling to the unwanted side effects in the respiratory and gastrointestinal systems (Bohn, Gainetdinov, Lin, Lefkowitz, & Caron, 2000; Raehal, Walker, & Bohn, 2005). This directed researchers to identify G protein-biased ligands that are devoid of β-arrestin activation to retain the analgesic effect while increasing safety and tolerability. Today, by deploying high-throughput screening and structure-based strategies for drug design, three novel μ-OR ligands have been identified (Rominger, Cowan, Gowen-MacDonald, & Violin, 2014; Violin, Crombie, Soergel, & Lark, 2014). One of those identified ligands, TRV130, was found to induces only 14% of β-arrestin-mediated signaling compared to morphine. Clinical testing of TRV130 in 2016 reported comparable analgesic effect to that of morphine, while causing fewer side effects (Viscusi et al., 2016), and TRV130 (marketed as Oliceridine) was finally approved by the FDA for intravenous clinical use for moderate to severe

pain relief in August 2020 (Markham, 2020). Oliceridine is the first FDA approved drug of the next-generation GPCR biased ligand and provides a major impetus for exploring GPCRs' functional bias for next-generation drug development.

AT1R was also exploited as a model receptor for the development of bias ligands. AT1R's G protein-mediated signaling was linked to vasoconstriction and cardiac hypertrophy (Fan et al., 2005; Touyz & Schiffrin, 2000). On the other hand, β-arrestin recruitment to AT1R was associated with positive inotropy and antiapoptotic signal activation. The classical use of AT1R blockers in acute heart failure blocks both G protein and β-arrestin pathways, with a beneficial net effect of vascular tone reduction. This is however achieved at the expense of decreasing β-arrestin's positive role in cardio-protection. From this basis, AngII analogues exhibiting β-arrestin bias were sought, which lead to the identification of a number of AT1R biased-ligands, including for TRV027 (Boerrigter et al., 2011; Boerrigter, Soergel, Violin, Lark, & Burnett, 2012). Studies on rats revealed that TRV027 diminished AngII-mediated hypertension while simultaneously promoting cardiomyocyte contraction and maintaining stroke volume (Violin et al., 2010). This contrasts the effect of AT1R-blockers which decreases cardiac contractility with a concomitant decrease in cardiac output and stroke volume. Despite those promising *in vitro* and *in vivo* findings, TRV027 failed in phase 2b clinical trials for the management of acute heart failure (Pang et al., 2017). This failure calls for further investigations regarding the basis of bias and the factors contributing to bias modulations at GPCRs.

#### *1.6.1.2 Biased Allosteric Modulators*

To this date, the G protein is the best characterized GPCR allosteric modulator through its ability to modulate ligand affinity. As detailed earlier in section 1.2.1, the "ternary complex model" was the first to describe this G protein allosteric effect in GPCR functional regulation (De Lean,

Stadel, & Lefkowitz, 1980). Similarly, allosteric ligands (interacting with an allosteric site in the GPCR different from the orthosteric site), may modulate active receptor response to its natural orthosteric ligand. In the recent years, GPCR allosteric modulation has been increasingly considered as a promising new approach for developing therapeutic agents, conferring enhanced selectivity targeting of GPCRs as compared to orthosteric ligands (Christopoulos & Kenakin, 2002; Foster & Conn, 2017; Kenakin & Miller, 2010). Allosteric modulators may affect orthosteric ligand binding to the receptor, the conformation assumed by the receptor upon it's binding to its natural ligand, and the downstream signaling responses of activated receptors. Based on their effect on GPCR signaling (whether through affecting ligand affinity, and/or efficacy or potency of responses), allosteric modulators are classified in three classes. 1) Positive allosteric modulators (PAMs), potentiating receptor response through it orthosteric ligand, 2) Negative allosteric modulators (NAMs), which inhibit receptor response, and 3) neutral allosteric agonists, which allosterically bind receptors but have no effect on their responsiveness (Conn, Christopoulos, & Lindsley, 2009).

To date, very few bias allosteric modulators that direct GPCR signaling have been identified. An allosteric modulator of the calcium-sensing receptor, cinacalcet, that potentiates Gαq-mediated intracellular $Ca^{2+}$ mobilization relative to Gαi-dependent MAPK activation was described (Davey et al., 2012). Moreover, an earlier study demonstrated that FP-mediated signaling through its cognate G proteins, Gαq/11 and Gα12/13, can be allosterically modulated by a peptide mimic of FP's ECL2, PDC113.824 (PDC). PDC biases PGF2α-mediated signaling by acting as a PAM on Gαq/11 pathway while acting as a NAM on Gα12/13 signaling (Goupil et al., 2010) (Fig. 12). Interestingly, despite the involvement of both Gαq/11 and Gα12/13 in regulating myometrial contractions, PDC administration was found to delay pre-term and normal labor in

mice (Goupil et al., 2010). Such finding opens more questions relating to Gαq/11 and Gα12/13 physiology and how bias regulation at GPCRs is achieved. While both Gαq/11 and Gα12/13 pathways regulate smooth muscle contraction, their relative contribution in FP-mediated uterine contraction remains poorly understood. Moreover, the noteworthy contrasting PAM and NAM effects of both cinacalcet and PDC on the different G protein pathways calls for further exploration of the mechanism by which bias regulation is achieved on GPCRs. Our findings in chapter 3 demonstrates a mechanism of G protein competitive regulation that mediates the function of PDC, and possibly other GPCR biased ligands.

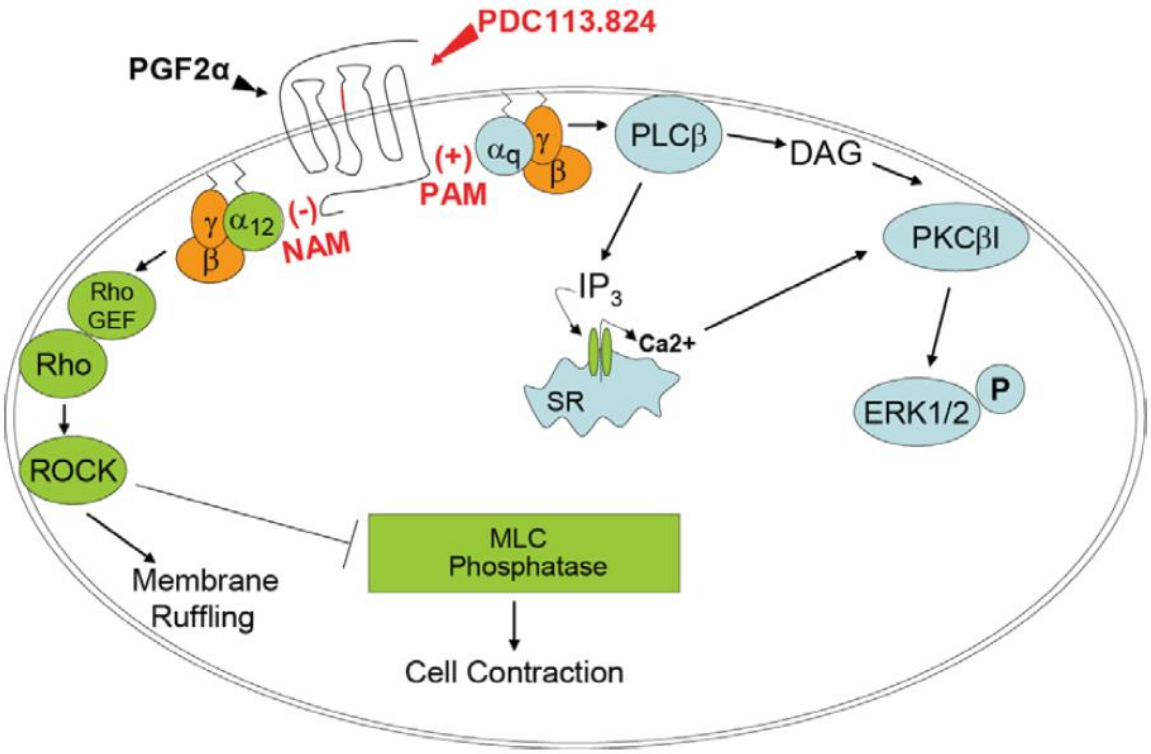


**Figure 12. FP biased allosteric modulation by PDC113.824.**

PDC113.824 increases FP coupling to Gαq, leading to an increase in PKC and ERK1/2 activity. In contrast, PDC113.824 negatively modulates Gα12-mediated cytoskeletal reorganization and cell ruffling. From (Goupil et al., 2010)

*1.6.1.3 Biasing Receptor Mutations*

A mutation within the receptor sequence can directly affect its active conformation and induce specific signaling pathways, different from that of the WT receptor. Indeed, naturally occurring mutations in numerous GPCRs were identified to bias receptors' signaling, many of which are associated with pathological disorders (reviewed in (Yang, Hou, & Tao, 2021)). Namely, a naturally occurring mutation within AT1R, T282M, alters AT1R's trafficking profile through β-arrestin and decreases it's coupling efficiency to AT1R compared to WT (Cao et al., 2020). Such basis for biasing receptor responses have only recently been appreciated, and more efforts are now being invested in characterizing biasing receptor mutations. Identifying such mutations provides a great tool to elucidate GPCR structure-function relationship and increase our understanding of bias GPCR modulation. With the hypothesis that functional selectivity could be achieved by site-specific mutations, mutagenesis studies were successful in identifying mutations within the receptor important in maintaining its active conformation, or capable of switching the receptor's functional selectivity. Indeed, an unpublished high throughput alanine scanning mutagenesis study employed on FP lead to the identification of FP mutations that bias the receptor responses between Gαq/11 and Gα12/13. Such screens allow for systemic analysis of structural functional determinants of GPCRs, which information ultimately support more rational in silico biased ligands design.

### 1.6.2 Selective G protein Coupling by Promiscuous GPCRs

As earlier stated, the ability of GPCRs to transduce extracellular stimulus to intracellular signaling depends principally on their interaction with G proteins and arrestins. It is now acknowledged that most GPCRs exhibit promiscuous G protein coupling behavior, engaging with

more than one G protein subtype (Woehler & Ponimaskin, 2009) (Fig. 13). More frequently, receptors couple to multiple Gα isoforms within the same G protein family due to their sequence similarity. However, more promiscuous couplers interact with G protein subtypes belonging to different families; AT1R interacts with Gαq/11, Gα12/13, and Gαi/o, and FP interacts with Gαq/11 and Gα12/13. The coupling efficiency of the different G protein partners to one receptor often differs, giving rise to unique signaling profiles for individual GPCRs (Avet et al., 2022; Inoue et al., 2019). Accordingly, the higher efficiency coupling is referred to as "primary coupling", while the lower efficiency coupling is referred to as "secondary coupling" (Inoue et al., 2019). Structural studies on the GPCR–G protein complex has revealed interactions between receptors and the nucleotide-free state of G proteins and uncovered novel insights regarding G protein coupling (Carpenter, Nehme, Warne, Leslie, & Tate, 2016; Liang et al., 2017; Rasmussen et al., 2011).

The pressing question of how GPCRs select for specific G protein coupling to achieve the functional selectivity is fundamentally important to understand GPCR signaling. Efforts in this regard led to the identification of molecular selectivity determinants at the GPCR–G protein interface (Flock et al., 2017). Moreover, structural selectivity determinants were also investigated and the distal part of the Gα carboxy terminus (helix 5) was identified as a major site of receptor interaction predominating at the interface with the intracellular TM core of the receptor (Rasmussen et al., 2011). The movement of TM6 within GPCRs to accommodate the helix 5 interaction represents a molecular signature for the active conformation of class A and B receptors. Several other studies also linked the Gα helix 5 with GPCR-selectivity; and mutations within this region were shown to alter the coupling selectivity, enabling individual receptors to couple noncognate heterotrimers (Conklin, Farfel, Lustig, Julius, & Bourne, 1993; Semack, Sandhu, Malik, Vaidehi, & Sivaramakrishnan, 2016). Nonetheless, other reports suggest that different

receptors recognize varying selectivity determinants with a G protein, highlighting other regions within the Gα core important for G protein recognition by GPCRs (Blahos et al., 2001; Flock et al., 2017; Okashah et al., 2019). This further confirms discrete interactions between individual GPCR and G protein pairs that could affect the coupling efficiency and the ensuing functional outcomes. Despite the great progress towards understanding the structural basis for GPCR–G protein interaction, the mechanisms inferring different GPCR coupling efficiencies remain not fully deciphered.

While structural understanding of the GPCR–G protein chemistry provides a basis for selective G protein interactions, the relative expression levels of GPCRs and the Gα subunits represent an important factor conferring GPCR's selectivity. Indeed, GPCR responses are greatly dependent on the cellular and physiological contexts (Neubig, 1994; Ritter & Hall, 2009). The relative abundance of receptors and the different G protein subtypes is considered an important factor governing cell specific GPCR responses. It has long been accepted that the stoichiometry of receptors and G protein interacting partners influences ligand efficacy and determine the signaling outcome (Kenakin, 1997). More recently, this view was further extended to include a direct effect of G protein expression on natural and biased ligands' signaling profiles (Onfroy et al., 2017). The mechanisms inferring how G protein availability regulate GPCR responses remain elusive and are thus investigated in chapter 3 of this thesis. Interestingly, a recent study showed that non-cognate G proteins may physically interact with receptors by virtue of "unproductive coupling", which may alter GPCR-mediated signaling independent of their own activation. Unproductive Gα12 coupling to V2R repressed basal Gα12-signaling by sequestering the Gα12 heterotrimers, and hindered β-arrestin recruitment to V2R and its subsequent internalization (Okashah et al., 2020). Another new concept pretrainin to the effect of individual G proteins on GPCR function is "GPCR

priming"; This is described as the influence of non-cognate GPCR–G protein interactions in stimulating canonical G protein signaling (Gupte, Malik, Sommese, Ritt, & Sivaramakrishnan, 2017). Together these studies highlight the influence of individual G proteins on GPCR signaling beyond the structural interface, and provide possible explanation of the differential tissue-specific responses of GPCRs. Further understanding of this regulation would provide valuable insight for the mechanisms mediating GPCR's effector selectivity.

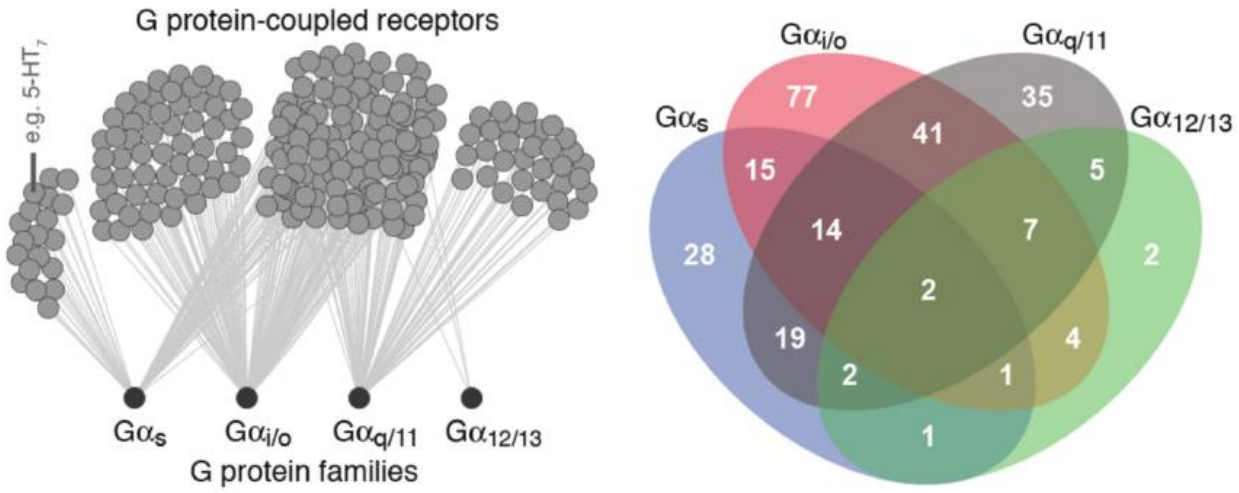


**Figure 13. Complex interactivity of GPCRs and G proteins.**

Network presentation of known GPCRs interaction with one or more members of the four G protein families is shown in the left panel. The numbers of GCRs coupling to the different G protein families is shown in the right panel. From (Flock et al., 2017)

### 1.6.3 Selective Regulation of β-arrestin Functional Outcomes

Interestingly, while a large number of GPCRs interact with the same β-arrestin, the functional outcomes for this interaction varies. To date, the mechanisms for such divergence are not completely understood. As detailed in section 1.3.2, β-arrestin changes conformation upon receptor binding (Charest, Terrillon, & Bouvier, 2005; Kang et al., 2015; Kim et al., 2013; Shukla et al., 2013). Moreover, it was revealed that GPCR–β-arrestin complexes can assume different arrangements; namely, the "partially engaged" complex which has lower β-arrestin affinity and involves its interaction with the phosphorylated tail of GPCRs, and the higher affinity "fully engaged complex" involving the interactions of various loops within β-arrestin with the receptor's TM domain (a.k.a. receptor core) (Ranjan, Dwivedi, Baidya, Kumar, & Shukla, 2017; Shukla et al., 2014). Interestingly, β-arrestin partial engagement with the receptor's C-terminus is sufficient to mediate GPCR endocytosis and ERK activation, but not G protein desensitization (Kumari et al., 2016; Kumari et al., 2017). On the other hand, the fully engaged GPCR–β-arrestin complex is required for fully arrest G protein activation (Cahill et al., 2017; Kumari et al., 2017).

A very attractive hypothesis for the differential regulation upon β-arrestin binding to GPCRs is that β-arrestins itself adopts a multitude of "active" conformations that dictates the functional outcome. The structural basis for β-arrestin conservation in inactive state and the multistep activation of process was detailed earlier in section 1.3.2. However, whether this active conformation differs relative to the functional outcome for β-arrestin recruitments remains elusive. Recently, it was shown that biased ligands not only altered the conformation of the receptor, but also that of β-arrestin. These manifold of GPCR–β-arrestin complex conformations were indeed translated to altered functional outcomes (Shukla et al., 2008). More recently, receptor-dependent variations in β-arrestin2 conformations have also been suggested (Lee et al., 2016). Interestingly,

a new link between the GPCR phosphorylation pattern (termed as “phosphorylation barcodes) and the signaling outcome has also been discovered, suggesting a model of phosphorylation code-dependant GPCR functional regulation (Fig. 14). A single GPCR could be phosphorylated by different sets of GRKs, resulting in varying phosphorylation profiles/signatures that directs the signaling outcome of GPCRs (Tobin, Butcher, & Kong, 2008). This provides a flexible mechanism to tailor GPCR responses based on cell-specific requirements, especially given the complex nature of GPCR phosphorylation, governed by one or more of the seven GRKs. Recently it was shown that the phosphorylation pattern not only altered arrestin’s signaling, but also its receptor binding affinity and conformational arrangement (Mayer et al., 2019; Sente et al., 2018; Yang et al., 2015). The scaffolding of MAPK components was also shown to be sensitive to β-arrestin’s conformation and receptor phosphorylation patterns (Latorraca et al., 2020). The discrepancies in GPCR–β-arrestin conformational arrangements thus require further exploration to explain how different receptors, bias ligands, and phosphorylation patterns, dictate β-arrestin functions.

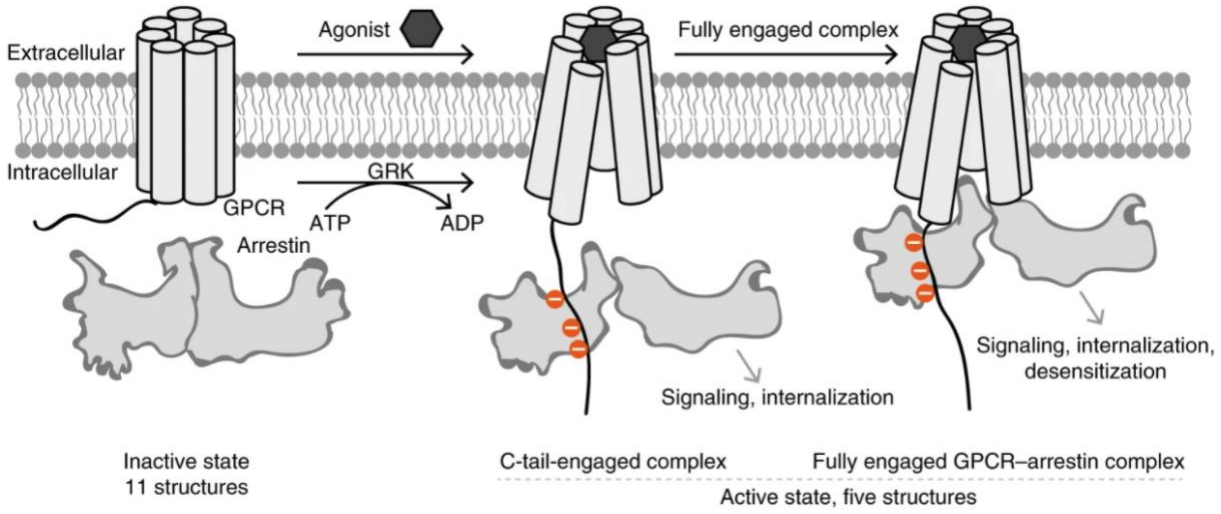


**Figure 14. Phosphorylation code-dependent β-arrestin functional regulation.**

In the absence of agonist stimulation, β-arrestin remains in its inactive state in the cytosol. Activation of GPCR leads to different phosphorylation profiles/signatures, depending on the implicated GRKs. The site-specific phosphates on the receptor mediates its interaction with select structural motifs on β-arrestin, leading to varying β-arrestin conformations and binding efficiencies, dependant on the phosphorylation pattern. This in turn directs the signaling outcome for the activated GPCR. From (Sente et al., 2018)

# CHAPTER 2: Rationale and Objectives

## 2.1 Rationale

The overall aim of this thesis is to expand our understanding of GPCR functional regulation by G proteins and β-arrestins, the two primary interacting effectors that dictate GPCR signaling. The initial view for GPCR activity represented a linear model, where a ligand activated GPCR engages with one G protein, leading to downstream signaling responses. However, extensive research efforts in this field are continuously exposing new concepts for GPCR functional regulation. GPCRs dynamic nature, their ability to signal through multiple cognate G proteins, and the differential outcomes for their β-arrestin interaction, are all examples for the complex regulatory control that fine-tunes GPCR responses in the different physiological and pathological conditions. Refining our understanding of the coordinated actions of GPCRs is fundamentally important given their prominent position as principal targets for drug development.

In humans, the ~800 GPCR members identified bind an astounding number of natural ligands. Despite the wide range of their physiological actions, the signaling activity of the majority of GPCRs is mainly conveyed by four Gα families, containing 16 members, as well as two β-arrestins. To fine-tune their signaling profiles in the different cellular contexts and physiological conditions, it is critical that GPCRs achieve delicate G protein selectivity. Moreover, the wide range of events associated with β-arrestin interaction suggests varying β-arrestin binding modalities with GPCRs to drive the distinct functional outcomes.

The first part of this thesis addresses the question of how G protein subtype selectivity is achieved at promiscuous GPCRs. While much research is focused on understanding the structural features underlying cognate G protein selectivity, not much is known about the factors driving receptors' preferential coupling between two cognate G proteins. In this study, I investigate the hypothesis that G protein availability influences GPCR binding to its cognate G proteins. FP and

AT1R receptors were chosen as prototypical promiscuous GPCRs to dissect their relative selectivity for binding their cognate Gαq/11 and Gα12/13 G protein families. The important role of FP and AT1R in mediating smooth muscle contraction renders these receptors as important targets for drug development for the control of preterm labor and hypertension. Moreover, understanding the functional regulation of Gαq/11 and Gα12/13 families at receptors is particularly appealing, given their intricate interplay and crosstalk to mediate their physiological functions. A better understanding of Gαq/11 and Gα12/13 functional selectivity provides insights for the development of better bias ligands that produce therapeutic advantages.

The second part of this thesis is devoted to providing a plausible explanation for the differential signaling outcomes of β-arrestin binding to receptors. How the interaction of one β-arrestin subtype leads to countless trafficking and signaling consequences remains poorly understood. This thesis tackles this phenomenon by hypothesizing differential GPCR–β-arrestin complex binding modalities that reflect on the functional outcome. It is appreciated that differential GPCR–β-arrestin complexes can exist. To date, two major modes of interaction are identified, the "partially engage complex" and the "fully engaged complex". While these modalities may dictate the coupling strengths and distinguish class A from class B GPCRs, they do not provide insights to the differential functional outcomes for β-arrestin interaction with class B receptors. Here bioorthogonal labeling of β-arrestin with unnatural amino acid (UAA) and photocross-linking is used to investigate β-arrestin binding modalities with three different class B GPCRs: AT1R, B2R, and V2R. Applying this method on β-arrestin allows us to identify important interacting residues within β-arrestin involved in the formation of GPCR-β-arrestin complexes and to appreciate varying β-arrestin binding modes assumed in different conditions.

## 2.2 Objectives

The main objectives of the work presented in this thesis are:

1. To understand the implication of G protein availability on Gα subtype selectivity by promiscuous GPCRs.
2. To identifying differential β-arrestin binding modalities in function of the GPCR partner and the cellular localization.

# CONNECTING TEXT

The following chapter entitled “Gq/11 and G12/13 Selectivity Regulation at Promiscuous GPCRs” addresses a mechanism for G protein selectivity binding between Gαq/11 and Gα12/13 at the promiscuous FP and AT1R receptors. Both receptors have been previously shown to exert functional selectivity when targeted by orthosteric or allosteric ligands. Particularly, a bias allosteric modulator of FP produces a PAM effect on Gαq/11 pathway while having a NAM effect on Gα12/13 (Goupil et al., 2010). Although this modulation delayed pre-term and term contractions in mice, it is still not clear how the modulator exerts these contrasting functions on G proteins’ coupling to FP. AT1R biased ligands that preferentially bind Gα12/13 have also been identified (Namkung et al., 2018). However, the physiological consequence of such bias is not yet determined. Given the interplay between Gαq/11 and Gα12/13 pathways in regulating smooth muscle contraction, this chapter seeks to develop a better understanding for these G proteins selectivity binding to FP and AT1R.

The effect of these G proteins binding to receptors on the alternate G protein activation was addressed using BRET biosensors that measure G protein activation (by recording Gα and Gβγ dissociation), or downstream intermediators activation (such as p63RhoGEF, PDZRhoGEF, and PKC) upon receptor stimulation. G protein binding to receptor was controlled by altering the expression levels in G protein knockout cells and by using a bias receptor mutation or biased ligands. A novel mechanism of regulation for G protein binding involving competitive receptor binding of individual G protein subtypes was reported. The competition between Gαq and Gα13 was unidirectional and receptor-dependent, suggesting an intrinsic mechanism for receptors to selectively bind their cognate G proteins. This study was submitted to *JBCs*, and is currently under peer-revision.

# CHAPTER 3: Selectivity Regulation of Gq/11 and G12/13 at Promiscuous GPCRs

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## 3.1 Abstract

Promiscuous G protein-coupled receptors (GPCRs) engage multiple Gα subtypes with different efficacies to propagate signals in cells. A mechanistic understanding of Gα selectivity by GPCRs is critical for therapeutic design because signaling can be restrained by ligand–receptor complexes to preferentially engage specific G proteins. Cognate G protein selectivity was investigated using the prototypical promiscuous Gαq/11 and Gα12/13 coupling receptors for angiotensin II type I (AT1R) and prostaglandin F2α (FP), BRET-based G protein and pathway-selective sensors, as well as G protein knockout cell lines. Competition between G proteins for receptor binding was characterized to occur in receptor- and G protein-specific manners for AT1R and FP, but not the bradykinin B2 receptor or the thromboxane A2 receptor alpha. Moreover, Gαq-mediated signaling regulated G protein coupling only at AT1R. The functional modulation of biased ligands at FP and AT1R was shown to be contingent upon Gα availability. Our findings suggest that despite preferential binding of similar subsets of G proteins, GPCRs follow distinct selectivity rules, which can be exploited to develop G protein-biased ligands.

## 3.2 Introduction

G protein-coupled receptors (GPCRs) are membrane proteins that control numerous physiological processes. Upon binding to extracellular stimuli, such as hormones and drugs, GPCRs relay signals by engaging intracellular signaling regulators, namely, heterotrimeric G proteins (αβγ subunits) and β-arrestins (Shenoy & Lefkowitz, 2011; Wettschureck & Offermanns, 2005). Ligand-mediated activation of GPCRs enables functional dissociation of the Gα subunit from the heterotrimeric G protein, triggering activation of downstream signaling effectors.

Based on the nature of the α subunits, G proteins are classified into four major families: Gαs, Gαi/o, Gαq/11, and Gα12/13. Different G proteins activate specific downstream effectors that ultimately produce diverse signaling events. While many receptors specifically couple to one G protein family, others show promiscuity and engage multiple different G protein subtypes. Recent pharmacological advances have revealed that signaling through promiscuous GPCRs can be directed by ligands to selectively engage specific G proteins, a strategy that can be useful therapeutically (Khoury, Clement, & Laporte, 2014; Slosky, Caron, & Barak, 2021). Therefore, understanding the mechanisms dictating preferred G protein coupling by promiscuous GPCRs in cells is pivotal for designing ligands with honed functional selectivity.

Despite considerable efforts, our knowledge remains limited regarding the fundamentals of GPCR-G protein selectivity. Information regarding the selectivity of GPCRs for different G proteins has been gleaned using various sensors, sequence homology analyses and structural comparisons of interacting domains of receptors and G proteins (Avet et al., 2020; Flock et al., 2017; Inoue et al., 2019; Masuho et al., 2015). For example, the G protein C-terminal α5 helix has been identified as a site dictating receptor selectivity (Conklin, Farfel, Lustig, Julius, & Bourne, 1993; Semack, Sandhu, Malik, Vaidehi, & Sivaramakrishnan, 2016). The orientation adopted by

the C-terminus of Gα upon receptor coupling correlates with the strength of the GPCR-G protein interaction, supporting cognate G protein interactions (Sandhu et al., 2019). Other regions within the G protein core have been shown to contribute to receptor selectivity (Alegre et al., 2021; Okashah et al., 2019). These findings provide valuable insights for understanding cognate vs. noncognate G protein recognition by GPCRs. However, few details are known concerning the selectivity between cognate G protein binding at promiscuous GPCRs. The study of the dynamic competition among cognate G proteins for promiscuous GPCRs is especially challenging due in part to variations in receptor and G protein expression in different cell and tissue types (Onfroy et al., 2017; Sriram, Moyung, Corriden, Carter, & Insel, 2019; Sungkaworn et al., 2017). Moreover, overexpression of G proteins has been shown to affect ligand efficacy and the biased signaling profile of GPCRs, such as angiotensin II (AngII) type 1 receptor (AT1R) (Kenakin, 1997, 2002; Onfroy et al., 2017). However, the rules governing the impact of one G protein on the coupling efficacy of another cognate G protein remain to be elucidated for different promiscuous GPCRs.

Here, we used the two promiscuous Gαq/11 and Gα12/13 receptors: AT1R and the prostaglandin F2α receptor (FP). These GPCRs, both found in smooth muscle cells, regulate contraction through these different G protein families and have exhibited ligand functional selectivity (Goupil et al., 2015, Goupil et al., 2010, Namkung et al., 2018). Using CRISPR/Cas9 cell lines depleted of Gαq/11 and Gα12/13 and the selective complementation of G proteins, we show that G proteins compete differentially at these promiscuous GPCRs by distinct mechanisms.

## 3.3 Materials and Methods

### Materials

PGFα, BK, and U46619 were from Cayman Chemical (Ann Arbor, MI). TRV (Sar Arg Val Tyr Ile His Pro D-Ala) and SVdF (Sar Arg Val Tyr Val His Pro D-Phe) ligands were from GenScript. AngII, Poly-L-ornithine, poly-L-lysine, and horseradish peroxidase–conjugated rabbit secondary antibodies were purchased from Sigma-Aldrich. Horseradish peroxidase–conjugated mouse secondary antibody was purchased from Bio-Rad. [$^{3}$H] PGF2α and chemiluminescence reagents were purchased from PerkinElmer Life Sciences. $^{125}$I-AngII (specific radioactivity~1000 Ci/mmol) was prepared with Iodo-GEN® (PerbioScience, Erembodegem, Belgium) as reported previously (Namkung et al., 2018). Polyethylenimine (PEI) was acquired from Polysciences (Warrington, PA). Coelenterazine was purchased from Nanolight Technology (Pinetop, AZ). YM-254890 was purchased from FUJIFILMWako Chemicals U.S.A. Gö6983 was acquired from Calbiochem. Y27632 is from Ascent. C3 exoenzyme is from Cytoskeleton. Anti Gαq (10) and anti Gα13 (A-20) antibodies were from Santa Cruz Biotechnology. Rabbit polyclonal anti-p-ERK and anti-total-ERK antibodies were from Cell Signaling. Az-PDC was synthesized at University of Montreal, Canada. Trypsin, phosphate-buffered saline (PBS), Dulbecco's modified Eagles medium (DMEM), fetal bovine serum (FBS), gentamycin and other cell culture reagents were acquired from Gibco, Life Technologies. Phusion DNA polymerase was from Thermo Scientific. Restriction enzymes, T4 DNA ligase, and Gibson assembly mix were obtained from New England Biolabs. Oligonucleotides were synthesized at Integrated DNA Technologies. All other reagents were obtained from Thermo Fisher Scientific (Waltham, MA) and were of analytical grade.

**Plasmids and constructs**

The polycistronic Gαq sensor, and the Gα12, Gα13, Gαi2 and Gαi3 BRET sensors were described elsewhere (Avet et al., 2020; Gales et al., 2005; Namkung et al., 2018). The Gα11 sensor was provided by Dr. Michel Bouvier (University of Montreal). Briefly, GNA11 internally tagged with RlucII at position 127 was generated by overlap PCR, similar to the GNAo constructs. Gα11-RlucII was then cloned into the Gαq polycistronic BRET vector, replacing the Gαq sequence with that of Gα11. BRET sensors for β-arrestin1 and β-arrestin2 recruitment, and PKC activation were described in (Namkung et al., 2016; Namkung et al., 2018). PDZRhoGEF and p63RhoGEF sensors were described previously (Avet et al., 2020). The cDNA clone for non-functional human Gαq mutant (Q209L/D27N) was previously described (Goupil et al., 2010). Mutations affecting FP coupling to Gαq such as I147A and M247A were identified from a whole receptor alanine mutagenesis screen (manuscript in preparation). The GαqNull (I147A/M247A) mutant FP receptor (GαqNull-FP) was engineered by the two-part PCR mutagenesis strategy described previously (Gagnon et al., 2019; Heydenreich et al., 2017). Briefly, site-directed mutagenesis primers with 18bp of Gibson homology for Gibson assembly recombination were generated and ordered from Integrated DNA Technologies (Coralville, IA). I147A mutation was first introduced and the I147A FP mutant vector was then used as the template to generate the double mutant (I147A/M247A) in FP. Mutations were introduced through a stepdown PCR; two separate PCRs were performed to split the vector in half. The two halves PCR samples were combined together, digested with DpnI, and purified. Samples of the two half vectors were then Gibson ligated. The re-annealed vector was then transformed into bacterial colonies, and one of the grown colonies was picked and amplified. The mutant was then verified by sequencing at Genome Québec, CES.

**Cell culture and transfections**

HEK293 cells depleted in Gαq/11 (ΔGαq/11) and Gα12/13 (ΔGα12/13) were obtained from Dr. Asuka Inou (Tohoku University, Japan) and previously described (Devost et al., 2017). Cells were cultured in DMEM supplemented with 10% fetal bovine serum and 20 ug/ml gentamycin at 37 °C in 5% CO2 and 90% humidity. Transient transfections were performed using the PEI method at a 3:1 ratio (w/w) with plasmid DNA on cells in suspension. For BRET experiments, cells were seeded onto polyornithine-coated white 96-well plates at a density of 20 × $10^3$ cells per well. Each 12 wells were transiently transfected with 150 ng receptor, along with one of the BRET sensors: 250 ng Gαq or Gα11 polycistronic sensors, or 15 ng Gα12-RlucII or Gα13-RlucII with 60 ng GFP10-Gγ1 and Gβ1 sensors, or 60 ng Gαi2-RlucII or Gαi3-RlucII with 60 ng GFP10-Gγ2 and Gβ2 sensors, or 15 ng of β-arrestin1-RlucII or β-arrestin2-RlucII with 60 ng of rGFP-CAAX sensors, or 60 ng PKC sensor, or 15 ng PDZRhoGEF-RlucII with 10 ng Gα13 and 60 ng of rGFP-CAAX sensor or 15 ng p63RhoGEF-RlucII with 10 ng Gαq and 60 ng of rGFP-CAAX sensor. For BRET experiments were Gα subunit is overexpressed; Gα13, Gαq or Gαs were transfected either at 10 ng or 20 ng per 12 wells. For binding experiments, cells were seeded onto poly-L-lysine-coated 24-well plates at a density of 80 × $10^3$ cells per well and were transiently transfected with 600 ng/well of the appropriate receptor. For immunoblotting experiments, cells were seeded at a density of 160 × $10^3$ cells per well in 12 well plates and were transiently transfected with 1 μg FP or AT1R receptors. In all experiments, the medium was replaced 18 hours post transfection, and the experiment were carried on 48 hours post transfection.

**Radioligand binding experiments**

Receptors abundance were assessed by ligand binding assays using [$^{3}$H]-PGF2α or [$^{125}$I]-AngII in saturation experiments. [$^{125}$I]-AngII was prepared using the Iodogen method, as previously described (Zimmerman et al., 2012). For binding experiments, HEK293T, ΔGαq/11, and ΔGα12/13 cells transiently expressing the appropriate receptor were washed once with ice cold PBS and incubated with or without 1 μM cold AngII or PGF2α and fixed concentrations of [$^{125}$I]-AngII (100,000 cpm at 2200Ci/mmol) or [$^{3}$H]-PGF2α (150–240 Ci/mmol) respectively, in a total volume of 0.5 ml of binding buffer (50 mM Tris (pH 7.4), 5 mM $MgCl_2$, 100 mM NaCl and 0.2% (w/v) BSA). Cells were incubated at room temperature for one hour for [$^{125}$I]-AngII or at 4 °C overnight for [$^{3}$H]-PGF2α. binding was stopped by washing cells three times with ice cold PBS, and cells were lysed with NP40 (for [$^{3}$H]) or 0.2M NAOH (for [$^{125}$I]) for 10 min at room temperature. Incorporated radioactivity was then measured by a β-counter [$^{3}$H] or a γ-counter [$^{125}$ I]. Receptor relative expression levels in the different cell lines was measured by subtracting the non-specific binding (determined by the addition of cold ligands) from the total binding.

**BRET assays**

Cells transfected with receptor and BRET sensors were washed once and incubated with Tyrode's buffer (140 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2$, 12 mM $NaHCO_3$, 5.6 mM D-glucose, 0.5 mM $MgCl_2$, 0.37 mM $NaH2PO_4$, 25 mM HEPES, pH 7.4) for 30 min at 37 °C with 5% CO2. For concentration-response and time course experiments, cells were stimulated with various concentrations of PGF2α or AngII in Tyrode for 2–6 min, or a single concentration of the ligand for various durations ranging between 5 and 30 min. For all BRET experiments, coelenterazine 400a was added at a final concentration of 5 μM 3–5 min prior and BRET measurements were

obtained using the Synergy2 (BioTek) plate reader with filter sets of 410/80 nm (donor) and 515/30 nm (acceptor). BRET ratio was then calculated as the ratio of the intensity of acceptor light emission over the intensity of donor light emission.

**Western blotting**

Two days post-transfection, cells were washed once with PBS, serum starved in hepes containing media for 30 min at 37 °C and stimulated with 1 μM of PGF2α or AngII. Stimulation was stopped by washing once with ice-cold PBS and lysing cells with laemmli buffer [250 mM tris-HCl (pH 6.8), 2% (w/v), SDS, 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue, and supplemented with 5% (v/v) β-mercaptoetanol]. For G protein overexpression experiments, cells were washed once with PBS and directly lysed by laemmli buffer. Protein samples were then resolved by SDS-polyacrylamide gel electrophoresis. Membranes were incubated with primary rabbit polyclonal anti-phospho-44/42 ERK1/2, or anti-total ERK1/2 antibodies diluted in 1:1000 ratio in 1% BSA, or with mouse monoclonal anti Gαq (10), or anti Gα13 (A-20) antibodies diluted in 1:500 ratio in 1% BSA. Antibody incubation was done overnight at 4 °C on a nutating mixer. Secondary anti-rabbit or anti-mouse antibodies conjugated to horseradish peroxidase were then used to detect bands by chemiluminescence (1:10,000). Chemiluminescence signals were detected using Chemidoc Imaging System (Bio-Rad), and Protein bands were quantified by densitometry analysis using Image Lab™ 6.0 (Bio-Rad). ERK phosphorylation was expressed as the relative ratio between the intensity of phospho-ERK1/2 to ERK1/2.

### Data analysis

All data were analyzed using Image LabTM 6.0 (Bio-Rad) and Prism 6.0 (GraphPad Software Inc., La Jolla, CA). Statistical analyses were performed using Student's t tests, one- or two-way analysis of variance (ANOVAs), and Dunnette's or Bonferroni's multiple comparisons test when appropriate. Statistical significance was considered when P values $<0.05$.

## 3.4 Results

### Gα13 impedes Gαq coupling and signaling through AT1R but not FP

We first evaluated G protein coupling profiles for FP and AT1R in HEK293 cells using Gαq/11, Gα12/13, and Gαi BRET-based sensors that measure Gα and Gβγ subunit dissociation (Namkung et al., 2018; Sauliere et al., 2012). For both receptors, we observed a more robust Gαq BRET signal compared with that of Gα11 (Fig. S1A). For AT1R, a similar efficacy in BRET response to Gα12 and Gα13 was observed, while the FP receptor showed better Gα13 activation (Fig. S1B). Only AT1R efficiently activated Gαi2 and Gαi3 (Fig. S1C). Because G protein coupling to receptors can be modulated by β-arrestin's interaction with receptors, we also assessed the ability of both FP and AT1R to engage β-arrestin1 and β-arrestin2 using a BRET-based membrane translocation assay (Namkung et al., 2016). As previously reported, only AT1R recruits β-arrestins (Goupil et al., 2012) (Fig. S1D).

Because FP and AT1R are both Gαq/11- and Gα12/13-coupled receptors, we next investigated how each G protein subtype influences the coupling and activation of its alternate cognate G proteins. We focused on Gαq and Gα13, as representatives of each of their respective G protein families, because of their high efficiency in binding both GPCRs. We also assessed the effect of Gα13 expression on FP- and AT1R-dependent Gαq activation in Gα12/13-depleted cells

(ΔGα12/13 cells) to mitigate any confounding effects of endogenous Gα12/13. Agonist dose–response curves with the Gαq BRET sensor were performed, and maximal responses between conditions were compared, which we reasoned would allow us to compare G protein coupling efficacy to fully engaged ligand–receptor complexes. FP coupling to Gαq was unaltered in these cells compared with parental cells, where FP was similarly expressed (Figs. 1A and S2A). Reintroducing Gα13 in ΔGα12/13 cells led to more FP signaling through this pathway as revealed by the recruitment at the plasma membrane (PM) of the Gα12/13-dependent BRET PDZRhoGEF sensor (Avet et al., 2022). However, reintroducing Gα13 did not show differences in FP coupling efficacy to Gαq compared to receptors expressed alone in ΔGα12/13 cells (Figs. 1B and S3). Remarkably, however, Gαq coupling to AT1R was significantly increased in ΔGα12/13 cells, compared with parental cells, despite AT1R being expressed at similar levels in both cell types (Figs. 1C and S2B). Moreover, reintroducing Gα13 in ΔGα12/13 cells impeded Gαq activation by AT1R (Fig. 1D), while overexpressing the noncognate Gαs protein had no effect on Gαq coupling to either FP or AT1R (Fig. 1B and D). The effect of Gα13 on receptor coupling and signaling through Gαq was also investigated by measuring the recruitment of its effector p63RhoGEF at PM and ensuing BRET signal (Namkung et al., 2018). Similar effects to those seen with the Gαq sensor were observed (Fig. S4). Gα13 had no effect on FP-mediated Gαq activity, unlike AT1R, where Gα13 expression significantly attenuated signaling through this pathway (Fig. S4).

To exclude the possibility that Gα13-mediated signaling regulates Gαq activity by AT1R, we also tested whether Gαq coupling was altered when Rho or Rho-associated protein kinase (ROCK) was inhibited using C3 exoenzyme and Y27632, respectively. Neither treatment influenced FP or AT1R coupling to Gαq (Fig. S5A and B). Together, these results suggest that the

effects (or lack thereof) observed herein reflect the distinctive intrinsic property of Gα13 to compete with Gαq at AT1R.

Because PKC and MAPK are known downstream effectors of Gαq-coupled receptors and are activated by FP and AT1R (Goupil et al., 2010; Namkung et al., 2018), we investigated the effect of Gα13 binding on the activation of these kinases at these receptors. Consistent with what we observed, the expression of Gα13 negatively affected PKC activation by AT1R but not FP, as revealed using the BRET sensor of this kinase (Namkung et al., 2018) (Fig. S5C and D). Moreover, Gαq-dependent MAPK downregulation by Gα13 was only observed for AT1R (Fig. S5E and F). In the absence of Gα12/13, AT1R-mediated MAPK activation increased, while reintroducing Gα13 in these cells significantly inhibited this response (Fig. S5F). Taken together, these data imply that Gα13 binding, independent of its downstream effectors, impedes receptor-Gαq coupling and signaling for AT1R but not FP.

**Gαq regulates receptor-Gα13 coupling and signaling for both FP and AT1R**

We next examined the extent to which Gαq affected Gα13 signaling by FP and AT1R using the Gα13 sensor in HEK293 cells bearing or lacking Gαq/11 (ΔGαq/11 cells). We confirmed that both receptors' expression levels were not altered between the two cell types (Fig. S2). Interestingly, we show that in the absence of Gαq/11, Gα13 coupling to both FP and AT1R was significantly potentiated compared with that in cells expressing Gαq/11, and this effect was reversed for both receptors following reintroduction of Gαq in ΔGαq/11 cells (Fig. 2). Gαq expression also restored FP-mediated signaling to p63RhoGEF and PKC (Fig. S6). However, expressing the noncognate Gαs subunit in ΔGαq/11 cells had no effect on FP- and AT1R-mediated Gα13 coupling (Fig. 2B and D). Gαq competition and the lack of Gαs effects on FP and AT1R

coupling to Gα13 were recapitulated when assessing the response of the downstream Gα13-mediated PDZRhoGEF sensor for these receptors (Fig. S7).

**Gαq binding to FP impedes Gα13 coupling, while signaling downstream of Gαq inhibits Gα13 signaling through AT1R**

PKC has been involved in GPCR desensitization (*i.e.,* reduced G protein coupling) through receptor phosphorylation, but the extent to which it regulates receptor-G protein selectivity is unclear (Lefkowitz, 1998). AT1R and FP both contain PKC phosphorylation sites (Qian, Pipolo, & Thomas, 1999; Srinivasan, Fujino, & Regan, 2002). As expected, activating PKC with phorbol 12-myristate 13-acetate (PMA) significantly reduced Gαq, Gα13, and Gαi2 activity at AT1R (Figs. 3B and S8). Surprisingly, however, it had no effect on FP coupling and signaling through Gαq and Gα13 (Figs. 3A, and S8A and B). Consistent with these observations, inhibiting PKC with Gö6983 (Gö) significantly increased AT1R coupling to its cognate G proteins, while FP coupling to Gαq and Gα13 remained unchanged (Figs. 3A and B, and S8). We next used the Gαq inhibitor YM254890 (YM), which prevents GDP release from the Gαq and the high affinity interaction between the Gα subunit and the agonist-bound receptor (Nishimura et al., 2010; Schrage et al., 2015). Interestingly, inhibiting receptor-dependent Gαq activation using YM significantly potentiated FP-Gα13 coupling efficacy and receptor signaling (Figs. 3A and S8B). Similarly, AT1R coupling to both Gα13 and Gαi2 was increased with YM treatment (Figs. 3B and S8B and C). Together, these results suggest that while AT1R signaling is regulated by Gαq-mediated PKC signaling, for FP, the extent of Gα13 coupling is independent of PKC activation but contingent on the ability of the Gαq to compete with Gα13 for receptor binding. To further support these observations, we used the Q209L/D277N Gαq mutant (Q/D-Gαq), which lacks the ability to

activate downstream effectors by mimicking the nucleotide-free Gα form (Yu, Gu, & Simon, 2000) but still binds receptors with high affinity, hence potentially competing with Gα13 for binding to FP. We also reasoned that because PKC should not be activated by AT1R when expressing Q/D-Gαq (Fig. S9), dampening of the receptor coupling to Gα13 should not be observed. As predicted, expressing Q/D-Gαq in ΔGαq/11 cells inhibited FP-mediated Gα13 binding and signaling (Fig. 3C), similar to what we observed when expressing a functional Gαq in these cells (Fig. S7B). Moreover, AT1R coupling and signaling through Gα13 was unaltered when Q/D-Gαq was overexpressed in these cells (Fig. 3D), markedly contrasting with the expression of a functional Gαq protein (Fig. S7D).

To further substantiate these observations, we next used an FP receptor mutant that is deficient in Gαq coupling (I147A/M247A; hereafter referred to as GαqNull-FP) (Fig. S10A). Despite GαqNull-FP being less well expressed than WT-FP in cells, it nonetheless showed a significant increase in Gα13 coupling and PDZRhoGEF activation, consistent with the lack of Gαq competition (Figs. 4A and B, and S10B). This finding is also in agreement with what we observed with WT-FP when Gαq/11 was absent from cells (ΔGαq/11 cells) or when Gαq was maintained in its inactive, low receptor affinity state (e.g., Gαq-GDP state following YM treatment) (Figs. 2A and 3A). The GαqNull-FP coupling to Gα13 was unchanged when the receptor was expressed in either HEK293 cells or ΔGαq/11 cells, which markedly contrasted with WT-FP signaling in these cells (Figs. 4C and D, and 2A). Furthermore, unlike what we observed for WT-FP, GαqNull-FP-mediated Gα13 coupling and signaling were unaffected by reintroducing Gαq in ΔGαq/11 cells (Figs. 2B, and 4E and F). Altogether, these findings suggest that a direct Gαq-FP interaction is required for competing with Gα13.

**Gα13 and Gαq competition are specific to FP and AT1R**

We further tested the extent to which Gαq and Gα13 competition and/or signaling regulated the coupling of other GPCRs to these G proteins. We used the bradykinin type 2 receptor (B2R) and the thromboxane A2 receptor alpha (TPα), which have been shown to couple to Gαq/11 and Gα12/13 (Avet et al., 2022; Inoue et al., 2019; Sauliere et al., 2012). We first confirmed that B2R and TPα coupled to and activated Gαq/11 and Gα12/13 (Fig. S11A and B). While loss of Gα13 competition with Gαq at AT1R increased receptor-mediated Gαq activation, consistent with our previous findings (Fig. 1C), it had no effects on either B2R or TPα signaling through Gαq, similar to FP (Fig. S11C). We also examined the effect of Gαq coupling and activation on Gα13-mediated signaling by these receptors using either the Gαq knockout cells, or the Gαq and PKC inhibitors. Unlike FP and AT1R, B2R and TPα signaling through Gα13 was not affected with the loss of Gαq expression (Fig. S11D). Moreover, while PKC inhibition potentiated Gα13 signaling by AT1R, it had no effect on the response mediated by neither B2R, TPα, nor FP (Fig. S11E). Furthermore, inhibiting Gαq with YM increased Gα13 signaling only for FP and AT1R (Fig. S11E), consistent with what we previously observed (Fig. 3A and B). Together, these results suggest that Gαq and Gα13 competition at GPCRs is receptor specific.

**Gα13 and Gαq availability influences the signaling profiles of FP and AT1R biased ligand**

We have previously reported the identification of an allosteric modulator, PDC113.824 (PDC), which biases FP signaling by inhibiting Gα12/13 coupling while concomitantly increasing Gαq/11 signaling by the receptor (Goupil et al., 2012). Considering our observation that Gαq competes with Gα13 coupling to FP, we reasoned that PDC exerts, in part, its bias function through such a mechanism. To investigate this possibility, we used a PDC analog, Az-PDC, which retained

its positive allosteric modulation (PAM) on the Gαq pathway as observed through the potentiation of FP-mediated MAPK signaling (Fig. S12A and B), as well as its negative allosteric modulation (NAM) on Gα13-mediated signaling by FP in HEK293 cells (Fig. 5A). As predicted, Az-PDC's NAM effect on FP coupling to Gα13 was completely lost in cells expressing the WT receptor and lacking Gαq, as well as in HEK293 cells expressing endogenous Gαq and overexpressing GαqNull-FP (Fig. 5B, C, and D). We also tested the effect of two AngII analogs (TRV and SVdF) that produced preferential coupling of AT1R to Gα12/13 compared to Gαq/11 (Namkung et al., 2018) (Fig. S12C and D). Similar to AngII, loss of Gα12/13 expression significantly increased the propensity of both TRV and SVdF to promote AT1R coupling to Gαq (Fig. 5E). These results further support the differential competition between Gαq and Gα13 at FP and AT1R and suggest a mechanism by which these ligands exert their bias function.

## 3.5 Discussion

Using the two prototypical Gαq/11 and Gα12/13 receptors, AT1R and FP, we show that the availability of G proteins at these promiscuous GPCRs and their ensuing downstream signaling, in some cases, differentially regulate receptors coupling to their cognate G proteins (Fig. 6). Such regulation is dependent on the nature of the receptor and the G protein.

The directional and opposite regulation in Gαq and Gα12/13 competition at receptors observed here for FP and AT1R supports a model where receptors bind their cognate G proteins with different affinities. Such behavior is also apparent when examining the effectors' responses downstream of these G proteins. These observations imply that G protein competition at these receptors neither results from an intrinsic property of the G protein sensors themselves nor is linked to the relative differences in endogenous Gαq/11 vs. Gα12/13 levels, which may still exist. The

lack of Gαq/11 vs. Gα12/13 competition observed at other GPCRs, such as the B2R and TPα receptors, also argues against these possibilities. It also suggests that affinities of these G protein subtypes for B2R and TPα receptors may not greatly differ. Whether this is the case for other receptors that bind different cognate G proteins will need further investigation. Our findings also suggest that G protein competition does not necessarily involve the coupling of functional G proteins to receptors, reminiscent of the recently reported non-productive G protein coupling to GPCRs (Okashah et al., 2020). For AT1R, our data suggest a competition between G proteins at the receptor level and the regulation of cognate G protein interactions through signaling by kinases like PKC, consistent with the receptor phosphorylation and desensitization (Qian et al., 1999; Tang, Shirai, & Inagami, 1995). However, our findings suggest that FP is neither subjected to such regulation nor that β-arrestin participates in this process, unlike for AT1R, where β-arrestin could have had differential effects on G protein subtype competition at the receptor, something we did not investigate herein. Whether receptor interactions with specific Gα subtypes trigger differential β-arrestin activity will also require further investigation. Although our findings with AT1R and FP suggest differential competition between cognate G proteins at the receptor level, we cannot exclude the possibility that the recruitment of effectors to a receptor-G protein complex interferes with the coupling of that receptor to its other cognate G proteins. Also, the re-localization of receptors and the compartmentalization of different signaling components, such as in lipid rafts or caveolae, could also alter receptor-coupling selectivity and contribute to the G protein competition observed (Ostrom and Insel., 2004). Lastly, we cannot exclude that in experiments using Gα-depleted cells or overexpressing Gα subunit, that the nature and absolute abundance of heterotrimers G complexes changes, which could also differentially affect cognate G proteins interaction with receptors.

Specific residues at the GPCR-G-protein interface not only play a role in determining selectivity (Flock et al., 2017; Okashah et al., 2019; Semack et al., 2016), they also likely regulate the coupling strengths of different cognate G proteins to their receptor. Selectivity in G protein coupling also likely emerges from the ability of GPCR-ligand complexes to differently sample distinct ensembles of conformations and to select for one G protein over another (Sandhu et al., 2019; Wingler et al., 2019). Such a model is supported by our observations that an allosteric modulator acting on FP, Az-PDC, which conceivably stabilizes conformations in the receptor favoring more efficient Gαq binding, reduces Gα13 coupling via a competition mechanism (Goupil et al., 2010; Harris et al., 2022). The absence of competition between Gαq and Gα13 coupling at B2R and TPα suggests that for these GPCRs, ligand–receptor complexes sample conformations that allow efficient binding of these two G protein families. Our findings also provide an explanation for how G protein availability in cells may alter not only the potency and efficacy of ligands but also their bias profiles (Kenakin, 1997, 2002; Onfroy et al., 2017). This is evidenced by our observation that AT1R ligands, which promote relatively better coupling to Gα12/13 over Gαq compared with AngII (Namkung et al., 2018), showed increased Gαq coupling in the absence of Gα12/13 competition. These findings also provide a potential mechanism regarding how AT1R ligands negatively bias Gαq signaling, in addition to their ability to more efficiently engage β-arrestins. We propose that such a differential competitive mechanism can be exploited to alter the G protein-biased profiles of allosteric and/or orthosteric drugs acting on AT1R and FP. This is indeed well illustrated with the use of PDC that likely allosterically increases Gαq coupling to the agonist-bound FP complex (i.e., through increase affinity) and competes Gα12/13 coupling, despite unaltered expression of these G protein subtypes in cells.

Gαq/11- and Gα12/13-mediated signaling by GPCRs, which contribute to myosin light chain phosphorylation through distinct and overlapping intermediate effectors such as Rho and ROCK, both contribute to coordinating smooth muscle contraction in vivo, although their relative involvement seems to differ in normal vs. pathological settings (Wirth et al., 2008). Our observation that Gαq and Gα13 differentially compete at AT1R and FP may have important implications in regulating smooth muscle and other cell contraction in normal physiology and pathophysiology, considering that G protein expression has been shown to vary in a cardiovascular disease model (Onfroy et al., 2017). Our results showing that biasing FP to increase its coupling to Gαq and ensuing competition with Gα13 binding is consistent with the observed inhibition of myometrial smooth muscle contraction *in vivo* (Goupil et al., 2010). This questions, however, the relative roles of Gαq/11- and Gα12/13-dependent signaling in the regulation of uterine smooth muscle contraction during parturition. Moreover, we previously showed that agonist activation of either FP or AT1R in vascular smooth muscles increases the pressor response promoted by agonist activation of the other receptor, a phenomenon that was attributed to receptor heterodimerization but could also have involved differential regulation of Gαq/11 and Gα12/13 availability at FP and AT1R (Goupil et al., 2015), as revealed herein. Interestingly, Gα12/13-dependent signaling by vasocontractile GPCRs such as AT1R following vascular injury has been shown to prevent vascular smooth muscle dedifferentiation and proliferation, which is mediated by Gαq/11 signaling, hence playing an antagonistic cardiovascular protective role (Althoff et al., 2012). It is therefore tempting to speculate that biased ligands such as TRV and SVdF that preserve Gα12/13 and β-arrestin coupling to AT1R (Namkung et al., 2018), hence further limiting the activation of Gαq pathways by the receptor, could have better cardioprotective effects than ligands that interfere with all pathways or only the Gαq/11 pathway.

In summary, our findings not only reveal distinctive coupling profiles in GPCRs engaging the same families of cognate G proteins but also suggest different mechanisms of competitive regulation for G protein binding to receptors. Such findings may have important ramifications in drug development given the potential role of cognate G protein competition in regulating the functional bias of orthosteric ligands and allosteric modulators.

## 3.6 Acknowledgements

We are grateful for the helpful discussions with all past and present members of the Laporte lab. We thank Drs. Michel Bouvier and Christian Le Gouill from the University of Montreal for providing the p63RhoGEF-RlucII, the PDZRhoGEF-RlucII, and the Gα11 polycistronic constructs. We also thank Dr. Asuka Inoue from Tohoku University for providing the ΔGαq/11 and ΔGα12/13 cells

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## 3.8 Figures and Legends

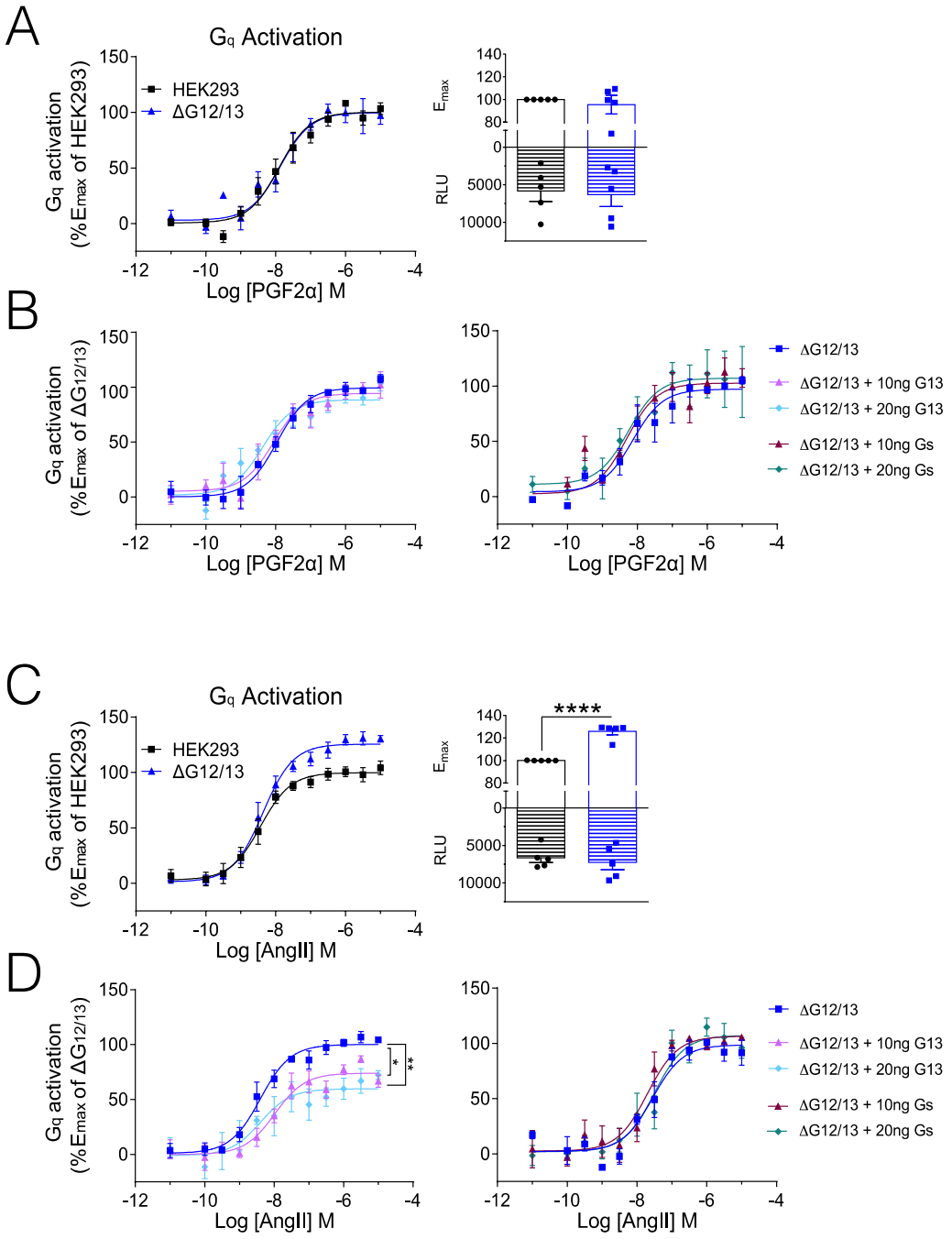


**Figure 1. Effect of Gα13 availability on Gαq activation by FP and AT1R.**

(A – D) Gαq activation following PGF2α stimulation of FP (A and B) or AngII stimulation of AT1R (C and D) assessed by the Gαq polycistronic sensor in HEK293 cells and ΔGα12/13 cells

+/- Gα13 (B and D left panels) or Gαs (B and D right panels) overexpression. BRET measurements are normalized to the maximal response in HEK293 cells (%Emax of HEK293) (A and C) or in ΔGα12/13 cells without Gα overexpression (%Emax of ΔG12/13) (B and D) in the same experiment. A and C insets show the expression levels of Gαq-RlucII (solid fill) and the Emax values of the dose-response curves (no fill).

Data information: all data represent means ± SEM of at least three independent experiments. In (A and C), Unpaired Student's t test was performed on the Emax values obtained from the nonlinear regression curves of the average data. ****$P < 0.0001$. In (B and D), two-way ANOVA followed by Dunnett's multiple comparisons tests were performed for the last time points. *$P < 0.05$, and **$P < 0.01$.

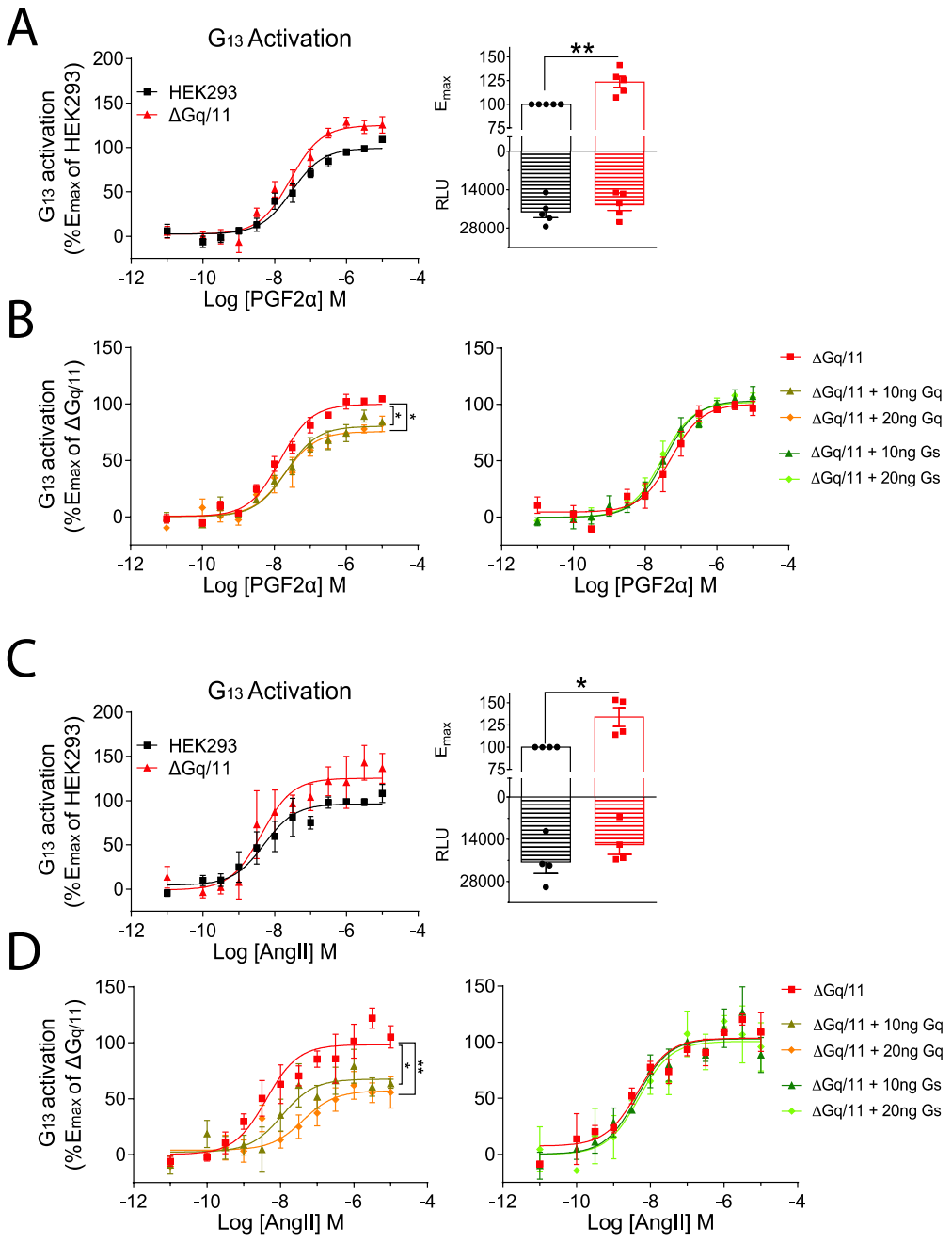


**Figure 2. Effect of Gαq availability on Gα13 activation by FP and AT1R.**

(A – D) Gα13 activation following PGF2α stimulation of FP (A and B) or AngII stimulation of AT1R (C and D) assessed by the Gα13 sensor in HEK293 cells and ΔGαq/11 cells +/- Gαq (B and D left panels) or Gαs (B and D right panels) overexpression. BRET measurements are normalized

to the maximal response in HEK293 cells (%Emax of HEK293) (A and C) or in ΔGαq/11 cells without Gα overexpression (%Emax of ΔGq/11) (B and D) in the same experiment. A and C insets show the expression levels of Gα13-RlucII (solid fill) and the Emax values of the dose-response curves (no fill).

Data information: all data represent means ± SEM of at least three independent experiments. In (A and C), Unpaired Student's t test was performed on the Emax values obtained from the nonlinear regression curves of the average data. *P < 0.05, and **P < 0.01. In (B and D), two-way ANOVA followed by Dunnett's multiple comparisons tests were performed for the last time points. *P < 0.05, and **P < 0.01.

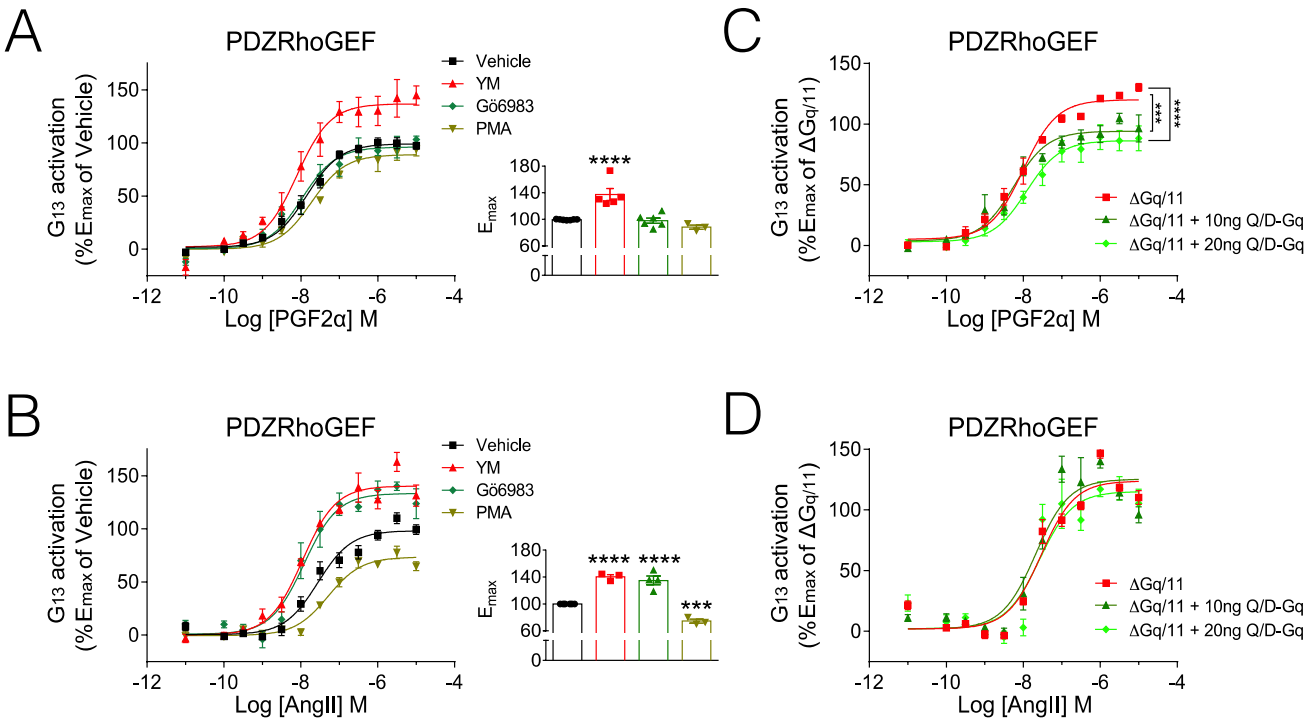


**Figure 3 Impact of Gαq downstream signaling on Gα13 activation by FP and AT1R.**

(A – D) Gα13-mediated PDZRhoGEF PM recruitment by FP (A and C) or AT1R (B and D) either in HEK293 cells treated with vehicle, 200 nM YM-254890 (YM), 1 μM Gö6983, or 1 μM PMA for 30 min (A and B), or in ΔGαq/11cells +/- inactive Gαq mutant (Q/D-Gαq) overexpression (C and D). Cells were stimulated with the indicated concentrations of PGF2α (A and C) or AngII (B and D). BRET measurements are normalized to the maximal response of vehicle treated cells (%Emax of Vehicle) (A and B), or in ΔGαq/11 cells without Q/D-Gαq expression (%Emax of ΔGq/11) in the same experiment.

Data information: all data represent means ± SEM of at least three independent experiments. In (A and B), One-way ANOVA followed by Dunnett's multiple comparison test was performed on Emax values obtained from the nonlinear regression curves of the averaged data. ***P < 0.001,

and ****P < 0.0001. In (C and D), two-way ANOVA followed by Dunnett's multiple comparisons tests were performed for the last time points. ***P < 0.001, and ****P < 0.0001.

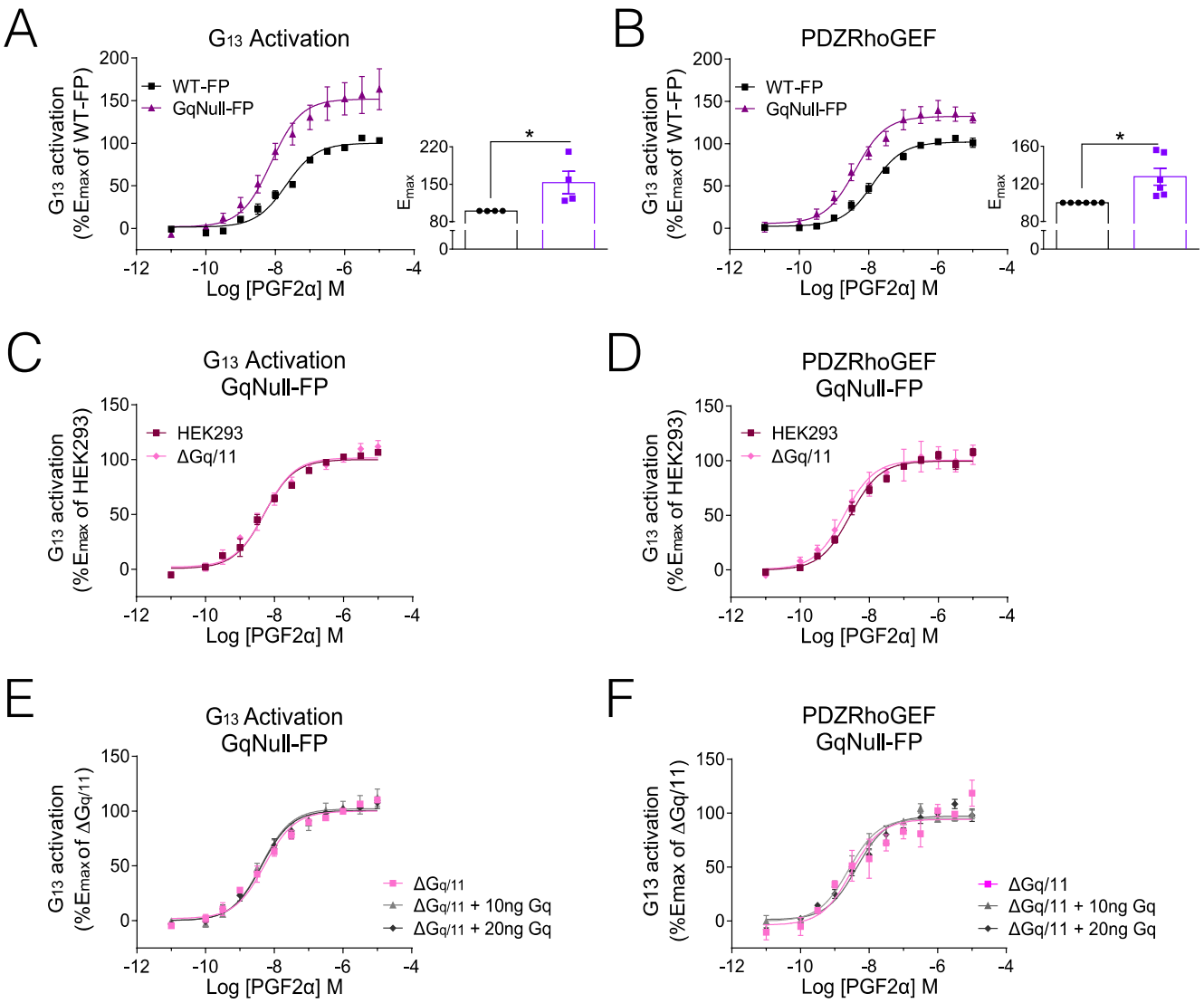


**Figure 4. Impact of Gαq availability on Gα13 signaling by the GαqNull mutant FP.**

Gα13 activation assessed by the Gα13 sensor (A, C, and E), or by Gα13-mediated PDZRhoGEF PM recruitment (B, D, and F) following PGF2α stimulation of WT-FP or GαqNull mutant FP (GαqNull-FP) in HEK293 cells (A and B), or of GαqNull-FP in HEK293 cells and ΔGαq/11 cells +/- Gαq overexpression (C – F). BRET measurements are normalized to the maximal response of

WT-FP (%Emax of WT-FP) (A and B) or of GαqNull-FP in ΔGαq/11 cells without Gαq expression (%Emax of ΔGq/11) in the same experiment.

Data information: all data represent means ± SEM of at least three independent experiments. In (A and B), Unpaired Student's t test was performed on Emax values obtained from the nonlinear regression curve of the averaged data. *P < 0.05.

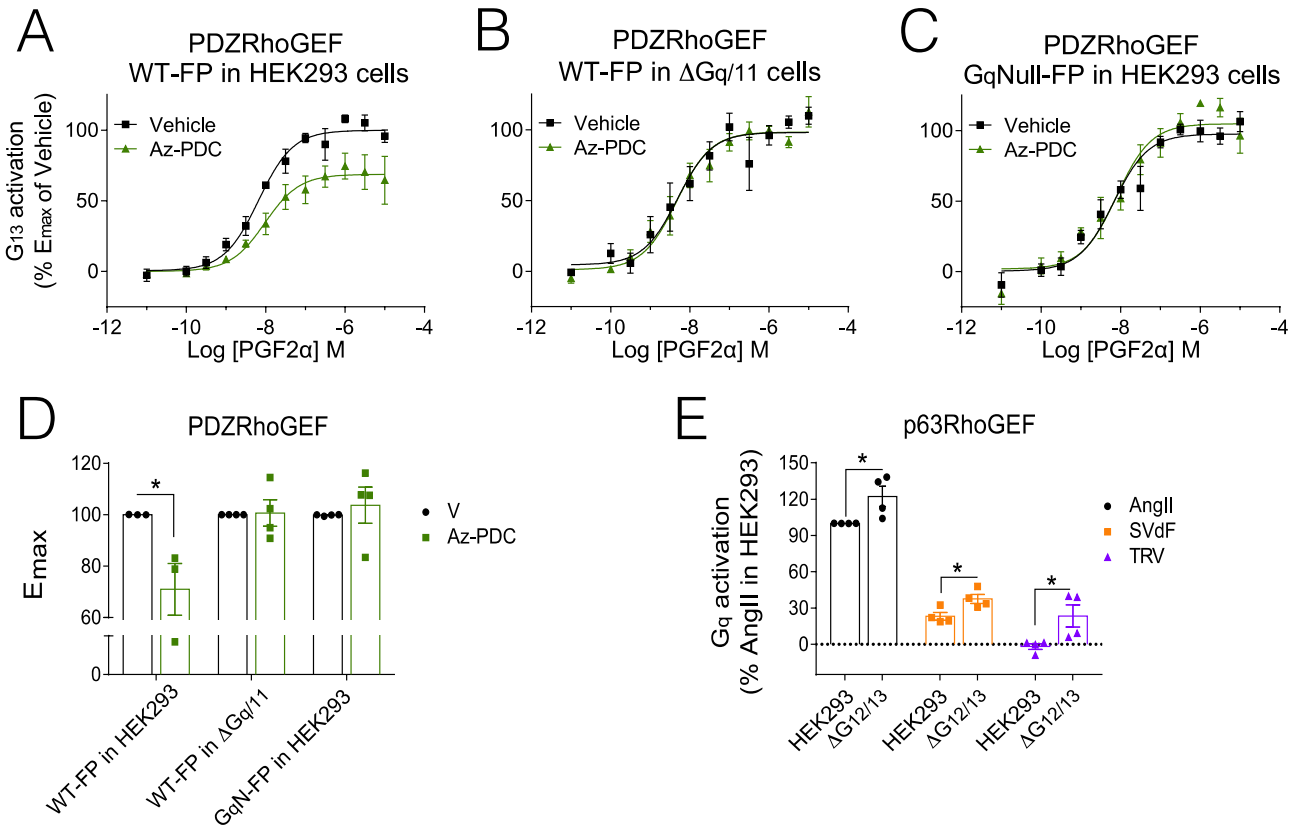


**Figure 5. Modulation of FP and AT1R biased ligands with altered receptor G protein binding availability.**

(A – C) Gα13-mediated PDZRhoGEF PM recruitment upon PGF2α stimulation of WT-FP (A and B) or GαqNull mutant FP (GαqNull-FP) (C) in parental HEK293 cells (A and C) or in ΔGαq/11 cells (B). Cells were pre-treated with vehicle or 10 μM Az-PDC for 30 min prior to PGF2α stimulation with the indicated concentrations. BRET measurements are normalized to the maximal response in the vehicle-treated condition (%$E_{max}$ of Vehicle). (D) Bar graph representation of $E_{max}$ values of the dose-response curves from A – C. (E) Gαq-mediated p63RhoGEF PM recruitment upon AT1R stimulation in HEK293 cells or in ΔGα12/13 cells. Cells were stimulated with 10 μM

of AngII, TRV, or SVdF. BRET measurements are normalized to the response of AngII (%$E_{max}$ of AngII).

Data information: all data represent means ± SEM of at least three independent experiments. In (D and E), Unpaired Student's t tests were performed. *$P < 0.05$.

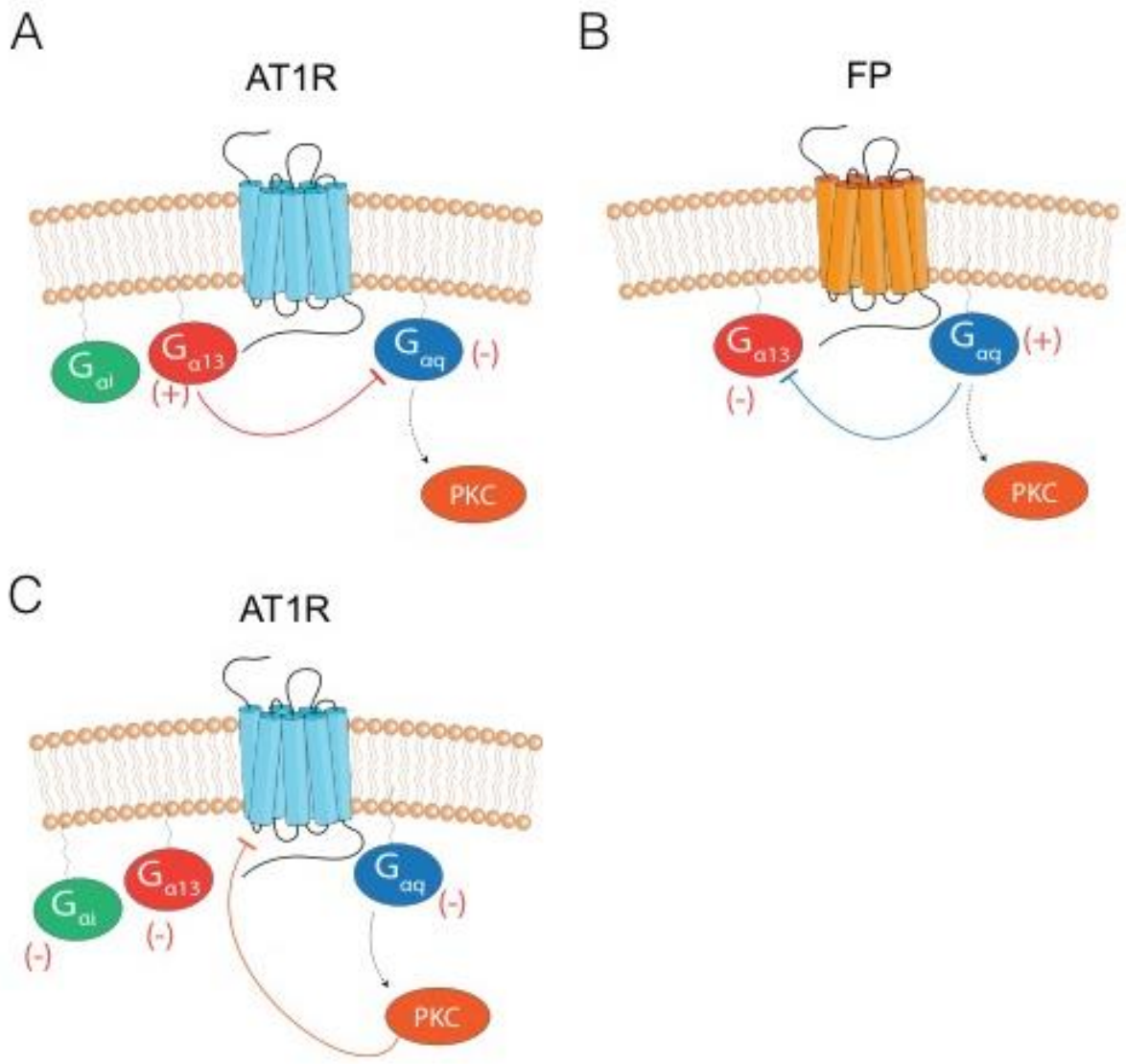


**Figure 6. Schematic representation of FP and AT1R selectivity regulation by a G protein competitive coupling and/or signaling.**

(A, B) G proteins compete for receptor binding in a receptor-dependent fashion. For AT1R, Gα13 binding restricts Gαq coupling and activation (A), while for FP, Gαq binding impedes Gα13 coupling and activation (B). (C) Indirect Gαq-mediated regulation of AT1R signaling through Gαq, Gα13, and Gαi2 is induced by an inhibitory feedback mechanism of AT1R signaling caused by PKC activation downstream of Gαq.

## 3.9 Supplemental Figures and Legends

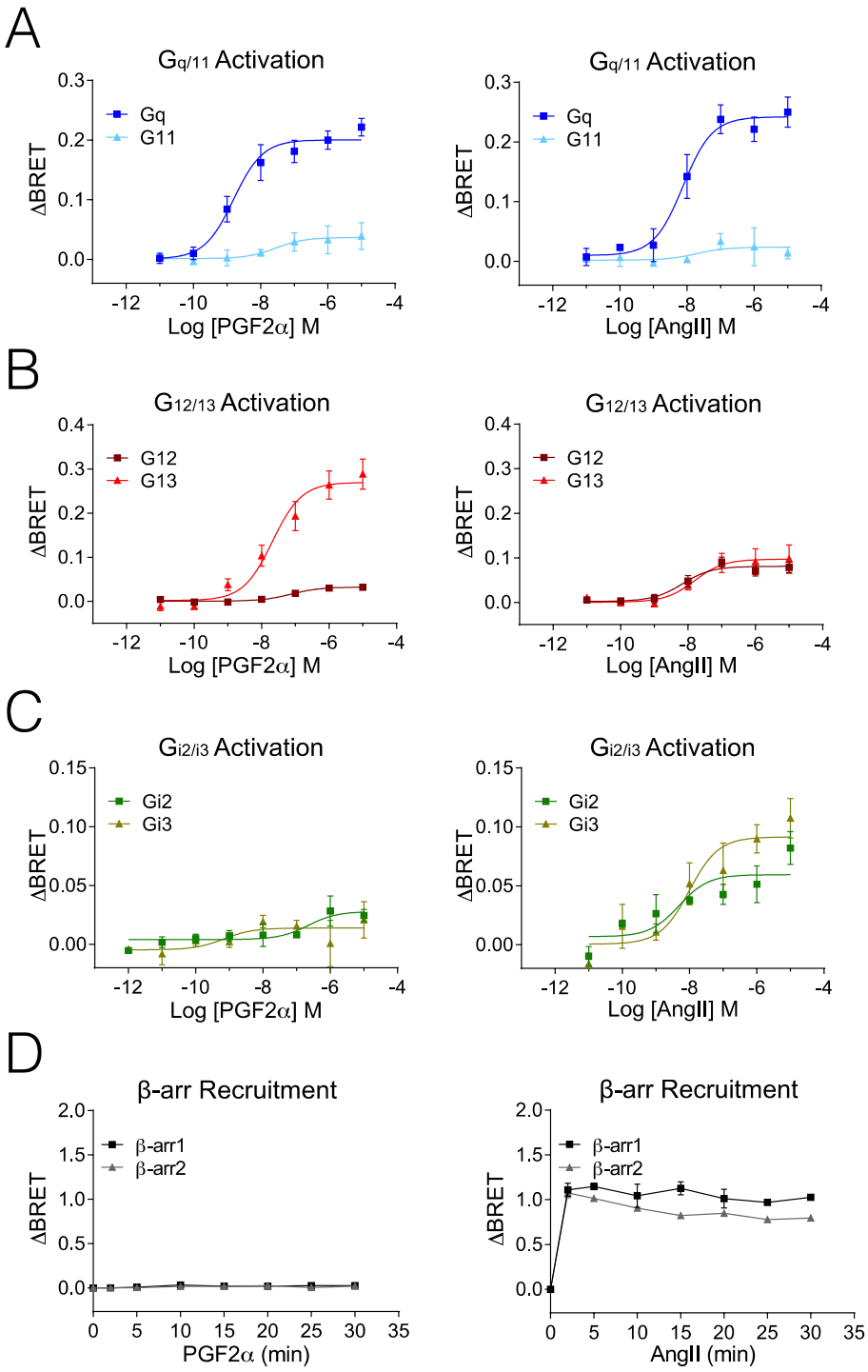


**Figure S1. FP and AT1R Signaling through G proteins and β-arrestins.**

(A – D) G protein and β-arrestin activation in HEK293 cells expressing FP (left panels) or AT1R (right panels). Cells were transfected with BRET sensors measuring the activation of Gαq, Gα11 (A), Gα12, Gα13 (B), Gαi2, or Gαi3 (C), β-arrestin1 or β-arrestin2 (D). Cells were stimulated with the indicated concentrations of PGF2α (left panel) or AngII (right panel) (A – C), or with 1 μM of the aforementioned ligands for the indicated times points.

Data information: all data represent means ± SEM of at least three independent experiments.

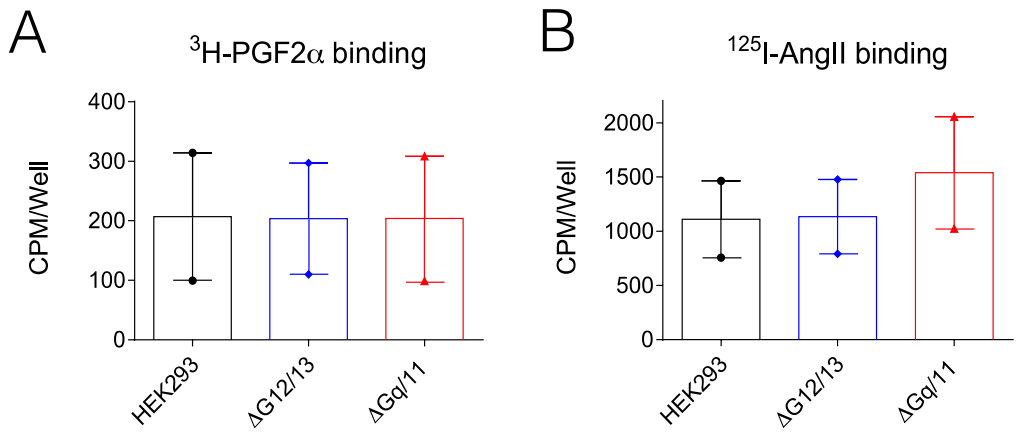


**Figure S2. FP and AT1R expression in parental and CRISPR Gαq/11 and Gα12/13 knockout cells.**

(A and B) Abundance of FP (A) and AT1R (B) in HEK293, ΔGαq/11 cells, and ΔGα12/13 cells reported by ligand binding assays using [3H]-PGF2α (A), or [125I]-AngII (B). Specific binding was determined as total minus nonspecific binding. Data represent means ± SEM of two independent experiments and presented as (CPM/Well). One-way ANOVA followed by Dunnett's multiple comparison test was performed.

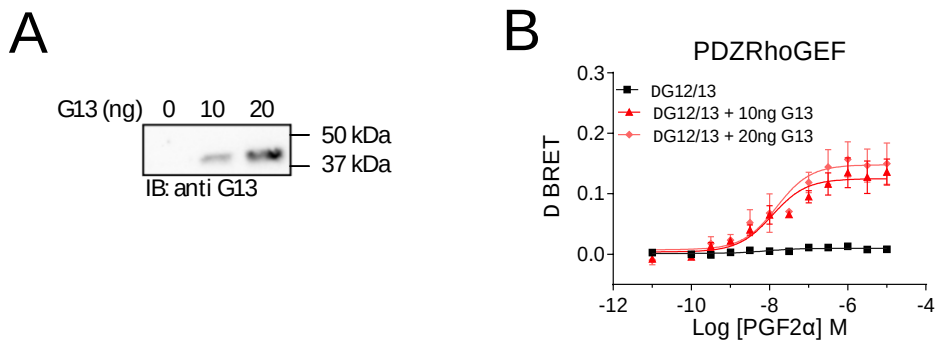


**Figure S3. Expression and activation of transfected Gα13 subunit in CRISPR-Cas9 Gα12/13 knockout cells.**

(A) Western blot analysis using anti-Gα13 antibody to detect the expression of transfected Gα13 in ΔGα12/13 cells. (B) PM recruitment of PDZRhoGEF by PGF2α-stimulated FP in ΔGα12/13 cells +/- increasing amounts of the Gα13 subunit. Change in BRET ratio is reported as means ± SEM of four independent experiments.

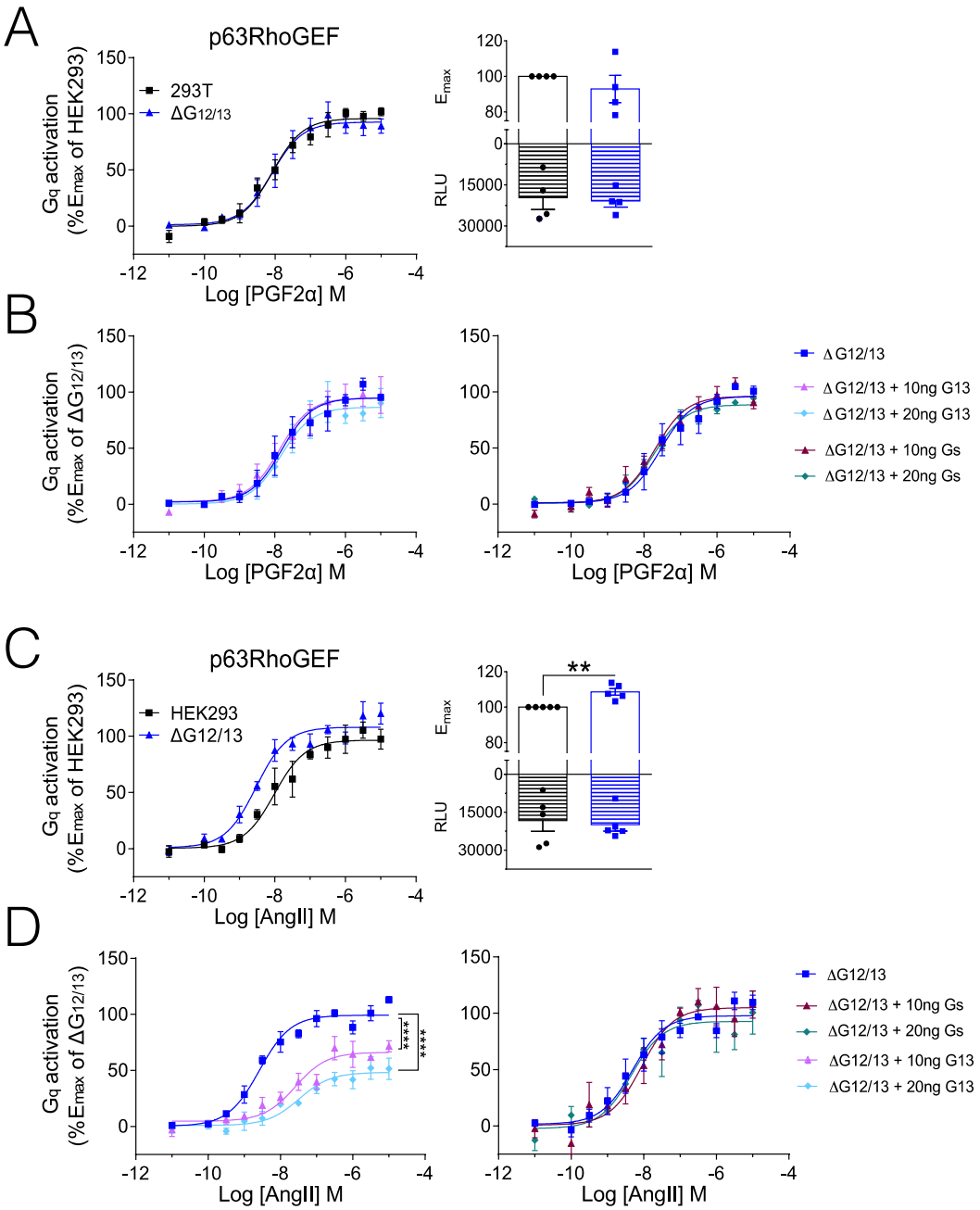


**Figure S4. Effect of Gα13 availability on Gαq-mediated signaling by FP and AT1R.**

(A – D) Gαq-mediated p63RhoGE PM recruitment following PGF2α stimulation of FP (A and B) or AngII stimulation of AT1R (C and D) in HEK293 cells and ΔGα12/13 cells +/- Gα13 (B and D

left panels) or Gαs (B and D right panels) overexpression. BRET measurements are normalized to the maximal response in HEK293 cells (%Emax of HEK293) (A and C) or in ΔGα12/13 cells without Gα overexpression (%Emax of ΔG12/13) (B and D) in the same experiment. A and C insets show the expression levels of p63RhoGEF-RlucII (solid fill) and the Emax values of the dose-response curves (no fill).

Data information: all data represent means ± SEM of at least three independent experiments. In (A and C), Unpaired Student's t test was performed on the Emax values obtained from the nonlinear regression curves of the average data. **P < 0.01. In (B and D), two-way ANOVA followed by Dunnett's multiple comparisons tests were performed for the last time points. ****P < 0.0001.

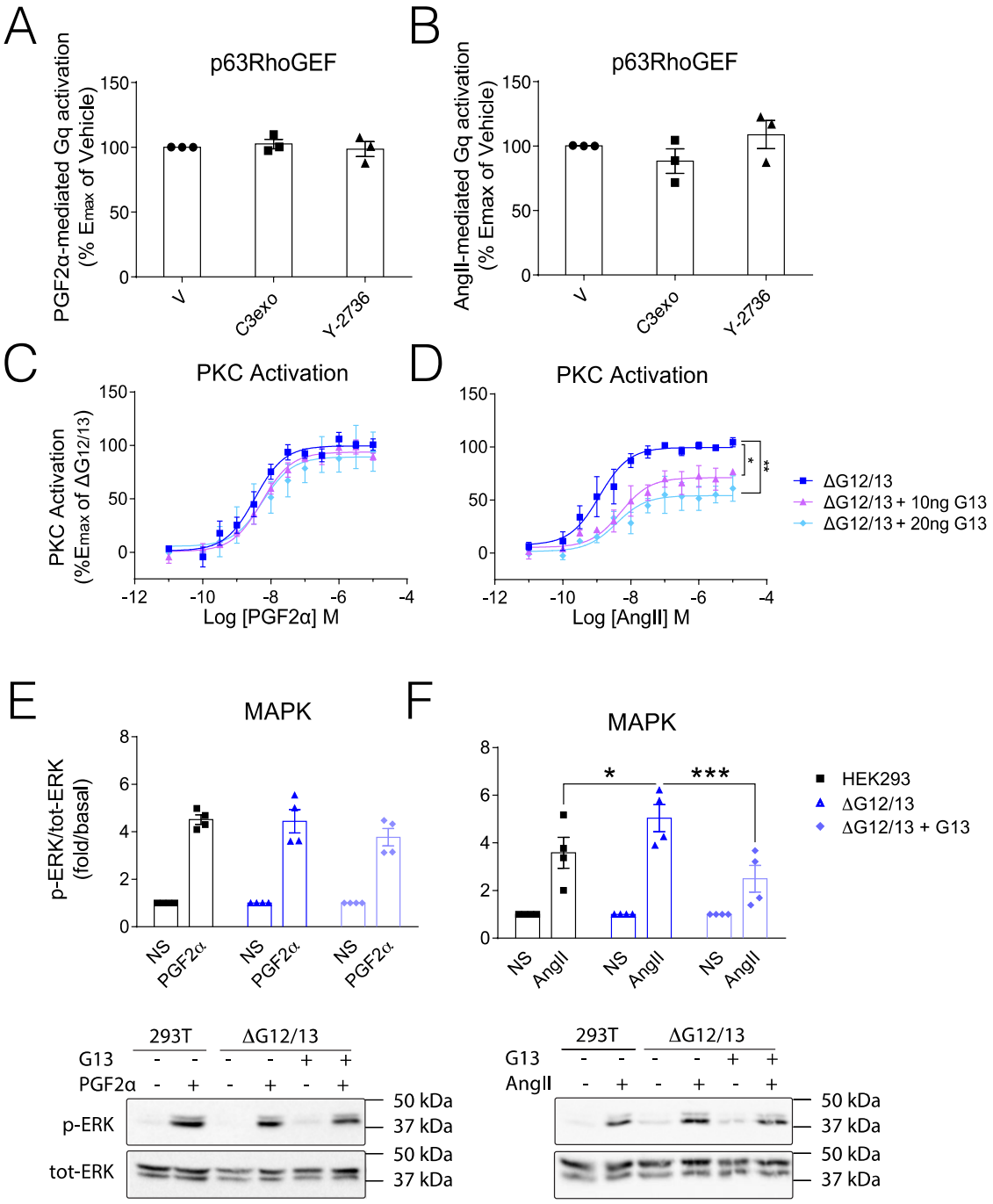


**Figure S5. Effect of Gα13 availability on Gαq downstream effectors activation by FP and AT1R.**

(A and B) Gαq-mediated p63RhoGEF PM recruitment in HEK293 cells transfected with FP (A) or AT1R (B). Cells were pre-treated or not with Rho inhibitor (C3 exoenzyme, 1 µg/ml) for 4 h at

37 °C, or ROCK inhibitor (Y27632, 1 μM) for 30 min at 37 °C prior to stimulation with 10 μM PGF2α (A) or AngII (B). BRET measurements are normalized to the maximal response of the vehicle-treated condition in the same experiment. (C and D) PKC activation in ΔGα12/13 cells +/- Gα13 overexpression. Cells were stimulated with the indicated concentrations of PGF2α (C) or AngII (D) and BRET measurements are normalized to the maximal response in ΔGα12/13 cells without Gα overexpression in the same experiment. (E and F) MAPK activation by FP (E) and AT1R (F) in HEK293 and ΔGα12/13 cells +/- Gα13 overexpression. Cells were stimulated with 1 μM of PGF2α (E) or AngII (F) and lysates were analyzed by Western blot analysis using anti-phospho-ERK and anti-total ERK antibodies. Bands were quantified by densitometry analysis (upper panel) and representative blots are shown (lower panel).

Data information: all data represent means ± SEM of three or four independent experiments. In (A and B), one-way ANOVA followed by Dunnett's multiple comparison tests was performed. In (C – F) Two-way ANOVA followed by Dunnett's multiple comparison tests were performed. *P <0.05, **P <0.01, and ***P <0.001.

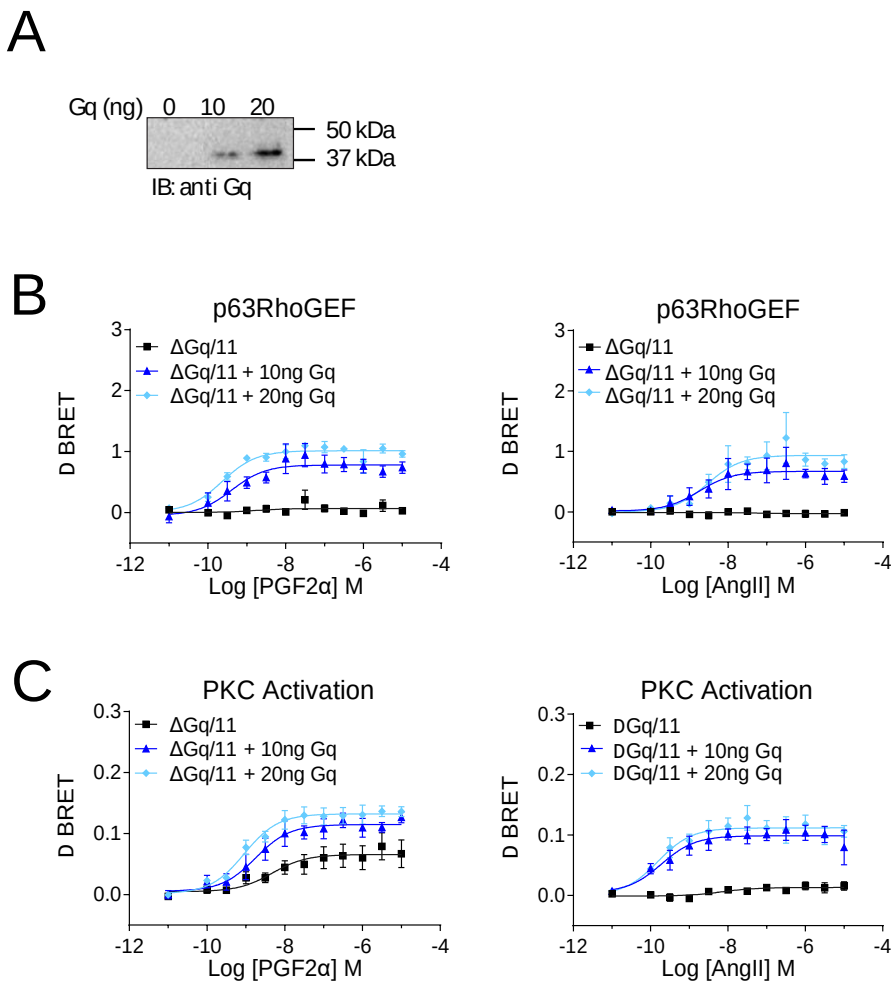


**Figure S6. Expression and activation of transfected Gαq subunit in CRISPR-Cas9 Gαq/11 knockout cells.**

(A) Western blot analysis using anti-Gαq antibody to detect the expression of transfected Gαq in ΔGαq/11 cells. (B and C) Activation of p63RhoGEF (B) and PKC (C) sensors following PGF2α or AngII stimulation of ΔGαq/11 cells expressing FP or AT1R, respectively, with or without Gαq overexpression. Change in BRET ratio is reported as means ± SEM of three or four independent experiments.

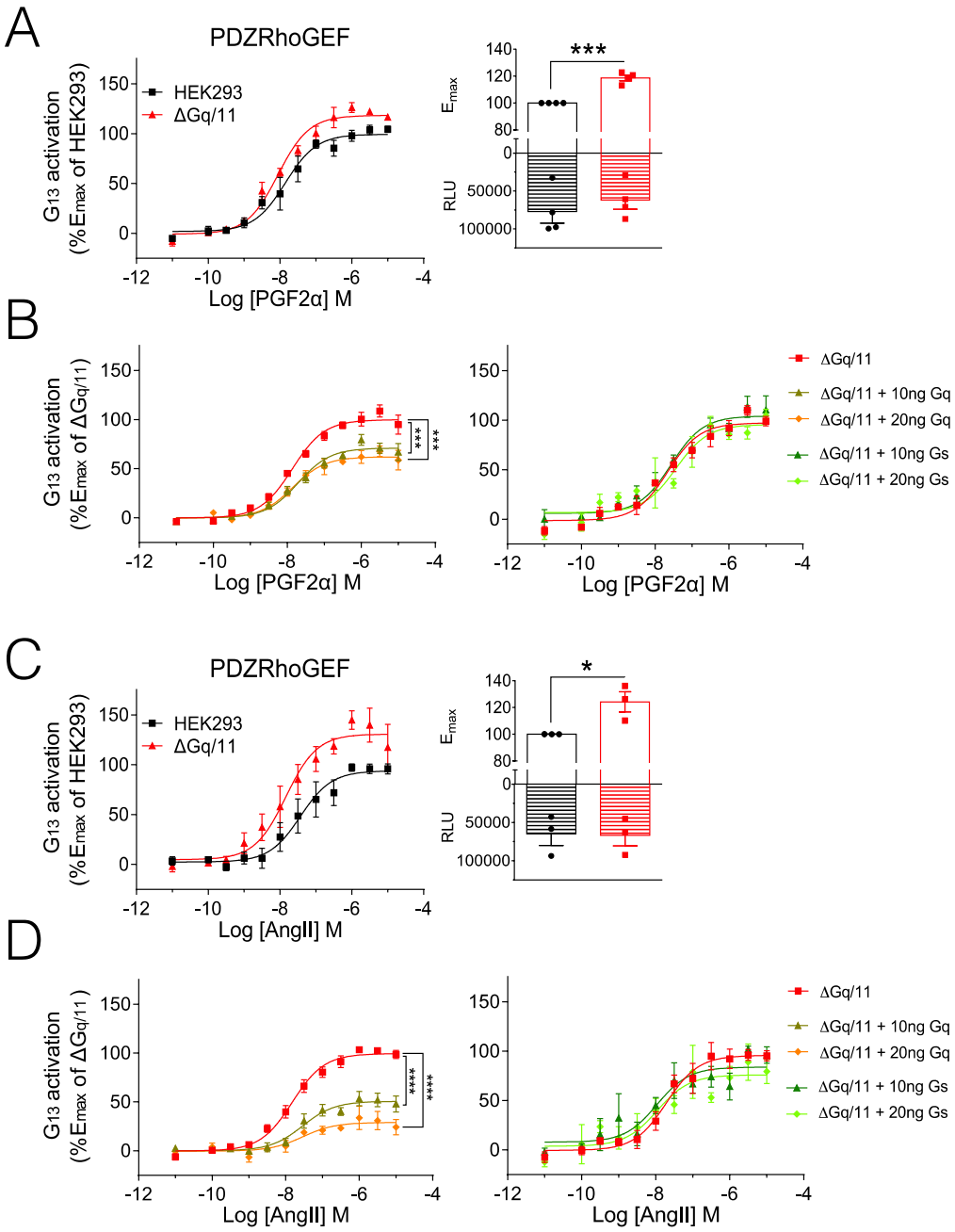


**Figure S7. Effect of Gαq availability on Gα13-mediated signaling by FP and AT1R.**

(A – D) Gα13-mediated PDZRhoGEF PM recruitment following PGF2α stimulation of FP (A and B) or AngII stimulation of AT1R (C and D) in HEK293 cells and ΔGαq/11 cells +/- Gαq (B and

D left panels) or Gαs (B and D right panels) overexpression. BRET measurements are normalized to the maximal response in HEK293 cells (%Emax of HEK293) (A and C) or in ΔGαq/11 cells without Gα overexpression (%Emax of ΔGq/11) (B and D) in the same experiment. A and C insets show the expression levels of PDZRhoGEF-RlucII (solid fill) and the Emax values of the dose-response curves (no fill).

Data information: all data represent means ± SEM of three to five independent experiments. In (A and C), Unpaired Student's t test was performed on the Emax values obtained from the nonlinear regression curves of the average data. *P < 0.05, and ***P < 0.001. In (B and D), two-way ANOVA followed by Dunnett's multiple comparisons tests were performed for the last time points. ***P < 0.001, and ****P < 0.0001.

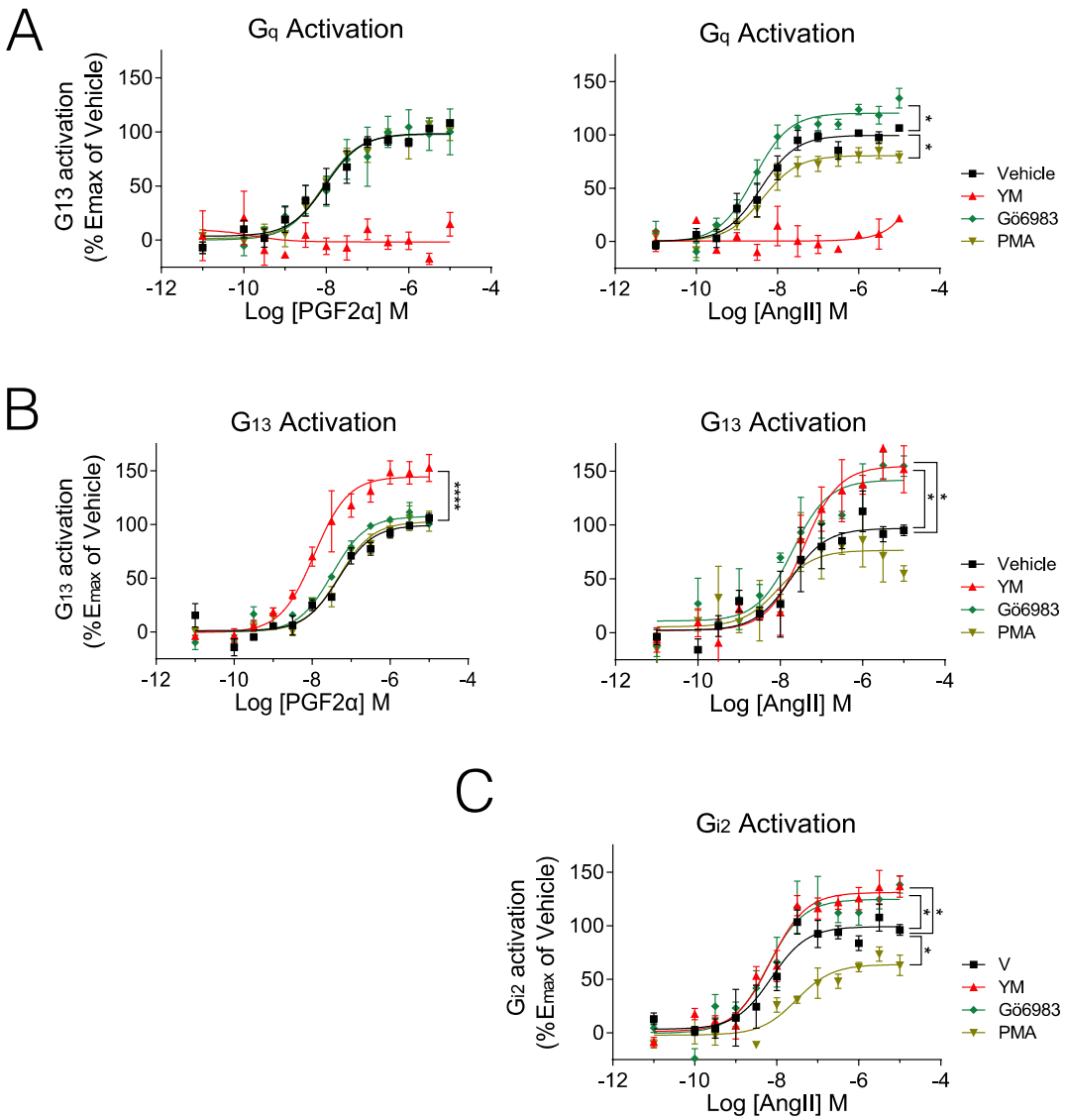


**Figure S8. Impact of PKC activation on FP and AT1R G protein signaling.**

(A – C) Effects of Vehicle, 200 nM YM-254890 (YM), 1 μM Gö6983, or 1 μM PMA treatments on Gαq (A), Gα13 (B), and Gαi2 (C) signaling by FP (left panels) and AT1R (right panels) in HEK293 cells. Cells expressing the BRET sensors for Gαq (polycistronic) (A), Gα13 (B), or Gαi2 (C) were stimulated with the indicated concentrations of PGF2α (left panel) or AngII (right panel).

BRET measurements are normalized to the maximal response of vehicle treated cells (%Emax of Vehicle)

Data information: all data represent means ± SEM of at least three independent experiments. Two-way ANOVA followed by Dunnett's multiple comparisons tests were performed for the last time points: *P < 0.05, and ****P < 0.0001.

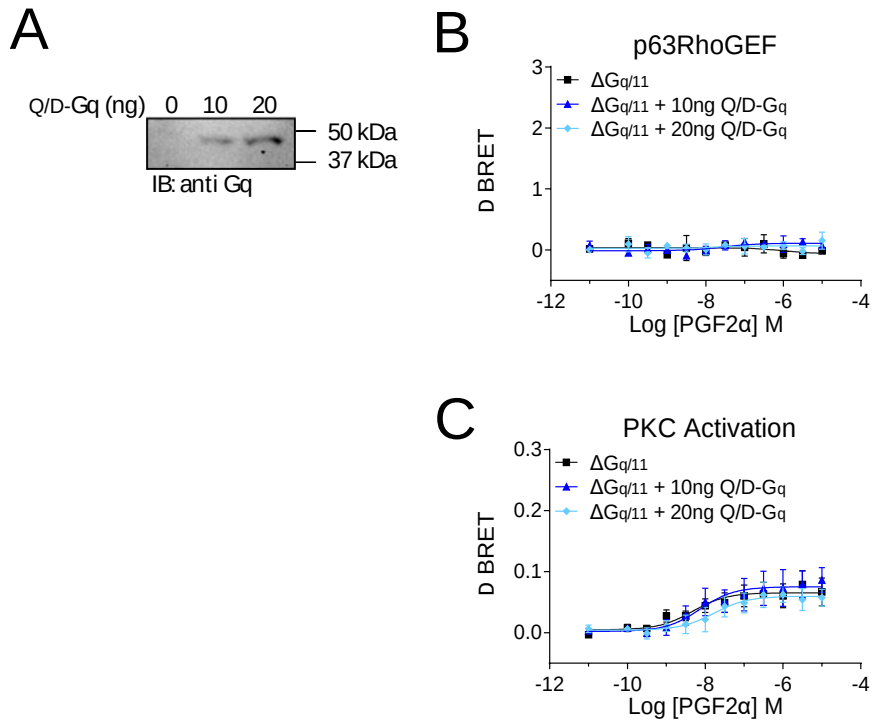


**Figure S9. Expression and activation of transfected Q/D-Gαq in CRISPR-Cas9 Gαq/11 knockout cells.** (A) Western blot analysis using anti-Gαq antibody to detect the expression of transfected inactive Gαq mutant (Q/D-Gαq) in ΔGαq/11 cells. (B and C) Activation of p63RhoGEF (B) and PKC (C) sensors following PGF2α-stimulation of FP in ΔGαq/11 cells +/- Q/D-Gαq overexpression. Change in BRET ratio is reported as means ± SEM of three or four independent experiments.

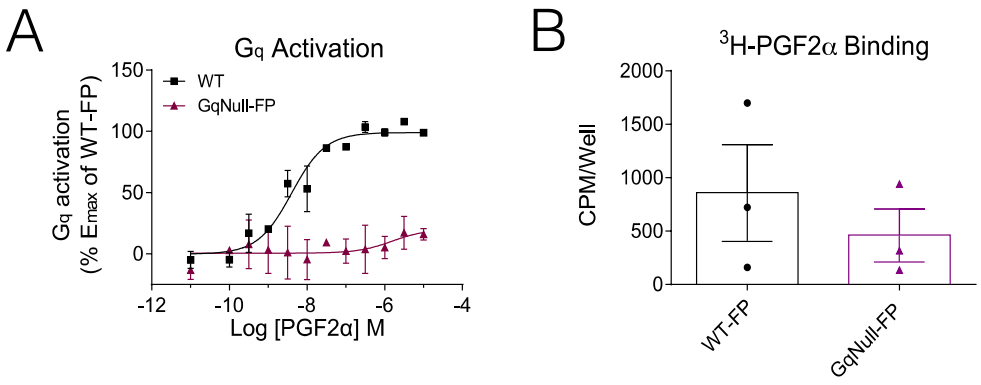


**Figure S10. Expression and Gαq activation of the Gαq inactive mutant FP (GαqNull-FP).**

(A) Gαq activation by WT-FP or GαqNull-FP in HEK293 cells assessed by the Gαq sensor. Cells were stimulated with the indicated concentrations of PGF2α and BRET measurements are recorded and normalized to the maximal response of WT-FP (%Emax of WT-FP). Data represent means ± SEM of three independent experiments. (B) WT-FP and GαqNull-FP abundance was assessed in HEK293 cells by ligand binding assay using [3H]-PGF2α. Specific binding was calculated as the difference between total and non-specific binding. Data represent means ± SEM of three independent experiments and presented as (CPM/Well). Unpaired Student's t test was performed on the averaged data.

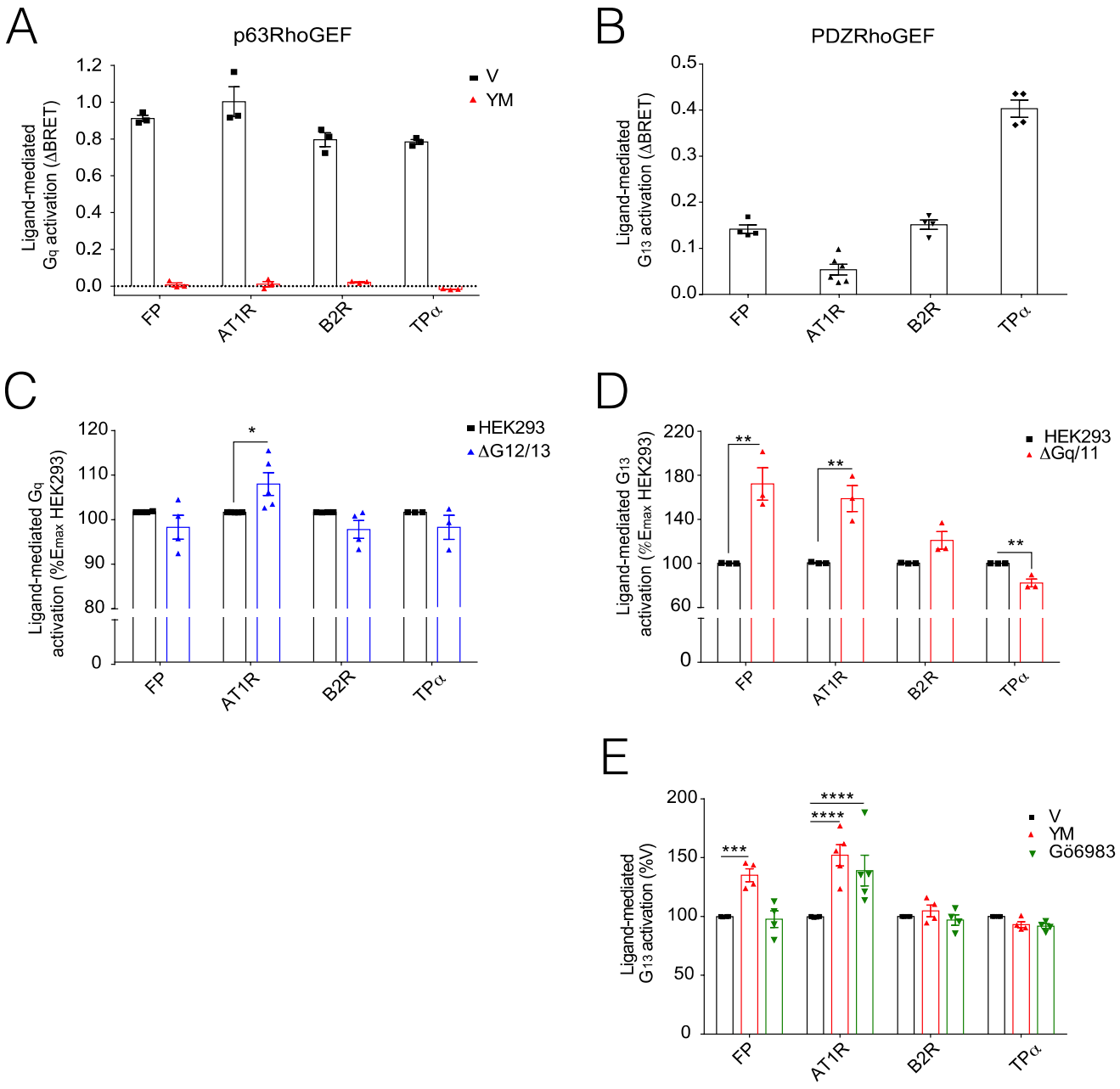


**Figure S11. Lack of Gαq vs. Gα13 competition or PKC-mediated regulation of B2R and TPα signaling.**

(A – E) Gαq-mediated PM recruitment of p63RhoGEF (A and C) orGα13-mediated PM recruitment of PDZRhoGEF (B, D, and E) was assessed in HEK293 (A, B, and E), and in ΔGα12/13 (C), or ΔGαq/11 (D) cells expressing FP, AT1R, B2R, or TPα receptors. Cells were pre-treated, or not, with vehicle, 200 nM YM-254890, or 1 μM Gö6983 for 30 min prior to ligand

stimulation. Cells were stimulated with 10 μM of PGF2α, AngII, BK, or U46619. BRET measurements are normalized to the maximal response in HEK293 cells (%$E_{max}$ of HEK293) (C and D), or in the vehicle treated condition (%$E_{max}$ of V) (E) in the same experiment.

Data information: all data represent means ± SEM of at least three independent experiments. In (C and D), Unpaired Student's t tests was performed. *P <0.05, and **P < 0.01. In (E), two-way ANOVA followed by Dunnett's multiple comparisons tests was performed. ***P <0.001, and ***P < 0.0001.

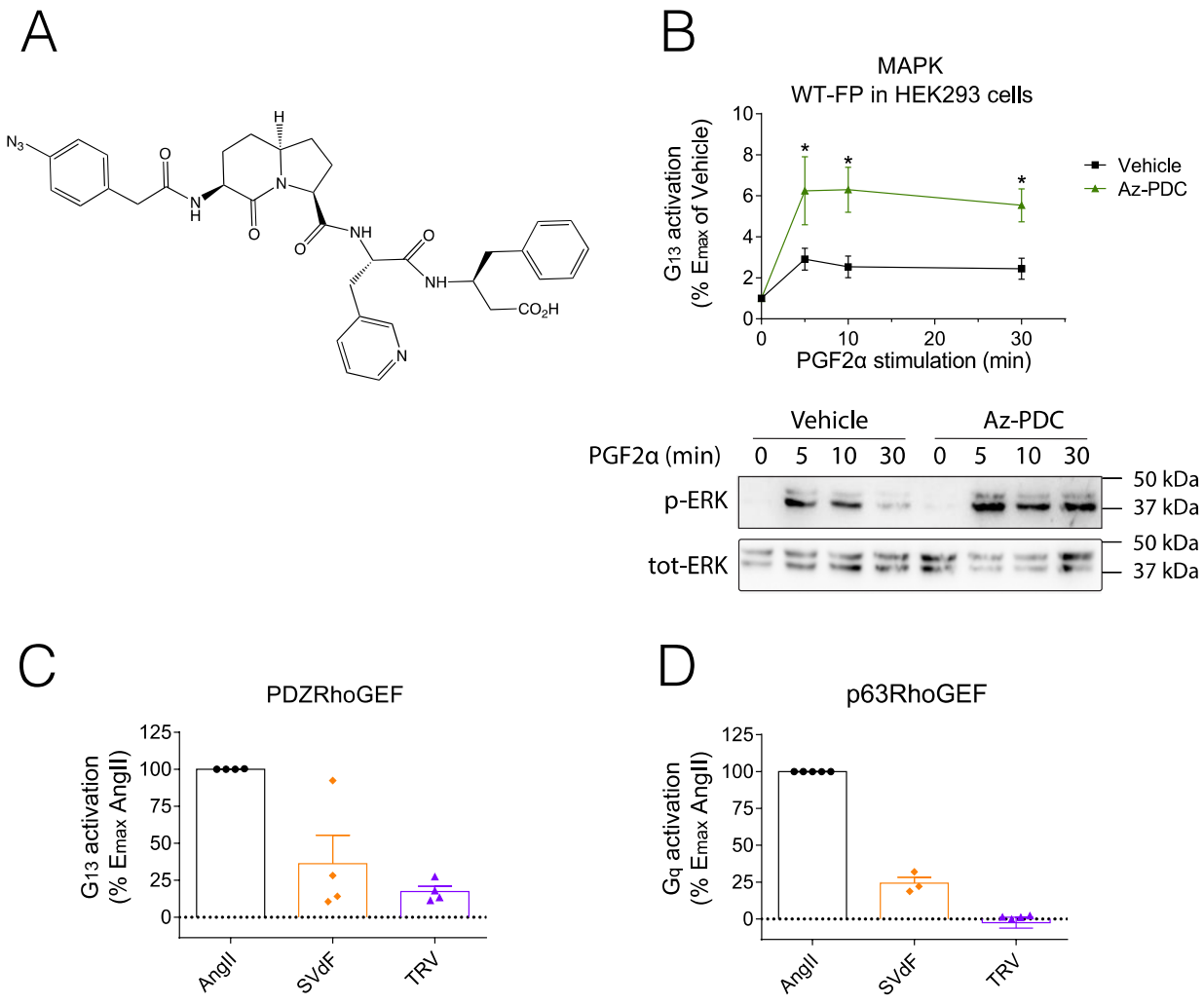


**Figure S12. G protein modulation by FP and AT1R biased ligands.**

(A) Structure of FP allosteric modulator, Az-PDC. (B) Western blot analysis of HEK293 cell lysates expressing FP and treated with Vehicle or 10 μM Az-PDC for 30 min prior to stimulation with 1 μM of PGF2α for 5 min using anti-phospho-ERK and anti-total ERK antibodies. Bands were quantified by densitometry analysis and represented as the means ± SEM of five independent experiments (upper panel) and representative blot are shown (lower panel). (C and D) Gα13-

dependent PDZRhoGEF (C) or Gαq-dependent p63RhoGEF activation in HEK293 cells expressing AT1R after stimulation with 10 μM AngII, SVdF, or TRV. BRET measurements are recorded and normalized to AngII-mediated response (%Emax of AngII) in the same experiment. Data information: all data represent means ± SEM of at least three independent experiments. In B, Two-way ANOVA followed by Bonferroni's multiple comparison tests was performed. *P <0.05.

## Connecting Text

The following chapter is entitled “Probing Differential β-arrestin active conformations Through Genetically Engineered Crosslinking”. This chapter falls under the overarching umbrella of this thesis in gaining insight regarding the fundamental factors conveying the diverse functional outcomes of GPCRs, despite interacting with the same receptor interacting partners. In the previous study presented in chapter 3, I highlighted distinctive mechanisms by which GPCRs, binding the same G protein partners, couple to their cognate G proteins. Here, I focus on the β-arrestin binding effector and investigate plausible differential β-arrestin conformations assumed upon binding with different receptors. Findings from this study broadens our understanding of the receptor-dependent interactions with their β-arrestin effector partner.

The main objective of this study was to demonstrate the use of azF-mediated crosslinking in distinguishing β-arrestin binding modalities. The use of such approach overcomes the inherent challenge of obtaining stable *in vitro* ligand–GPCR–β-arrestin complexes due to the highly dynamic nature of these interactions. Moreover, this approach provides valuable information relating to molecular contact sites and the global β-arrestin binding modalities. Genetic azF incorporation within β-arrestin sequence via amber suppression technology and photoaffinity cross-linking approach was implemented to study β-arrestin interaction with three GPCRs: AT1R, B2R, and V2R in live cells. Findings from this chapter not only reveal unique signatures for β-arrestin interactions with the different receptors, but they also report unique conformational regulation of the GPCR–β-arrestin complexes in function of the cellular localization. This manuscript is in preparation.

# CHAPTER 4: Probing Differential β-arrestin Active Conformations Through Genetically Engineered Crosslinking

**Dana Sedki**, Yubo Cao, Yoon Namkung, Stéphane A. Laporte

*In preparation*

## 4.1 Abstract

β-arrestins are multifunctional adaptor proteins that play a central role in regulating G protein-coupled receptors (GPCRs). They are recruited to active phosphorylated GPCRs to desensitize G protein-mediated signaling, promote receptor endocytosis, and initiate their own signal transduction. The active form of β-arrestin associates with conformational changes induced by the bound GPCR. Thus, structural characterization of differential GPCR–β-arrestin binding modalities is important to better understand the multiplicity of β-arrestin-mediated functions. Here, we used bioorthogonal labeling to genetically introduce the unnatural amino acid, p-azido-L-phenylalanine (azF) within different sites of the β-arrestin sequence. AzF-β-arrestin mutants were used to preform photocross-linking experiments with ligand-activated angiotensin II type 1 receptor, bradykinin B2 receptor, and vasopressin receptor 2. We identified unique β-arrestin contact sites for its interaction with each of the three receptors, consistent with a change in β-arrestin conformation. Moreover, we reveal that the binding modality of GPCR–β-arrestin complexes varies depending on the complex's cellular localization. Overall, our findings highlight the dynamic nature of GPCR–β-arrestin interactions that vary not only in function of the receptor in complex, but also on the internalization stage.

## 4.2 Introduction

The two nonvisual arrestins (Arrestin-2 and Arrestin-3, also known as β-arrestin1 and β-arrestin2, respectively), are adaptor proteins integral for the regulation of G protein-coupled receptors (GPCRs). Typically, ligand activation of GPCRs is followed by phosphorylation of their C-terminus by GPCR kinases (GRKs), which leads to the recruitment of β-arrestin proteins at the plasma membrane (PM). β-arrestins in turn promote receptor desensitization and terminate the signaling mediated by heterotrimeric G proteins (for review, see Refs. (Gurevich & Gurevich, 2019; Lefkowitz, 2004)). Besides this classical role, β-arrestins serve as versatile adaptor proteins mediating other functions, such as receptor endocytosis and initiating G protein-independent signaling.

These multifaceted responses are mostly linked to the ability of β-arrestins to act as scaffolding proteins. For example, scaffolding components of the clathrin-coated vesicles (CCV) (otherwise referred to as clathrin-coated pits (CCPs)) like AP-2 and clathrin, promotes GPCR–β-arrestin complex internalization to endosomes (DeWire, Ahn, Lefkowitz, & Shenoy, 2007; Laporte et al., 1999; Lefkowitz & Shenoy, 2005). Interestingly, while many receptors interact with β-arrestin at the PM, such interaction leads to diverging trafficking and recycling profiles between different receptors. This has been typically correlated with the strength of receptors' interaction with β-arrestin, thereby dividing GPCRs into class A and B receptors (Luttrell & Lefkowitz, 2002). Class A GPCRs, such as the β2-adrenergic receptor (β2AR), transiently interacts with β-arrestin and forms short-lived complexes that rapidly dissociate, allowing for rapid receptor recycling. On the other hand, class B GPCRs, including the angiotensin II type 1 receptor (AT1R), the bradykinin B2 receptor (B2R), and the vasopressin receptor 2 (V2R), bind β-arrestins with higher affinity and form stable, long-lived complexes that persist throughout endosomal internalization, leading to

delayed receptor recycling (Oakley, Laporte, Holt, Caron, & Barak, 2000). β-arrestins can also scaffold elements of the mitogen-activated protein kinase (MAPK) cascade, such as Raf, MEK, and ERK to promote G protein-independent signal transduction (Cao et al., 2020; Luttrell et al., 2001; Meng et al., 2009). Interestingly, the outcomes of β-arrestin-mediated signaling diverge between receptors. Indeed, β-arrestin biased ligands often lead to distinctive signaling outcomes with different receptors (Jean-Charles, Kaur, & Shenoy, 2017). The remarkable functional multiplicity of β-arrestins prompted much interest in the mechanisms of GPCR–β-arrestin interactions and how these translate into specific cellular responses. It is expected that binding of the different scaffolding partners is sensitive to β-arrestin's conformation; thus, suggesting that similar to receptors, β-arrestins can also adopt multiple conformations that specify the final functional outcome for their activation.

Resolving GPCR–β-arrestin complex conformation has been technically challenging due to the dynamic nature of β-arrestin interactions with activated receptors. To date, only few arrestin structures have been resolved; these include the rhodopsin with visual arrestin (Kang et al., 2015), and the muscarinic acetylcholine M2 (M2R) and the neurotensin 1 (NTS1R) receptors with β-arrestin-1 (Huang et al., 2020; Staus et al., 2020; Yin et al., 2019). These structures, along with live cells biophysical studies (Charest, Terrillon, & Bouvier, 2005), confirm that arrestins undergo conformational rearrangements when bound to GPCRs. Moreover, GPCR–β-arrestin complexes were found to generally conform two interaction modes: one involving the GRK-phosphorylated cytoplasmic tail of the activated receptor to form the "tail conformation", and another additionally involving receptors' intracellular core regions to form the "core conformation" (Kang et al., 2015; Shukla et al., 2014). Interestingly, a recent study proposes a relationship between these distinct β-arrestin interaction modes and its functional outcome, where internalization and signaling was

linked to the “tail conformation”, while G protein desensitization was linked to the “core conformation” (Cahill et al., 2017). Moreover, numerous studies on GPCRs including for AT1R and V2R, reveal that specific “phosphorylation codes” at the same GPCR regulate β-arrestin binding mode and downstream responses (Kaya, Perry, Gurevich, & Iverson, 2020; Mayer et al., 2019; Nobles et al., 2011; Ren et al., 2005; Tobin, Butcher, & Kong, 2008). Thus, dissecting the conformational arrangements that β-arrestin assumes in different conditions is necessary towards understanding its functional regulation. While structural studies provide valuable information on the GPCR–β-arrestin complex arrangements, it remains necessary to define the precise molecular determinants for β-arrestin interactions in varying contexts and within the native cellular environment.

We previously utilized the photocross-linking approach to map important contact sites for AT1R–β-arrestin complex within different regions of the receptor (Gagnon et al., 2019). Here, we used this approach to generate photoactivable β-arrestin-1 variants through site-specific incorporation of the unnatural amino acid (UAA), p-azido-l-phenylalanine (azF), at 12 distinct sites with β-arrestin’s structure. These azF-β-arrestin mutants allowed investigating the binding interface of β-arrestin with the three class B receptors, AT1R, B2R, and V2R. Despite all receptors strongly interacting and trafficking with β-arrestin, we show substantial differences in the modality of β-arrestin binding to these receptors.

## 4.3 Materials and Methods

### Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), gentamycin, and other cell culture reagents were acquired from Gibco, Life Technologies. Polyethylenimine (PEI) was purchased from Polyscience, Inc (Warrington, PA). Human AngII, BK, AVP, Poly-L-ornithine, 2,3-dimercapto-1-propanol, anti-HA Affinity Matrix, anti-FLAG M2 Affinity Gel, and horseradish peroxidase–conjugated rabbit secondary antibody, SIGMAFAST OPD and 16% paraformaldehyde (PFA) was purchased from Sigma-Aldrich (St. Louise, ML). Anti-HA-peroxidase rat antibody (3F10) and anti-HA affinity matrix were purchased from Roche (Manheim, Germany). Chemiluminescence reagents were purchased from Perkin-Elmer (Waltham, MA). BSA was purchased from Fisher BioReagents (Hampton, NH). Horseradish peroxidase–conjugated mouse secondary antibody was purchased from Bio-Rad. Anti-c-Myc antibody was purchased from cell signaling. β-arrestin-1 A1CT antibody was kindly provided by Dr. Robert J. Lefkowitz (Duke University). AzF was purchased from Chem Impex International (Wood Dale, IL). Chemiluminescence reagents were purchased from PerkinElmer Life Sciences. Coelenterazine was purchased from Nanolight Technology (Pinetop, AZ). Q5 high fidelity DNA polymerase, restriction enzymes, and Gibson Assembly Mix, and other PCR reagents were purchased from New England Biolabs (Ipswich, MA). Oligonucleotides were synthesized at Integrated DNA Technologies. All other reagents were obtained from Thermo Fisher Scientific (Waltham, MA) and were of analytical grade.

**DNA constructs and β-arr1 mutagenesis**

Suppressor tRNA, azF aaRS plasmids, and N-terminal FLAG-tagged, C-terminal RlucII-labeled human AT1R amber mutants (Coin et al., 2013; Gagnon et al., 2019), β-arrestin-1-RlucII (Cao et al., 2019), HA-B2R (Simaan, Bedard-Goulet, Fessart, Gratton, & Laporte, 2005), HA-V2R (Beautrait et al., 2017), rGFP-FYVE, rGFP-CAAX (Namkung et al., 2016), and β-arrestin1 and its N-terminally tagged YFP version (β-arrestin1-YFP) (Oakley et al., 2000), were previously described. HA-AT1R in pcDNA3.1 vector was generated by PCR with overlapping ends and Gibson assembly using the signal peptide–Flag–tagged human AT1R (sp-Flag-hAT1R) (Goupil et al., 2015) as the template for PCR amplification. Primers for AT1R gene amplification were designed to introduce the HA tag at the N terminus and generate 20 bp sequence homology with Not I–Hind III–cleaved pcDNA3.1: 5′-CCAAGCTGGCTAGCGTTTAAACTTAAGCTTACC-ATGTACCCATACGACGTCCCAGACTACGCCATCCTTAACTCTTCTACTGAAGATGGC-3′, and 5′-AAACGGGCCCTCTAGACTCGAGCGGCCGCCTCACTCAACCTCAAAACATG-GTGCAGG-3′. The PCR product was then subcloned in pcDNA3.1 vector using Not I and Hind III sites and the NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs). The N-terminal Myc-tag and C-terminal RlucII-tag WT human β-arrestin1 construct (3xMyc-β-arrestin1-RlucII) was created and used as the template for site-directed β-arrestin1 mutagenesis. It was constituted in the pCMV-3Tag-2B vector by PCR with overlapping ends and Gibson assembly using the β-arrestin1-RlucII (Cao et al., 2019) as the PCR template. PCR amplification of the β-arrestin1-RlucII was performed using the following primers: 5′-TGAGGAAGATCTGAGCCCG-GGCGGGATCCTCTCGATGGGCGACAAAGGG-ACC-3′, and 5′-CCCCTCGAGGTCGACG-GTATCGATAAGCTGTTACTGCTCGTTCTTCAG-CAC-3′. These primers introduced the 3xMyc-tag at the N-terminus of β-arrestin1-RlucII and generated sequence homology with Bam

HI–Hind III–cleaved pCMV-3Tag-2B vector. The PCR product was then subcloned in pCMV-3Tag-2B vector using Bam HI and Hind III sites and the NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs). Subsequently, site-directed β-arrestin1 mutagenesis was carried on by introducing the amber codons (TAG) in the desired location within 3xMyc-β-arrestin1-RlucII using the two-fragment PCR approach previously described (Gagnon et al., 2019; Heydenreich et al., 2017). HA-AT1R, 3xMyc-β-arrestin1-RlucII, and all generated β-arrestin1 amber mutants were validated by sequencing at Genome Québec.

**Transfections and cell culture**

HEK293T cells described previously in (Namkung et al., 2016) were cultured in DMEM supplemented with 10% fetal bovine serum and 20 μg/ml gentamicin. Cells were grown at 37 °C in 5% CO2 and 90% humidity. For expression and photocross-linking, transient transfections were performed using PEI (3:1 PEI:DNA ratio) on cells 24 h after seeding. Cells were transfected with suppressor tRNA, aaRS, and either the WT β-arrestin1 or the different β-arrestin1 amber-mutants, at a ratio of 10:1:1 or 10:1:5, respectively (supplemented with pcDNA3.1 to complete the final DNA amounts to 1.1 μg/well of 12-well plate) for azF-β-arrestin1 mutants' expression experiments. For photocross-linking experiments with azF-β-arrestin mutants, cells were transfected with the HA-tagged receptor, suppressor tRNA, aaRS, and amber-mutant β-arrestin1, at a ratio of 6:10:1:5 (supplemented with pcDNA3.1 to complete the final DNA amounts to 2.2 μg/well of 6-well plate). As for cross-linking experiments with the azF-AT1R mutants, transfections were carried out as described previously (Gagnon et al., 2019). For BRET and Elisa experiments, transient transfections were performed in suspension while seeding using PEI (3:1 PEI:DNA ratio). DNA transfection ratios for BRET experiments were as follows: HA-tagged

receptor, suppressor tRNA, aaRS, WT or amber mutant β-arrestin1, and either rGFP-CAAX or rGFP-FYVE at 6:10:1:1:3 for WT β-arrestin1, and 6:10:1:5:3 for amber mutant β-arrestin. DNA was supplemented with pcDNA3.1 to complete the final DNA amounts to 1 μg for 12 wells in 96-well plate). For Elisa experiments, each 16 wells/96-well plate were transiently transfected with HA-tagged receptor (600 ng) and pcDNA (1600 ng). For microscopy experiments, cells were transiently transfected 24 h after seeding with HA-B2R (400 ng), β-arrestin1-YFP (75 ng), and pcDNA3.1 (525 ng). In all experiments, medium was replaced 18 h after transfection with DMEM, supplemented or not with 0.5 mM azF. All assays were carried out 48–72 h post transfection.

**Intact cell ELISA**

Cell surface abundance of HA-AT1R, HA-B2R, and HA-V2R was assessed by cell surface ELISA. Cells were plated into polyornithine-coated transparent 96-well plates at a density of $2 \times 10^4$ cells per well and transfected as described earlier. At ~48 h post-transfection, cells were washed once with PBS and fixed with 4% paraformaldehyde for 10 min at room temperature. This was followed by two washes with PBS and incubation with 2% BSA in PBS for 1 h at room temperature for blocking. Next, cells were incubated with HRP-conjugated HA antibody (1:1000 in PBS/BSA) for 1 h at room temperature on nutating mixer. Cells were then washed four times with PBS and supplemented with colorimetric HRP substrate (SIGMAFAST OPD) for 10 min. The reaction was stopped by adding 3M HCl, and the absorbance was measured at 492 nm with a microplate reader (Synergy2, Biotek). Basal signal from mock pcDNA-transfected cells was subtracted and the obtained values were normalized to protein amounts measured by the Bradford assay after cell lysis with 0.01% SDS.

**Photocross-linking**

HEK293T cells were seeded onto poly-L-lysine-coated plates at a density of $4 \times 10^5$ cells per well in 6-well plates and transfected as detailed earlier. At ~72 h post-transfection, medium was replaced with 1 μM AngII, BK, or AVP, each prepared in Tyrode's buffer (pH 7.4, 140 mM NaCl, 2.7 mM KCl, 1 mM, $CaCl_2$, 12 mM $NaHCO_3$, 5.6 mM D-glucose, 0.5 mM $MgCl_2$, 0.37 mM $NaH_2PO_4$, and 25 mM Hepes, and 0.1% (w/v) BSA). Cells were incubated for 20–30 min either at room temperature (21 °C), 4 °C, or 37 °C. Cells were then placed on ice and subjected to UV irradiation for 20 min with a Blak-Ray B-100AP/R UV light (Analytik Jena). Cells were then washed once with ice-cold PBS and lysed for 1 h, at 4 °C on a nutating mixer with lysis buffer (pH 7.4, 50mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40 (v/v), 0.5% sodium deoxycholate (w/v), and 0.1% sodium dodecyl sulfate (w/v)) supplemented with protease inhibitors: leupeptin (20 μg/ml), aprotinin (10 μg/ml), pepstatin A (2 μg/ml), and phenylmethylsulfonyl fluoride (10 mM). Samples were then centrifuged at 14,000 rpm (18,407 × g) for 20 min at 4 °C and the solubilized proteins (supernatant fraction) were isolated from the cellular debris (pellet).

**Immunoprecipitation**

HA-tagged receptors (AT1R, B2R, or V2R), or Flag-tagged AT1R amber mutants were immunopurified from solubilized cell lysates by incubating them either with anti-HA Affinity gel, or anti-FLAG M2 Affinity Gel, respectively, for 3 h, at 4 °C on a nutating mixer. Samples were then centrifuged at 5,000 rpm (2,348 × g) for 1 min and beads were washed three times with ice-cold lysis buffer. Finally, beads were incubated with laemmli buffer for 1 h at 37 °C and samples were ready to be loaded on SDS-polyacrylamide gels.

**Western blotting**

Acquired total cell lysates, or immunopurified proteins used for receptor–β-arrestin1 complex detection, were resolved on one or multiple 8% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. An anti-Myc antibody (1:1000 dilution) or anti-β-arrestin1 A1CT (1:4000 dilution) was then used to incubate the membranes overnight at 4 °C on a nutating mixer. A secondary anti-rabbit antibody conjugated to horseradish peroxidase was used to detect the bands by chemiluminescence (1:10,000). Chemiluminescence signals were detected using ChemiDoc Touch Imaging System (Bio-Rad) and densitometry analysis of immunoblots was performed using the Image LabTM 6.0 software (Bio-Rad). To determine the relative expression of β-arrestin1 variants, measurements of the optical density of chemiluminescence signals were normalized to the WT-β-arrestin1 sample within the same experiment. Whereas the crosslinking of receptor–β-arrestin1 complexes was quantified by normalizing either to L68azF-β-arrestin1, to F75azF-β-arrestin1, or A221azF-AT1R bands within the same experiment.

**BRET experiments**

HEK293T cells were seeded at a density of $2 \times 10^4$ cells per well in white 96-well polyornithine coated plates, and transiently transfected using PEI as detailed above. On the day of the experiment, cells were washed and incubated with 90 μL of Tyrode's buffer for 30 min in 5% CO2 either at room temperature (21 °C), at 37 °C, or at 10 °C. Cells were then stimulated with 1 μM of the appropriate ligand for another 20–30 min. Cell-permeable substrate coelenterazine 400a at a concentration of 5 mM was added 3–5 min prior to BRET measurements, which were performed in triplicates using the Victor X (PerkinElmer) plate reader with a filter set of 460/25nm (donor) and 535/25 nm (acceptor). BRET signals were calculated by dividing the intensity of light

emitted by the acceptor over the intensity of light emitted by the donor. All BRET experiments were done in triplicates.

**Fluorescence microscopy**

HEK293T cells were seeded at a density of $1.5 \times 10^5$ cells per 35-mm glass bottom dish one day prior to transfection. At ~48 h post-transfection, cells were serum-starved in DMEM for 1 h, either at 37 °C, or at 10 °C. Images were acquired before and after 30 min stimulation with 1 μM BK using a Zeiss LSM-510 Meta laser-scanning microscope through a 633/1.4 oil objective lens. A laser with 514-nm excitation and 530–600 nm (band pass) emission was used to detect YFP signals. Image processing was performed with the Zen software from Zeiss.

**Data analysis and statistics**

All data analysis was performed by Image LabTM 6.0 software and Prism 6.0 (GraphPad Software Inc., La Jolla, CA). Statistical analysis was done using unpaired Student t-tests, one- or two-way analysis of variance (ANOVA), Dunnett's post hoc multiple comparisons test, Tukey's post hoc multiple comparisons test, or Bonferroni's post hoc multiple comparisons test when appropriate. P values $\leq 0.05$ were considered significant.

## 4.4 Results

### *Expression and functionality of azF-incorporated β-arrestin mutants*

To identify specific β-arrestin residues important for its interaction with GPCRs, we selected 12 positions within the structure of β-arrestin1 to introduce the photoreactive UAA, azF, and generate 12 photoactivable β-arrestin mutants. The residues selected for azF incorporation where chosen considering the crystal structure of the active β-arrestin1, which implies substantial structural rearrangements within the N domain and central loops (Shukla et al., 2013). Accordingly, most of the selected residues were allocated in the N domain, 4 of which were included within the central loops (finger loop residues: D67, L68, and F75, and lariat loop residue: G291), while only 2 residues were selected in β-arrestin's C domain (A254, and S330) (Fig. 1A). Moreover, to minimize the effect of mutagenesis on β-arrestin expression and activity, we preferentially selected nonpolar and uncharged amino acid residues. Mutagenesis was done on β-arrestin1 containing an N-terminal Myc tag and a C-terminal RlucII epitope (WT-β-arrestin), which was confirmed for its ability to effectively translocate to the PM and internalize to endosomes upon AT1R activation (Cao, Namkung, & Laporte, 2019) (Fig. S1). To achieve azF incorporation, an amber stop codon (TAG) was introduced at each of the 12 specific sites within the WT-β-arrestin. Subsequently, amber mutant β-arrestins were expressed in cells along with the engineered suppressor $tRNA_{CUA}$ and specific aminoacyl-tRNA synthetase (aaRS), to allow for the substitution of the amber residue with the supplemented azF, as previously described (Grunbeck et al., 2012; Valentin-Hansen et al., 2014). We confirmed successful azF incorporation for all amber β-arrestin mutants, which allowed for their expression in cells (Fig.1, B and C). Most of the azF-β-arrestin mutants expressed to comparable levels to that of the WT-β-arrestin, except for

T58azF, D67azF, F80azF, and S330azF mutants, which showed significant reduced expression (Fig.1C).

Using the bioluminescence resonance energy transfer (BRET) assays previously described (Namkung et al., 2016; Namkung et al., 2018), we verified the functionality of the azF-β-arrestin-RlucII mutants in terms of their ability to translocate to the PM and endosomes upon ligand stimulation of AT1R, B2R, and V2R. To maximize the detection of β-arrestin at the PM or in endosomes, agonist-stimulation of cells expressing the azF-β-arrestin mutants with one of the aforementioned receptors was performed either at room temperature for 2 min, or at 37 °C for 30 min, respectively. We show that all 12 azF-β-arrestin mutants were able to translocate to the PM and traffic to endosomes upon receptor activation, and the majority retained more than 50% of WT-β-arrestin activity (Fig. S2). The reduced functionality of some of the azF-β-arrestin mutants did not always correlate with their level of expression. For example, the S330azF mutation significantly influenced β-arrestin's expression, but not it's PM and endosomal translocation with either of the receptors (Fig.1C, and S2). Moreover, while the P14azF mutant expressed to similar levels as the WT-β-arrestin, its PM translocation was reduced, especially with AT1R and B2R (Fig.1C, and S2A). The effects of azF mutations on β-arrestin's ability to translocate to the PM vs. endosomes was generally consistent, although some exceptions were observed. F80azF and K160azF mutations inhibited B2R-mediated β-arrestin trafficking more than its PM translocation (Fig. S2). On the other hand, some mutations such as P14azF, had a stronger negative effect on AT1R-mediated β-arrestin translocation to the PM compared to endosomes (Fig. S2). Overall, we confirm that azF incorporation to β-arrestin is mostly well tolerated, and all azF-β-arrestin mutants are capable of expressing, binding, and internalizing with activated receptors.

***Photocross-linking of GPCR–β-arrestin complexes reveals receptor-specific β-arrestin binding modalities***

We then explored β-arrestin binding to AT1R, B2R, and V2R, by performing photocross-linking experiments in HEK293 cells expressing the different HA-tagged receptors and azF-incorporated β-arrestin mutants. Cells were stimulated with angiotensin II (AngII), bradykinin (BK), or vasopressin (AVP) at room temperature (RT) for 20 min, to maximize the formation of GPCR–β-arrestin complexes and allow their detection. Photolysis was then performed, and azF-mediated ligations between β-arrestin1 and the three GPCRs was determined as detailed in the methods section (Fig. 2A). Interestingly, we identified five residues within β-arrestin common for its interaction with all three receptors: P14, T58, L68, F75, and K160 (Fig. 2). Moreover, despite all receptors being expressed to similar levels (Fig. S3), we observed receptor-specific ligations, such as D67, L100, and A254 with B2R, and G291 and S330 with V2R (Fig. 2). Interestingly, the relative levels of photocross-linked complexes varied with the three receptors; the most prominent complexes were obtained with P14azF for AT1R, L68azF for B2R, and L68azF and F75azF for V2R (Fig. 2). The observed receptor-specific cross-linking patterns reflect distinctive β-arrestin binding modalities. We show that the levels of azF-mediated cross-linking did not always correlate with the expression of mutant β-arrestins nor their functionality in terms of PM recruitment upon receptor activation (Fig. S4). For instance, L100azF mutant showed similar levels of WT-β-arrestin expression (Fig. 1C) and functionality (Fig. S4), yet, yielded minimal cross-linking with the three GPCRs (Fig. 2). Additionally, P14azF and D67azF β-arrestin mutants formed strong cross-linked complexes with B2R, despite having reduced levels of BK-mediated PM and endosomal translocation compared to the WT-β-arrestin (Figs. 2 and S4). Overall, these findings identify discrete β-arrestin interactions with AT1R, B2R, and V2R.

***β-arrestin binding modalities in function of the cellular localization***

β-arrestin is known to bind specific effectors in the different cellular compartments. For instance, AP-2 and clathrin bind β-arrestin specifically at the PM (Gurevich & Gurevich, 2014; Tian, Kang, & Benovic, 2014). Binding to the different scaffolding proteins may reflect on β-arrestin conformation either by coordinating its phosphorylation (Perry et al., 2019; Tobin et al., 2008; Vishnivetskiy et al., 2007), or through allosteric interactions. Thus, we reasoned that the binding modality of β-arrestin may be altered between the two stages of internalization: initial complex interaction at the PM and the subsequent targeting of complexes into CCVs in the endosome. To restrain GPCR–β-arrestin complexes in the different compartments, we relied on the temperature-sensitive nature of the receptor internalization process, which requires the activation of the dynamin GTPase enzyme to internalize receptors from the PM. Ligand stimulation for 30 min at 10 °C would thus be expected to restrict complexes at the PM, whereas stimulation for 30 min at 37 °C would allow efficient targeting of complexes to endosomes. Using BRET biosensors, we confirmed the inhibition of endocytosis and the accumulation of GPCR–β-arrestin complexes at the PM upon AT1R, B2R, and V2R stimulation at 10 °C (Fig. S5). Moreover, we observed significant endosomal prevalence and reduced PM localization for the formed complexes when stimulations were done at 37 °C, compared to 10 °C (Fig. S5). This altered complex localization between the two stimulation conditions (i.e., PM vs. endosomes at 10 °C and 37 °C, respectively), was also observed when using confocal microscopy performed on cells transfected with B2R and YFP-tagged β-arrestin (Fig. 3A).

To identify alterations in β-arrestin conformations between the two compartments, we compared the photocross-linking patterns obtained when receptors where stimulated at 10 °C vs. at 37 °C. Interestingly, we observed marked differences between the photocross-linking patterns

attained with the two stimulating conditions for AT1R and B2R, but not V2R (Fig. 3 B–D). The ligation level of P14azF and T58azF were significantly reduced when AngII stimulation of AT1R was performed at 37 °C, compared to at 10 °C (Fig. 3B). These two mutants also showed an altered B2R photocross-linking pattern (Fig. 3C). BK stimulation at 37 °C resulted in an increase in P14azF and a decrease in T58 ligation, compared to BK stimulation at 10 °C (Fig. 3C). These distinctive cross-linking patterns did not always correlate with the differential β-arrestin mutants' ability to be recruited at the PM vs. in endosomes (Fig. S2). P14azF and T58azF β-arrestin mutants were equally recruited at the PM and in endosomes following B2R activation (Fig. S2), yet, showed variations in the ligation with the receptor in each compartment (Fig. 3C). Together, these data reveal that β-arrestin interaction with AT1R and B2R, but not V2R, varies in function of the receptor–β-arrestin complex localization between the cellular compartments.

***Specific β-arrestin conformations are reflected on AT1R intracellular interface***

The observed variations in β-arrestin conformation assumed when complexed with AT1R in the different cellular compartments is expected to associate with parallel alteration in AT1R interactions. To detect this, we performed photocross-linking experiments on six azF-labeled AT1R mutants, where azF was incorporated within AT1R's intracellular loop 2 and 3 (ICL2 and ICL3) (Fig. 4A), and which were previously identified to form strong cross-linked complexes with β-arrestin (Gagnon et al., 2019). Cells expressing WT-β-arrestin with each of the six Flag-tagged azF-AT1R mutants were stimulated with AngII, either at 10 °C or 37 °C, to restrain the formed AT1R–β-arrestin complexes at the PM or in the endosomes, respectively. Photolysis was then performed, and photocross-linked complexes were detected as earlier described (Gagnon et al., 2019). As expected, our data show a clear divergence in the relative band intensities corresponding

with different binding modalities of AT1R–β-arrestin complexes in the two stimulating temperatures (Fig. 4B). Two AT1R azF mutants, M134azF and I228azF, showed significant reduction in the formed AT1R–β-arrestin complexes at the 37 °C stimulation condition, compared to the 10 °C condition (Fig. 4B). These findings confirm that AT1R–β-arrestin complexes adopt different binding conformations between the PM and endosomes, which involves differential contacts within both complex interacting partners.

## 4.5 Discussion

The knowledge of differential β-arrestin interactions with receptors, especially in the native cellular environment, is key to our understanding of the multiple functions associated with β-arrestin activation. In this study, we site-specifically incorporated azF at 12 distinct sites with β-arrestin sequence to perform azF-mediated photocross-linking with AT1R, B2R, and V2R. This allowed us to map critical β-arrestin1 residues involved in its binding with each of the three receptors. Despite belonging to the same class B family of GPCRs, we show that these receptors induce distinct β-arrestin activation modes. Moreover, the binding modality of β-arrestin to the same receptor may vary in function of the trafficking stage.

Distinct sets of residues were identified as important contacts for β-arrestin interaction with AT1R, B2R, and V2R, giving rise to receptor-specific β-arrestin binding signatures. We identified two residues within the finger loop region, L68 and F75, as important in β-arrestin interaction with all three receptors. The fact that the finger loop plays a pivotal role in β-arrestin's interaction with the core of GPCRs (Shukla et al., 2014; Sommer, Farrens, McDowell, Weber, & Smith, 2007; Vishnivetskiy, Hosey, Benovic, & Gurevich, 2004), together with the previously identified involvement of AT1R's ICL2 and ICL3 in β-arrestin binding (Gagnon et al., 2019), suggests that

all three receptors form “fully engaged” complexes with β-arrestin. Nonetheless, our findings of distinct photocross-linking patterns amongst receptors suggest that altered β-arrestin binding modalities occur within the “fully engaged” complex model. Other common β-arrestin interacting residues detected include P14, T58, and K160, all of which are located in the N-terminal region of β-arrestin. These three residues were also previously shown to interact with the ICL3 of NTSR1 by cysteine cross-linking (Yin et al., 2019). We also identified receptor-specific binding interactions, including that of B2R with β-arrestin’s D67 residue, located in the finger loop region. Another example is G291 interaction with V2R, which is consistent with V2R–β-arrestin molecular modeling predicating interactions between β-arrestin’s gate loop (i.e., residues 289 – 298) and V2R’s ICL3 (Bellucci, Felline, & Fanelli, 2020).

A recent study using p-benzoyl-L-phenylalanine (Bpa) incorporation and photocross-linking to investigate β-arrestin’s finger loop interaction with GPCRs was conducted (Bottke et al., 2020). In this study, four of the β-arrestin residues explored here: D67, L68, F75, and F80, were mutated to Bpa and cross-linked with V2R. By comparing our findings, we noted that both Bpa- and azF-incorporation in place of F80 did not result in the detection of V2R–β-arrestin cross-linked complexes. Moreover, substituting L68 and F75, either with azF or Bpa, lead to efficient ligation with V2R. However, unlike with D67azF, V2R efficient cross-linking was achieved with D67Bpa (Bottke et al., 2020). Such discrepancy could be linked to the differences of the incorporated UAA size, which would affect the proximity of interacting residues. Thus, the lack of D67azF cross-linking with V2R is possibly owing to the smaller size of the azF, relative to the bulkier Bpa (Poulsen, Poshtiban, Klippenstein, Ghisi, & Plested, 2019).

Relying on the temperature-sensitive nature of the trafficking process, we investigated the activation modes of β-arrestin with receptors at the PM and in endosomes. Interestingly, our data

show β-arrestin's contacts with AT1R, and B2R, but not with V2R, were altered between the different temperature stimulating conditions (10 °C vs. 37 °C). It is unlikely that altering the stimulating-temperature in itself has an effect on β-arrestin binding modality, given the lack of effect on V2R cross-linking. Thus, β-arrestin active conformations are contingent on β-arrestin localization and the specific receptor partner. Interestingly, we noted that β-arrestin conformational signatures obtained after ligand stimulation of AT1R and B2R for 20 min at RT were similar to those obtained when receptors were stimulated for 30 min at 10 °C, but not at 37 °C. Moreover, the active AT1R signature reported using azF-AT1R mutants after AngII treatment at RT for 20 min (Gagnon et al., 2019) was also comparable to that obtained when stimulation was performed at 10 °C, rather than at 37 °C. Given that receptor activation at 10 °C completely inhibits internalization, RT stimulation is thus expected to result in a similar impediment of the endocytosis process, thus confining the majority of receptor complexes at the PM. On the other hand, 37 °C stimulation for 30 min promotes efficient endocytosis and significantly reduces initial GPCR–β-arrestin complex PM localization (Namkung et al., 2016). Nonetheless, we show that a proportion of PM complexes are still detected, possibly owing to GPCR recycling. Since class B GPCRs recycle at a slow rate, the smaller proportion of PM complexes, relative to that of endosomal complexes, is unlikely to interfere with obtaining accurate signatures of endosomal β-arrestin active conformations.

Our results showing unaltered interactions between β-arrestin and V2R at the PM and in endosomes suggests that β-arrestin binding mode with V2R remains consistent throughout endocytosis. However, we cannot exclude the possibility that other β-arrestin residues, not tested here, could be differentially involved in V2R interactions in the different compartments. Additionally, while it was previously shown that β-arrestin1 and β-arrestin2 finger loop

interactions with V2R were relatively similar (Bottke et al., 2020), it remains to be determined whether β-arrestin2 interactions with V2R and other receptors diverge between different cellular compartments. Variations in β-arrestin interactions between the PM and endosomes, such as those reported in this study with AT1R and B2R, potentially reflect on β-arrestin's signaling and functions. Especially since β-arrestin-mediated ERK1/2 activation varies depending on the subcellular compartmentalization. Indeed, it was shown that efficient β-arrestin-mediated ERK1/2 signaling correlates with the formation of stable endosomal AT1R–β-arrestin complexes (Cao et al., 2020; Zimmerman et al., 2012). An interesting hypothesis is that scaffolding partners binding β-arrestin in different compartments may have a role in regulating β-arrestin's conformation and signaling. It has been previously reported that selective inhibition of β-arrestin and AP-2 interactions not only blocks V2R–β-arrestin internalization, but also ERK1/2 signaling (Beautrait et al., 2017). Exploitation of the photocross-linking approach to investigate whether AP-2 and/or other β-arrestin-interacting partners alter β-arrestin's active conformation would provide valuable insights to how these partners may influence β-arrestin signaling.

Overall, our findings with azF-labeled β-arrestins and photocross-linking allowed us to identify varying conformational signatures of β-arrestin interactions with different receptors and in different cellular compartments. This highlights the differences between GPCRs in relaying information to their interacting β-arrestin binding partner, potentially resulting in the varying functional outputs for β-arrestin recruitment.

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## 4.8 Figures and Legends

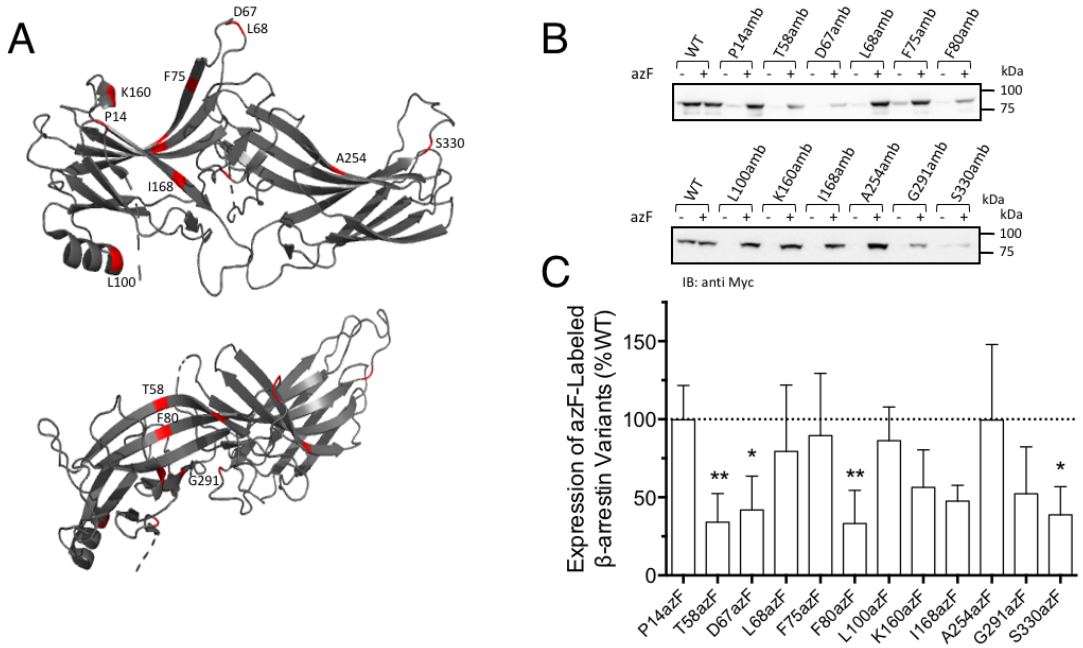


**Figure 1. Expression of azF-incorporated β-arrestin mutants.**

A. The 12 sites selected for azF incorporation are shown on the crystal structure of the activated β-arrestin1 (PDB entry 4JQI) (Shukla et al., 2013). Upper panel highlights 9 of the selected residues on the frontal view of β-arrestin, while lower panel shows the other 3 selected residues on the upper view of β-arrestin. B. HEK293 cells transiently expressing the different β-arrestin1 amber mutants in the absence (-) or presence (+) of 0.5 mM azF were assessed by western blotting and antibody detection of the N-terminal Myc tag. Quantification from blots is represented as means ± S.D. (error bars) of the optical density of the band from three to five independent experiments and expressed as percentage of WT-β-arrestin (dashed line; lower panel). One-way ANOVA followed by Dunnett's multiple comparison tests was performed: * = $p<0.05$, and ** = = $p<0.01$

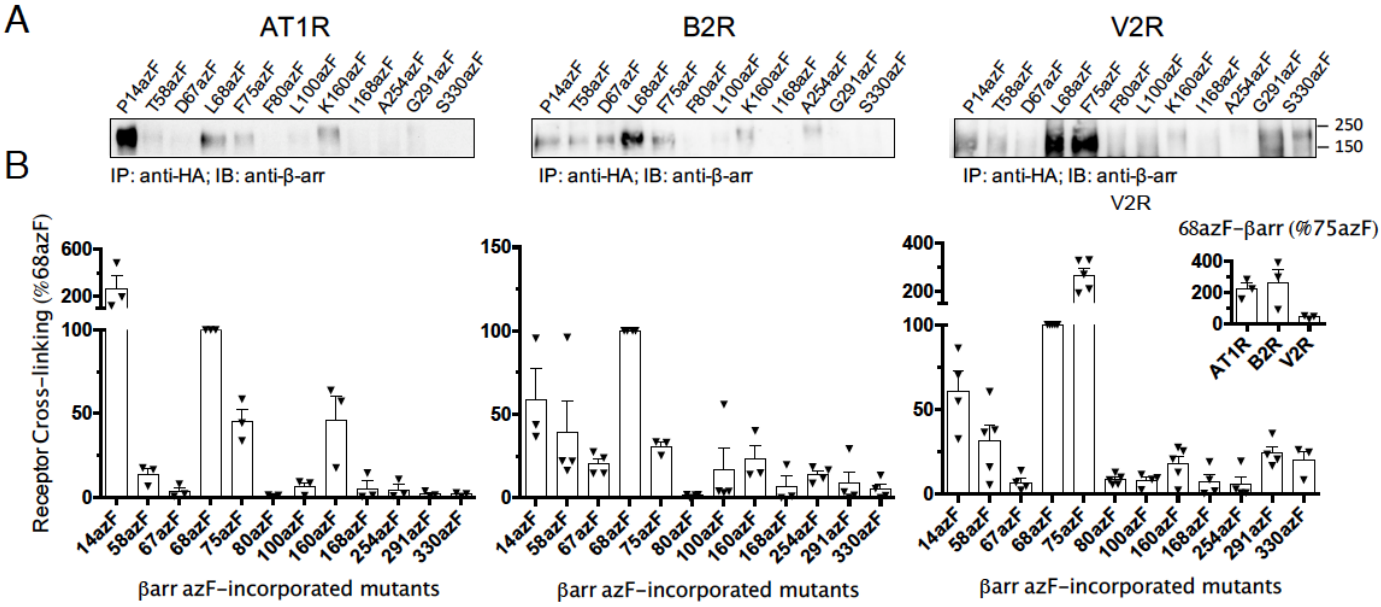


**Figure 2. GPCR binding to azF-incorporated β-arrestin mutants.**

A, HEK293 HEK293 cells transiently expressing each of the β-arrestin amber mutants with one of the HA-tagged receptors: AT1R *(left panel)*, B2R *(middle panel)*, or V2R *(right panel)*, were supplemented with 0.5mM azF and were stimulated with 1 μM of the appropriate ligand for 20 min before UV exposure as described under "materials and methods". Total cell lysates were then immunoprecipitated using an anti-HA antibody to isolate receptors, and immunoprecipitated proteins were resolved by SDS-Page. An anti β-arrestin antibody was then used to detect cross-linked complexes. Shown are representative blots from three independent experiments. *B,* Quantification of optical densities of GPCR–β-arrestin complexes' bands from *A* are represented as means ± S.E.M. (error bars), normalized to the L68azF variant within the same experiment.

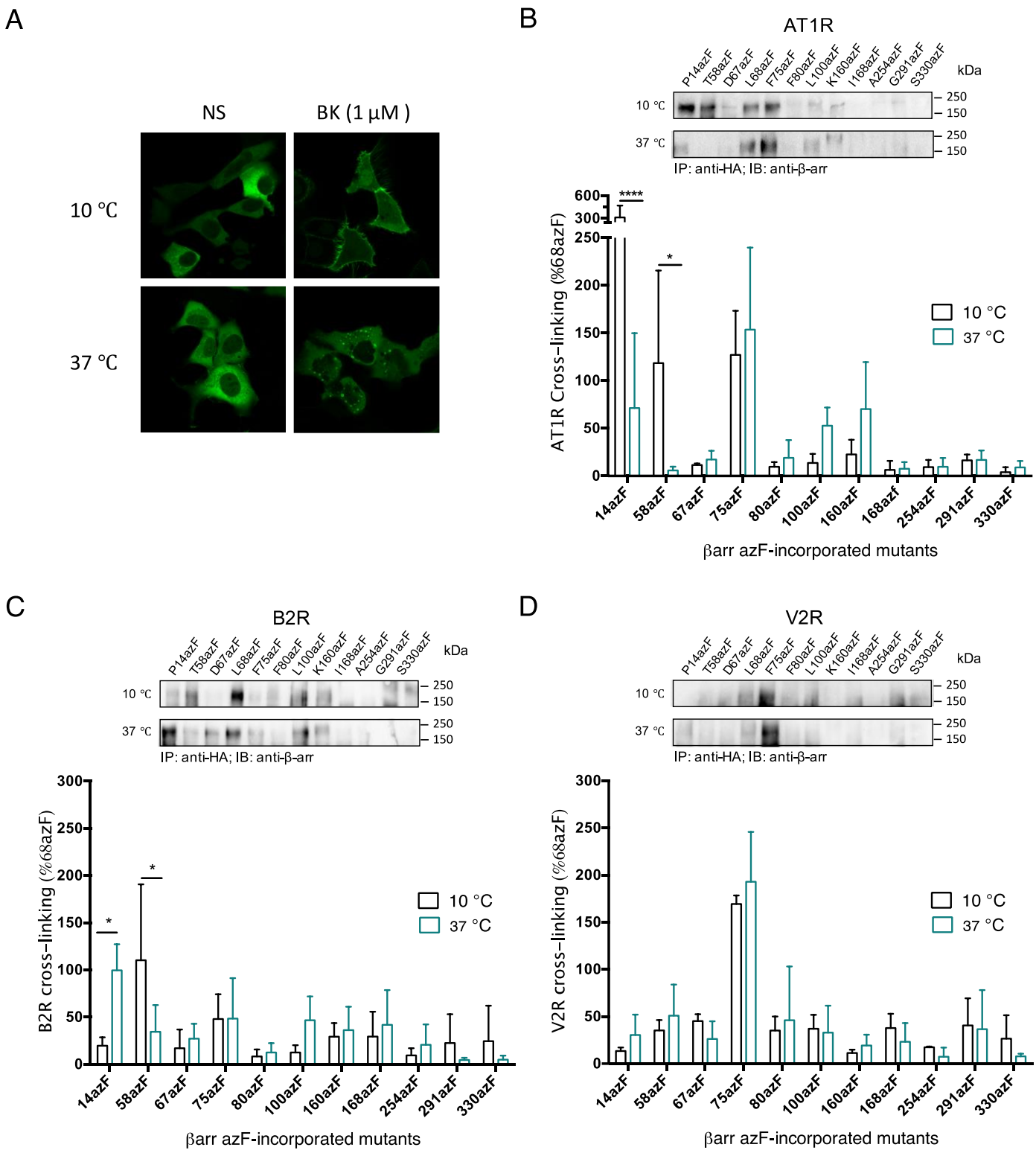


**Figure 3. Modulation of β-arrestin binding modalities at the PM vs. in endosomes.**

A, Internalization of β-arrestin following B2R stimulation is shown by fluorescence microscopy. HEK293 cells transfected with YFP-tagged β-arrestin and B2R were imaged 48 h post transfection. Images were acquired before and after stimulation with BK for 30 min either at 10 °C or 37 °C. Shown images are representatives of three independent experiments. Scale bar, 20 μM. B–D, Photocross-linking of GPCR–azF-β-arrestin in different temperature stimulating conditions. HEK293 cells transiently expressing each of the 12 azF β-arrestin variants with HA-tagged AT1R (B), B2R (C), or V2R (D), in the presence of 0.5 mM azF, were stimulated with 1 μM of the appropriate ligand at 10 °C or at 37 °C for 30 min. Photocross-linking was performed as described in the "materials and methods" section. Total cell lysates were then immunoprecipitated using an anti-HA antibody to isolate receptors and immunoprecipitated proteins were resolved by SDS-Page. An anti β-arrestin1 antibody was used to detect cross-linked complexes. Shown are representative blots from three or four independent experiments (upper panels). Quantifications of blots represent the means ± S.D. (error bars) of optical densities of the bands after normalization to the L68azF variant within the same experiment (lower panels). Two-way Anova followed by Bonferroni's post hoc multiple comparisons tests were performed: *** = $P < 0.001$, and **** = $P < 0.0001$.

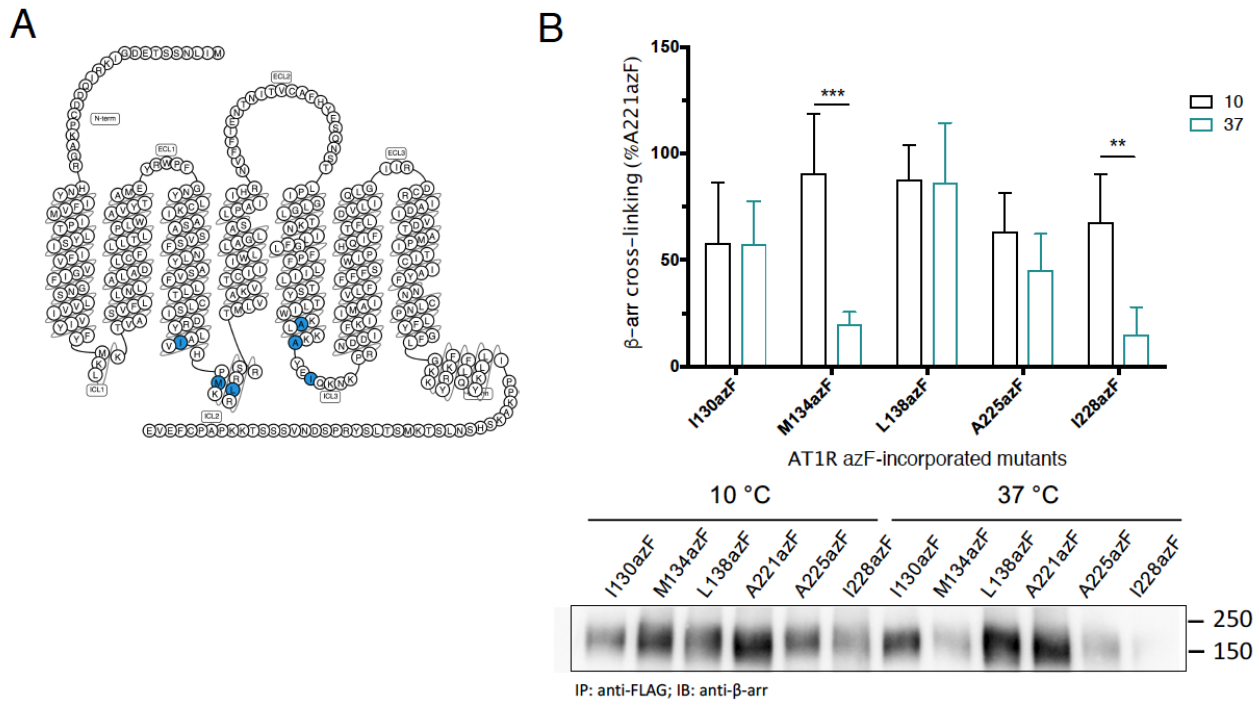


**Figure 4. Altered AT1R–β-arrestin intermolecular contact points within AT1R at the PM vs. endosomes.**

A, Schematic structure of AT1R showing the intracellular localization of 6 amino acids identified for their involvement in AT1R–β-arrestin complex formation (Gagnon et al., 2019). B, AngII-mediated photocross-linking of azF-AT1R mutants with β-arrestin. HEK293 cells transiently expressing β-arrestin and each of the 6 AT1R amber mutants were supplemented with 0.5 mM azF and stimulated with 1 µM of AngII for 30 min either at 10 °C or 37 °C prior to UV exposure as described under "materials and methods". Total cell lysates were then immunoprecipitated using an anti-Flag antibody to isolate receptors, and immunoprecipitated proteins were resolved by SDS-Page. AT1R–β-arrestin complexes were detected with an anti β-arrestin1 antibody. Shown are representative blots from four independent experiments (upper panel). Quantifications represent the means ± S.D. (error bars) of optical densities of the bands after normalization to A221azF

within each condition (lower panel). Two-way Anova followed Bonferroni's multiple comparisons tests were performed: ** = $P < 0.01$, and *** = $P < 0.001$.

## 4.9 Supplemental Figures and Legends

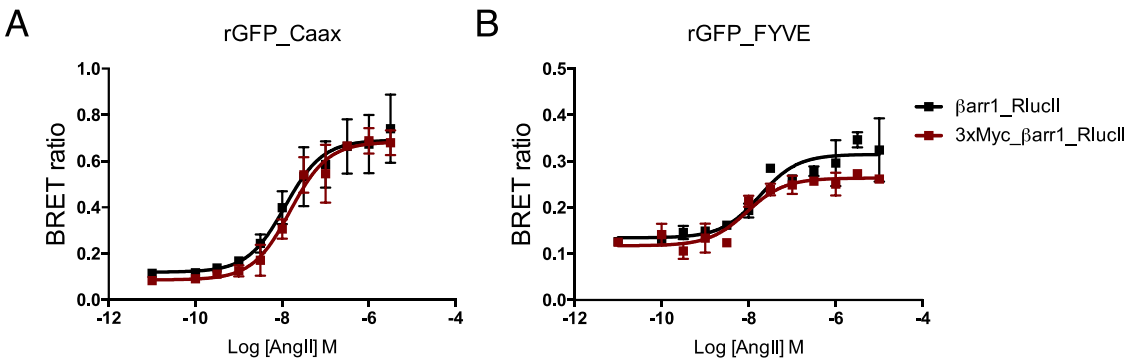


**Figure S1. Myc-β-arrestin-RlucII activation.**

HEK293 cells transiently expressing AT1R and RlucII-tagged β-arrestin or 3xMyc-β-arrestin-RlucII along with rGFP-CAAX (A) or rGFP-FYVE (B) were stimulated with the indicated concentrations of AngII before BRET measurements. Data represents means ± S.E. (error bars) of three independent experiments.

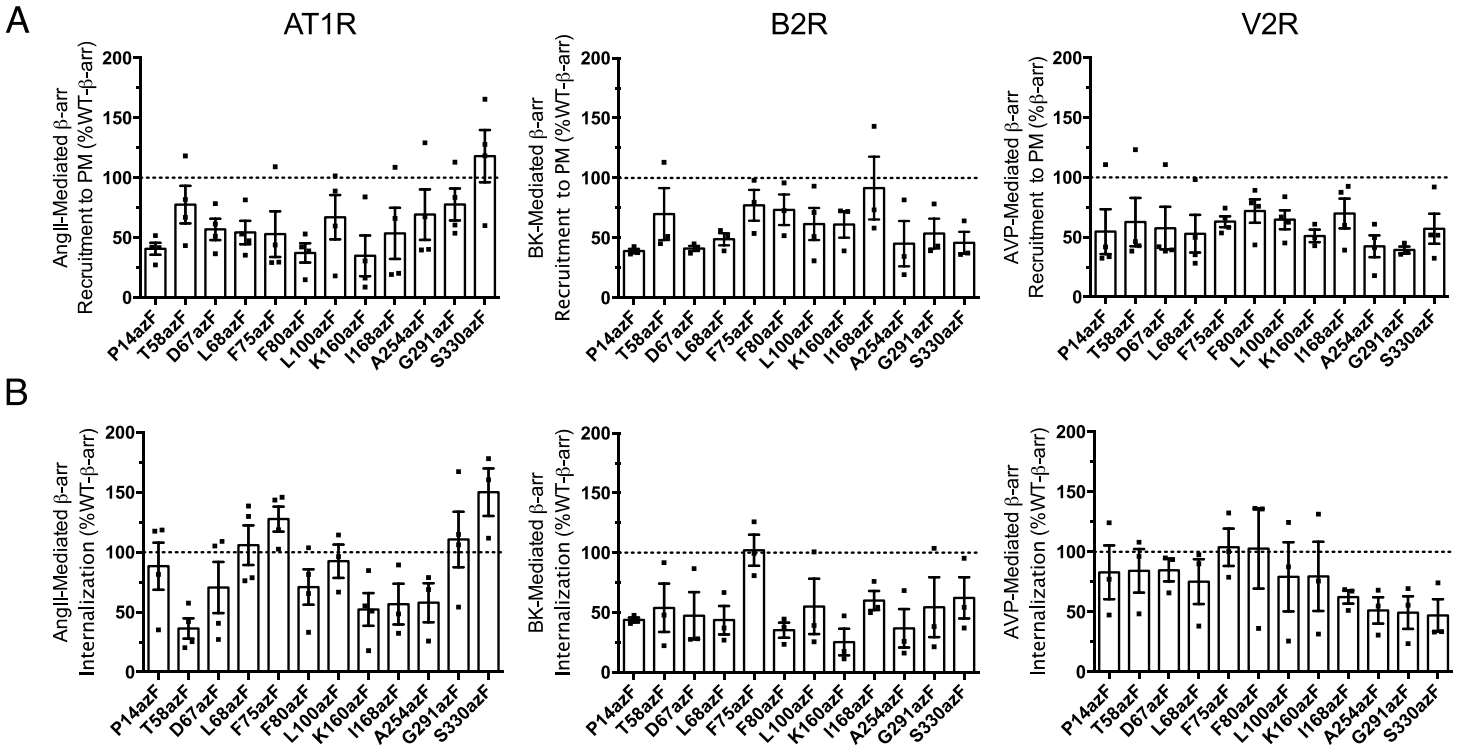


**Figure S2. Efficacies of azF-β-arrestin mutants' recruitment to the PM and endosomes by three activated GPCRs.**

A and B, HEK293 cells transiently expressing rGFP-CAAX (A) or rGFP-FYVE (B) with the 3xMyc-RlucII-tagged β-arrestins (WT or amber mutants), and either AT1R (left panel), B2R (middle panel), or V2R (right panel), in the presence of 0.5 mM azF were stimulated with 1 μM of the appropriate ligand before BRET measurements. BRET signals are normalized to the response of WT (%WT-βarr) and averaged. Data represents means ± S.E. (error bars) of at least three independent experiments.

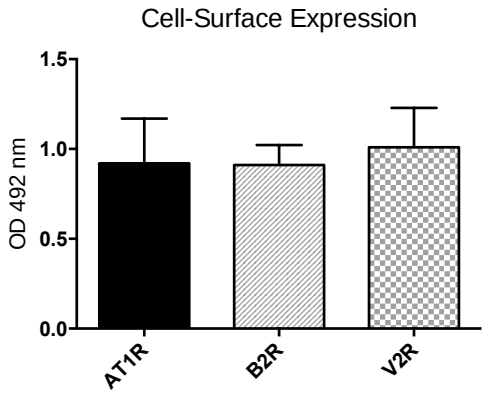


**Figure S3. Cell surface expression of GPCRs.**

Surface expression of receptors was assessed by whole cell Elisa assay. HEK293 cells were transfected either with an empty vector (pcDNA 3.1), or with HA-tagged AT1R, B2R, or V2R. Surface expression level of receptors was measured using HRP-coupled anti-HA antibody following the whole-cell ELISA protocol. The ratio of absorbance of 492 nm was calculated, and the baseline empty vector absorbance level was subtracted.

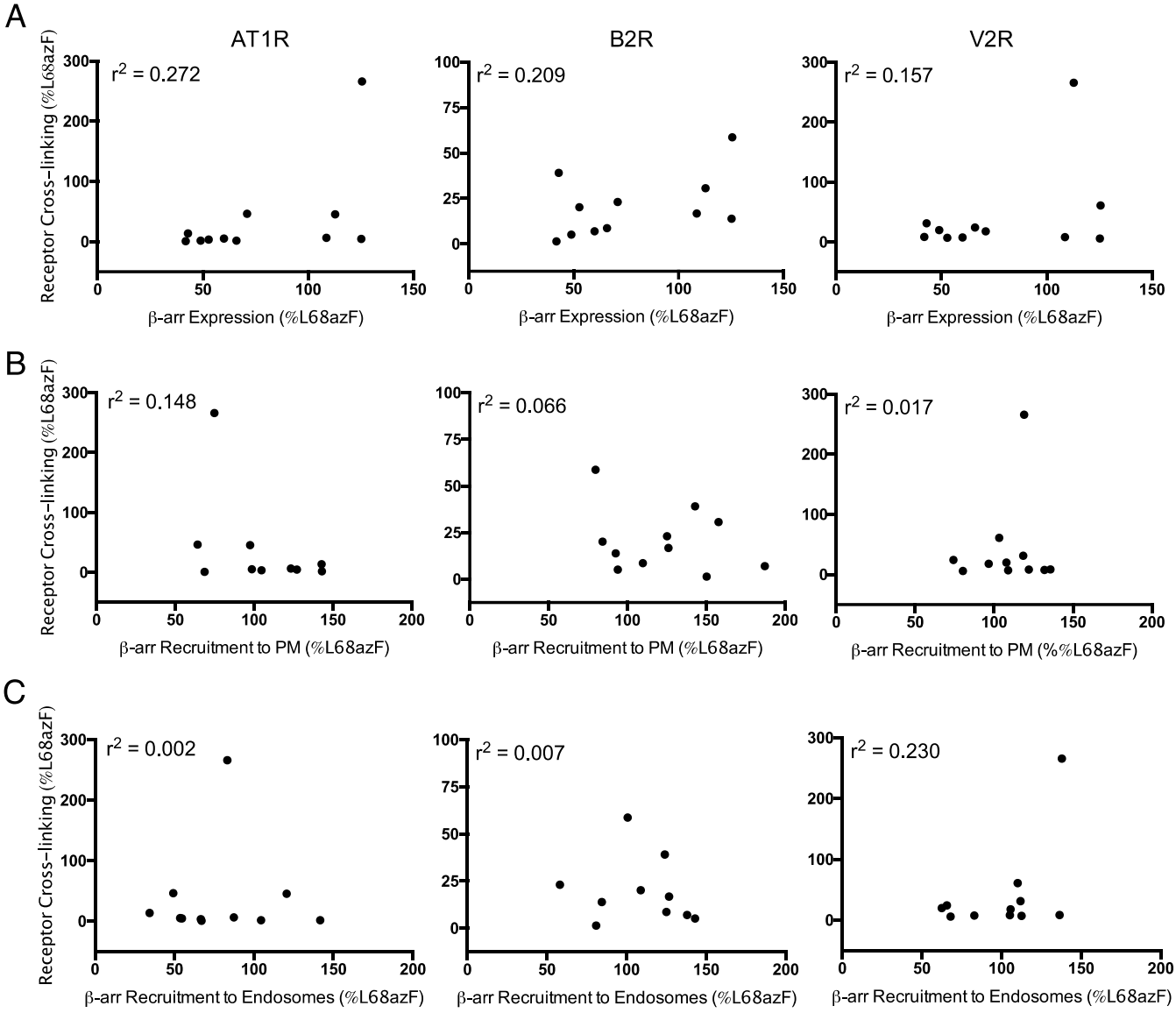


**Figure S4. Correlation analysis between cross-linked GPCR–βarrestin complexes vs. the expression levels of azF β-arrestin variants, and their ability to be recruited to the PM and endosomes upon agonist stimulation**

A – C, β-arrestin cross-linking data from figure 2 were plotted as scatter plot graphs with the expression data from figure 1C (A), or the PM translocation data from figure S2A (B), or the endosomal translocation data from figure S2B (C). Linear regression analysis was used to establish the correlation ($r^2$).

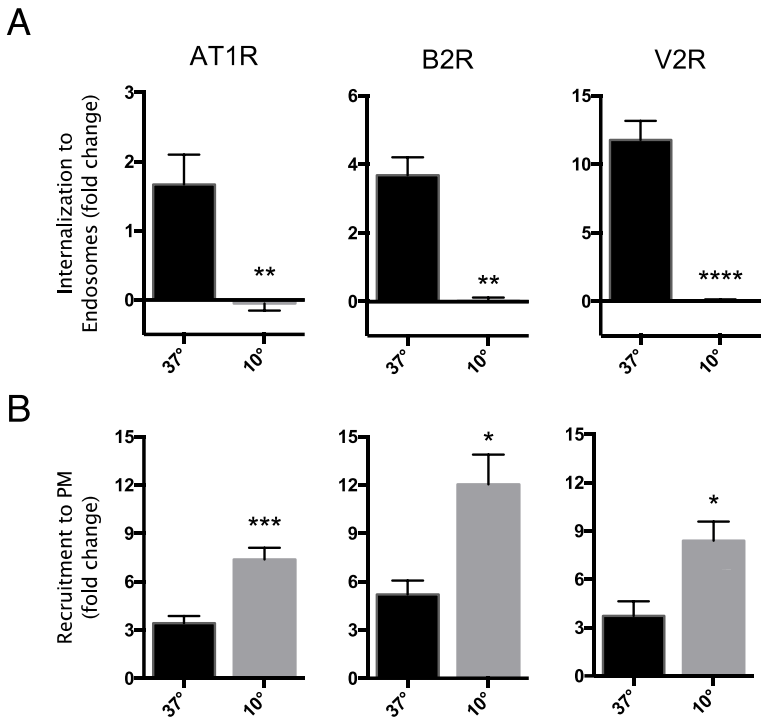


**Figure S5. β-arrestin translocation to the PM and endosomes**

A and B, HEK293 cells were transfected with RlucII-tagged β-arrestin and endosomal-anchored rGFP-FYVE (A) or PM-anchored rGFP-CAAX (B), along with AT1R (left panel), B2R (middle panel), or V2R (right panel). Cells were stimulated with 1 uM of the appropriate ligand for 30 min either at 10 °C or at 37 °C. The fold change in basal BRET before and after ligand stimulation is reported, and data is represented as means ± S.E (error bars) of triplicated from at least three independent experiments. Unpaired student t-test was performed: * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, and **** = $P < 0.0001$.

# CHAPTER 5: General Discussion

## 5.1 Contributions to Original Research

The goal of this thesis was to gain insight of the mechanisms employed by GPCRs to produce countless signaling responses through their interaction with a fairly limited number of effectors. Two main aspects were considered: 1) G protein coupling selectivity by promiscuous GPCRs, and 2) β-arrestin differential binding modalities when in complex with receptors. Both aspects provide mechanistic regulation for the eventual signaling outcomes of activated GPCRs.

Chapter 3 – Selectivity Regulation of Gq/11 and G12/13 at Promiscuous GPCRs.

In this chapter, BRET biosensors that measure G protein and downstream effectors' activation were used to vet the effect of individual cognate G protein availabilities on FP and AT1R signaling selectivity. Results from this study reveal that G proteins may compete with one another for GPCR binding in a unique receptor-specific direction. This competitive G protein binding renders receptors sensitive to the relative expression levels of G proteins in specific physiological contexts. The direction of this competition may also provide a reference for receptors' preferential G protein coupling, which prediction may be otherwise missed when using systemic screens and not accounting for the level of G protein expression. Moreover, this study highlights the role of G protein downstream effectors in mediating receptor responses, where PKC activation downstream of Gαq desensitized AT1R and inhibited its G protein-mediated signaling. Such effect also depends on the activated receptors and does not occur for all Gαq/11-coupled receptors. Thus, providing another mechanism for which the availability of G proteins may alter GPCR responses. Finally, competitive G protein receptor binding was further underlined as a mechanism regulating the bias effects of ligands targeting FP and AT1R, thereby providing a potential approach for the design of biased ligands.

Chapter 4 – Probing Differential β-arrestin Active Conformations Through Genetically Engineered Crosslinking.

The aim of this chapter was to glean information regarding the details of GPCR–β-arrestin complex formation in the native cellular environment. This prospect poses a technical challenge due to the dynamic nature of β-arrestin interactions with GPCRs. A set of 12 azF-incorporated β-arrestin variants were generated by site-directed incorporation of the photoactivable UAA, azF. The ability of azF to cross-link with receptors upon UV exposure allows us to appreciate regions and residues within β-arrestin involved in its interaction with receptors. Indeed, photocross-linking of the generated β-arrestin variants with AT1R, B2R, and V2R, revealed distinct active β-arrestin binding modalities when in complex with each of the three receptors. Moreover, β-arrestin conformation was not only dependent on the GPCR in complex, but also on the cellular localization between the plasma membrane and endosomes. This subcellular-dependent change in β-arrestin conformation was also reflected on AT1R intracellular conformation, suggesting an overall rearrangement of the complex interface. Findings of this study demonstrate the use of the photocross-linking approach to study the interface of β-arrestin with GPCRs, and potentially other interacting partners. Moreover, this study reveals how receptor activation dictates β-arrestin binding modality and provides an explanation for how GPCRs utilize a common β-arrestin partner for various purposes. Further identification of the factors that dictate β-arrestin conformations as well the reflection of such alteration on signal transduction remains important for a better understanding of the multifaceted functions of β-arrestin adaptor proteins.

## 5.2 General Discussion

### 5.2.1 Directional G protein Competition

The main finding of Chapter 3 of this thesis was the identification of Gαq and Gα13 competition for FP and AT1R binding as a mechanism of G protein selectivity regulation at these receptors. A specific direction for which G proteins compete for receptor binding was observed (i.e., Gα13 competes with Gαq for AT1R binding, whereas Gαq competes with Gα13 for binding FP). The GPCR–G protein cocrystal structure infers a large G protein surface area compared to that of the GPCR interface (Rasmussen et al., 2011); and this may explain the competitive inhibition for simultaneous G protein interactions on receptors. However, this alone does not justify why G protein competition is unidirectional and receptor dependent. Moreover, recent studies argue for simultaneous GPCR interactions with two effectors, either two G proteins (cognate and non-cognate) (Gupte, Malik, Sommese, Ritt, & Sivaramakrishnan, 2017), or G protein and β-arrestin (Thomsen et al., 2016), to form supercomplexes that contribute to GPCR signaling properties. However, results from this thesis instead suggest a competitive mode of G protein binding in a direction that is dependent on the G protein and receptor pair. The selectivity in G protein coupling is thus expected to emerge from the ability of GPCR–ligand complexes to sample an ensemble of distinct conformations that selects for different G protein subtype binding. The lack of Gαq and Gα13 competition at B2R and TPα shown in chapter 3 suggests that ligand binding to these two receptors yields conformations that produce efficient coupling to both G proteins. However, it remains unidentified how these receptors interact with their other G protein partners, and whether G protein competition will be observed when testing different Gα subtypes.

The discrete direction of G protein competition likely reflects a stronger propensity of GPCRs to bind with the "competing" G protein compared to the "competed" one. Such hypothesis requires further investigation to identify the relative affinities of FP and AT1R binding to Gαq and Gα13. Efforts in the fields are currently invested to identify accurate ligand–receptor signaling profiles. The ability to predict GPCR's preferential G protein coupling through identifying the direction of G protein competition will thus aid in decoding GPCR signaling signatures through their cognate G proteins.

### 5.2.2 G protein Competition in Bias Signaling Regulation

One of the findings of chapter 3 is how G protein competition provides a mechanism for the bias activity of ligands targeting FP and AT1R. The previously identified PDC compound presents clinical significance given its role in inhibiting myometrial contractions in mice (Goupil et al., 2010). Its effect has been previously described in inhibiting PGF2α-mediated Gα12/13 activity while simultaneously acting as a PAM on Gαq/11 responses (Goupil et al., 2010). Yet, the mechanism for such modulation remained unidentified. Results from chapter 3 imply that PDC-mediated increase in PGF2α–FP interaction with Gαq leads to an increase in Gαq competition with Gα13 for FP binding, thereby competitively inhibiting Gα13 signaling. This was confirmed by the loss of PDC effect in inhibiting the Gα13 pathway when Gαq competition was relieved (using ΔGαq/11 cells, or GαqNull-FP mutant). As for AT1R, a recent systematic study identified bias between Gαq/11 and Gα12/13 signaling through AT1R (Namkung et al., 2018). While none of the identified AT1R biased ligands show a potentiation for G protein coupling, TRV and SVdF showed bias towards Gα12/13 pathway relative to Gαq/11. Chapter 3 findings suggest that Gα13 competition with Gαq for AT1R binding plays a mechanism as to which these two ligands exert their functional bias. Altogether, G protein competition is suggested to partake in mediating biased

ligands' functional selectivity. Molecular simulations and mutagenesis studies that focus on identifying contact points for GPCR interactions with specific G proteins could provide insights for the development of biased ligands by directing receptor responses towards a specific pathway rather than focusing solely on selective pathway inhibition.

In the past decade, research in the field was focused on developing numerous GPCR effector biosensors to monitor signaling events in living cells; and these were heavily exploited to perform high throughput screens and to probe functionally selective compounds. The role of directed G protein competition in altering the bias modulation of ligands highlights the importance of adapting signaling assays in the relevant cellular context. This will ensure accurate prediction of GPCR responses in the diverse cell types. Chapter 3 findings suggest that the differential expression of the Gα subtypes may alter GPCR signaling, either through direct G protein competition, or through downstream signaling responses. Such bias GPCR regulation is referred to as "system bias" and is an important consideration in the identification of druggable biased ligands (Smith, Lefkowitz, & Rajagopal, 2018). A relevant example of the importance of system bias in mediating ligands' responses is the targeting of dopamine-2 receptor which has differential effects between the striatum and prefrontal cortex, relating to the altered levels of GRKs and β-arrestins expression in the brain regions (Urs et al., 2016).

### 5.2.3 The Importance of Cellular Context

While the structural determinants underlying G protein coupling are extensively explored and discussed in further detail in sections 1.6.2 and 3.2, identifying G protein coupling for promiscuous GPCRs in native cellular environment remains vital for our understanding of GPCR signaling. Chapter 3 highlights G protein availability as an important factor regulating G protein selectivity binding. This has a great implication on GPCR signaling in physiological and

pathophysiological conditions, where G protein expression varies. Indeed, alterations in G protein expression were reported in cardiomyopathies (Evora & Nobre, 1999; Onfroy et al., 2017) and in cancer (Yajima et al., 2012). Moreover, diseases such as type I diabetes mellitus causes alterations in Gαi2 expression and worsens insulin resistance (Moxham & Malbon, 1996). Gαi2 expression was also associated with atherosclerosis contributing to the impairment of endothelium-dependent relaxation in this disease (Harrison, 1994). Accordingly, the change in G proteins' availability in pathological cellular context would therefore be expected to impact GPCR–G protein selectivity and bias ligand modulation.

The direction of AT1R G protein competition uncovered in Chapter 3 indicates that an increase in Gα13 expression inhibits Gαq responses. This would be valuable when designing biased drugs targeting AT1R, especially given the reports of altered G protein expressions in cardiac conditions. In particular, the gene encoding for Gα12 was shown to be upregulated in hypertrophy and heart failure, while that encoding for Gα13 was downregulated (Kong et al., 2005; Onfroy et al., 2017). The decrease in Gα13 expression would thus be expected to mitigate its competitive inhibition of Gαq binding to AT1R, leading to an increase in Gαq-mediated responses. Whether Gα12 infers competitive binding to AT1R was not investigated in this thesis but would be interesting to uncover. Despite belonging to the same family of G proteins, Gα12 and Gα13 may have differential mechanisms for receptor interactions. Accordingly, future studies dissecting the impact of increased Gα12 expression on AT1R signaling will help better understand the physiological consequences of AT1R activation in cardiac pathologies.

β-arrestin is an important player in mediating AT1R signaling responses. Moreover, it is commonly considered when designing AT1R bias ligands, due to its cardioprotective role (detailed in section 1.6.1.1). A reciprocal regulation between interacting G proteins and β-arrestins is very

likely, especially given the recent report showing that Gαq may encode GRK selectivity and direct β-arrestin-mediated responses (Kawakami et al., 2022). Therefore, identifying whether and how G protein availability for receptor binding plays a role in regulating β-arrestin functions represents an interesting future direction for this study. Investigating β-arrestin interactions with AT1R in the different G protein knockout cells and exploring potential G protein-dependent mechanisms influencing its selectivity for AT1R binding would provide valuable information to broaden our understanding of AT1R-mediated signaling.

In addition to G proteins, agonist bound GPCRs interact with GRKs, arrestins, and other interacting partners. Those partners regulate various aspects of G protein signaling such as ligand binding, trafficking, and subcellular GPCR anchoring (Ritter & Hall, 2009). For example, calmodulin interaction with 5-HT2A and the μ-opioid receptors leads to impaired G protein coupling (Turner & Raymond, 2005; Wang, Sadee, & Quillan, 1999). Another example is PDZ protein $Na^+$–$H^+$ exchange regulatory factor 1 (NHERF1), which interaction with β2AR promotes receptor recycling (Cao, Deacon, Reczek, Bretscher, & von Zastrow, 1999). Although not addressed in this thesis, varying patterns of expression for these partners in the different tissues could have a significant impact on GPCR regulation. Interacting partners may also modulate the functional outcomes for arrestin recruitment following receptor activation.

Following their activation, the majority of GPCRs are phosphorylated by one or multiple GRKs that may be differentially expressed in the different cell-types. While it is well expected that the primary output of receptor phosphorylation is arrestin binding, the resulting functional outcomes for such binding amongst the ~800 GPCRs considerably varies. One explanation for this variation lies in the heterogeneity of GPCR phosphorylation patterns by the different GRKs. In the case of AT1R and V2R, it was shown that GRK2 and GRK3 inhibition had little effect on ERK1/2

signaling by arrestin, while inhibiting GRK5 and GRK6 significantly reduced arrestin-mediated ERK1/2 activity (Kim et al., 2005; Ren et al., 2005). A similar distinction for arrestin-mediated signaling promoted by GRK2/3 vs. GRK5/6 has also been reported for the β2AR (Shenoy et al., 2006), and the follicle-stimulating hormone receptor (Kara et al., 2006). The argument presented for such phosphorylation-dependent signaling outcomes is the presence of varying β-arrestin conformations, that may or may not be conductive of signaling responses. Results from Chapter 4 with azF-mediated crosslinking indeed identify the presence of such differences in the binding modality of β-arrestin1 to GPCRs in different conditions. Although not investigated here, it will be interesting to utilize this approach in the future to identify the effects of specific GRKs on β-arrestin binding modalities. It would also be of interest to assess the effects of different GPCR and/or β-arrestin interacting partners in inducing specific β-arrestin conformations.

### 5.2.4 PKC-Mediated AT1R Desensitization Confirmed with the Gαqi Chimera

The C-terminal tail (Helix 5) of the Gα subunit is believed to be the primary structural determinant for G proteins' selectivity. It predominates at the GPCR–G protein interface and accounts for ~70% of the complex interface (Flock et al., 2017; Rasmussen et al., 2011; Sullivan et al., 1987). Interchanging the last 4–6 residues of the G protein C-terminus produces chimeric G proteins with altered receptor selectivity. While the C-terminus infer G protein selectivity, the core of the G protein produces downstream signaling responses. A Gαqi chimera, where the last 5 residues of Gαq C-terminus were substituted with those of Gαi, was previously used for monitoring Gαi signaling (Conklin, Farfel, Lustig, Julius, & Bourne, 1993). Such concept has also been employed for detecting GPCR selectivity for G protein coupling by substituting the last 6 amino acids residues of Gαq with those of other Gα subunits, allowing the identification of various GPCRs' coupling selectivity by testing for one signaling Gαq output (Inoue et al., 2019). Utilizing

a G protein chimera can validate Chapter 3 results showing that PKC activation, rather than Gαq binding, desensitizes AT1R responses including for Gα13. A chimeric Gαqi construct, where the last 5 residues of Gαq were substituted with those of Gαi is expected to bind Gαi-coupled receptor and activate Gαq downstream signaling responses. Indeed, overexpressing the Gαqi chimera in ΔGαq/11 cells activated Gαq downstream effectors, p63RhoGEF and PKC, upon ligand stimulation of the Gαi-coupled AT1R, but not the non Gαi-coupled FP (Fig. 1A and B). Moreover, Gαqi downregulated Gα13 activation by AT1R but not FP (Fig. 1C). This is consistent with ability of Gαqi to activate PKC, leading to AT1R desensitization. However, the lack of effect of receptor-binding to the Gαi C-tail on Gα13 coupling to AT1R remains to be experimentally validated. It would be interesting as a future direction to generate different types of chimeras to validate the effects of G protein binding vs. downstream effectors in regulating GPCR signaling. For instance, a Gαsq chimera would be expected to bind both FP and AT1R (by virtue of the Gαq C-tail), and to activate effector downstream of Gαs rather than Gαq. Since Gαq binding to FP competes that of Gα13, the Gαsq chimera should inhibit FP-mediated Gα13 signaling. On the other hand, AT1R-mediated Gα13 binding is not competed by direct Gαq interaction, and PKC activation is required to desensitize AT1R signaling. Thus, Gαsq chimera, incapable of PKC activation, should not have an effect on Gα13 signaling by AT1R.

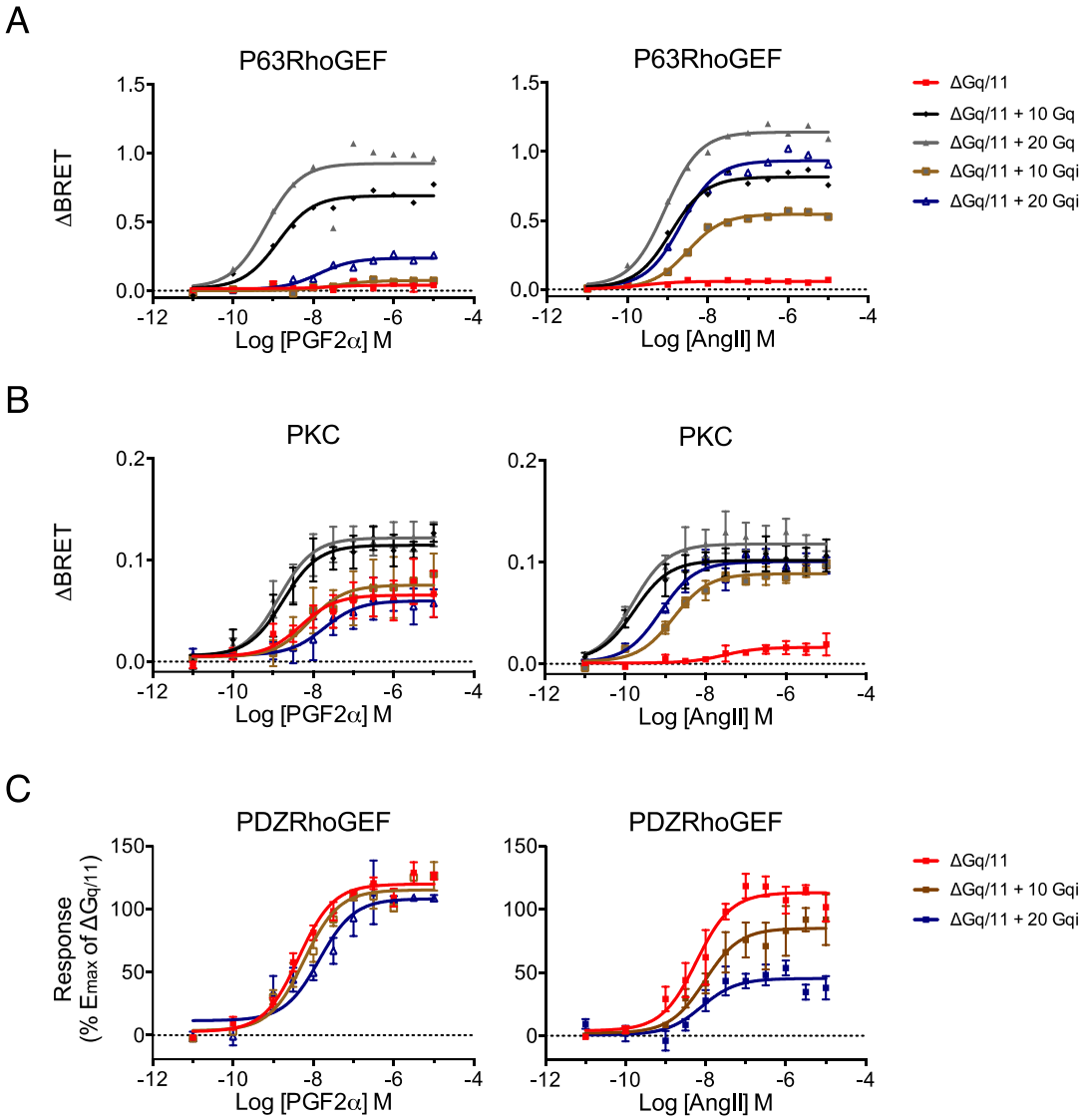


**Figure 1. Effect of Gαqi chimera on FP and AT1R signaling.**

A – C, ΔGαq/11cells expressing FP (left panels) or AT1R (right panels) with the BRET sensors for p63RhoGEF (A), PKC (B), or Gα13-mediated PDZRhoGEF (C), were transfected, or not, with increasing amounts of the Gαq or Gαqi subunits. Cells were stimulated with the indicated concentrations of PGF2α (left panels) or AngII (right panels), and BRET measurements are recorded. Data is represented as means ± S.E (error bars).

### 5.2.5 YM254890 (YM) Inhibition of Gαq

The findings of Chapter 3 with the Gαq inhibitor, YM, shows that YM treatment led to the same Gα13 potentiation by FP observed in the Gαq/11 knockout cells. The mechanism proposed for such potentiation is the relief of Gα13 competitive inhibition by Gαq binding to FP, irrespective of its activation. This direct Gαq binding competition was confirmed by multiple means: 1) the lack of PKC-mediated desensitization of FP's Gα13 activation, 2) the loss of Gαq inhibitory competition of Gα13 with the GαqNull FP mutant, and 3) the ability of the non-functional Gαq mutant (Q/D-Gαq) to compete with and inhibit FP-mediated Gα13 signaling. The nucleotide binding domain of the Q/D-Gαq is selective for xanthine nucleotides, as opposed to guanosine nucleotides (Barren & Artemyev, 2007; Yu, Gu, & Simon, 2000; Yu & Simon, 1998). Thus, the lack of xanthine nucleotides in cells renders the Q/D-Gαq mutant as an empty Gα subunit. This nucleotide free state of G protein forms stable complexes with activated receptors, a phenomenon that has been exploited to stabilize and study receptor interactions with G proteins (Gregorio et al., 2017; Yu & Simon, 1998). Therefore, despite Q/D-Gαq not undergoing the nucleotide exchange required for activation, it is expected to associate and bind with the receptor in high affinity. The fact that Q/D-Gαq still competes with Gα13 for FP binding greatly substantiated our finding that direct Gαq FP interaction competitively inhibits Gα13 signaling.

The effect of YM treatment in potentiating Gα13 coupling to FP is presumably linked to alleviating the inhibitory competitive effect of Gαq binding. Accordingly, YM treatment should decouple Gαq from the receptor, though this has not been experimentally proven. The mechanism for Gαq inhibition by YM is by intercalating within Gαq subunit and preventing domain separation required for GDP release and GTP exchange, and hence heterotrimer activation (Schrage et al., 2015). Gαq is thus retained in its heterotrimeric (αβγ), which does not form stable interactions with

receptors. The data in Chapter 3 show that Gα13 coupling was potentiated by YM treatment and inhibited with Gαq overexpression (Fig. 2). Interestingly, Gαq overexpression does not have the same inhibitory effect when cells are treated with YM (Fig. 2). This suggests that YM treatment is decoupling the overexpressed Gαq subunit and not allowing its competitive inhibition of Gα13 binding. This however will be interesting to further explore by investigating Gαq association with receptors upon YM treatment. While this thesis did not focus on examining the mechanism for YM-mediated Gαq inhibition, distinguishing the ability of G protein inhibitors to prevent the binding or the activation of G proteins could have an impact on receptors' signaling outcome.

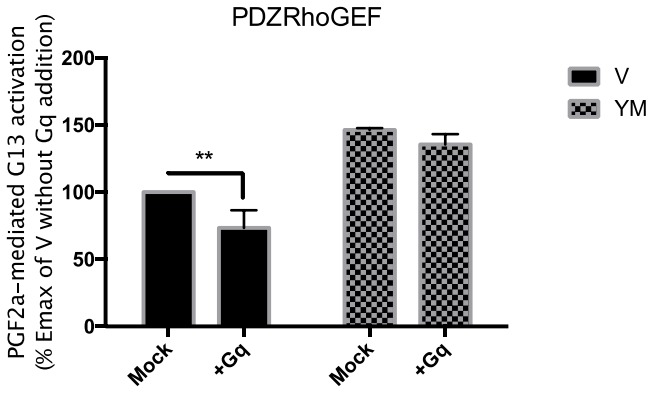


**Figure 2. Effect of Gαq overexpression with YM treatment.**

HEK293 cells are transfected with FP and the Gα13-PDZRhoGEF BRET sensor, with or without overexpressing the Gαq subunit. Cells are treated with Vehicle or 200 nM YM for 30 min and stimulated with 1 μM PGF2α. BRET measurement are recorded and normalized to the BRET response in the Vehicle treated condition without Gαq overexpression. Data is represented as means ± S.E (error bars) of three independent experiments. Two-way Anova followed by Dunnett's comparison tests were performed. **P <0.01.

### 5.2.6 Investigating GPCR Complexes by Genetic UAA Incorporation and Photocross-linking

The study of GPCR complexes presents a technical challenge due to their highly dynamic nature, especially when considering their interactions in the native cellular environment. While crystal structures, negative-stain electron microscopy, and homology modeling, give valuable structural information about GPCR complexes, these may not reflect accurately the structural modalities assumed in cells. In the past few years, newer methods were developed to gain information regarding the structural dynamics of GPCR complexes. The approach used in chapter 4, incorporating the UAA, azF, into β-arrestin, have been recently exploited by our group to map the binding sites of β-arrestin on AT1R (Gagnon et al., 2019). Initially, it was developed to map the ligand binding site on receptors, including for the AT1R (Fillion et al., 2013), neurokinin-1 receptor (Valentin-Hansen et al., 2014), the glucagon-like peptide-1 receptor (Koole et al., 2017), amongst others. Recently, this approach was also used to assess GPCR heterodimerization and delineate interacting residues at the 5-HT2AR-mGluR2 complex interface (Shah, Toneatti, Gaitonde, Shin, & Gonzalez-Maeso, 2020). The power of this method lies in the ability to site specifically introduce the UAA in the protein of interest directly in living cells. Moreover, covalent cross-linking with the introduced azF only occurs when the formed C-H bonds falls within a very small distance of 2–4 A°, allowing the identification of direct interactions with the complexed protein partner (Ray-Saha, Huber, & Sakmar, 2014; Sato et al., 2011).

Other methods deployed for studying β-arrestin conformations in cells include the intramolecular fluorescent arsenical hairpin (FlAsH) BRET and mutagenesis studies (structure–activity relationship studies, SAR) (Kaiser & Coin, 2020; Lee et al., 2016; Strungs, Luttrell, & Lee, 2019). An advantage of the photaffinity cross-linking approach lies in its ability to not only distinguish different global binding modes of β-arrestin, but to also give information regarding the

interacting contact sites at the complex interface. The approach of azF-incorporation into β-arrestin and photocross-linking holds great potential for various future applications. For instance, it may be used to probe for the different β-arrestin interacting partners, such as endocytic or signaling proteins, in the different compartments or in different conditions. It would also be interesting to optimize this approach to investigate GPCR interactions with G protein partners. GPCR–G protein interactions have only been successfully explored for the M3 muscarinic acetylcholine receptor (M3R) with Gαq by using chemical disulfide crosslinking with cysteine mutagenesis (Hu et al., 2010). It is important to note that cysteine crosslinking requires the substitution of highly conserved native cysteine residues with other polar or nonpolar amino acids, leading to significant impact on the structure and the signaling properties of the tested protein. This highlights an advantage for using azF-mediated cross-linking over the cysteine crosslinking approach for testing such interactions.

### 5.2.7 Pharmacological Inhibition of Endocytosis Reveals Altered β-arrestin Interaction with AT1R

An interesting finding of chapter 4 is the differential β-arrestin binding modalities between the plasma membrane (PM) and the endosome. This was assessed by cross-linking azF-β-arrestin mutants with receptors after ligand stimulation at different temperatures, relying on the temperature specific nature of the endocytosis process. Ligand stimulation at 10 °C was shown to restrict GPCR–β-arrestin complexes at the PM, while that at 37 °C allowed for receptor endocytosis. Relying on other methods to block endocytosis would substantiate chapter 4 findings and would confirm that the observed alteration in β-arrestin conformation is driven by the complexes' localization rather than the change in stimulating temperature. Endocytosis was previously shown to be blocked by the small compound inhibitor, Rasarfin, which blocks Arf6

activity, and hence AP-2 and clathrin recruitment to β-arrestin (Giubilaro et al., 2021). Indeed, β-arrestin endosomal translocation upon AT1R, B2R, and V2R stimulation was potently blocked by rasarfin treatment (Fig. 3A – C), similar to what was previously shown with agonist stimulation at 10 °C in chapter 4. Interestingly, crosslinking of azF-β-arrestin mutants with agonist stimulated AT1R after rasarfin treatment results in an increase in P14azF ligation with AT1R, compared to DMSO treatment (Fig. 3D). This is consistent with chapter 4 finding of an increase in P14 ligation when AngII-stimulation was performed at 10 °C, compared to 37 °C (Fig. 3E). However, the increase in T58 ligation observed at 10 °C, compared to 37 °C, was not observed by rasarfin treatment (Fig. 3D and E). The lack of rasarfin effect on T58 ligation is potentially due to the incomplete inhibition of endocytosis, as compared to the 10 °C ligand-stimulation condition. Further controlling the conditions for performing the cross-linking experiments with rasarfin may allow the detection of altered T58azF ligation with AT1R. It remains equally possible that rasarfin treatment and performing ligand stimulation at 10 °C have varying consequences on the trafficking properties of the AT1R–β-arrestin complex. Notwithstanding, these data conclude that inhibiting receptor internalization, whether through rasarfin treatment, or by performing ligand stimulation at 10 °C, results in altered AT1R–β-arrestin binding modality.

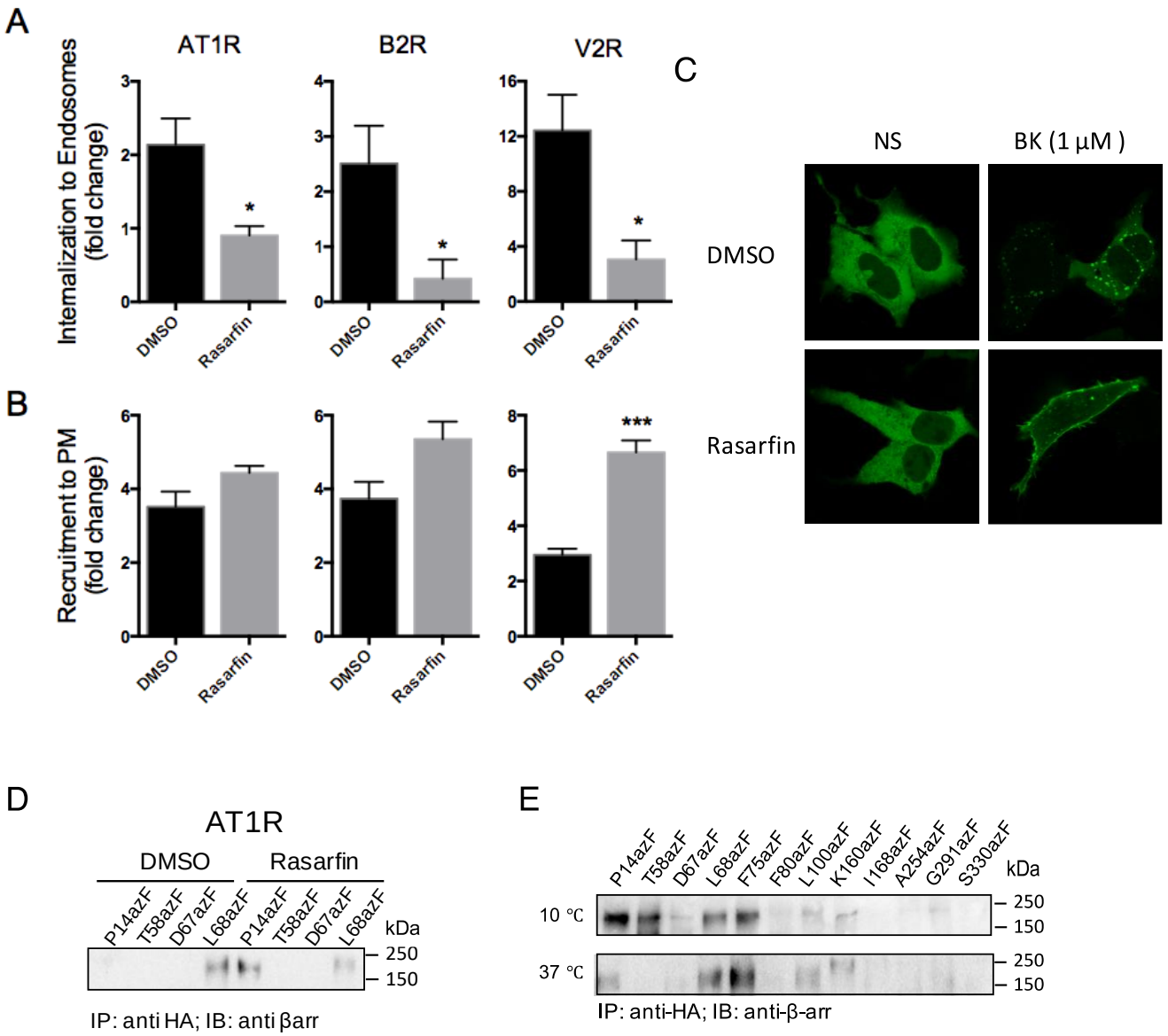


**Figure 3. Effect of rasarfin treatment on β-arrestin trafficking and binding modality.**

A–B, HEK293 cells were transfected with RlucII-tagged β-arrestin and endosomal-anchored rGFP-FYVE (A) or PM-anchored rGFP-CAAX (B), along with AT1R (left panel), B2R (middle panel), or V2R (right panel). Cells were treated with DMSO or 50 µM rasarfin for 30 min before stimulation with 1 uM of the appropriate ligand for 30 min at 37 °C. The fold change in basal BRET before and after ligand stimulation is reported, and data is represented as means ± S.E (error

bars) of triplicated from at least three independent experiments. Unpaired student t-test was performed: * = $P < 0.05$. C, Internalization of β-arrestin following B2R stimulation is shown by fluorescence microscopy. HEK293 cells transfected with YFP-tagged β-arrestin1 and B2R were imaged 48 h post transfection. Cells were treated with DMSO or 50 μM rasarfin for 30 min, Images were acquired before and after stimulation with BK for 30 min at 37 °C. Scale bar, 20 μM. D, HEK293 cells transiently expressing each of the indicated azF β-arrestin variants with HA-tagged AT1R in the presence of 0.5 mM azF were treated with DMSO or 50 μM rasarfin for 30 min before stimulation with 1 μM of AngII for 30 min at 37 °C. Photocross-linking and co-immunoprecipitation were performed as described in chapter 4. E. Replication of Fig. 3B from chapter 4 showing the photocross-linking pattern of AT1R when AngII stimulation was performed at 10 °C or at 37 °C.

### 5.2.8 Subcellular Organization of β-arrestin Functions

Principally, altered β-arrestin active conformation could directly reflect on the functional outcome for β-arrestin recruitment. A supporting evidence for that is the finding that mutations destabilizing the complex interface within the TM core prevent G protein desensitization, while preserving β-arrestin-mediated internalization and signaling (Cahill et al., 2017; Kumari et al., 2016; Kumari et al., 2017). In those studies, an association between the conformational arrangement of β-arrestin with the functional outcome of its recruitment is established; the "core-conformation" complex was linked to G protein desensitization, while the "tail-conformation" complex was deemed responsible for promoting receptor internalization (Cahill et al., 2017) (Fig. 4). Our data in Chapter 4 however show that the AT1R–β-arrestin complex interface, both at the PM and in endosomes, involved residues within the AT1R ICL2 and ICL3 (i.e., core engagement), although the level of these residues' involvement in β-arrestin interaction varied. These data infer that core interaction with β-arrestin may still promote AT1R endocytosis. The diverging conformational arrangement of the endocytosed AT1R-β-arrestin however may impose varying consequences in terms of signaling or trafficking.

The altered β-arrestin binding modality with receptors at the PM and in endosomes is also expected to have significant impact on β-arrestin-mediated signaling in these compartments. With the improved understanding of GPCR trafficking, it is now accepted that GPCR signaling is not restricted to the PM and signaling from internal membrane locations is now evident (Di Fiore & von Zastrow, 2014; Irannejad, Tsvetanova, Lobingier, & von Zastrow, 2015). Despite the current progress in the field, our knowledge remains limited of the spatio-temporal organization of GPCR signaling, the physiological consequences of such organization, and the factors driving specificity signaling in the different loci. Chapter 4 reveals that β-arrestin binding modality is altered between

the PM and the endosome when in complex with AT1R or B2R, while it remains consistent with V2R. The implication of persistent β-arrestin conformation when bound to V2R at the PM and in endosomes remains open for investigation. An earlier study showed that blocking β-arrestin interaction with AP-2, and hence receptor internalization, completely inhibited ERK1/2 signaling by V2R (Beautrait et al., 2017). It will be interesting to test whether the specific interaction with AP-2, or potentially other interacting partners, alters β-arrestin conformations. Overall, many questions remain to be addressed. For instance, how does the spatio-temporal β-arrestin-mediated signaling vary between AT1R, B2R, and V2R? Does β-arrestin interaction with the different receptors lead to the recruitment of distinct scaffolding partners? How does β-arrestin2 conformation compare to those of β-arrestin1 in the different cellular compartments? The application of the photocrosslinking approach together with biochemical assays would help address these questions and enhance our understanding of the functional regulation of β-arrestin.

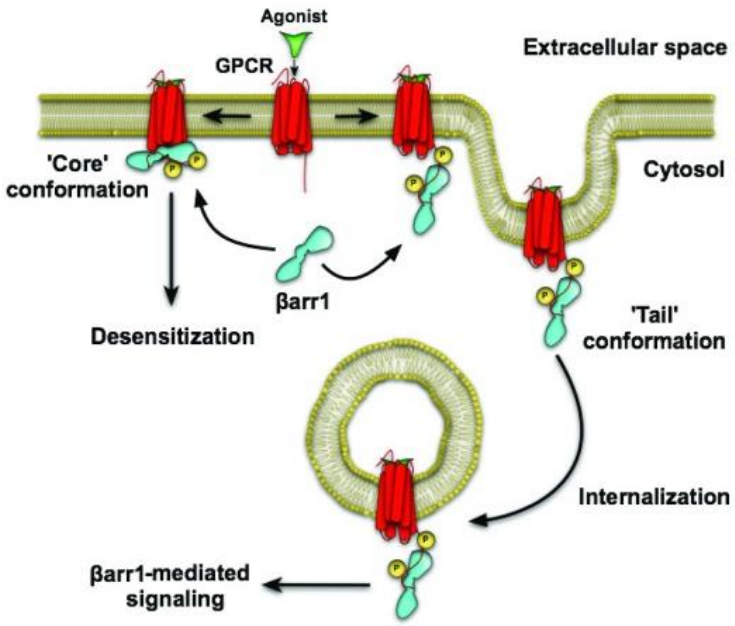


**Figure 4. Varying functional outcomes with different GPCR–βarrestin complex conformations.**

From (Cahill et al., 2017**)**

### 5.2.9 Limitations of the Study

It is important to address some of the limitations of this study and the techniques used. For instance, some of the BRET biosensors require over-expression of Gα subunits, with or without the Gβγ dimer. This ultimately can change the stoichiometry of the Gα and Gβγ subunits and lead to differences in the G protein heterotrimer assembly and activation. Moreover, for experiments with Gα overexpression, an excess of GDP-bound Gα which has high affinity to the Gβγ subunits, leads to the sequestration of the pool of Gβγ dimers. Such sequestration suppresses Gβγ-mediated functions (Jeong and Ikeda, 1999), which may indirectly have an effect on GPCR signaling. This can also result in the inhibition of Gα-mediated signaling by GPCRs. Indeed, such mechanism is exploited by some dominant-negative Gα mutations to exert their inhibitory functions (Barren and Artemyev, 2007).

Another limitation relating to the use of BRET-based biosensors lies in the restriction to the use of HEK293 cells in all experiments. Until now, the number of GPCR signaling biosensors that are suitable for use in primary cells or in native tissues remain limited. However, research in the field is progressing towards investigating GPCR signaling in more physiologically relevant models. For instance, DAG, PKC, and Rho BRET sensors were successfully used in vascular smooth muscle cells (Namkung et al, 2018). Moreover, Maziarz et al recently developed a type of biosensors that detect endogenous G protein activity in cells (Maziarz et al., 2020). It constitutes a unimolecular BRET sensor with an ER/K linker and YFP (BERKY), which binds the GTP-bound form of Gα protein on the plasma membrane (Maziarz et al., 2020). Likewise, another BRET-based biosensor used for the detection of cyclic guanosine monophosphate activation was also reported this year (Valkovic et al., 2022). Chapter 3 findings highlights the importance of testing receptors' signaling in the relevant tissue types and to identify the relative expression levels of

GPCRs and G proteins in the different cell types as well as in pathological conditions. Progress in this area would significantly increase our capacity to study GPCR signaling and functional selectivity.

Compartmentalization of the variable components of GPCR signaling is also an important factor to consider when investigating GPCR signaling and G protein coupling. GPCRs and signaling components are enriched in lipid rafts and caveolae (Pike, 2003). Such compartmentalization could vary in different cell-types and physiological context and is expected to have a significant impact on G protein coupling to GPCRs (Ostrom & Insel, 2004). Accordingly, the compartmentalization between different receptors and G proteins could vary and contribute to the differences in G protein selectivity. While not investigated in this thesis, it is important to acknowledge the different players that can contribute to GPCR signaling regulation and functional selectivity.

The use of the genetic code expansion and photocross-linking technique in this thesis presents a promising approach for the study of GPCR-G protein interactions. While the incorporation of the azF photoprobe overcomes many limitations that arise with other UAAs (Braun et al., 2021), azF mutations may still disrupt the protein and could have an impact on their folding, expression, and/or functions. In chapter 4, azF mutant arrestins were confirmed to tolerate the azF substitution in terms of expression and functionality, albeit to different extents. Nonetheless, variations in those two parameters were shown not to correlate with the cross-linking efficiency. Another limitation for the study in chapter 4 lies in the use of different temperatures to detect GPCR–β-arrestin complexes at different trafficking stages. Given that the formation of a protein complex may depend on temperature, it becomes challenging to separate the impact of lowering the stimulating temperature on trafficking and protein complex formation. For this, an

alternative approach is to be used to confirm the findings observed at 10 °C vs. at 37 °C. Earlier in section 5.2.7. I begin to tackle this caveat by using a pharmacological inhibitor of endocytosis. Nonetheless, different inhibitors could impact β-arrestin interaction with endocytic proteins, leading to differential engagement with receptors. Thus, this possibility should be carefully considered when analyzing photocross-linking results with endocytosis inhibitors.

## 5.3 Conclusion

In conclusion, results obtained from this thesis provide insights regarding the mechanistic regulation of GPCR signaling selectivity in the native cellular environment, either by 1) selecting for specific G protein interacting effectors, or by 2) inducing different conformational arrangements of the multifaceted β-arrestin adaptor protein. The findings on Gαq and Gα13 coupling selectivity and bias modulation at FP and AT1R expands our understanding of those receptors' signaling. They also propose a novel rationale for the design of biased ligands targeting these receptors. Moreover, this thesis underscores structural insights of β-arrestin binding with different GPCRs and in the distinct cellular compartments, which enhances our understanding for how β-arrestins remarkably achieves their great functional versatility.

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