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Multi-modal Signaling and Regulation of the Adhesion G Protein-coupled Receptor ADGRG1 (GPR56)

by

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Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences

Molecular and Systems Pharmacology

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Abstract

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G protein-coupled receptors (GPCRs) are important drug targets due to their tissue expression profiles and wide-ranging involvement in human physiology. The adhesion GPCRs (aGPCRs) comprise a subfamily of GPCRs that have been implicated in a number of human diseases but still remain mysterious in many ways. ADGRG1 (G1 or GPR56) is an aGPCR of special interest because loss-of-function mutations to the receptor underlie a devastating human neurological disease called bilateral frontoparietal polymicrogyria (BFPP). A characteristic feature of aGPCRs, including G1, is their ability to autocatalytically cleave at the first transmembrane domain. Multiple aGPCRs have been shown to exhibit significantly increased constitutive activity when truncated to the point of cleavage (ΔNT), and a general model of aGPCR activation has been proposed in which the new post-cleavage N-terminal stalk directly stimulates receptor activity. We tested whether G1 adheres to this 'cryptic agonist' model by engineering a mutant version of the receptor that lacks the entire NT including the stalk (G1-SL) and broadly assessing receptor signaling. G1-SL displayed robust activity in several assays (including activation of NFAT-luciferase and βarrestin recruitment) but lost the ability to activate SRF-luciferase, a classic measure of $G\alpha_{12/13}$-mediated activity. We also examined the effects of two different BFPP-inducing extracellular loop mutations (R565W & L640R) on multi-modal signaling by both full-length (FL) and ΔNT versions of G1. Similar to stalk deletion, the disease-associated mutations ablated receptor-mediated SRF activation but had no effect on receptor-mediated NFAT activation. Given these differential signaling results, we sought to further elucidate G1-mediated signaling to NFAT and found that it does not involves $G\alpha_{q/11}$ or βarrestins but rather involves liberation of Gβγ subunits and activation of calcium channels. These data support a model in which G1 is capable of at least two distinct modes of signaling: stalk-dependent and stalk-independent, with the downstream intermediates being distinct for the two modes of signaling. The findings presented in this dissertation improve understanding of G1 signaling and regulation, and make significant contributions to the larger debate on the mechanisms of aGPCR activation. By providing insights into the fundamental biology of G1, these studies set the stage for future drug development efforts aimed at G1 and other aGPCRs.

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CHAPTER 1: Introduction[1]

[1] A portion of this chapter is adapted from 1. Kishore, A. and R.A. Hall, *Versatile Signaling Activity of Adhesion GPCRs.* Handb Exp Pharmacol, 2016. **234**: p. 127-146.

1.1 G protein-coupled receptors

The ability of cells to detect and respond to changes in the extracellular environment is vital for their survival. Cell surface receptors mediate the flow of information from the extracellular space to the cell by transducing extracellular stimuli to intracellular signals. G protein-coupled receptors (GPCRs) represent the largest superfamily of cell surface receptors in the human genome [2, 3]. With approximately 800 members, GPCRs participate, either directly or indirectly, in most physiological processes of the human body [4]. This is possible partly because GPCRs can respond to an impressive and diverse array of biological stimuli, including hormones, neurotransmitters, growth factors, odorant molecules, ions, photons, proteases, lipids, and nucleotides [5].

All GPCRs possess a conserved architecture, including seven hydrophobic anti-clockwise alpha helical transmembrane domains (TMs) of about 25-35 amino acids [6]. Compared to their extracellular N-terminal (NT) and intracellular C-terminal regions, the transmembrane regions of GPCRs are highly conserved [6]. The seven transmembrane domains (TMI-VII) are connected via three alternating intracellular and extracellular loops (ICL1-3; ECL1-3). The extracellular and transmembrane regions of GPCRs are often involved in ligand binding while the cytoplasmic regions are essential for signal transduction and interactions with cytoplasmic scaffolding and/or regulatory proteins [7]. There are often multiple glycosylation sites on GPCR N-termini, which may be vital for proper receptor function and folding [8]. Additionally, many GPCRs possess two cysteine residues that form a disulfide bridge between ECLs 1 and 2 that is important for normal receptor folding [9]. The amino acid sequence of glutamic acid/aspartic acid-arginine-tyrosine (E/DRY) on TMIII is conserved in most GPCRs. The E/DRY motif is

believed to stabilize the receptor's inactive state as mutations to the motif frequently result in constitutive receptor activity [9].

While there are several GPCR classification systems that organize receptors based upon structural or physiological features, the two most widely used are the A-F and GRAFS systems. The A-F system was introduced in 1994 and was designed for both vertebrate and invertebrate GPCRs, dividing them into six families (A to F) of which three (A, B and C) contain the majority of human GPCRs [10]. General uniformity of this classification scheme, however, remains elusive as in some instances there can exist large sequence differences between mammalian and invertebrate receptors [10, 11]. The more recent GRAFS system phylogenetically organizes the human GPCR superfamily into five main sub-families: Glutamate, Rhodopsin, Adhesion, Frizzled/Taste2, and Secretin [6]. The Rhodopsin sub-family is by far the largest, comprising about 90% of all human GPCRs [12]. They serve as molecular targets for neurotransmitters such as dopamine, serotonin, acetylcholine, histamine, adrenaline, and norepinephrine [13]. Generally speaking, Rhodopsin-like GPCRs have very short N-termini compared to GPCRs from the other sub-families. A common characteristic of Rhodopsin-like GPCRs is the aforementioned highly conserved E/DRY motif, which is located at the border between TMIII and ICL2 and that regulates receptor activation, at least for many members of this sub-family [6, 9]. The Glutamate sub-family contains metabotropic glutamate receptors (mGluRs), γ aminobutyric acid$_B$ ($GABA_B$) receptors, Ca^{2+}-sensing (CaS) receptors and taste receptors type 1 (TSR1) [6]. Members of this sub-family have exceptionally large extracellular domains which contain a Venus flytrap (VFT) module and a cysteine rich domain (CRD, except in $GABA_B$ receptors) [14]. Glutamate receptors play exceedingly important roles in learning, memory formation, neural communication and have been implicated in multiple neurological disorders. The Frizzled

receptors of the Frizzled/Taste 2 receptor family are known to activate the Wnt signaling pathway and mediate cell fate, proliferation and polarity [6]. Taste 2 receptors are expressed on the tongue and palate epithelium and function as bitter taste receptors. The Secretin receptors possess long N-termini, which contain the ligand binding domains. Specifically, members of this sub-family bind large peptide hormones such as secretin and glucagon and therefore play essential roles in the endocrine system [6]. The Adhesion receptors are related to the secretin receptors and possess extremely long N-termini, which can span several thousand amino acids in length. They are involved in many diverse roles such as immunity, neural development, and cell polarity [6].

1.2 G protein-coupled receptor signaling

G proteins are heterotrimers composed of three subunits: an α subunit (39-46 kDa), β subunit (37 kDa) and γ subunit (8 kDa) [15]. The β- and γ-subunits are tightly associated and effectively function as a single unit. The G protein subunits are tethered to the inner leaflet of the plasma membrane by lipid modifications such as prenylation, palmitoylation and myristoylation, such that they are readily accessible to their cognate receptors. While there are numerous receptors within the GPCR superfamily, there exists only 16 known mammalian Gα subunits (not counting splice variants), which are grouped by sequence homology into four families: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12}$ [15]. The members of the $G\alpha_s$ family, consisting of $G\alpha_s$ and $G\alpha_{olf}$, stimulate the enzyme adenylate cyclase to raise intracellular cyclic AMP levels. The members of the $G\alpha_i$ family, consisting of $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{io}$, two isoforms of $G\alpha_t$ or transducin, $G\alpha_{gust}$ or gustducin, and $G\alpha_z$. Most members of this family inhibit the function of adenylate cyclase. The members of the $G\alpha_q$ family, consisting of $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, and $G\alpha_{15}$, stimulate phospholipase C activity to elevate IP3 levels and intracellular Ca^{2+} concentration. The $G\alpha_{12}$ family consists of

$G\alpha_{12}$ and $G\alpha_{13}$ and activates RhoGEFs, like p115RhoGEF to modulate the cell's actin cytoskeleton [7].

In the classical tripartite model of GPCR signaling, the receptor binds an agonist via its extracellular and/or transmembrane domains and undergoes a conformational change, which promotes coupling of the receptor's cytoplasmic regions to heterotrimeric guanine nucleotide-binding proteins (G proteins) [16]. The ligand-stimulated GPCR acts as a guanine nucleotide exchange factor (GEF) and catalyzes the exchange of GDP for GTP on the Gα subunit. Subsequently, the GTP-bound Gα subunit dissociates from the Gβγ subunits. It is at this point that the dissociated G protein subunits are able to regulate the activity of various effector proteins within the cell to affect changes in protein phosphorylation, ion channels, cyclic AMP, intracellular Ca^{2+} levels and other aspects of cellular physiology [7].

Growing recognition of the importance of Gβγ subunits for both their potential roles in Gα activation as well as mediators of intracellular signaling cascades distinct from Gα has placed the heterodimer under intense scientific scrutiny. There exist 5 Gβ subtypes (as well as an alternatively spliced version of β5 known as β5-long) and 12 Gγ subtypes [17]. Gβ subunits 1-4 share greater than 80% amino acid sequence identity with each other while Gβ5 shares only ~50% identity with the other subtypes [17, 18]. There is significantly less homology amongst the Gγ subtypes. The various β and γ subunits can pair up to form unique Gβγ combinations. The functional significance of this is not well-understood and remains an active area of investigation [17].

While it is generally well-accepted that the presence of Gβγ subunits are required for Gα activation, the precise mechanistic role that they play in guanine nucleotide exchange is currently unclear [19]. Some models of receptor activation view Gβγ subunits as simple scaffolds for Gα

subunits, whereas other models portray Gβγ subunits as more active participants in Gα activation [17]. Two models promoting the view that Gβγ subunits play more active roles are the lever hypothesis and the gearshift model [17]. The lever hypothesis proposes that the receptor uses Gβγ as a lever to 'pry open' the guanine nucleotide binding pocket of Gα to facilitate GTP for GDP exchange [20]. In contrast, the gearshift model suggests that the activated receptor, Gα subunit and Gβγ subunits interact with each other in distinct ways such that they move in lock-step fashion to push the helical and Ras-like domains of Gα apart to provide an exit route for GDP [21].

There is a growing list of examples of the influence of Gβγ subunits on intracellular signaling events [22]. Gβγ subunits have been shown to be involved in the direct stimulation of the inwardly rectifying K^+ channel Kir3 [23, 24], inhibition of voltage-gated Ca^{2+} channels [25] and T-type Ca^{2+} channels [26], modulation of adenylyl cyclase [27, 28], direct activation of phospholipase C (PLC) [29], activation of phosphoinositide-3 kinases (PI3K) [30], and activation of mitogen-activated protein kinases/extracellular signal-regulated kinases (MAPK/ERK) [31, 32].

1.3 Regulation of G protein-dependent signaling

G protein-dependent signaling can be negatively regulated at many different levels. At the level of G proteins, this can take place in multiple ways. For example, the Gα subunit possesses intrinsic GTPase activity to hydrolyze bound GTP and effectively limit its own signaling life. However, for many Gα subunits this may be a relatively slow process. To accelerate this process there exist GTPase-activating proteins (GAPs), which catalyze Gα GTPase activity through allosteric interactions. These G protein GAPs can accelerate hydrolysis by >2,000-fold [33].

Interestingly, early research on GAPs revealed that effector proteins could also act as GAPs and thus both amplify G protein-mediated signaling as well as negatively regulating them. Examples include PLCβ, a major $G\alpha_{q/11}$ effector and GTPase regulator [34], cyclic GMP phosphodiesterase, $G\alpha_t$ effector and GTPase regulator [35] and p115 RhoGEF, which is both an effector and GTPase for $G\alpha_{12/13}$ [36]. Beyond G protein effectors, another important class of multi-functional proteins with GAP activity is the regulators of G protein signaling or RGS proteins. They possess the RGS domain that binds the Gα subunit to accelerate hydrolysis. Currently, there are more than 30 mammalian RGS proteins [37]. While some of the RGS members selectively interact with a specific class of Gα proteins (such as the RGS domain of p115RhoGEF for $G\alpha_{12/13}$) most appear to be at least somewhat promiscuous [37].

Gβγ subunits also play a critical role in terminating GPCR signal transduction. They do so by recruiting and activating GPCR kinases 2 & 3 (GRK2 & GRK3) [38, 39]. GRKs are specialized serine/threonine kinases that phosphorylate the intracellular loops and C-termini of active GPCRs with the help of Gβγ. Subsequently, β-arrestins (βArr), a ubiquitously expressed class of adaptor proteins, bind the GRK-phosphorylated receptor and oppose further G protein-dependent signaling by sterically hindering GPCR and G protein interactions as well as by initiating receptor internalization via clathrin-coated pits [40]. Specifically, β-arrestins act as scaffolds to recruit adaptor protein AP-2 and other proteins (ARF6, ARNO, *N*-ethylmaleimide-sensitive fusion protein, etc.) to clathrin-coated pits for receptor endocytosis in a mechanism dependent on the GTPase dynamin [41]. Within endosomes, GPCRs are sorted by at least 3 different molecular sorting machineries: the ubiquitin-ESCRT (“endosome sorting complex required for transport”) machinery, the GPCR-associated sorting protein (GASP) machinery and the actin-sorting nexin 27-retromer tubule (ASRT) machinery [42-44]. The internalized GPCR

has two basic fates: to be recycled to the plasma membrane via ASRT machinery in the process of receptor re-sensitization or to be shunted toward lysosomal degradation through ubiquitin-ESCRT and/or GASP machinery in the process of receptor downregulation [42, 45, 46].

1.4 G protein-independent signaling by G protein-coupled receptors

In addition to coupling to G proteins, GPCRs can also signal via G protein-independent mechanisms. Many examples of this phenomenon have been described over the past two decades [47], but perhaps the most intensively-studied example has been GPCR signaling through β-arrestins. Beyond the mediation of receptor internalization, as described above, it is increasingly clear that β-arrestins also play important roles as scaffolding proteins for various signaling complexes. This was first demonstrated for the proto-oncogene Src (c-Src) and the subsequent activation of MAP kinases ERK1 and ERK2 [48]. Since then there have been many more reported instances of the β-arrestins mediating the ERK pathway [49-51]. Additionally, the β-arrestins have been implicated in the activation of c-Jun amino-terminal kinase 3 (JNK3) [52], the activation of p38 MAPK [53], the inhibition of NFκB [54] and the activation of protein kinase B (Akt) pathways [55].

The physiological significance of this mode of G protein-independent signaling is also beginning to be appreciated. For instance, it was demonstrated that βArrestin-biased signaling of the β2-adrenergic receptor promotes cardiomyocyte contraction and survival with the obvious potential clinical application of treating congestive heart failure by selectively blocking G protein signaling but not βArrestin-based signaling [56]. In another report, it was shown that the protease-activated receptor-2 (PAR2) relies upon βArrestin-dependent MAPK signaling to reorganize the cell's actin cytoskeleton and to regulate chemotaxis of immune cells—a result with clear immune-modulatory implications [57]. Adding another layer of complexity, there is

evidence now that distinct patterns of GRK-mediated phosphorylation found on GPCRs may constitute unique 'barcodes' that influence the conformation of bound βArrestins and thus their functional capabilities [58, 59]. Additionally, several biased ligands have been discovered that are able to selective activate βArrestin-mediated GPCR activity over traditional G protein-dependent pathways [60]. Biased ligands for non-classical GPCR signaling will remain an exciting area for future drug development for years to come.

1.5 Regulation by G protein-coupled receptor interacting proteins

GPCR interacting proteins (GIPs) provide the cell with a greater capacity to fine-tune receptor signaling [47]. One major class of GIPs are PDZ scaffold proteins, which are cytoplasmic adaptors that regulate the localization and activity of many GPCRs. PDZ proteins contain one or more PDZ domains which are named after the first three PDZ proteins that were discovered: Post-synaptic density protein 95 (PSD95), Drosophila disc large tumor suppressor (Dlg) and Zona occludens-1 protein (ZO-1) [61]. There are estimated to be 200-300 members of the PDZ family [62]. PDZ domains are 80-90 amino acids in length and makes up a globular protein structure comprised of 6 β-sheets and 2 α-helices [61]. These domains mediate binding to GPCRs (and other proteins) that possess PDZ-binding motifs on their extreme C-terminal tails.

One prominent example of PDZ regulation of the localization of a cell surface protein is the cystic fibrosis transmembrane conductance regulator-associated ligand (CAL; also known as GOPC, PIST, and FIG) and the beta1-adrenergic receptor. CAL was shown to bind the beta1-adrenergic receptor via its PDZ-binding motif and reduce its surface expression [63]. PDZ proteins can also have a more direct effect upon the activity of cell surface proteins. For example, PSD95 was shown to enhance ligand-stimulated activity by the 5-HT_{2A} receptor,

potentially by acting as a scaffold to facilitate interactions between the receptor and its signaling effectors [64].

1.6 G protein-coupled receptors are outstanding drug targets

An estimated 30% of all marketed drugs modulate GPCRs [65]. There are several factors that explain why GPCRs make such attractive drug targets. First, they regulate wide-ranging human physiological processes [65]. Moreover, GPCR expression profiles can be restricted to specific tissues, thus reducing off-target effects [66]. Additionally, the cell surface expression of these receptors makes them more accessible to drugs and eliminates the need for cell-penetration.

Orphan GPCRs are receptors whose natural ligands are unknown. Orphan receptors with demonstrated roles in physiology and/or disease states thus represent enticing targets for future drug development. Currently there are no FDA-approved drugs targeting the sub-family of Adhesion GPCRs, whose members are mostly orphan receptors [67]. Not surprisingly, this sub-family has received much attention in recent years as many investigators have worked to de-orphanize and screen these receptors for potential ligands in addition to striving to understand the fundamental biology of these receptors.

1.7 Adhesion G protein-coupled receptors

According to the GRAFS classification system, the Adhesion GPCRs (aGPCRs) make up the second largest class within the GPCR superfamily, encompassing 33 members in humans. These receptors are broadly expressed and involved in many diverse processes including neural development, immunity, myelination and angiogenesis [68]. At one time, the aGPCRs were included in the secretin-like GPCR sub-family and were thought to have evolved from the secretin-like GPCRs. However, it is now believed that aGPCRs probably pre-date the secretin

GPCRs since it has been discovered that primitive organisms such as *Monosiga brevicollis* (choanoflagellate) and *Dictyostelium discoideum* (soil-dwelling amoeba) possess aGPCRs in their genomes but not secretin GPCRs [69].

Other terms that were once used to describe this sub-family include Family B [10], Family B2 [70], epidermal growth factor-seven span transmembrane (EGF-TM7) receptors [71], and the long N-terminal seven transmembrane receptors related to family B (LNB-TM7) family [72]. The term 'Adhesion' was aptly applied to this sub-family due to the fact that the members of this sub-family all possess a number of extracellular domains that resemble domains known to mediate cell-to-cell and cell-to-extracellular matrix (ECM) interactions.

Previously, aGPCRs were either given names based on their functional significance (e.g. brain angiogenesis inhibitors 1-3; BAI1-3) or temporary identifiers (i.e. GPR#). Advances in genomics technologies in recent years have necessitated harmonization of aGPCR nomenclature to clearly illustrate the relationship between aGPCR genes and proteins. In 2015 the IUPHAR Committee on Receptor Nomenclature and Drug Classification in conjunction with the Adhesion GPCR Consortium introduced a new naming system for the aGPCRs [73]. First, each aGPCR is given the pre-fix 'ADGR' which signifies 'Adhesion GPCR'. 'ADGR' is followed by a letter relating the receptor to its previous name and to one of the nine phylogenetic aGPCR subclasses and a number to denote different receptors within that subclass [73]. In this new nomenclature BAI1, for example, is "ADGRB1".

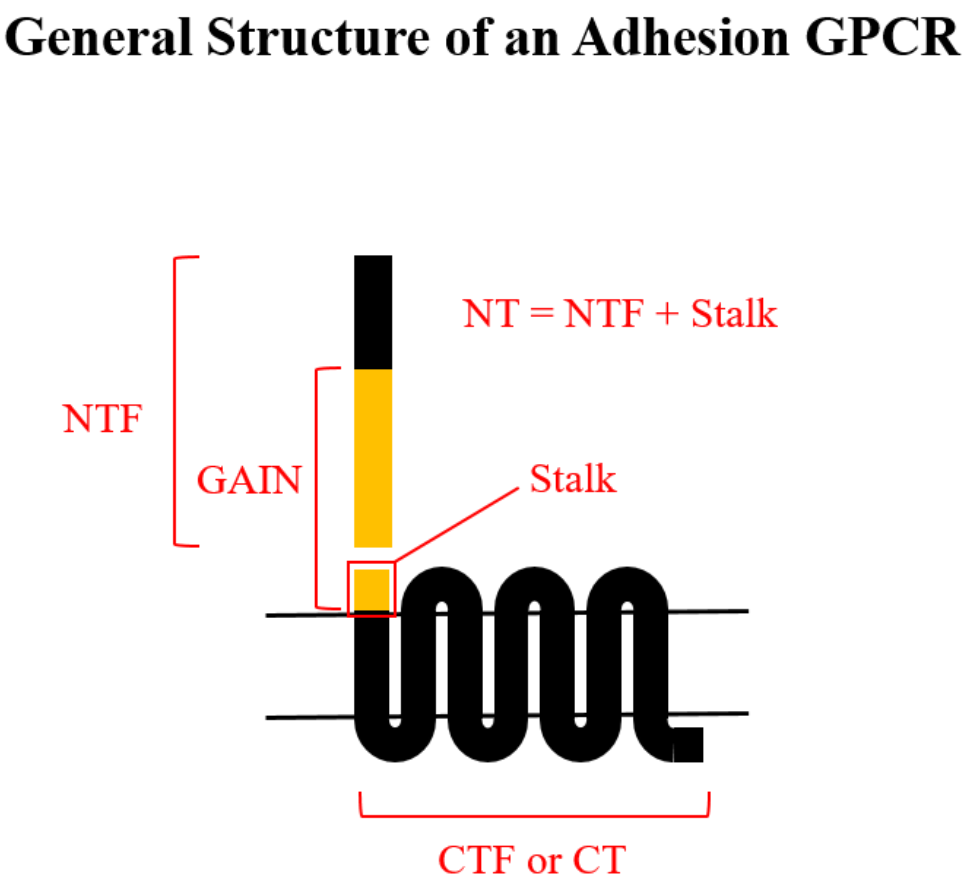
1.8 Adhesion G protein-coupled receptor structure

Adhesion GPCRs possess extremely large extracellular N-terminal domains (NT) that can reach several thousand amino acids in length and harbor multiple protein-protein interaction

domains. In fact, the tremendous diversity in the NT regions has led to the further categorization of the ~33 aGPCRs into 9 distinct subfamilies [73]. The most commonly-shared protein-protein interaction domain(s) found in the N-termini of each of the 9 aGPCR families are olfactomedin (OLF) and rhamnose binding lectin-like (RBD) domains (subfamily I), epidermal growth factor (EGF)-like repeats (subfamily II), leucine-rich repeats (LRRs; subfamily III), cadherin repeats (subfamily IV), pentraxin domains (PTX; subfamily V), sea-urchin sperm protein, enterokinase and agrin (SEA) domains (subfamily VI), thrombospondin type 1 repeats (TSRs; subfamily VII), pentraxin domains (subfamily VIII) and calx-β repeats (subfamily IX) [73].

Another unique feature of the aGPCRs is their autoproteolytic activity at a membrane-proximal motif of the NT called the GPS or GPCR Proteolysis Site motif [74, 75]. This ~40-50 amino acid, cysteine- and tryptophan-rich motif is located within a much larger functional domain that is both necessary and sufficient for aGPCR self-cleavage called the GPCR Autoproteolysis Inducing (GAIN) domain [76]. The GAIN domain is the only commonly shared domain in the NT of aGPCRs (with the exception of ADGRA1/GPR123) [77]. Moreover, the GAIN domain is also one of the most ancient domains found in aGPCRs, existing in the genomes of more primitive organisms such as *Dictyostelium discoideum* and *Tetrahymena thermophile* [76, 78]. Structural studies by Arac and colleagues showed that the GAIN domain stays intact following cleavage through an extensive network of hydrogen bonding and hydrophobic side-chain interactions [76]. These insights confirmed prior biochemical observations that autoproteolysis does not necessarily result in the dissociation of the N-terminal fragments (NTFs) and C-terminal fragments (CTFs) that result from GAIN domain cleavage.

Figure 1: A schematic depicting the general structure of an adhesion G protein-coupled receptor. The NTF (N-terminal fragment) refers to the extracellular N-terminal portion of the receptor up to the site of GAIN domain cleavage. The NT (N-terminal domain) refers to the entire N-terminal domain of the receptor up to TMI. The CTF or CT (C-terminal fragment) refers to the 7TM structure of the receptor, including the stalk, proceeding from the site of GAIN domain cleavage.



The GAIN domain of aGPCRs is always located near the end of the extracellular NT, preceding the start of the first TM domain. Autocatalytic proteolysis was first proposed due to the sequence similar between the GPS motif and the N-terminal nucleophile hydrolases [75]. Cleavage is believed to occur at the tripeptide sequence H-L*T/S ('*' represents the site of cleavage) within the GPS motif of the GAIN domain. In the proposed mechanism, histidine pulls a proton from the hydroxyl group of threonine/serine to yield a negatively charged oxygen group [75]. This oxygen performs a nucleophilic attack on the carbonyl carbon of leucine producing a transient tetrahedral intermediate that eventually results in cleavage of the protein between leucine and threonine/serine [75]. Moreover, there is evidence to support the notion that receptor autoproteolysis occurs in the endoplasmic reticulum (ER) during normal protein processing [75, 79, 80] .

1.9 Evidence of G protein-mediated signaling by Adhesion G protein-coupled receptors

Notwithstanding their N-terminal diversity, all members of the aGPCR family share a similar seven-transmembrane (7TM) domain architecture, which is the molecular signature of GPCRs. However, in the early years of aGPCR research, it was not known whether these proteins were bona fide GPCRs. In studies that were facilitated by the serendipitous discovery of a potent and high affinity agonist, ADGRL1 (Latrophilin-1) was one of the first aGPCRs characterized in terms of its signaling activity [81]. It was found that α-latrotoxin (α-LTX), a component of black widow spider venom, stimulated increases in intracellular cAMP and IP3 levels in ADGRL1-transfected COS7 cells in a receptor-dependent manner [82]. However, in addition to binding to ADGRL1, α-LTX can also form calcium-permeable pores in the plasma membrane and trigger exocytosis [83]. Therefore, a mutant version of the toxin was generated, α-LTX^{N4C}, which does not cause exocytosis but still binds to and activates ADGRL1 [83]. Further

studies showed that ADGRL1 could activate phospholipase C (PLC) and increase intracellular Ca^{2+} within minutes of α-LTX^{N4C} treatment, suggesting coupling of the receptor to $G\alpha_q$ [84]. Moreover, ADGRL1 could be co-purified with $G\alpha_o$ [82, 85] and $G\alpha_{q/11}$ [85] using α-LTX affinity chromatography.

Unlike ADGRL1, the majority of aGPCRs do not have known ligands. Thus, a common method of discerning the signaling pathways downstream of aGPCRs has been to overexpress the receptors in heterologous systems and measure their constitutive activities in assays of specific G protein signaling. For example, overexpression of ADGRD1 (GPR133) has been shown to result in large increases in cAMP, which is suggestive of coupling to $G\alpha_s$ [86, 87]. Similarly, overexpression of ADGRG1 (GPR56; hereafter referred to as 'G1'), a receptor that is critically involved in the development of the cerebral cortex [88, 89], was shown to robustly stimulate the activation of RhoA via coupling to the $G\alpha_{12/13}$ signaling pathway [90, 91]. Subsequent studies have demonstrated that G1 expression can upregulate the activity of a variety of downstream transcription factors, including NFκB [92], PAI-1 [92], TCF [92], SRE [90, 93-95], SRF [96] and NFAT [93, 96]. Other outputs influenced by G1 include PKCα [97], VEGF [95] and TGFα-shedding [96]. In addition to these results, direct evidence of receptor G protein coupling has been provided by several groups. Little et al. demonstrated that $G\alpha_{q/11}$ could be co-immunoprecipitated with G1 in heterologous cells [98]. This interaction, however, depended on the presence of the tetraspanin CD81, which may be acting as a scaffold for a signaling complex. In agreement with those data, Ohta et al. showed recently that stimulation of G1 in U87-MG cells could raise intracellular Ca^{2+} levels [99]. More recently, Stoveken et al. showed that G1 can activate $G\alpha_{13}$ in a reconstituted GTPγS assay [94] and additionally an association between G1 and $G\alpha_{13}$ was also shown via a co-immunoprecipitation approach [96].

In addition to G1, evidence for G protein coupling has also been provided for several other members of aGPCR subfamily VIII. For example, ADGRG2 (GPR64) expression in transfected cells has been demonstrated to stimulate the SRE & NFκB pathways [100] and raise intracellular cAMP levels [101]. Another member of the subfamily, ADGRG6 (GPR126), which plays an important role in regulating peripheral nerve myelination [102], was also found to raise intracellular cAMP [87, 103, 104] as well as stimulate IP3 accumulation [104]. Thus, both ADGRG2 and ADGRG6 may couple to $G\alpha_s$ to raise cAMP levels while also exhibiting coupling to other G proteins to mediate pleiotropic effects on cellular physiology.

ADGRB1 (BAI1), a receptor that regulates phagocytosis [105-108], myogenesis [109] and synaptic plasticity [110, 111], has been shown to constitutively activate RhoA [112], Rac1 [108], ERK [112], SRF [96], NFAT [96] and TGFα-shedding [96] when overexpressed in heterologous cells. ADGRB1 signaling to most of these downstream readouts can be greatly attenuated by co-expression of the RGS domain of p115-RhoGEF, suggesting a predominant coupling of the receptor to $G\alpha_{12/13}$. Expression of ADGRB2 (BAI2), a close relative of ADGRB1, was found to also stimulate the NFAT pathway and moreover induce IP3 accumulation in HEK293T cells, indicating a likely coupling to $G\alpha_{q/11}$ [113].

In terms of evidence for G protein coupling to other aGPCRs, ADGRE5 (CD97), a receptor highly expressed in immune cells, was found to activate the SRE pathway in transfected COS7 cells in a manner that was blocked by RGS-p115RhoGEF, suggesting receptor coupling to $G\alpha_{12/13}$ [114]. ADGRV1 (VLGR1), a receptor that has a crucial role in hearing and vision and whose dysfunction is associated with the human disease Usher syndrome, was shown to inhibit isoproterenol-induced cAMP levels in HEK293 cells, indicative of $G\alpha_i$-coupling [115]. Moreover, the use of a chimeric G protein, $G\alpha_{qi5}$, was able to re-rout receptor activity toward a

$G\alpha_{q/11}$ read-out, NFAT activation, thereby providing further evidence for $G\alpha_i$ coupling. Finally, Gupte et al. showed that ADGRE2 (EMR2), ADGRF1 (GPR110) and ADGRF4 (GPR115) were all able to stimulate IP3 accumulation in transiently-transfected HEK293 cells [116]. Additionally, expression of either ADGRG5 (GPR114) or ADGRD1 potentiated cAMP levels, an effect which could be blocked by knocking down endogenous $G\alpha_s$ or by overexpressing the chimeric G protein $G\alpha_{qs4}$, which converts $G\alpha_s$ signaling into $G\alpha_q$-mediated activity. In separate studies that confirmed some of these findings, ADGRF1was shown to activate $G\alpha_q$ in a GTPγS assay [94] and ADGRD1 was shown to raise intracellular cAMP levels [87]. Lastly, Gupte et al. also demonstrated that ADGRG3 (GPR97) could stimulate IP3 accumulation only in the presence of chimeric G protein $G\alpha_{qo3}$, which converts $G\alpha_o$ signaling into $G\alpha_q$ activity, suggesting a natural coupling of this receptor to $G\alpha_o$.

1.10 Adhesion G protein-coupled receptor ligands

As mentioned previously, α-LTX is a high-affinity agonist of ADGRL1 that has been shown to stimulate several read-outs of receptor activity. Another reported ligand for ADGRL1 is teneurin-2, a large (~2,800 residue) glycoprotein with a single transmembrane region that is found predominantly in the brain [117]. Teneurin-2 was first identified as a binding partner of ADGRL1 through pull-down studies in which rat brain lysates were subjected to α-LTX affinity chromatography [117]. Treatment of cultured neurons expressing ADGRL1 with a soluble, C-terminal fragment of teneurin-2 was found to trigger the release of intracellular Ca^{2+}, possibly through a G protein-dependent mechanism [117]. In another study, co-culturing cells expressing either ADGRL1 or teneurin-2 resulted in the formation of large cell aggregates, indicating that the specific interaction between the two proteins may mediate cell adhesion [118]. In the brain, ADGRL1 and teneurin-2 are enriched in the presynaptic and postsynaptic membranes,

respectively. The extracellular NT of ADGRL1, however, may be large enough to span the synaptic cleft to mediate inter-neuronal contact through its high-affinity interaction with teneurin-2.

ADGRL1 has also been shown to interact with neurexin, a presynaptic protein implicated in synaptogenesis and function [119]. Neurexin is a binding partner of α-LTX, as is ADGRL1 [120]. A particular neurexin isoform (1α) binds α-LTX in a Ca^{2+}-dependent fashion, while the α-LTX-ADGRL1 interaction is Ca^{2+}-independent [120]. Interestingly, in the absence of Ca^{2+}, knock-down of neurexin in cultured hippocampal neurons significantly diminished the α-LTX response compared to wild-type neurons, suggesting that while both proteins can independently associate with α-LTX, their interaction may synergistically enhance ADGRL1-mediated α-LTX responses [121]. Moreover, co-culture of cells expressing either ADGRL1 or neurexin resulted in numerous cell aggregates, providing evidence that the interaction promotes adhesion complexes [122]. More work must be done, however, to demonstrate whether neurexins can directly stimulate receptor signaling activity.

The fibronectin leucine-rich repeat transmembrane (FLRT) proteins are an additional class of ligands for ADGRL1 and the related receptor ADGRL3 (Latrophilin-3) [123]. Direct interactions between the NT of ADGRL3 and FLRT3 were demonstrated in a non-cell-based assay [123]. *In vivo*, both proteins are enriched in cell-to-cell junctions and regulate synaptic density [123]. In another study, a high affinity interaction was demonstrated for ADGRL3 and FLRT2 [124]. This interaction is mediated by the OLF domain on the ADGRL3 NT and, intriguingly, promoted either adhesion of FLRT2-expressing HeLa cells or repulsion of FLRT2-expressing cultured cortical neurons. These results potentially highlight the influence that the cellular environment may have on the relationship between receptor and ligand. At present,

however, there is no evidence that FLRT proteins can instigate signaling by the latrophilin receptors.

A number of ligands have been identified for subfamily VII aGPCRs. ADGRB1 was found to bind externalized phosphatidylserine on apoptotic cells through the thrombospondin type 1 repeat domains on its NT [105]. This interaction promoted the engulfment of the apoptotic cells in a mechanism reliant on the adaptor protein ELMO1 and signaling by the small GTPase Rac1 [105]. Another receptor from this subfamily, ADGRB3 (BAI3), was shown to bind to C1q-like (C1ql) proteins [125, 126]. Similar to the interaction of ADGRB1 and phosphatidylserine, the interaction between ADGRB3 and C1ql3 was found to be mediated by thrombospondin repeats on the receptor's NT [125]. In cultured neurons, submicromolar C1ql3 treatment significantly reduced synaptic density, an effect readily blocked by exogenous addition of purified ADGRB3 NT [125]. In a similar study, it was shown that ADGRB3 binds C1ql1 via its N-terminal CUB domain and that both proteins were necessary for normal spine density of cerebellar neurons [127]. Furthermore, the interaction between C1ql1 and ADGRB3 was demonstrated to regulate pruning in mice cerebellum, with knock-out of either protein resulting in severe motor learning deficits [128]. Future studies in this area will likely examine whether C1ql proteins have similar binding affinities for other members of subfamily VII and whether those interactions can stimulate receptor-mediated activity.

Several ligands have been identified for G1, including tissue transglutaminase 2 (TG2), a major cross-linking enzyme of the extracellular matrix implicated in cancer progression [129, 130]. TG2 binds a ~70 residue region on the NT of G1; deletion of this TG2-binding region was found to enhance receptor-mediated VEGF production *in vitro* and significantly increase tumor growth and angiogenesis *in vivo,* whereas expression of the wild-type receptor reduced both

measures [97]. In a more recent study, it was demonstrated that the antagonistic relationship between G1 and TG2 may be attributed to internalization and lysosomal degradation of extracellular TG2 in a receptor-dependent mechanism [131]. It is unclear at present whether interaction with TG2 stimulates G protein-mediated signaling by G1.

Collagen III is another ligand for G1 [132]. Remarkably, knock-out of collagen III in mice results in a cortical phenotype similar to that observed in mice lacking G1 as well as human patients with a neurological disease (bilateral frontoparietal polymicrogyria) that is caused by loss-of-function mutations to G1 [133]. Moreover, nanomolar concentrations of collagen III have been shown to significantly reduce migration of mouse neurospheres (masses of cells containing neural stem cells) in a receptor-dependent fashion [132]. Biochemical studies revealed that collagen III could stimulate RhoA signaling in a mechanism dependent on receptor expression and likely mediated by $G\alpha_{13}$ [132].

Another subfamily VIII receptor, ADGRG6, has also been shown to be stimulated by collagen interactions, albeit with a distinct type of collagen. The association between ADGRG6 and collagen IV was found to be mediated by a region of the ADGRG6 NT containing the CUB and PTX domains [103]. Furthermore, the association was shown to be specific, as other types of collagen, including collagen III, did not bind the receptor. In heterologous cells, collagen IV stimulated receptor-dependent cAMP elevation. The half-maximal effective concentration for this response was 0.7 nM, indicating that collagen IV is a potent agonist for ADGRG6.

An additional ligand for ADGRG6 is laminin-211, an extracellular matrix protein that is involved in Schwann cell development and peripheral nervous system myelination [134]. Interestingly, laminin-211 was found to antagonize receptor-mediated cAMP elevation in a dose-dependent fashion in heterologous cells. Furthermore, cAMP inhibition was due to antagonism

of receptor-mediated $G\alpha_s$ activity rather than through differential activation of $G\alpha_i$. Remarkably, laminin-211 treatment under the condition of mechanical shaking had the opposite effect of boosting receptor-mediated cAMP levels. Thus, laminin-211 may serve as a unique ligand that can differentially modulate receptor activity depending upon other physical cues and mechanical forces in the extracellular environment.

The prion protein PrP^C was shown to be yet another ligand of ADGRG6 [135]. Interestingly, loss of PrP^C results in a chronic demyelinating polyneuropathy. It was demonstrated that a short peptide derived from PrP^C was able to stimulate cAMP levels in a receptor-dependent manner in both a Schwann cell line with endogenous receptor expression and in HEK293T cells with heterologous receptor expression. Moreover, the agonistic peptide was able to induce increased levels of cAMP in mouse sciatic nerve (where *Adgrg6* is highly expressed) and increased myelination in mutant *adgrg6* hypomorphic zebrafish.

The association between ADGRE5 and CD55 was one of the first confirmed protein-protein interactions involving an aGPCR [136]. This interaction was found to be mediated by the EGF domains on the receptor's NT [137]. Recently, it was shown that CD55 does not modulate ADGRE5-mediated signaling to ERK or Akt [138]. It remains to be determined whether CD55 can modulate other receptor-controlled pathways, such as perhaps the RhoA signaling pathway. ADGRE2 is a close relative of ADGRE5 with highly homologous EGF domains, but nonetheless ADGRE2 has been found to have a much lower binding affinity for CD55 than ADGRE5 [139]. Both ADGRE5 and ADGRE2 have also been shown to bind to extracellular matrix (ECM) components known as chondroitin sulfates [140]. These interactions are generally low-affinity and Ca^{2+}-dependent and have not yet been demonstrated to instigate G protein-mediated signaling for either receptor.

Most of the putative aGPCR endogenous ligands described thus far are large, ECM-derived molecules. Nonetheless, it has been shown that small molecules can be developed as aGPCR ligands. For example, screening studies revealed beclomethasone dipropionate as a ligand for ADGRG3 [116]. Beclomethasone dipropionate is a glucocorticoid steroid that can stimulate ADGRG3 with nanomolar potency. The region of the receptor that interacts with beclomethasone is unknown, but considering the molecule's hydrophobicity it would not be surprising if it were found in future studies to directly interact with the receptor's 7TM region to modulate receptor activity. Another example is the small molecule rotenoid derivative dihydromunduletone (DHM), which was shown to antagonize (with low micromolar potency) signaling by G1 and ADGRG5, but not ADGRF1 [67].

An intriguing observation made for several aGPCRs has been that these receptors may be activated by antibodies directed against their NT regions. Antibodies may be able to mimic the binding of endogenous ligands to aGPCRs, and thus may represent powerful research tools for studying aGPCR signaling, especially for those receptors with no identified ligands. An N-terminal activating antibody of G1 was first described in 2008 by Itoh and colleagues. Studies in heterologous cells revealed that antibody treatment could dose-dependently stimulate receptor signaling in the SRE-luciferase assay (a commonly used assay for $G\alpha_{12/13}$ activity) [90]. Moreover, stimulation was readily blocked by exogenous addition of the receptor's NT, which presumably competed for antibody binding. Moreover, in a later study it was shown that other newly-generated N-terminal antibodies for G1 could inhibit cell migration in a manner that was sensitive to inhibition of either $G\alpha_q$ or $G\alpha_{12/13}$ signaling [99]. In another example, an antibody directed against the N-terminal region of ADGRE2 was shown to dose-dependently increase inflammatory cytokine production in receptor-mediated neutrophil activation [141].

1.11 Adhesion G protein-coupled receptors in human disease

Genetic analyses have implicated several aGPCRs in human diseases. A prominent example is ADGRV1, mutations of which cause cochlear and retinal defects in humans [142]. Missense mutations to ADGRC1 impair surface trafficking of the receptor and result in a severe human neural tube defect known as craniorachischisis [143]. Additionally, several aGPCRs have been implicated in the progression of malignancies including glioblastoma multiforme [92, 144], metastatic melanoma [145], breast cancer [146] and acute myeloid leukemia [147].

The involvement of G1 mutations in causing human neurological disease has been particularly well-studied. Loss-of-function mutations to G1 cause the human brain malformation bilateral frontoparietal polymicrogyria (BFPP). This recessively inherited disorder is characterized by abnormally numerous and small gyri of the brain—especially affecting the frontal lobes. BFPP patients exhibit mental retardation, epilepsy, motor deficits and language impairment [88].

Early studies revealed that G1 is highly expressed on neural precursor cells (NPCs) during development and mediates their migration [90, 148]. During this critical period, NPCs migrate from proliferative zones in the ventricles to build circuits and functionally integrate with each other. This highly orchestrated series of molecular events in early childhood are critically dependent upon normal migration of NPCs and any abnormality can have serious consequences [149].

To date, there are at least 26 BFPP-causing mutations to the receptor [150]. Eight missense mutations, in particular, are the best characterized in terms of their effect on receptor function and regulation [151]. These include mutations found on the distal portion of the

extracellular NT; R38Q, R38W, Y88C, and C91S, the GAIN domain; C346S and W349S, and on ECLs 2 and 3; R565W and L640R, respectively.

According to one study, which utilized the cell surface biotinylation approach to determine the relative surface expression of BFPP-associated G1 mutants in regards to their 7TM or CT protomer, GAIN domain mutants C346S & W349S, and ECL2 mutant R565W displayed essentially no expression on the cell's surface [152]. In contrast, distal NT mutants R38Q, R38W, Y88C and C91S displayed reduced surface expression and ECL3 mutant L640R displayed normal surface expression, in regards to the CT protomer [152]. When looking at the surface expression of the NTF protomer, it was noted that all mutants were significantly reduced at the cell surface with the exceptions of C346S and W349S, which displayed no surface expression of the NT [152].

A separate study demonstrated that mutations R565W and L640R cause aggregation and/or increased receptor oligomerization in lipid raft-containing membrane fractions [153]. A subsequent report examining G1 activation by a proposed ligand, collagen III, found that while stimulation resulted in NT release and increased presence of the CT protomer in lipid raft-containing membrane fractions for both wild-type and mutant receptors, that only the wild-type receptor displayed enhanced RhoA activation [154]. This finding suggests that the L640R mutation detrimentally affects the activated NT-dissociated (ΔNT) form of G1 but not the basal heterodimeric receptor. The authors speculate that the L640 mutation may be essential for ligand interactions and therefore the mutation might interfere with receptor activation.

In addition to causing reduced surface expression and altering receptor signaling dynamics, some BFPP-causing mutations also disrupt ligand binding. The collagen III binding domain of G1 exists between amino acids 27-160 [155]. The BFPP-causing mutations within that

region, namely R38Q, R38W, Y88C and C91S, abolishes the ability of the receptor's NT to bind to collagen III [155].

An important point to clarify in regards to the reduced surface expression of BFPP mutants is whether this may be attributed to a deficit in forward trafficking or receptor recycling following internalization. Several lines of evidence from multiple studies strongly suggest that most BFPP mutations cause protein misfolding and reduced forward trafficking of the receptor. For example, confocal immunofluorescence staining against the NT protomer of mutants R38W, C346S, R565W and L640R revealed strong co-localization with an endoplasmic reticulum (ER) marker [153]. Another point of support provided by the same study demonstrated that R38W, C346S and R565W are much more sensitive to endogycosidase H (EndoH), a glycosidase that cleaves N-linked high mannose-type glycans added in the ER but not the more complex carbohydrate chains added later on in Golgi apparatus, than the wild-type receptor [153, 156, 157]. Another line of evidence demonstrated that the presence of the dominant-negative form of dynamin (K44A), a GTPase involved in the endocytosis of membrane proteins, enhanced surface expression of the wild-type receptor but not R38W, R38Q, Y88C and C91S mutants [152]. These findings taken together suggest that the aforementioned mutations mainly influence forward trafficking of G1 and not receptor internalization/recycling.

1.12 Adhesion G protein-coupled receptor models of activation

With the idea that aGPCR ligands mainly bind to the large extracellular NT regions, and that the NT regions are cleaved in the GAIN domain and may be removed at some point following ligand binding, a number of groups have generated truncated versions of aGPCRs lacking most of their NT regions up to the sites of predicted GAIN cleavage ('ΔNT' mutants). The first studies of this type were performed independently for a trio of receptors - ADGRB2

[113], G1 [91] and ADGRE5 [114] - and in each case the truncation was found to result in a substantial increase in the receptors' constitutive signaling activity. Subsequently, this phenomenon has been reported for a number of other aGPCRs, including ADGRB1 [112], ADGRG6 [103], ADGRG2 [100, 101], ADGRD1 [87], ADGRF1 [94], and ADGRV1 [115]. In light of these findings, a general model of aGPCR activation was proposed wherein the tethered NTF behaves as an antagonist of CTF-mediated signaling, with N-terminal deletion mimicking ligand-mediated removal of the NTF to result in receptor activation [158]. This model of activation, termed the *disinhibition model*, was a general model that left open the mechanistic question of precisely how removal of aGPCR NT regions might activate receptor signaling

Subsequently, a more mechanistically specific model of aGPCR activation, termed the *tethered agonist model*, was proposed (Fig. 2). In this model, GAIN domain autoproteolysis results in the creation of a cryptic agonist sequence that is unveiled following receptor self-cleavage and subsequent NTF dissociation. This mechanism of activation is conceptually similar to that of the protease-activated receptors for which proteolysis of the N-terminal domain by an extracellular protease unveils an agonist in the remaining NT [159]. Evidence in favor of the cryptic agonist model was provided by two independent groups: Liebscher et al. and Stoveken et al. First, Liebscher et al. showed that deletion of the remaining NT (i.e. the stalk or "stachel" region) from constitutively-active NTF-lacking versions of ADGRG6 and ADGRD1 ablated activity of both receptors in cAMP accumulation assays [87]. Moreover, synthetic peptides corresponding to the stalk regions of each receptor were able to restore activity of the stalkless mutants with varying degrees of efficacy. The most potent peptides displayed half-maximal effective concentrations in the high micromolar range. Further studies from Liebscher et al. along similar lines provided evidence for tethered agonist-mediated activation of ADGRG2

[101] and ADGRG5 [160]. Additionally, Stoveken et al. showed that stalkless versions of G1 and ADGRF1 lacked activity in reconstitution assays examining GTP binding to purified $G\alpha_{13}$ and $G\alpha_q$, respectively [94]. Synthetic peptides fashioned after the stalk of each receptor were shown to resuscitate their cognate stalkless receptors in a dose-dependent manner, with the most potent peptides displaying submicromolar half-maximal effective concentrations. Moreover, the most potent stalk peptide of G1 was shown to stimulate receptor-mediated activity in cellular SRE-luciferase assays in addition to the $G\alpha_{13}$ reconstitution studies.

1.13 Adhesion G protein-coupled receptor N-termini as sensors of mechanical forces

There is emerging evidence that aGPCRs may be involved in sensing mechanical forces. For example, it was shown that the ADGRE5 NTF is released from the CTF after engagement with the ligand CD55, but only under mechanical shaking conditions that are meant to recapitulate the shear stress associated with circulating blood [138]. In a similar vein, laminin-211, a ligand of ADGRG6, was found to only stimulate the receptor under shaking conditions, and actually antagonized receptor activity under static conditions [134]. In these studies, the mechanical forces may have helped laminin-211 to disengage the NTF from its CTF, whereas without shaking, the ligand binding may have actually stabilized the inhibitory NTF-CTF interaction. These examples support the idea that, for at least some ligand-receptor pairs, mechanical force may be a key determinant of the signaling output that results from the interaction. In a key *in vivo* study on aGPCR-mediated mechanosensation, Scholz et al. demonstrated that *Drosophila* larvae lacking the ADGRL1 ortholog lat-1 exhibited diminished sensitivity to mechanical stimuli [161]. The role of aGPCRs in sensing mechanical force is likely to be an active area of research in the coming years.

1.14 Adhesion G protein-coupled receptor associations with signaling proteins other than G proteins

In addition to the aforementioned examples of aGPCR coupling to G proteins, there have also been a number of cytoplasmic proteins other than G proteins that have been found to interact with aGPCRs. In some cases, these interactions appear to modulate G protein-mediated signaling, while in other cases these associations appear to mediate G protein-independent signaling. One example of the regulation of G protein signaling comes from work on ADGRV1, which was found to interact with the PDZ domain-containing protein PDZD7, a key scaffold protein in the USH2 protein complex that is known to be pivotal for stereocilial development and function [115]. Association with PDZD7 was found to antagonize ADGRV1 activity, likely by competitively disrupting receptor association with $G\alpha_i$ [115, 162]. ADGRB1 is another aGPCR that has been found to associate with PDZ scaffold proteins. One such PDZ protein, MAGI-3, was found to potentiate receptor-mediated ERK signaling, possibly by recruiting positive regulators of the pathway [112].

In terms of G protein-independent signaling by aGPCRs, ADGRB1 and ADGRB3 have both been shown to bind to the intracellular adaptor protein ELMO1 [105, 163]. For ADGRB1, this interaction has been demonstrated to result in the formation of a complex at the plasma membrane capable of activating the small GTPase Rac1 in a G protein-independent manner [105]. ADGRB1-mediated activation of Rac1 has been implicated in phagocytosis and myoblast fusion [105, 109]. Intriguingly, ADGRB1 can also activate Rac in a distinct G protein-independent manner through association with the RacGEF Tiam1 [110]. Other examples of G protein-independent signaling by aGPCRs include ADGRB2 interaction with GA-binding protein (GABP) gamma to regulate VEGF expression [164], ADGRC1 association with

dishevelled, DAAM1 and PDZ-RhoGEF to regulate neural tube closure [165], and ADGRA3 (GPR125) interaction with dishevelled to mediate the recruitment of planar cell polarity components [166].

1.15 Dissertation Aims

The aims of this dissertation work have been to elucidate the molecular determinants of G1 signaling activity and regulation. In the studies described in Chapter 2, I investigated whether the cryptic agonist model of activation applied to G1. To this end, I created a stalk-less version of the receptor and found that the stalk is essential for some but not all signaling outputs downstream of the receptor. In particular, the stalk was necessary for receptor-mediated activation of serum-response factor (SRF), a well-characterized output of $G\alpha_{12/13}$-coupled GPCRs, but not for activation of nuclear factor of activation T-cells (NFAT). Moreover, receptor-mediated activation of NFAT appeared to only be possible if the NTF was absent. These findings prompted us to formulate a more nuanced model of aGPCR activation: the allosteric antagonist model. In the allosteric antagonist model, the tethered NTF antagonizes multi-modal receptor activity in two distinct ways: by masking the stalk to indirectly antagonize stalk-dependent receptor signaling and by directly inhibiting the constitutive stalk-independent activity of the CTF protomer.

Chapter 3 describes investigations of the G1 extracellular loops (ECLs) as essential structural determinants of receptor function and regulation. The approach taken was to look at two disease-causing mutations that occur on the receptor's ECLs: R565W & L640R. I characterized the effect of these two mutations on both the heterodimeric full-length and the constitutively active ΔNT receptors. I found that the mutations reduced surface expression of the full-length receptor but not the ΔNT receptor, hinting at hitherto unknown NTF-ECL

interactions. The mutations also had differential effects on receptor signaling—disrupting receptor activation of SRF luciferase but not NFAT luciferase. These findings implicate the ECLs as essential structural determinants controlling receptor signaling. Finally, through a series of inhibitor studies, I determined that G1-mediated NFAT signaling is mediated through Gβγ liberation and downstream activation of calcium channels.

Table 1: Comprehensive List of Adhesion GPCR Ligands.

In each row of this comprehensive table a proposed ligand, ligand binding region and brief description of the supporting experimental data along with corresponding reference(s) are provided for each listed Adhesion GPCR member. Moreover, the listed Adhesion GPCR members are organized by subfamily.

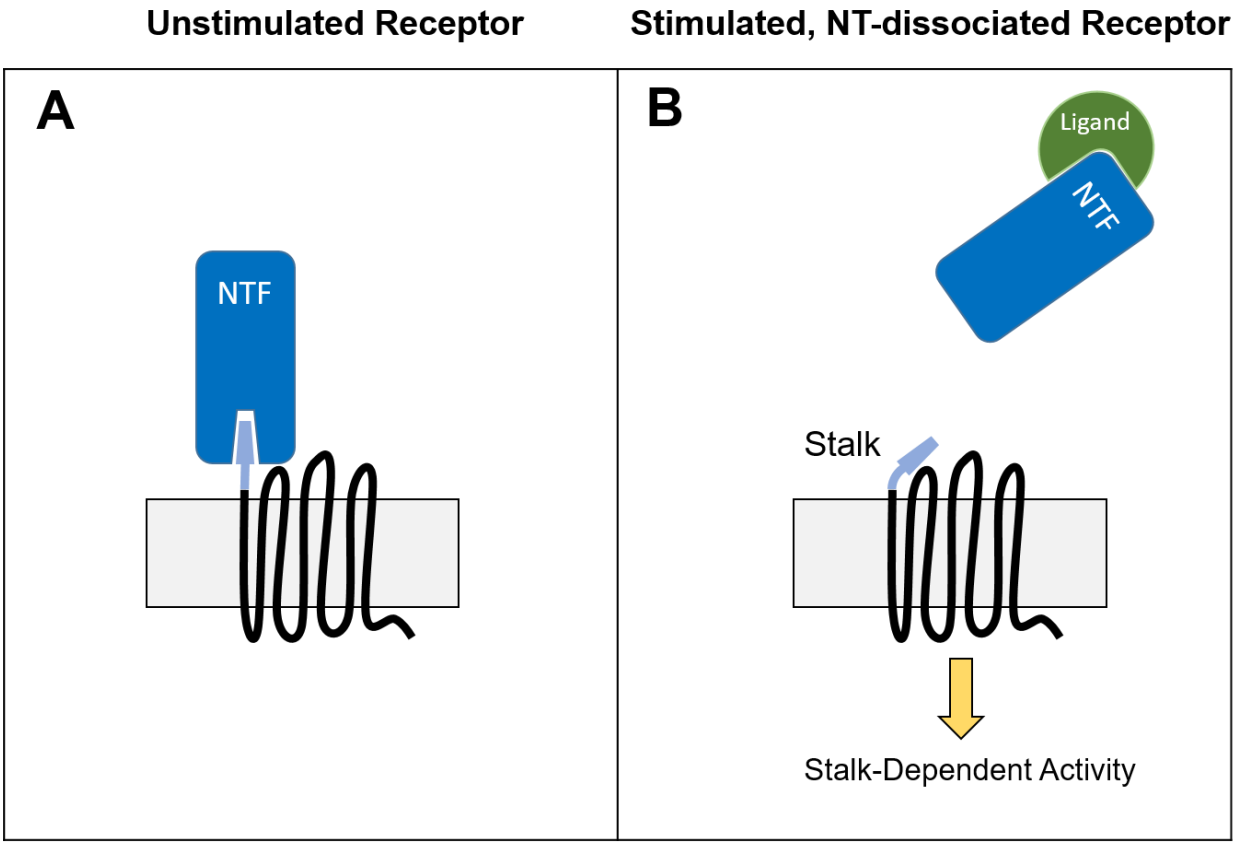
Table 1. Adhesion GPCR Ligands

Receptor	Ligand	Binding region	Downstream Activity
Family I			
ADGRL1	α-latrotoxin	NT (GAIN domain)	Increased cAMP [82], IP3 [82], Ca^{2+} [84] and PLC activation [84]
ADGRL1	teneurin-2	NT	Increased Ca^{2+} in cultured hippocampal neurons [117]
ADGRL1	neurexin1α	NT	Regulation of α-latrotoxin-mediated glutamate release [121]
ADGRL3	FLRT3	NT	Regulation of synaptic density [123]
ADGRL3	FLRT2	NT (OLF domain)	Regulation of cell adhesion/repulsion [124]
Family II			
ADGRE2	NT antibody (2A1)	NT	Increased production of inflammatory cytokines [141]
ADGRE5	CD55	NT (EGF domains)	Alteration in ADGRE5 NT-CTF interaction [138]
Family V			
ADGRD1	Stalk peptide(s)	? (likely 7TM region)	Increased cAMP levels [87]
Family VI			
ADGRF1	Stalk peptide(s)	? (likely 7TM region)	Increased GTPγS binding [94]
Family VII			
ADGRB1	Phosphatidylserine	NT (TSR domains)	Enhanced Rac1-dependent uptake of apoptotic cells [106]
ADGRB3	C1ql1	NT (CUB domain)	Regulation of dendritic spine density [127]
ADGRB3	C1ql3	NT (TSR domains)	Regulation of synaptic density [125]
Family VIII			
ADGRG1	Tissue transglutaminase 2	NT (STP region)	Regulation of VEGF secretion [97]
ADGRG1	Collagen III	NT (aa 27-160)	Stimulation of RhoA activation [132]
ADGRG1	NT antibody	NT	Stimulation of SRE and RhoA activity [90]
ADGRG1	Stalk peptide(s)	? (likely 7TM region)	Stimulation of SRE luciferase [94]
ADGRG2	Stalk peptide(s)	? (likely 7TM region)	Increased cAMP and IP3 accumulation [101]
ADGRG3	Beclomethasone dipropionate	?	Increased GTPγS binding [116]
ADGRG5	Stalk peptide(s)	? (likely 7TM region)	Increased cAMP levels [160]
ADGRG6	Collagen IV	NT (CUB and PTX domains)	Increased cAMP levels [103]

ADGRG6	Laminin-211	NT (aa 446-807)	Increased cAMP levels upon mechanical shaking [134]
ADGRG6	Stalk peptide(s)	? (likely 7TM region)	Increased cAMP levels [87]
ADGRG6	PrP^{C}	?	Increased cAMP levels [135]

Figure 2: A schematic describing the Tethered Cryptic Agonist Model of Adhesion GPCR Activation. *A*, according to this model, the unstimulated receptor is inactive due to the masking of an agonistic region of the stalk by the NTF. *B*, following ligand binding to the NTF, the NTF is released from the seven-transmembrane CTF to unveil a new N-terminal stalk, which then stimulates G protein-dependent signaling activity.

Tethered Cryptic Agonist Model



CHAPTER 2: Stalk-dependent and Stalk-independent Signaling by ADGRG1[2]

[2] Portions of this chapter have been adapted from 167. Kishore, A., et al., *Stalk-dependent and Stalk-independent Signaling by the Adhesion G Protein-coupled Receptors GPR56 (ADGRG1) and BAI1 (ADGRB1).* J Biol Chem, 2016. **291**(7): p. 3385-94.

2.1 Introduction

As described in Chapter 1 nearly all aGPCRs have an N-terminal juxtamembrane GPCR Autoproteolysis-Inducing (GAIN) domain, which can cleave the receptor into two non-covalently associated protomers [76]. N-terminal cleavage is thought to be a critical activation step because a number of groups have reported that aGPCR truncated mutants that mimic post-cleavage receptors exhibit enhanced constitutive activity; these include ADGRB1/BAI1 [112], ADGRB2/BAI2 [113], ADGRD1/GPR133 [87], ADGRE5/CD97 [114], ADGRF1/GPR110 [94], G1/GPR56 [91, 94], ADGRG2/GPR64 [100, 101], ADGRG6/GPR126 [103] and ADGRV1/VLGR1 [115]. These data prompted the proposal of a disinhibition model of aGPCR activation. In this model, the N-terminal fragment (NTF) inhibits the constitutive signaling ability of the 7TM protomer (also known as the C-terminal fragment or CTF) until the NTF is engaged by a large extracellular ligand, which results in a conformational change and/or removal of the NTF to relieve inhibition and unleash maximal receptor activity [91].

The disinhibition model is a general model that leaves open the mechanistic question of precisely how aGPCR NTF regions inhibit receptor signaling. At least two more mechanistically specific models have been discussed, one in which the NTF acts as a tethered antagonist to suppress signaling by the CTF and another model in which the NTF lacks antagonist activity *per se* but instead masks a cryptic agonist that becomes unveiled upon cleavage and removal of the NTF (Fig. 2) [73]. Several recent reports have provided evidence in support of the cryptic agonist model [87, 94, 101]. Liebscher and colleagues found that peptides mimicking the remaining post-cleavage NT stalk (also known as the "*stachel*") can activate ADGRG6/GPR126, ADGRD1/GPR133 and ADGRG2/GPR64 [87, 101]. Similarly, Stoveken *et al.* demonstrated that ADGRF1/GPR110 and G1/GPR56 can also be activated by stalk-mimetic peptides [94].

In the studies described here, we performed a series of tests of the cryptic agonist model for G1. To explore the importance of the stalk region for receptor signaling, we took two approaches. First, since the cryptic agonist model is largely dependent on GAIN domain cleavage, we engineered a cleavage-deficient form of G1 by mutating the catalytic threonine (Thr-383) to alanine [76]. Second, we created a mutant form of G1 that lacks almost the entire NT, including the stalk region. According to the cryptic agonist model, this deletion should render the receptor completely inactive due to a lack of the tethered agonist that is necessary for receptor activation. Since most if not all GPCRs can couple to multiple downstream pathways that may be differentially activated by distinct receptor active conformations [168], the signaling activities of the G1 stalkless mutant was assessed in a battery of different assays to provide a panoramic view of the importance of the stalk region for receptor signaling.

2.2 Experimental Procedures

Constructs

Human ADGRG1ΔNT (383–693) and ADGRG1-SL (404–693) were subcloned into pcDNA3.1 between 5′ HindIII (G1ΔNT: GCA AAG AAG CTT ATG ACC TAC TTT GCA GTG CTG ATG; G1-SL: GCA AAG AAG CTT ATG AGC CTC CTC TCC TAC GTG GG) and 3′ XbaI (GCA AAG TCT AGA CTA GAT GCG GCT GGA CGA GGT).

FLAG-βarrestin2 was purchased from Addgene, the RGS domain of p115RhoGEF (RGSp115) was a gift from Tohru Kozasa (Univ. of Illinois Chicago), and HA-ubiquitin was kindly provided by Keqiang Ye (Emory University). These constructs have been described previously [112]. Internal EE-tagged $G\alpha_{13}$ was acquired from the cDNA Resource Center (cdna.org).

Cell Culture

HEK-293T/17 cells were acquired from ATCC (Manassas, VA) and maintained in DMEM (Life Technologies) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a humid, 5% CO_2, 37 °C incubator. Cells were transfected using Mirus (Madison, WI) TransIT-LT1 according to the manufacturer's protocol.

Western Blot

Protein samples were reduced and denatured in Laemmli buffer, loaded into 4–20% Tris-Glycine gels (Bio-Rad) for SDS-PAGE, and then transferred to nitrocellulose membranes (Bio-Rad). Blots were blocked with 5% milk (in 50 mM NaCl, 10 mM HEPES, pH 7.3 with 1% Tween-20 (Sigma) and incubated with primary antibodies for 1 h at room temperature or overnight at 4 °C. The anti-ADGRG1 C-terminal antibody was developed by Orbigen, Inc. via injection of rabbits

with a peptide (CSNSDSARLPISSGSTSSSRI) derived from the ADGRG1 C terminus, and has been characterized previously [91]. The biotinylated anti-ADGRG1 N-terminal antibody was purchased from R&D Systems. Rat anti-HA (Roche), mouse HRP-conjugated anti-FLAG (Sigma), and mouse anti-Glu Glu (Abcam) antibodies were used to detect epitope-tagged proteins. HRP-conjugated secondary antibodies were purchased from GE Healthcare and antibody labeling of specific bands was visualized using Thermo Scientific SuperSignal West solutions.

Cell Surface Biotinylation

HEK-293T cells were transfected with 2 μg of DNA (empty vector or receptor). At 24-h post-transfection, cells were placed on ice and washed with ice-cold PBS+Ca^{2+} three times. Cells were then incubated with 10 mM Sulfo-NHS-Biotin (Thermo Scientific) in PBS+Ca^{2+} on ice for 30 min and then washed three more times with PBS+Ca^{2+} + 100 mM glycine. Cells were resuspended in 250 μl of lysis buffer (1% Triton X-100, 25 mMHEPES, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, protease inhibitor mixture (Roche Diagnostics), and 2% glycerol) and lysed by slowly rotating on a spinning wheel for 30 min at 4 °C. Cell debris was cleared by centrifugation, and soluble cell lysates were incubated with 50 μl of streptavidin agarose beads (Thermo Scientific) for 1 h at 4 °C. Beads were washed three times with lysis buffer and resuspended in 60 μl of Laemmli buffer. Biotinylated proteins were detected via Western blot, as described above.

β-Arrestin Binding Assay

HEK-293T cells were transfected with a total of 6 μg of DNA (empty vector, receptor, FLAG-βArr2). The next day, cells were washed with cold PBS+Ca^{2+} and lysed in harvest buffer (150 mM NaCl, 25 mM HEPES pH 7.3, 1 mM EDTA, 10 mM $MgCl_2$, 1% Triton X-100, Roche EDTA-free complete protease inhibitor mixture tablet). Lysates were rotated at 4 °C for 45 min to solubilize integral membrane proteins and membranes were cleared by centrifugation (15 min at 17,000 × *g*, 4 °C). Solubilizates were added to magnetic anti-FLAG beads (Sigma) and rotated at 4 °C for 1 h. Beads were washed 3× in harvest buffer and proteins were eluted in Laemmli buffer at 37 °C for 10–15 min and loaded in 4–20% Tris-glycine gels for SDS-PAGE and Western blotting. Western blot bands were quantified using Image Studio software (Licor, Lincoln, NE).

G Protein Co-immunoprecipitation

HEK-293T cells were transfected with 1 μg of EE-tagged $G\alpha_{13}$ and 1–4 μg of receptor DNA). EE-tagged G proteins were immunoprecipitated with anti-EE antibody (1:200, Abcam) and protein A/G beads (Thermo) as described above. Beads were washed 3×, and proteins were eluted in 2× Laemmli buffer.

Ubiquitination Assays

HEK-293T cells were plated and transfected as described above with 3 μg of receptor and 1 μg of HA-ubiquitin DNA. Four hours after transfection, cells were treated with 100 nM MG-132 (Tocris) to inhibit the proteasome overnight. The following day, cells were washed and harvested

as described above. Cleared lysates were incubated with anti-HA agarose beads (Sigma) for 1 h, washed, and eluted in Laemmli buffer.

Luciferase Reporter Assays

HEK-293T cells were seeded in 96-well plates 20–24 h prior to transfection. Each well was transfected with 50 ng of firefly reporter, 1 ng of *Renilla* luciferase, and 10 ng of receptor or mock DNA. All reporter constructs (NFAT: pGL4.30, SRF: pGL4.34, *Renilla* pRLSV40) were acquired from Promega (Madison, WI). 24–48 h later Dual-Glo luciferase assays (Promega) were performed according to the manufacturer's protocol and plates were read on either a Biotek Synergy 3 or BMG Omega plate reader. Results were calculated for each assay by determining the luminescence ratio of firefly:*Renilla* luciferase counts, normalized to empty vector (EV) transfected wells. Error bars for all EV-transfected conditions were represented as the standard errors of the normalized raw value means.

AP-TGFα-shedding Assays

HEK-293T cells were seeded in 96-well plates 20–24 h prior to transfection. Each well was transfected with 50 ng of AP-TGFα plasmid (kindly provided by Shigeki Higashiyama, Ehime University) and 10 ng of receptor or mock DNA. Twenty-four hours later, the plate was incubated with *p*-nitrophenyl phosphate (New England BioLabs) and read on either a Biotek Synergy 3 or BMG Omega plate reader as per the protocol described by Inoue *et al.* [169].

2.3 Results

2.3.1 ADGRG1 autoproteolysis is not necessary for signaling activity

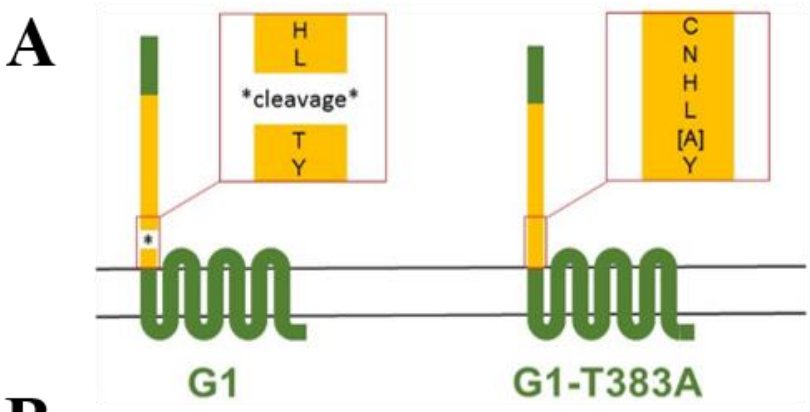
According to the cryptic agonist model, signaling activity depends upon efficient GAIN domain cleavage followed by dissociation of the NTF to unveil the agonistic peptide found on the remaining N-terminal stalk of the 7TM protomer or CTF. Thus, we tested whether GAIN domain cleavage was indeed necessary for G1 basal constitutive activity.

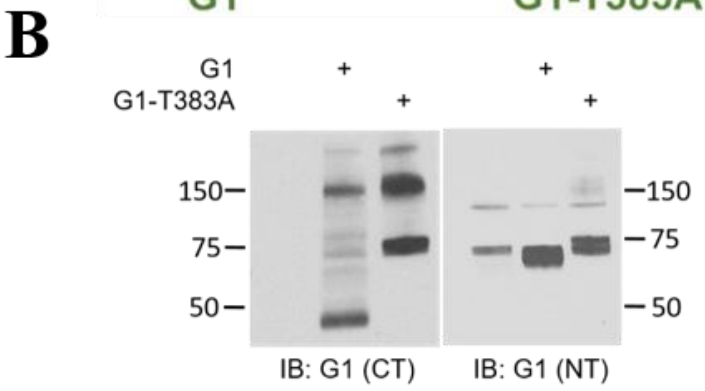
As G1 is efficiently cleaved in transfected HEK-293T cells [91], we introduced a point mutation to the G1 GAIN domain at the site of cleavage (T383A) to create a cleavage-deficient version of the receptor (Fig. 3A). Human G1 mutations (C346S and W349S) that abrogate GAIN domain cleavage have been shown to result in the devastating neurological condition bilateral frontoparietal polymicrogyria, which led to speculation that autoproteolysis may be necessary for proper aGPCR function [152, 153]. However, more recent crystallographic studies provided insights as to how GAIN domain cleavage can be abrogated without inducing misfolding of the GAIN domain [76]. Using a cell surface biotinylation approach, we found that indeed G1-T383A (T383A) traffics to the plasma membrane, albeit at a somewhat reduced level compared with the wild-type receptor (Fig. 3B). To validate whether T383A is indeed cleavage-deficient, we probed for expression of the mutant receptor in Western blots using both CT- and NT-specific antibodies (Fig. 3C). The left panel of Fig. 3C displays the expression patterns of the wild-type and T383A receptors using a C-terminal specific antibody. For wild-type G1, there is a prominent band at ~45 kDa, which represents the monomeric, cleaved 7TM protomer. As expected, the T383A mutant lacks the ~45 kDa band and instead displays a prominent band at ~75 kDa, which is the predicted molecular weight of full-length, uncleaved G1. Higher order bands for either the wild-

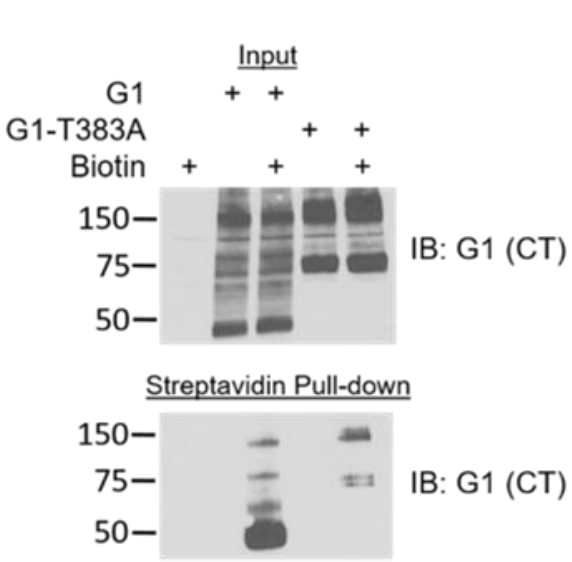
type or T383A receptors are likely to be unresolved, oligomeric complexes. The right hand panel of Fig. 3C displays the expression patterns of both receptors as detected by an N-terminal specific antibody. Here bands are found at ~70 kDa and ~75 kDa for the wild-type receptor and T383A, respectively. The ~75 kDa band of T383A is both C-terminally and N-terminally reactive, providing strong evidence that the point mutation does indeed abrogate cleavage to result in a single, uncleaved protein.

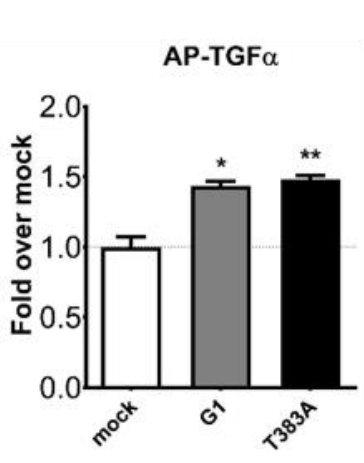
Figure 3: GAIN domain cleavage is not necessary for G1 activity.

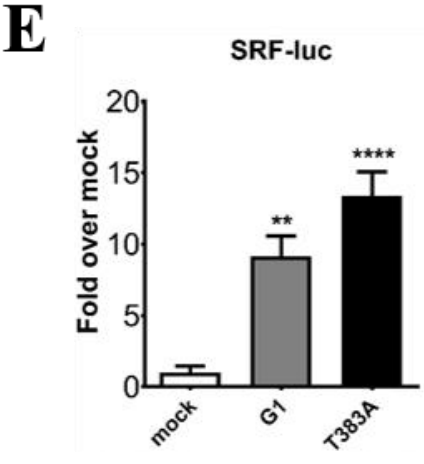
A, schematic of T383A point mutation in G1. *B*, G1-T383A is expressed on HEK cell surface, albeit at a reduced level compared with the wild-type receptor. Molecular weight markers (in kDa) are shown on the left side of the blots. *C*, Western blots of G1 and G1-T383A reveal a ~75 kDa band for G1-T383A that is both N-terminally and C-terminally reactive, suggesting non-cleavage of the mutant receptor. Equal amounts of protein (10-20 ug) were loaded in each lane for the blot shown in panels *B* and *C*, and these experiments were performed 3-4 times each. *D* and *E*, G1 and G1-T383A produce comparable activity in the AP-TGFα shedding and SRF-luciferase assays. Results for TGFα and SRF-luc are from 3-6 independent experiments (± S.E. shown, *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ versus cells transfected with a mock vector).


A
H
L
cleavage
T
Y
C
N
H
L
[A]
Y
*
G1
G1-T383A


B
G1
G1-T383A
150
75
50
150
75
50
IB: G1 (CT)
IB: G1 (NT)


C
Input
G1
G1-T383A
Biotin
150
75
50
IB: G1 (CT)
Streptavidin Pull-down
150
75
50
IB: G1 (CT)


D
AP-TGFα
Fold over mock
2.0
1.5
1.0
0.5
0.0
*
**
mock
G1
T383A


E
SRF-luc
Fold over mock
20
15
10
5
0
**
****
mock
G1
T383A

We next assessed the basal constitutive activity of the T383A mutant using two distinct downstream readouts: TGFα shedding and activation of SRF luciferase. G1 has previously been reported to couple to $G\alpha_{12/13}$ [90, 91, 94], and GPCRs that activate $G\alpha_q$- or $G\alpha_{12/13}$-mediated pathways stimulate the ectodomain shedding of TGFα from the plasma membrane, with the amount of TGFα released into the conditioned media serving as a proxy for receptor activity [169]. We observed that wild-type G1 and the T383A mutant displayed an equal level of activity in the TGFα shedding assay (Fig. 3D). Next, we compared the activities of wild-type G1 and T383A in a serum response factor (SRF)-luciferase reporter assay, another well-described readout for $G\alpha_{12/13}$-coupled receptors that has previously been shown to be activated by G1 [93]. In agreement with our TGFα shedding data, the T383A mutant mediated an approximately equal level of signaling activity to wild-type G1 in the SRF-luciferase assay (Fig. 3E). Taken together, these data provide evidence that GAIN domain cleavage is not necessary for activity, at least for G1.

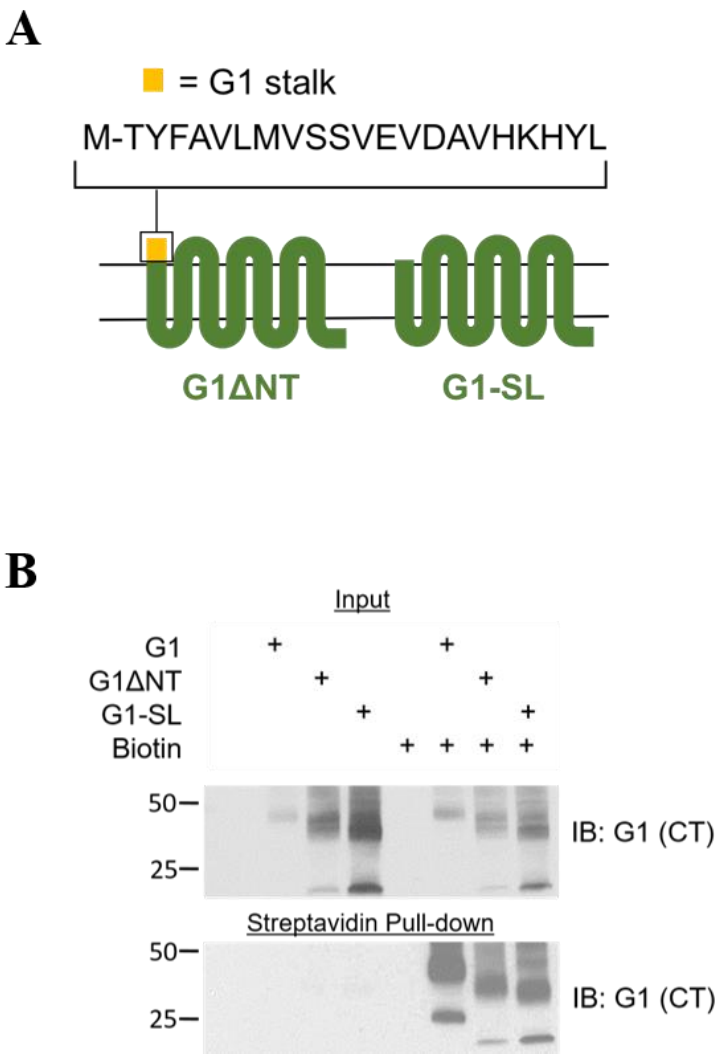
2.3.2 Stalk-less ADGRG1 retains activity in some signaling assays but not others

It is plausible that an agonistic peptide region of the stalk could still be important for activation of aGPCR signaling even in the absence of GAIN autoproteolysis, as cleavage-independent conformational changes that expose the cryptic agonist could conceivably be responsible for receptor activity. Therefore, to definitively answer the question of whether the stalk does indeed contain a requisite agonist for G1 signaling, we created a mutant version of the receptor that lacks the entire NT (including the stalk) such that the mutant protein begins very close to the start of the predicted first transmembrane domain (Fig. 4A). The stalkless ("SL") mutant receptor expressed and was trafficked to the plasma membrane at levels comparable to

ΔNT version of G1 (Fig. 4B), revealing that removal of the stalk region did not result in impairments in receptor expression or trafficking.

Figure 4: Generation of the G1 Stalk-less Receptor.

A, schematic of G1-SL alongside ΔNT counterpart. *B*, SL mutant exhibits comparable surface expression in HEK cells to ΔNT. Molecular weight markers (in kDa) are shown on the left side of the blots. For G1, prominent C-terminally reactive bands between ~40-45 kDa (for full-length G1) and ~20 kDa (for ΔNT and SL) likely represent further cleaved forms of the protein and/or differential conformations. Equal amounts of protein (10-20 ug) were loaded in each lane for the blots shown here, and the data shown in this figure are representative of 3-4 experiments for each pair of mutants.

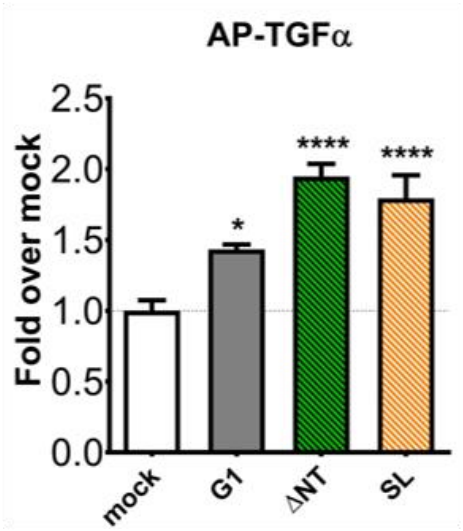


It has previously been reported that deletion of the NT up to the site of GAIN cleavage results in significant increases in the constitutive activity of aGPCRs, including G1 [91, 94, 112]. According to the cryptic agonist model, further NT deletions that remove some or all of the stalk region should render the receptors inactive. To assess the activity of G1-SL, we utilized a battery of signaling assays. In the TGFα shedding assay, G1-SL mediated significant signaling compared with mock-transfected cells (Fig. 5A). The results were different, though, in the SRF-luciferase assay, in which G1ΔNT exhibited robust activity but G1-SL did not (Fig. 5B). Given these contrasting results, we measured receptor activity using a third signaling output: transcription of the nuclear factor of activated T-cells (NFAT), a readout that has been shown to be downstream of some $G\alpha_{12/13}$-coupled receptors [93, 170] including G1 [93]. We found that G1ΔNT signals strongly in the NFAT-luciferase assay but the full-length receptor does not (Fig. 5C). Interestingly, we found that G1-SL also strongly activated the NFAT pathway, achieving an extent of activation comparable to G1ΔNT (Fig. 5C). This signaling was sensitive to inhibition by the broad-spectrum $G_{\beta\gamma}$ inhibitor gallein [171] (Fig. 6C), demonstrating that it is significantly mediated by heterotrimeric G proteins. Another interesting observation was that while co-expression of the RGS domain of p115RhoGEF completely blocked SRF signaling by both G1 and G1ΔNT by >90%, it was only able to inhibit G1ΔNT and G1-SL signaling to NFAT by 64% and 51%, respectively (Fig. 6A-B). This may indicate a G protein-independent component of the receptor-mediated NFAT signal.

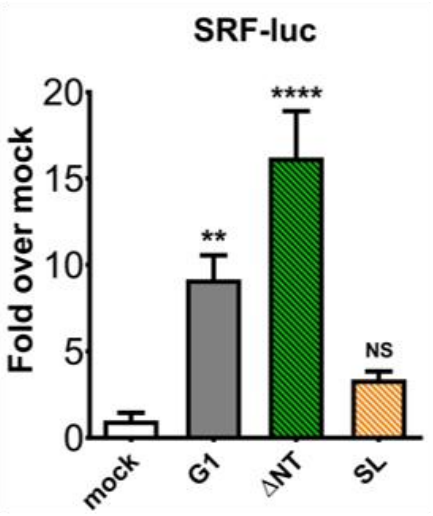
Figure 5: G1-SL exhibits differential levels of signaling activity in distinct assays.

G1-SL exhibited significant signaling activity in the TGFα-shedding (*A*) and NFAT luciferase (*C*) assays but was found to not be significantly active in the SRF-luciferase assay (*B*). All experiments performed in HEK cells. TGFα, SRF-luc, and NFAT-luc results are from 4-6 independent experiments (± S.E. shown, * $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$ versus cells transfected with a mock vector).

A


AP-TGFα
Fold over mock
2.5
2.0
1.5
1.0
0.5
0.0
*
****
****
mock
G1
ΔNT
SL

B


SRF-luc
Fold over mock
20
15
10
5
0
**
****
NS
mock
G1
ΔNT
SL

C

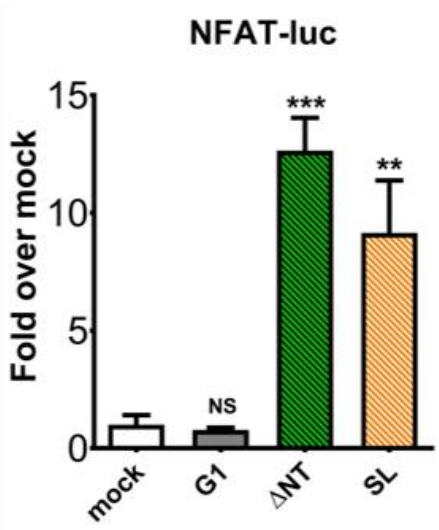
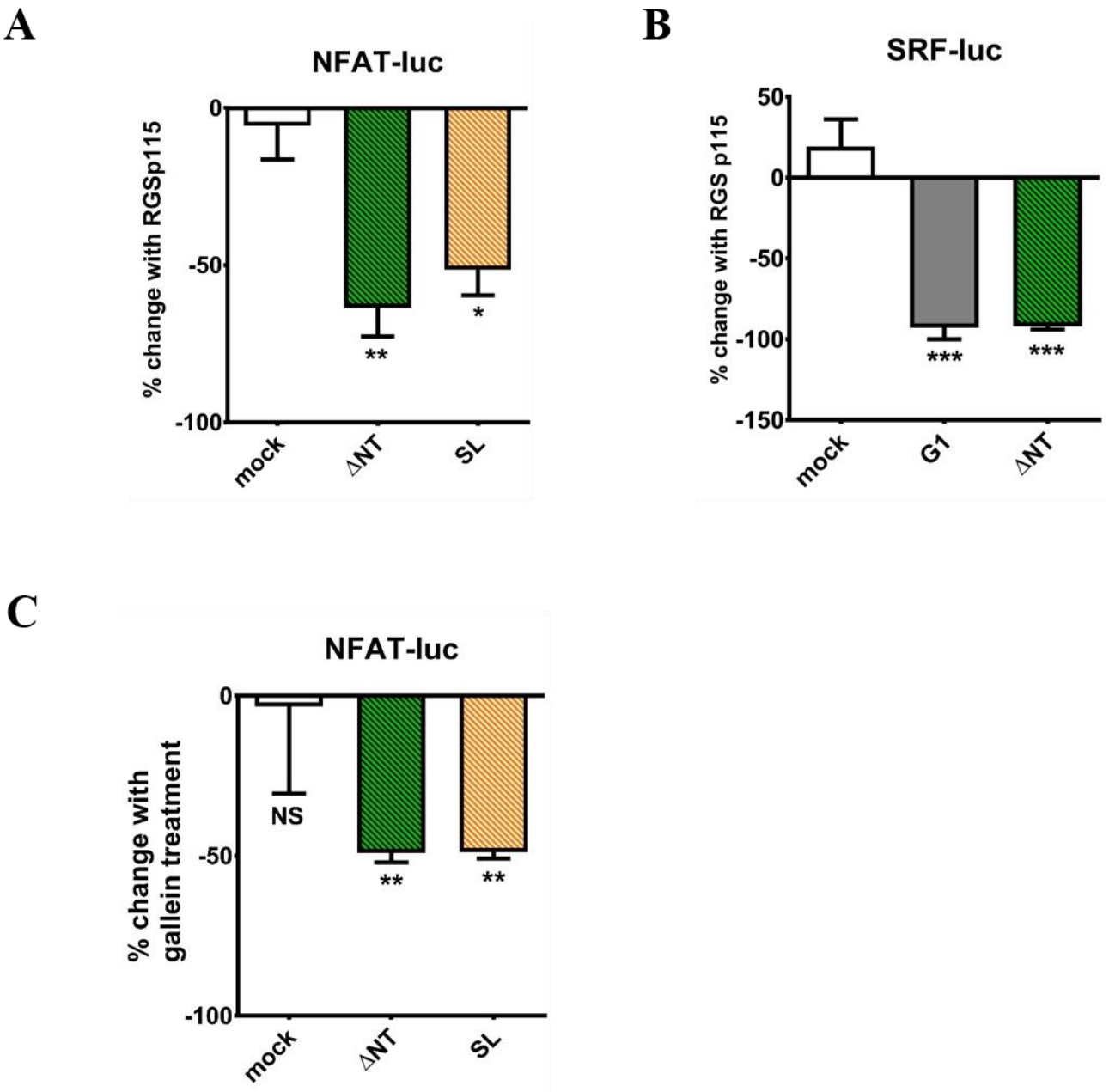

NFAT-luc
Fold over mock
15
10
5
0
NS
***
**
mock
G1
ΔNT
SL

Figure 6: Inhibitors of Gα$_{12/13}$ and Gβγ block ΔNT and SL signaling activity.

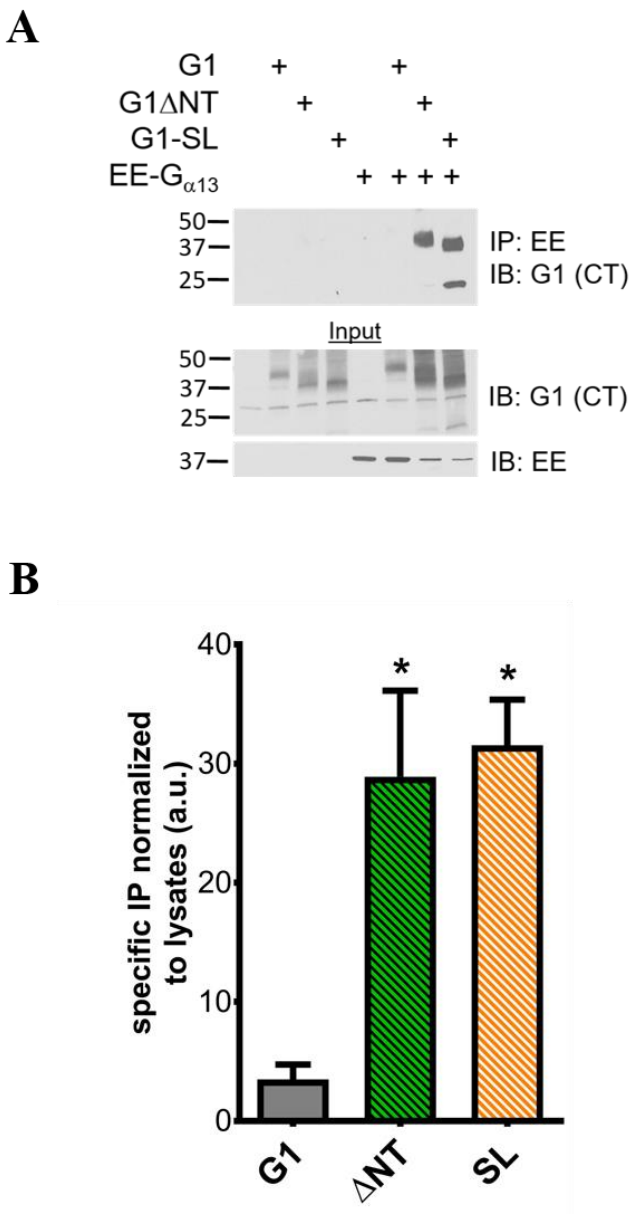
A & *C*, the RGS domain of p115RhoGEF, a Gα$_{12/13}$ inhibitor, as well as the Gβγ inhibitor gallein, significantly blocked ΔNT and SL signaling to NFAT. *B*, the RGS domain of p115RhoGEF completely blocked G1 signaling to SRF. Results are from 3-6 independent experiments (±S.E. shown, *, p<0.05; **, p<0.01; ***, p<0.01 versus receptor control).



Finally, to further assess the abilities of the SL mutant to couple to heterotrimeric G proteins, we performed co-immunoprecipitation experiments with the receptor and co-transfected EE-tagged $G\alpha_{13}$. In these experiments, the tagged G protein was immunoprecipitated and Western blots were performed to detect any co-immunoprecipitated receptor. We found that the truncated ΔNT and SL forms strongly and significantly co-immunoprecipitated with $G\alpha_{13}$ but the full-length form of the receptor did not (Fig. 7A-B).

Figure 7: G1-SL couples to G proteins.

A & *B*, Western blots of co-immunoprecipitation experiments in HEK cells demonstrating that ΔNT and SL receptors robustly associate with Gα13 whereas the full-length receptor does not. Equal amounts of protein (10-20 ug) were loaded in each lane for the blots shown here, and the results shown are from 3 independent experiments (±S.E. shown, *, $p<0.05$ versus the full-length receptor).

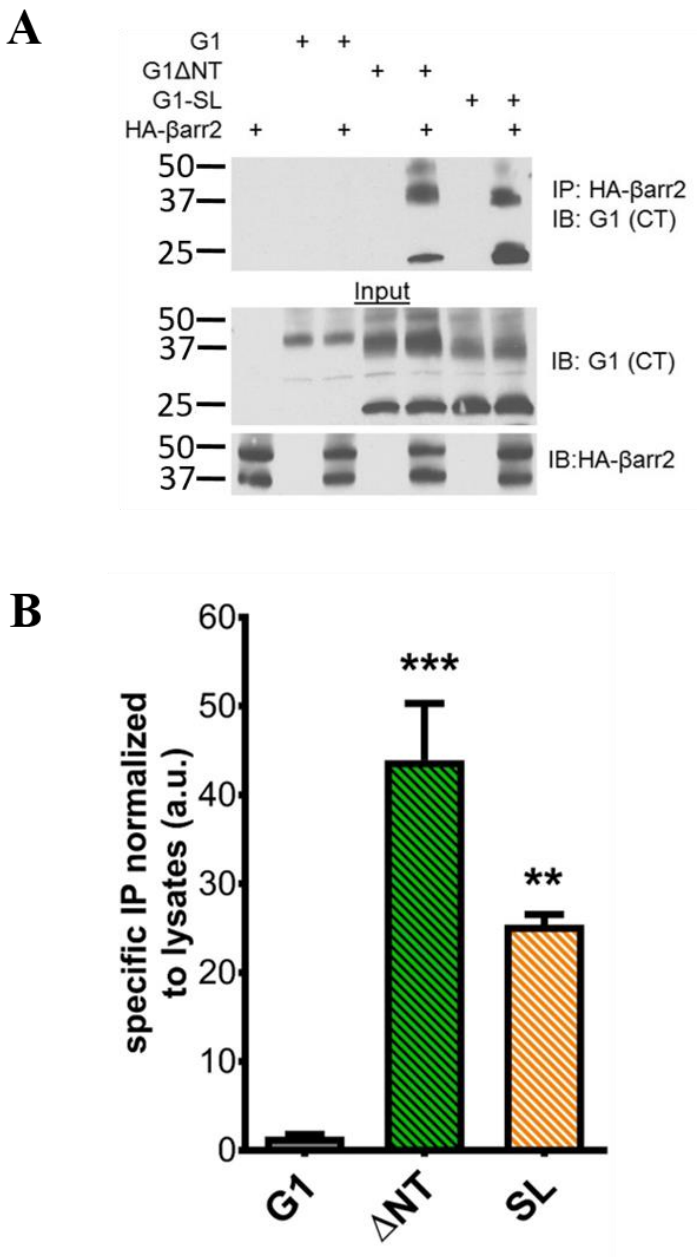


2.3.3 Stalk-less ADGRG1 exhibits robust β-Arrestin association and ubiquitination, two correlates of enhanced GPCR activity

To further assess the activity of the stalk-deficient receptor, we employed a β-arrestin2 recruitment assay. β-Arrestin recruitment is a classical hallmark of highly active GPCRs [172]. In support of its constitutively-active nature, ΔNT has been reported to strongly bind β-arrestin2 whereas its full-length counterpart does not [91, 112]. In the present study, we confirmed that ΔNT robustly co-immunoprecipitates with β-arrestin2, and additionally found that the SL mutant binds β-arrestin2 to a comparably robust extent as ΔNT (Fig. 8).

Figure 8: G1-SL binds robustly to βArrestin2.

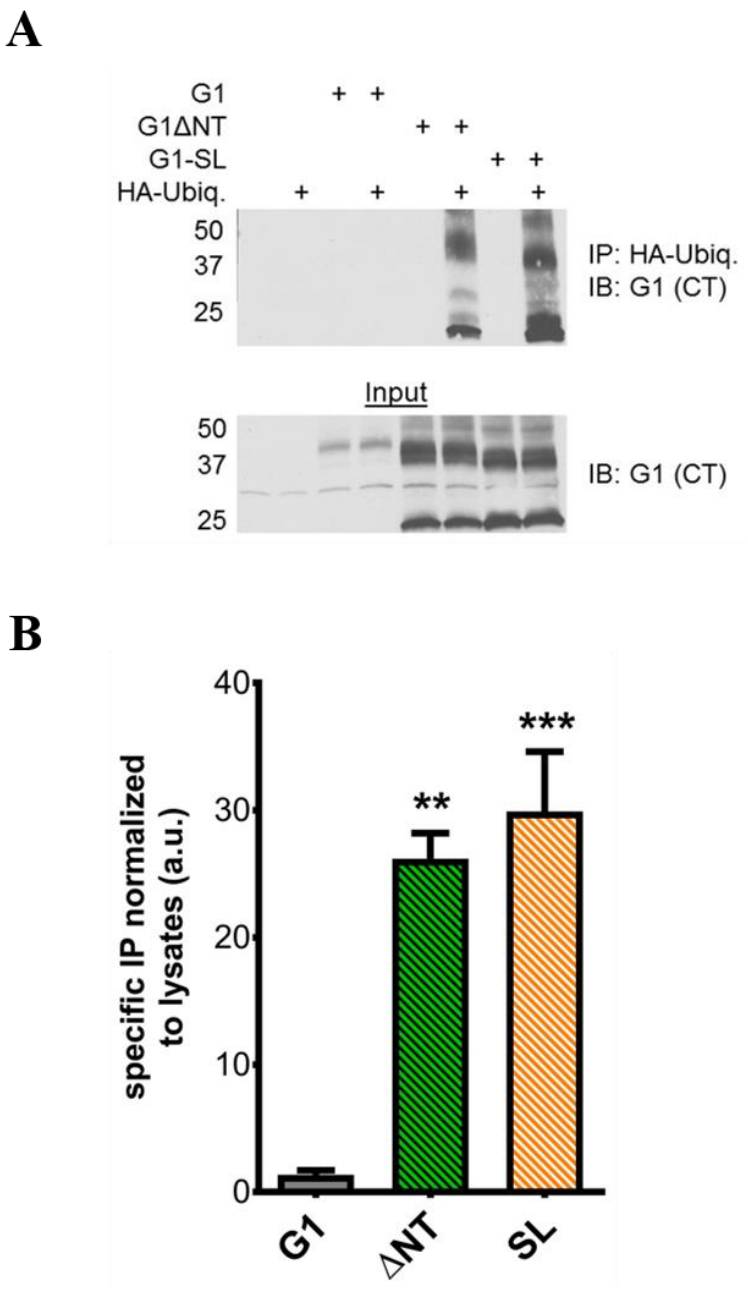
A & *B*, Western blots of co-immunoprecipitation experiments in HEK cells with HA-tagged βArrestin2 revealed that ΔNT and SL receptors bound to βArrestin2 significantly more than the full-length receptor. Equal amounts of protein (10-20 ug) were loaded in each lane for the blot shown here, and the results shown are from three independent experiments (±S.E. shown, *p<0.05; **, p<0.01; ***, p<0.001 versus the full-length receptor).



In addition to binding to β-arrestins, highly active GPCRs are often ubiquitinated prior to down-regulation and degradation [173]. As with β-arrestin association, ΔNT has previously been found to be heavily ubiquitinated whereas the full-length receptor is not [91]. As shown in Fig. 9, we found that SL is ubiquitinated to a similar extent as ΔNT, which provides further support for the idea that the SL mutant is a highly active receptor.

Figure 9: G1-SL is heavily ubiquitinated.

Western blots of co-immunoprecipitation experiments with HA-ubiquitin demonstrated that ΔNT and SL receptors were significantly more ubiquitinated than the full-length receptor. Equal amounts of protein (10-20 ug) were loaded in each lane for the blot shown here, and the results are from three independent experiments (± S.E. shown, *, p<0.05; **, p<0.01; ***, p<0.001 versus the full-length receptor).



2.4 Discussion

Substantial recent progress has been made in understanding the mechanisms of aGPCR activation. These mechanisms are important to understand, given the association of these receptors with several human diseases and the potential value of these receptors as drug targets [174]. Specifically, it has been found for a number of aGPCRs that truncation of the receptor N termini up to the point of predicted GAIN domain cleavage leads to increased constitutive activity [87, 91, 94, 100, 101, 103, 112-115]. These observations led to the proposal of the disinhibition model of aGPCR activation, which posits that the NTF exerts an inhibitory constraint on signaling by the CTF, with this inhibitory constraint being removed following engagement of the NTF with a large extracellular ligand that results in either dissociation of the NTF from the CTF and/or a conformational change that reduces NTF-mediated inhibition [158]. Subsequently, more mechanistically specific variations of the disinhibition model have been proposed, including the cryptic agonist model [87, 94, 101], wherein GAIN domain cleavage and NTF dissociation result in the unveiling of a cryptic agonist peptide on the post-cleavage stalk of the CTF in a manner analogous to protease-activated receptors [159].

In the studies described above, we tested the cryptic agonist model for G1 by deleting the stalk region and broadly assessing receptor activity using a variety of downstream outputs. Our results provide evidence that the stalk region is not a requisite agonist for the receptor, as we observed that deleting the stalk does not affect signaling to most pathways measured. In particular, removal of the stalk largely abrogated the receptor's ability to stimulate SRF luciferase but had little effect on the other readouts examined. Additionally, G1-SL retained the ability to robustly co-immunoprecipitate with $G\alpha_{13}$.

Stoveken *et al.* (2015) suggested that the NT stalk of G1 is necessary for signaling activity [94]. This study reported signaling data from SRE luciferase experiments in transfected cells and GTP loading experiments in a reconstitution system. Our data reported here are in agreement with the findings of Stoveken *et al.*, as we found that the activity of G1-SL was sharply reduced compared with G1ΔNT in the SRF luciferase assay, which is very similar to the SRE luciferase assay. However, in other assays in which we assessed G1 activity (TGFα shedding, NFAT luciferase, β-arrestin recruitment and receptor ubiquitination), we found G1-SL to be in an active conformation and capable of mediating receptor signaling to a similar extent as G1ΔNT. These results suggest that the stalk region of G1 is necessary for certain aspects of receptor signaling activity but dispensable for others.

There have been prior indications that the cryptic agonist model may represent an incomplete description of aGPCR activation. For example, studies on the *Caenorhabditis elegans* aGPCR lat-1 demonstrated that mutations blocking cleavage of the receptor's GAIN domain exerted no effect on the *in vivo* function of the receptor [175]. These *in vivo* data find a parallel in the *in vitro* findings reported here regarding the non-cleaving G1-T383A mutant, which we found to exhibit no change in signaling activity relative to wild-type G1. Similarly, a recent report from Peeters *et al.* demonstrated that non-cleavable versions of GPR64/ADGRG2 can still activate downstream signaling [100]. According to the cryptic agonist model, GAIN-mediated cleavage should be essential for exposure of the agonistic peptide sequence on the stalk region. Thus, observations that the activity of at least some aGPCRs is not modulated by GAIN cleavage obviously run counter to this model. Moreover, there is convincing evidence that some aGPCRs do not undergo GAIN-mediated cleavage [176], an observation that needs to be taken into account in general models of aGPCR activation.

Another challenge faced by the cryptic agonist model is the uncertainty surrounding how aGPCR NTF regions become dissociated from their cognate 7TM regions. In the cryptic agonist model, it is envisioned that the N-terminal portion of a cleaved GAIN domain can be released from the receptor's stalk region in a regulated manner, thereby exposing the agonistic stalk peptide sequence [87, 94, 101]. However, the first crystal structures of GAIN domains reported by Arac *et al.* have cast doubt on whether GAIN domains can actually exist as stable folded protein units in the absence of the hydrophobic stalk peptides [76]. Thus, while it is clear that aGPCR NTF regions can become dissociated from their cognate CTF regions, it is uncertain whether dissociated GAIN domains leave the stalk behind or take the stalk with them. Interestingly, studies on ADGRL1/CIRL/latrophilin-1 provided evidence that the release of this receptor NTF region is dependent on two proteolytic steps, with GAIN domain cleavage followed by a second cleavage event that cleaves the receptor's stalk region to release the GAIN domain and stalk together [177]. According to the cryptic agonist model, the resultant 7TM region of such a twice-cleaved aGPCR would be devoid of signaling activity, as the stalk region containing the agonistic peptide would have been lost with the second cleavage event. However, our studies presented here on the stalkless version of G1 demonstrates that stalkless receptors can still exert significant downstream signaling.

Further work will be needed to elucidate the differences in signaling intermediates that presumably exist between the stalk-dependent *versus* stalk-independent signaling activities observed in our studies. As shown above, the activation of NFAT luciferase by G1 was significantly blocked by the Gβγ inhibitor gallein and also blocked to a similar extent by the RGS domain of p115RhoGEF, which would be expected to attenuate signaling by $G\alpha_{12/13}$ as well as any Gβγ subunits released from activated $G\alpha_{12/13}$. Insofar as the SRF luciferase assay

represents a more pure readout of $G\alpha_{12/13}$ activity, the differential change in activity observed for G1-SL in the SRF *versus* NFAT luciferase assays may represent a difference in the relative importance of $G\alpha_{12/13}$- *versus* Gβγ-dependent pathways [17]. Yet another possibility is that β-arrestins may contribute to G1 signaling, with the presence of the stalk having little effect on β-arrestin-mediated signaling. Indeed, we found that the ΔNT and SL versions of G1 exhibited strong interactions with β-arrestin2. However, the specific contributions of the various G protein subunits and β-arrestins to signaling by G1 and other adhesion GPCRs will require further investigation to truly assess whether the stalk regions might confer bias toward certain receptor-initiated pathways and away from others [178].

CHAPTER 3: Disease-Associated Extracellular Loop Mutations Differentially Regulate Signaling Pathways Downstream of ADGRG1[3]

[3] Portions of this chapter adapted from 179. Kishore, A. and R.A. Hall, *Disease-Associated Extracellular Loop Mutations Differentially Regulate Signaling Pathways Downstream of ADGRG1 (GPR56).* Ibid.Manuscript submitted.

3.1 Introduction

ADGRG1 (G1; also known as GPR56) has been one of the most intensely studied aGPCRs, as mutations to G1 were shown more than a decade ago to underlie the human disease bilateral frontoparietal polymicrogyria (BFPP) [88]. Subsequent studies have revealed G1 to be involved in many diverse physiological processes including neurodevelopment [88, 90, 180, 181], myelination [182], tumorigenesis [92, 97, 130, 145], pancreatic function [183], immune function [184, 185], muscle hypertrophy [93, 186] and hematopoietic stem cell maintenance [187]. To date, there are more than two dozen distinct BFPP-causing mutations [150]. While most BFPP-causing missense mutations to G1 occur on the NTF, at least 5 disease-associated missense mutations have been found to occur on the CTF: C418W, S485P, E496K, R565W and L640R [88, 150, 188]. In terms of functional effects, the last of those mutations (L640R) was found to ablate G1-mediated activation of RhoA following stimulation with the G1-interacting protein collagen III [154].

As discussed previously, the activation mechanisms of aGPCRs have garnered much attention in recent years [1, 77, 174]. Studies by several groups have delineated a model of activation termed the cryptic agonist model, wherein dissociation of the NTF from the membrane-embedded CTF unveils the agonistic properties of the remaining extracellular stalk (also termed the ‘stachel’) [87, 94, 101, 160].

In the data shown in Chapter 2, we investigated this model for G1 and found that the stalk was indeed essential for some but not all signaling outputs [167]. Moreover, for other aGPCRS, such as ADGRB1 (BAI1), the presence of the extracellular stalk does not appear to matter at all for receptor signaling activity [167]. These findings led us to posit that aGPCRs may be capable of at least two distinct modes of signaling activity: stalk-dependent and stalk-independent. For

G1, the stalk was found to be required for activation of serum response factor (SRF) luciferase, a traditional measure of activity for $G\alpha_{12/13}$-coupled receptors [189]. The stalk-independent activation of nuclear factor of activated T cells (NFAT) luciferase, however, was found in those studies to rely on both G protein-dependent and -independent components and has not been clearly defined in terms of the relevant signaling cascade.

In the present study, we investigated the effects of two BFPP-causing mutations, R565W & L640R, on receptor surface expression and signaling. These studies were performed on both full-length G1 and the ΔNT truncated receptor that mimics the cleaved, active receptor. Moreover, the signaling studies assessed both stalk-dependent and stalk-independent signaling activity. The results of these studies have provided new insights into the regulation of G1 signaling and the mechanisms by which these mutations cause human disease.

3.2 Experimental Procedures

Constructs

Human G1ΔNT, G1-SL and FLAG- and HA-βarrestin2 constructs have been previously described [167].

R565W mutant receptors were generated using the following primers:

5'-CCATGTGCTGGATCTGGGACTCCCTGGTC-3'

5'-GACCAGGGAGTCCCAGATCCAGCACATGG-3'

L640R mutant receptors were generated using the following primers:

5'-TGATGCTGAAAAGGTAGCGGACGACAAGCTGGAAG-3'

5'-CTTCCAGCTTGTCGTCCGCTACCTTTTCAGCATCA-3'

S690A mutant receptors were generated using the following primers:

5'-AGATGCGGCTGGCCGAGGTGCTGCC-3'

5'-GGCAGCACCTCGGCCAGCCGCATCT-3'

All mutant receptors were generated using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent; Cat # 210519) according to the manufacturer's protocol.

Reagents

SKF 96365 was purchased from Cayman Chemicals (Item: 10009312) and U73122 was purchased from Tocris Biosciences (Cat #1268). All other general reagents were from Sigma.

Cell Culture

HEK-293T/17 cells were acquired from ATCC (Manassas, VA) and maintained in DMEM (Life Technologies) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a

humid, 5% CO_2, 37 °C incubator. Cells were transfected using Mirus (Madison, WI) TransIT-LT1 according to the manufacturer's protocol.

Western Blot

Protein samples were reduced and denatured in Laemmli buffer, loaded into 4–20% Tris-Glycine gels (Bio-Rad) for SDS-PAGE, and then transferred to nitrocellulose membranes (Bio-Rad). Blots were blocked with 5% milk (in 50 mM NaCl, 10 mM HEPES, pH 7.3 with 0.1% Tween-20) and incubated with primary antibodies for 1 h at room temperature or overnight at 4 °C. The anti-GPR56 C-terminal antibody was developed by Orbigen, Inc. via injection of rabbits with a peptide (CSNSDSARLPISSGSTSSSRI) derived from the GPR56 C terminus, and has been characterized previously [91]. Rat anti-HA (Roche) and mouse HRP-conjugated anti-FLAG (Sigma) antibodies were used to detect epitope-tagged proteins. HRP-conjugated secondary antibodies were purchased from GE Healthcare and antibody labeling of specific bands was visualized using Thermo Scientific SuperSignal West solutions.

Cell Surface Biotinylation

HEK-293T cells were transfected with 2 μg of DNA (empty vector or receptor). At 24-h post-transfection, cells were placed on ice and washed with ice-cold PBS+Ca^{2+} three times. Cells were then incubated with 10 mM Sulfo-NHS-Biotin (Thermo Scientific) in PBS+Ca^{2+} on ice for 30 min and then washed three more times with PBS+Ca^{2+} + 100 mM glycine. Cells were resuspended in 250 μl of lysis buffer (1% Triton X-100, 25 mM HEPES, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, protease inhibitor mixture (Roche Diagnostics), and 2% glycerol) and lysed by slowly rotating on a spinning wheel for 30 min at 4 °C. Cell debris was cleared by

centrifugation, and soluble cell lysates were incubated with 50 µl of streptavidin agarose beads (Thermo Scientific) for 1 h at 4 °C. Beads were washed three times with lysis buffer and resuspended in 60 µl of Laemmli buffer. Biotinylated proteins were detected via Western blot, as described above. Western blot bands were quantified using Image Studio software (Li-Cor, Lincoln, NE).

β-Arrestin Binding Assay

HEK-293T cells were transfected with a total of 6 µg of DNA (empty vector, receptor and/or HA-βArr2 or FLAG-βArr2). The next day, cells were washed with cold PBS+Ca^{2+} and lysed in harvest buffer (150 mM NaCl, 25 mM HEPES pH 7.3, 1 mM EDTA, 10 mM $MgCl_2$, 1% Triton X-100, Roche EDTA-free complete protease inhibitor mixture tablet). Lysates were rotated at 4 °C for 45 min to solubilize integral membrane proteins and membranes were cleared by centrifugation (15 min at 17,000 × *g*, 4 °C). Solubilizates were added to anti-HA (Sigma) or anti-FLAG agarose beads (Sigma) and rotated at 4 °C for 1 h. Beads were washed 3× in harvest buffer and proteins were eluted in Laemmli buffer at 37 °C for 10–15 min and loaded in 4–20% Tris-glycine gels for SDS-PAGE and Western blotting. Western blot bands were quantified using Image Studio software (Li-Cor, Lincoln, NE).

Luciferase Reporter Assays

HEK-293T cells were seeded in 96-well plates 20–24 h prior to transfection. Each well was transfected with 50 ng of firefly reporter, 1 ng of *Renilla* luciferase, and 10 ng of receptor or empty plasmid DNA. All reporter constructs (NFAT: pGL4.30, SRF: pGL4.34, *Renilla* pRLSV40) were acquired from Promega (Madison, WI). At 24–48 h later,

Dual-Glo luciferase assays (Promega) were performed according to the manufacturer's protocol and plates were read on a BMG Omega plate reader. For inhibitor studies, U73122 (10 μM, diluted from a stock in DMSO) or SKF96365 (50 μM, diluted from a DMSO stock) were added to wells for 8 hours before the plates were read. Vehicle control wells received an equivalent amount of DMSO (0.1% final). Results were calculated for each assay by determining the luminescence ratio of firefly:*Renilla* luciferase counts, normalized to empty vector-transfected wells. Error bars for all empty vector-transfected conditions were represented as the standard errors of the normalized raw value means.

3.3 Results

3.3.1 BFPP-causing mutations R565W & L640R differentially affect surface expression of full-length vs. ΔNT versions of ADGRG1

We generated four mutants version of G1: full-length (FL) and ΔNT receptors harboring either the R565W or L640R mutations (Fig. 10). The ΔNT versions of G1 lack most of the N-terminus, up to the site of predicted GAIN domain cleavage, and therefore mimic the C-terminal fragment (CTF) of G1 that is cleaved at the GAIN domain and undergoes dissociation from the N-terminal fragment (NTF). We assessed the surface trafficking of each mutant in HEK-293T cells in relation to its wild-type counterpart via a cell surface biotinylation approach. As shown in Figure 11A-B, we observed that the surface and total expression of both FL mutants were drastically reduced in comparison to the wild-type FL receptor. Surprisingly, though, the ΔNT mutants displayed no significant deficits in surface expression compared to the wild-type ΔNT receptor (Fig. 11C-D).

Figure 10: Schematic diagrams of full-length and ΔNT versions of R565W and L640R ADGRG1 mutant receptors.

The illustrations depict the predicted transmembrane architecture and relative positions of mutations on the extracellular loops for A) the full-length (FL) R565W G1 mutant, B) the truncated ΔNT-R565W mutant, C) the FL-L640R mutant and D) the ΔNT-L640R mutant.

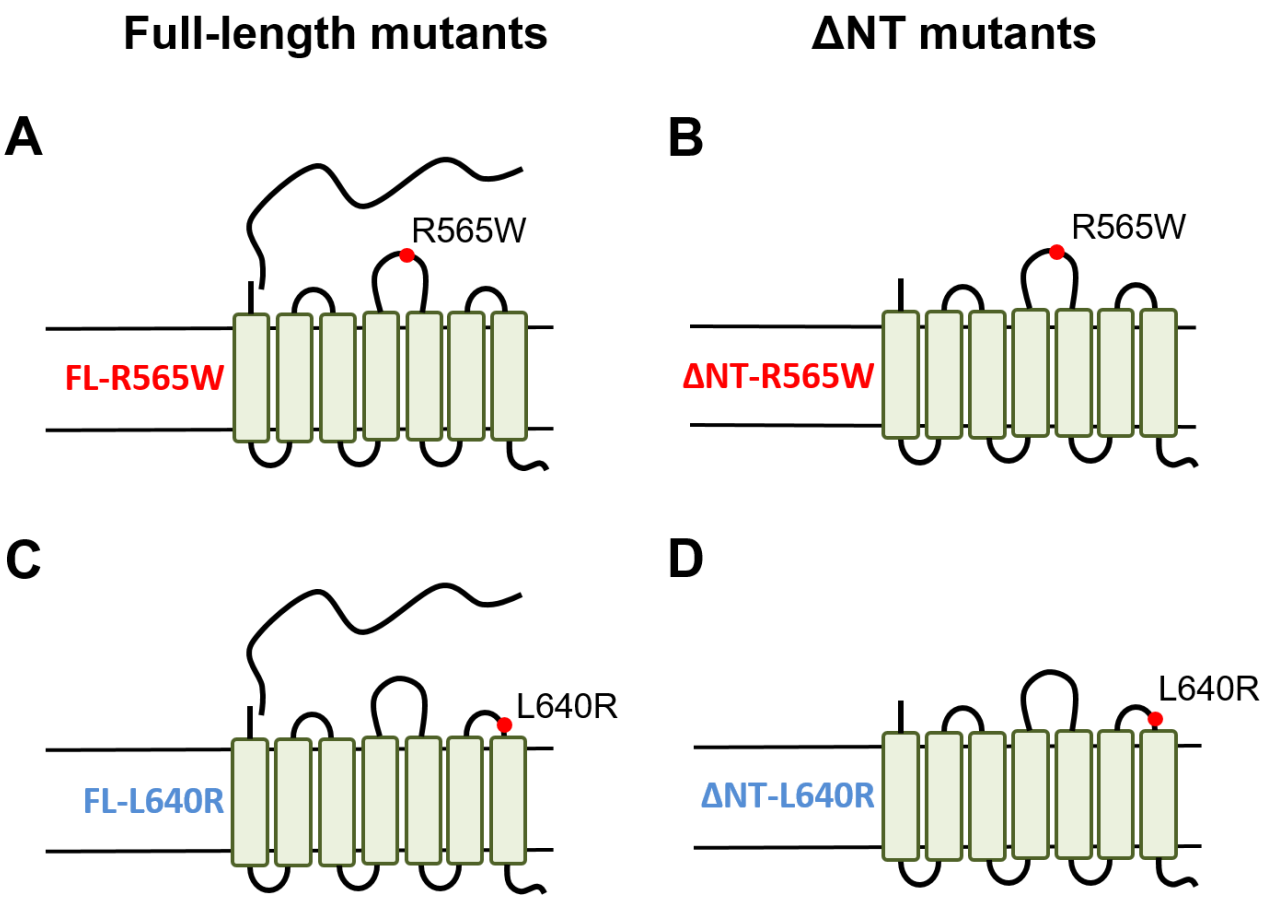
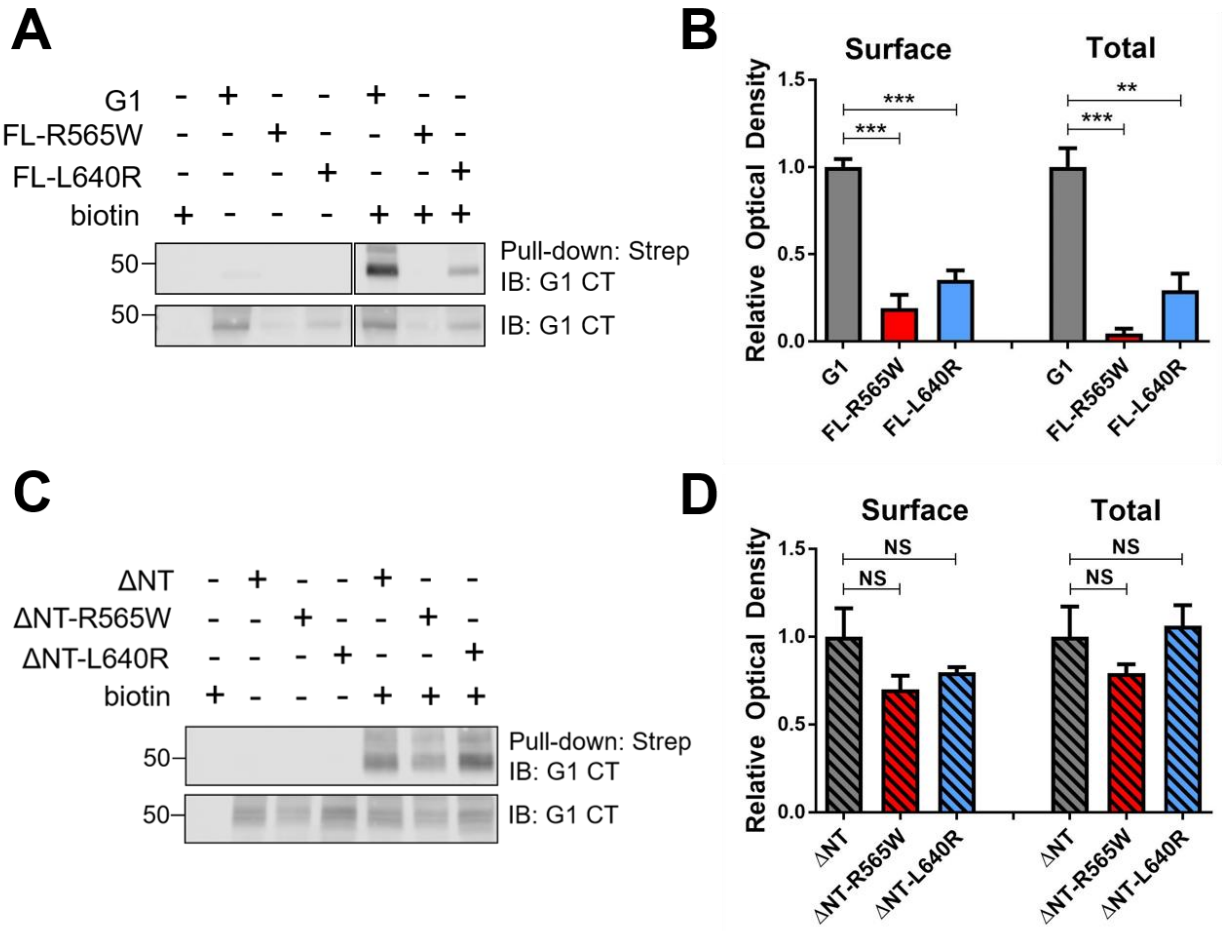


Figure 11: R565W & L640R mutations have differential effects on the surface expression of full-length vs. ΔNT ADGRG1.

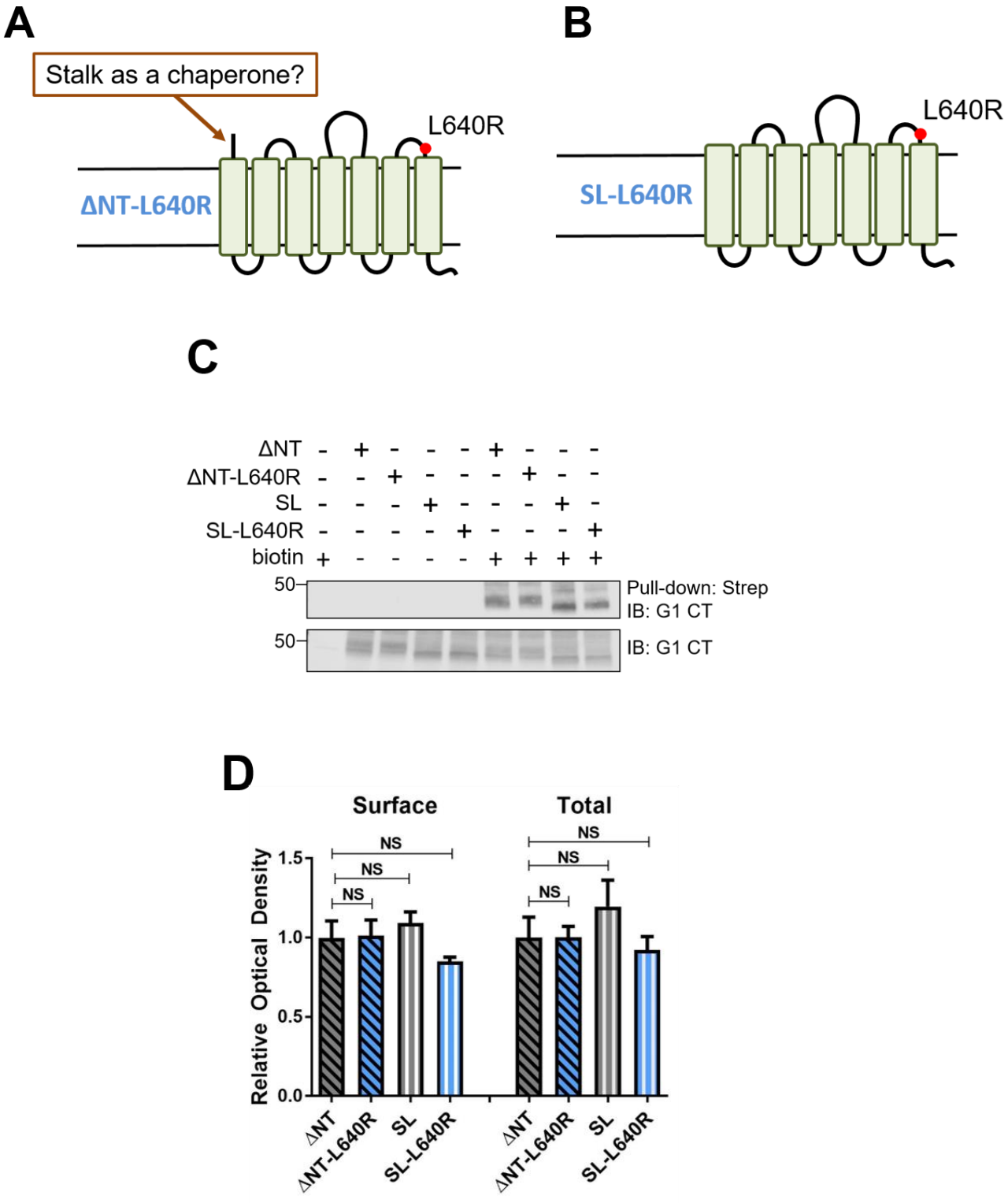
A & *C*, representative Western blots showing surface & total expression of R565W & L640R mutant receptors compared to their wild-type counterparts. The lower blot in each panel represents total receptor expression, whereas the upper blot in each panel represents the amount of receptor pulled down by streptavidin beads ("strep") following biotinylation of surface-expressed proteins. *B* & *D*, Quantified results of three independent Western blot experiments demonstrating that both full-length mutants exhibit markedly reduced surface & total expression while ΔNT mutants do not, relative their wild-type counterparts (S.E.M. shown, One-way ANOVA analysis, **, $p < 0.01$; ***, $p < 0.001$ for indicated comparisons).



A key difference between the full-length and ΔNT receptors is that G1-ΔNT has a fully exposed extracellular stalk, whereas the stalk of the full-length receptor is mostly hidden, either due to lack of GAIN cleavage or masking by the associated NTF. The exposed stalk of G1 has agonistic properties [94, 167] and therefore may serve as a pharmacological chaperone for the receptor, counteracting the trafficking deficits conferred by the R565W & L640R mutations in a manner analogous to pharmacological chaperones for other misfolded GPCRs [190]. To test the hypothesis of the stalk as a pharmacological chaperone, we generated a stalk-less version of the L640R G1 mutant (SL-L640R; Fig. 12B). As shown in Figure 12C-D, however, SL-L640R retained normal surface expression and trafficked to the plasma membrane at levels comparable to WT ΔNT, L640R-ΔNT and the wild-type stalk-less receptor. These data suggest that the extracellular stalk (stachel) of G1 does not act as a pharmacological chaperone, as its presence made no difference for trafficking of the L640R mutant.

Figure 12: The exposed stalk of ADGRG1 does not act as a pharmacological chaperone.

A & *B*, to test the idea that the exposed stalk of ΔNT might act as a pharmacological chaperone to counteract surface trafficking deficits conferred by mutations to the G1 extracellular loops, a stalk-less version of ΔNT-L640R (B; SL-L640R) was developed. A representative Western blot (*C*) and the quantified results of three independent experiments (D) demonstrate that deletion of the ΔNT-L640R stalk does not impair receptor surface expression (n = 3; S.E.M. analyses shown).


A
Stalk as a chaperone?
L640R
ΔNT-L640R
B
L640R
SL-L640R
C
ΔNT - + - - - + - - -
ΔNT-L640R - - + - - - + - -
SL - - - + - - - + -
SL-L640R - - - - + - - - +
biotin + - - - - + + + +
50
Pull-down: Strep
IB: G1 CT
50
IB: G1 CT
D
Surface
Total
Relative Optical Density
1.5
1.0
0.5
0.0
NS
NS
NS
NS
NS
NS
ΔNT
ΔNT-L640R
SL
SL-L640R
ΔNT
ΔNT-L640R
SL
SL-L640R

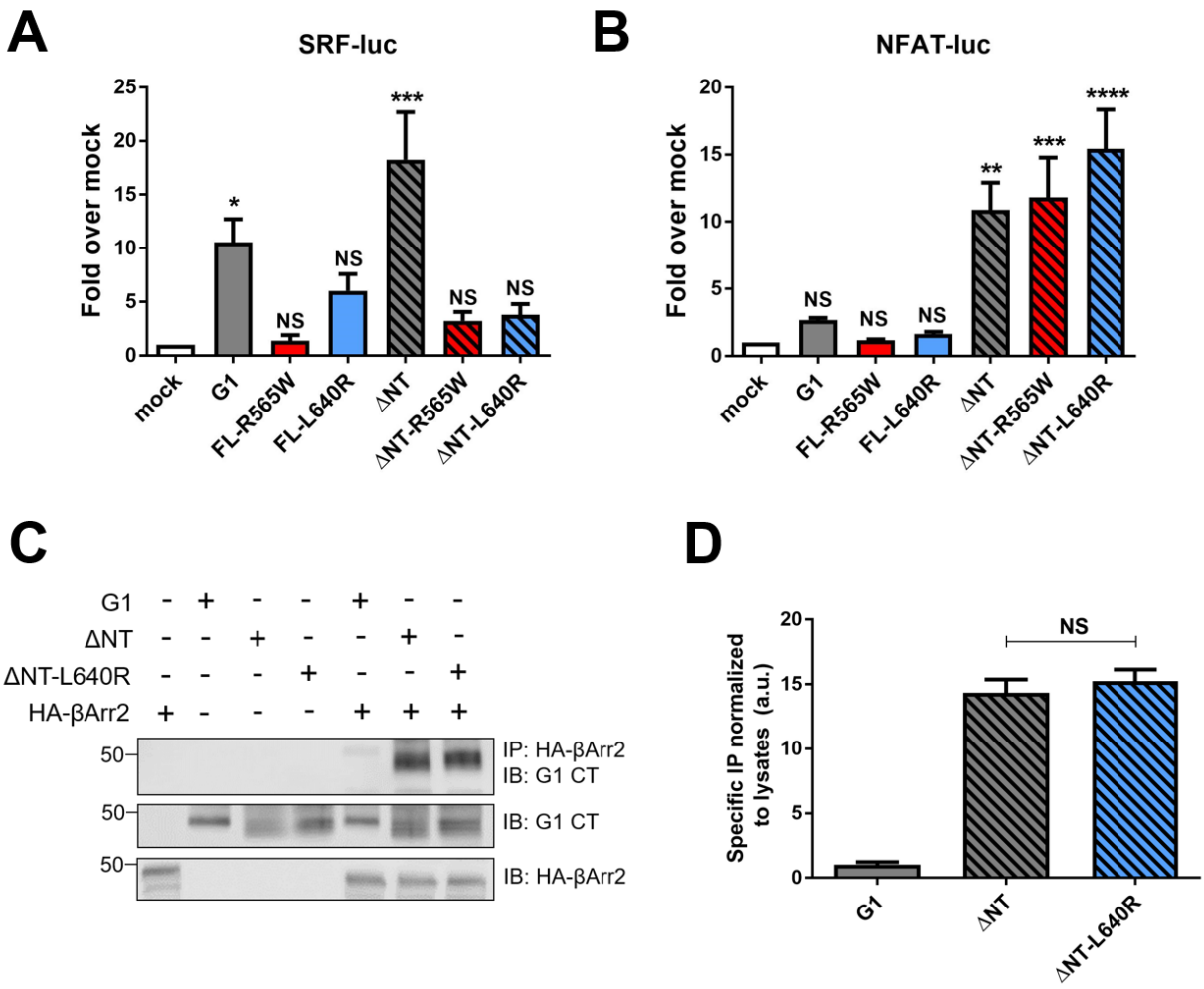
3.3.2 R565W & L640R mutations disrupt ADGRG1-mediated activation of SRF luciferase but not NFAT luciferase

We next assessed the signaling activity of the mutant receptors in HEK-293T cells in two distinct gene reporter assays: serum response factor (SRF) luciferase and nuclear factor of activated T-cells (NFAT) luciferase. In the SRF luciferase assay (Fig. 13A), none of the mutant receptors elicited significant levels of activity. In contrast, expression of the ΔNT mutant receptors resulted in substantial activation of NFAT luciferase that was comparable to the activity induced by wild-type G1-ΔNT (Fig. 13B).

Another measure of GPCR activity is association with βarrestins [172]. We previously showed that G1-ΔNT associates robustly with βarrestin2 while the full-length receptor does not [91, 167]. Therefore we assessed whether ΔNT-L640R could also associate with βarrestin2 even though it is deficient in signaling to SRF luciferase. As shown in Figure 13C-D, co-immunoprecipitation studies revealed that ΔNT and ΔNT-L640R associate with βarrestin2 to a similar extent, thereby providing further evidence that the mutant receptor is capable of achieving an active conformation.

Figure 13: R565W & L640R mutations have differential effects on ADGRG1 signaling.

A, Full-length ("FL") and ΔNT R565W & L640R mutants failed to elicit significant signaling to SRF luciferase compared to mock-transfected cells, whereas wild-type G1 and ΔNT elicited substantial signaling. *B*, ΔNT and ΔNT-R565W/L640R displayed signaling to NFAT luciferase comparable to their wild-type counterparts. All signaling data shown here are from at least 5 independent experiments (S.E.M. shown, **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ versus cells transfected with a mock vector). A representative Western blot (*C*) and quantified results from 3 independent experiments (*D*) demonstrate that both wild-type ΔNT and ΔNT-L640R robustly co-immunoprecipitate with HA-tagged βarrestin2 ("HA-βArr2").

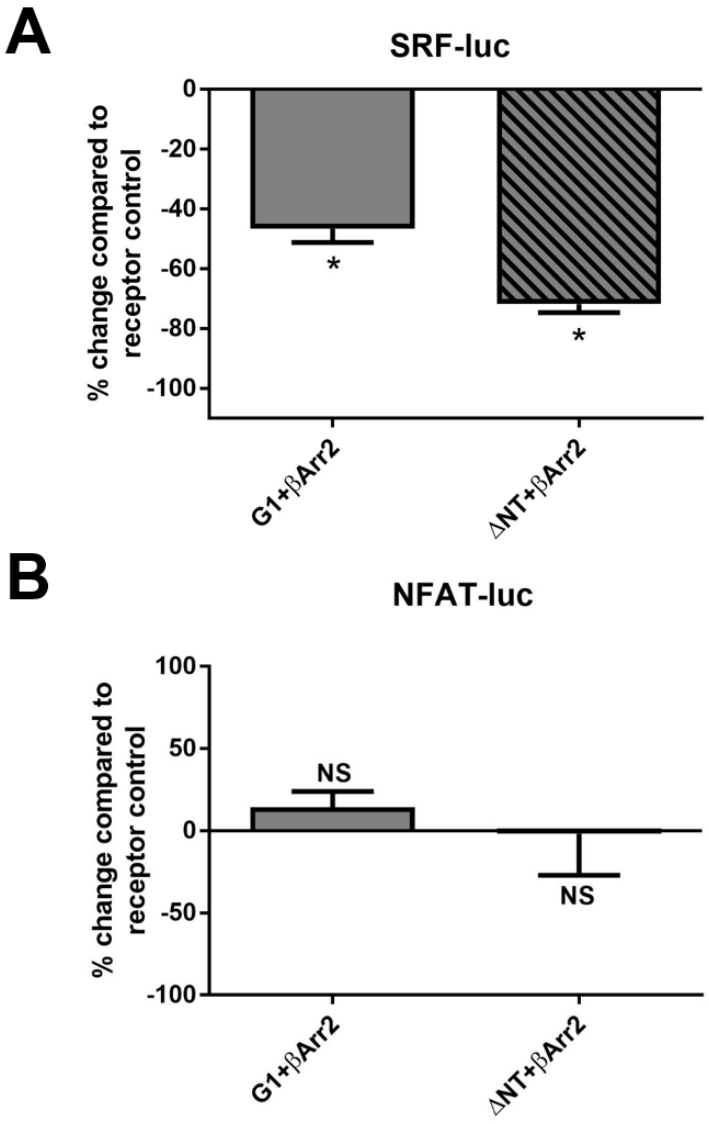


3.3.3 ADGRG1 signaling to NFAT luciferase does not involve βarrestins or $G\alpha_{q/11}$ but does involve Gβγ and calcium channels

Given that the R565W and L640R mutations disrupted signaling to SRF luciferase but preserved signaling to NFAT luciferase and interaction with βarrestins, we explored whether βarrestins might be involved in mediating G1 signaling to NFAT luciferase. Overexpression of βarrestins typically arrests G protein-dependent signaling by GPCRs but enhances βarrestin-dependent signaling activity [191], and thus we studied G1 signaling in the absence and presence of βarrestin overexpression. As shown in Figure 14A, overexpression of βarrestin2 significantly impaired signaling to SRF luciferase by full-length G1 and G1-ΔNT but had no significant effect on the ability of either version of G1 to activate NFAT luciferase (Fig. 14B). These data suggest that βarrestins can arrest G1 signaling to SRF luciferase but are not significantly involved in G1 signaling to NFAT luciferase.

Figure 14: βarrestin2 overexpression dampens ADGRG1-mediated activation of SRF but not NFAT luciferase.

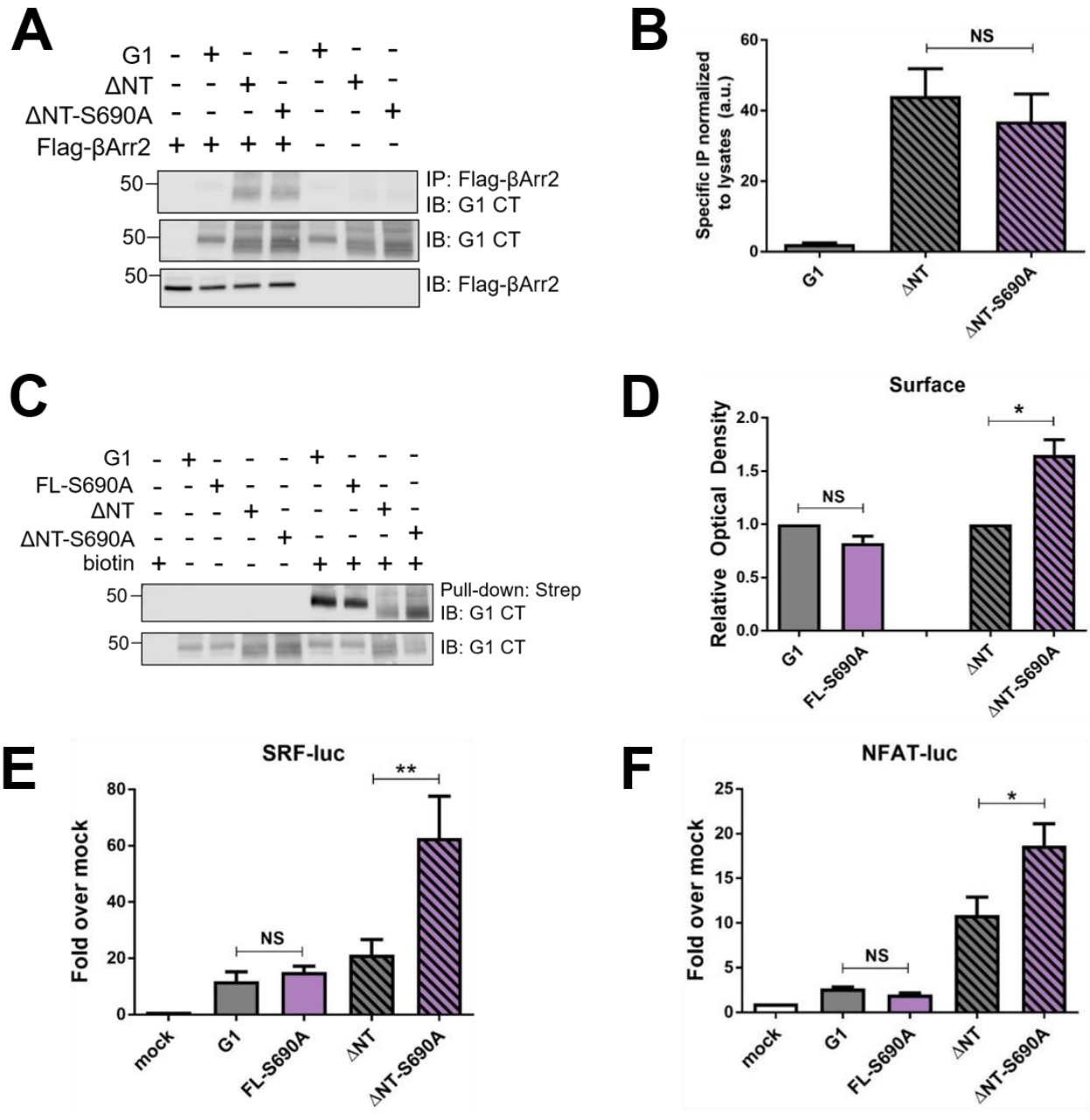
A, Overexpression of Flag-tagged βarrestin2 with full-length or ΔNT G1 resulted in significant reductions in receptor-mediated activation of SRF luciferase. *B*, Overexpression of Flag-βarrestin2 full-length or ΔNT G1 had no significant effect upon G1-mediated signaling to NFAT luciferase. Results are from 5 independent experiments (S.E.M. shown, *, $p < 0.05$ compared to the corresponding receptor condition without Flag-βarrestin2).



To further explore the potential role of βarrestins in G1 signaling, we sought to remove key phosphorylation sites from the C-terminus of G1, as phosphorylation of GPCR C-termini is typically required for βarrestin association [41]. As a starting point for these studies, we focused on S690, which is predicted by phosphorylation motif prediction algorithms to be a GPCR kinase (GRK) phosphorylation site [192] and has been identified in phosphoproteomic studies to be a highly phosphorylated reside on the G1 C-terminus [193]. We mutated this serine to an alanine (S690A) in both FL and ΔNT versions of G1, but subsequent co-immunoprecipitation studies revealed that ΔNT-S690A associated with βArrestin2 to the same extent as wild-type ΔNT (Fig. 15A-B). These data suggest that this residue is not essential for βarrestin recruitment. Nonetheless, in the course of performing these experiments we noted that this mutation markedly enhanced surface expression of the ΔNT mutant (Fig. 15C-D) and also enhanced G1-ΔNT signaling to both SRF and NFAT luciferase (Fig. 15E-F). Thus, these findings demonstrate that G1-mediated signaling to both SRF and NFAT luciferase is not saturated under our assay conditions, which as discussed below has important implications for interpreting the differential changes in signaling induced by the R565W and L640R mutations in the different pathways downstream of G1.

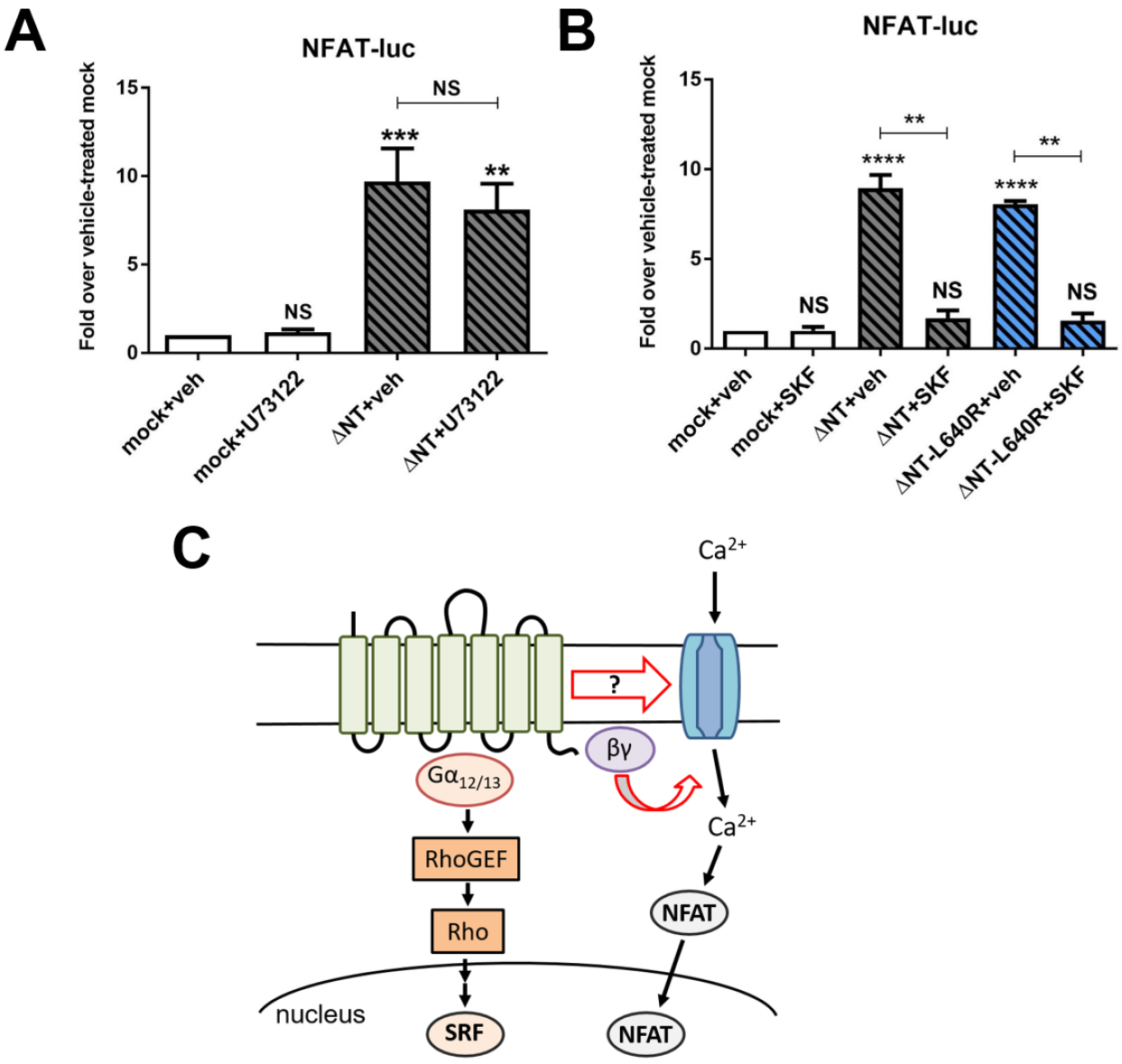
Figure 15. Mutation of a putative phosphorylation site (S690A) on the C-terminus of ADGRG1 enhances surface expression and signaling by the ΔNT mutant but does not abolish binding to βarrestin2.

A representative Western blot (*A*) and quantified results from 3 independent experiments (*B*) demonstrate that there was no significant difference in co-immunoprecipitation with βarrestin2 between wild-type G1-ΔNT and ΔNT-S690A. A representative Western blot (*C*) and quantified results from 3 independent experiments (*D*) reveal that the S690A mutation enhanced the surface expression of the ΔNT mutant but not the full-length mutant. The ΔNT-S690A mutant also displayed significantly higher levels of SRF (*E*) and NFAT luciferase (*F*) activation compared to the wild-type ΔNT receptor (S.E.M. shown, *, $p < 0.05$; **, $p < 0.01$ for indicated comparisons). Results shown are from at least 4 independent experiments.


A
G1
ΔNT
ΔNT-S690A
Flag-βArr2
50
IP: Flag-βArr2
IB: G1 CT
IB: G1 CT
IB: Flag-βArr2
B
Specific IP normalized to lysates (a.u.)
NS
G1
ΔNT
ΔNT-S690A
C
G1
FL-S690A
ΔNT
ΔNT-S690A
biotin
Pull-down: Strep
IB: G1 CT
IB: G1 CT
D
Surface
Relative Optical Density
NS
*
G1
FL-S690A
ΔNT
ΔNT-S690A
E
SRF-luc
Fold over mock
NS
**
mock
G1
FL-S690A
ΔNT
ΔNT-S690A
F
NFAT-luc
Fold over mock
NS
*
mock
G1
FL-S690A
ΔNT
ΔNT-S690A

To shed further light on G1 signaling to NFAT luciferase and how this pathway may be mechanistically distinct from G1 signaling to SRF luciferase, we performed a set of inhibitor studies. First, we assessed whether G1 might be capable of activating the $G\alpha_{q/11}$ pathway in addition to coupling to $G\alpha_{12/13}$. However, as shown in Figure 16A, we observed that U71322, an inhibitor of phospholipase Cβ and therefore a blocker of the $G\alpha_{q/11}$ signaling cascade, had no effect on G1 activation of NFAT luciferase. Another mechanism by which GPCRs can increase cellular calcium levels to activate NFAT luciferase is via activation of plasma membrane calcium channels Thus, we assessed G1 signaling to NFAT in the presence of SKF96365, a relatively non-specific calcium channel inhibitor [194, 195]. Treatment with SKF96365 resulted in a dramatic decrease in G1-ΔNT signaling to NFAT luciferase for both G1-ΔNT and G1-ΔNT-L640R (Fig. 16B). Taken together with our previous observations that Gβγ inhibitors antagonize G1-ΔNT-mediated signaling to NFAT luciferase [167], these findings suggest that G1-ΔNT can activate NFAT luciferase via a pathway involving the liberation of Gβγ subunits and activation of calcium channels (Fig. 16C) and moreover demonstrate that the disease-associated mutations to the G1 extracellular loops do not impair this signaling.

Figure 16. ADGRG1-mediated signaling to NFAT luciferase involves activation of calcium channels but not receptor coupling to $G\alpha_{q/11}$. *A*, Treatment with the phospholipase Cβ inhibitor U73122 (50 μM; 8 hours) had no effect on ΔNT-mediated activation of NFAT luciferase. *B*, Treatment with the calcium channel inhibitor SKF96365 (10 μM, 8 hours) ablated activation of NFAT luciferase by both G1-ΔNT and ΔNT-L640R. Results shown are from at least 4 independent experiments (S.E.M. shown, **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ for indicated comparisons) *C*, Schematic model depicting the putative signaling pathways by which G1 stimulates SRF or NFAT luciferase activity. The N-terminal fragment is shown interacting with the extracellular stalk and also potentially the extracellular loops of the transmembrane C-terminal fragment to modulate receptor signaling activity.


A
NFAT-luc
Fold over vehicle-treated mock
15
10
5
0
NS
***
**
NS
mock+veh
mock+U73122
ΔNT+veh
ΔNT+U73122
B
NFAT-luc
Fold over vehicle-treated mock
15
10
5
0
**
**
****
****
NS
NS
NS
mock+veh
mock+SKF
ΔNT+veh
ΔNT+SKF
ΔNT-L640R+veh
ΔNT-L640R+SKF
C
Ca2+
?
βγ
Gα12/13
RhoGEF
Rho
SRF
nucleus
Ca2+
NFAT
NFAT

3.4 Discussion

In the data shown in this chapter, we assessed the effects of the disease-causing mutations R565W & L640R on G1 surface expression and signaling. One important observation was that the extracellular loop mutations reduced the surface expression of full-length G1 but not the ΔNT receptor, which suggests that the tethered NTF may interact with the extracellular loops of G1. In this scenario, the R565W & L640R mutations may corrupt the normal interaction between the NTF and extracellular loops to cause protein misfolding. It is well-accepted that aGPCR NTF and CTF protomers interact via hydrophobic stalk interactions within the cleaved GAIN domain [76, 78], but there has also been speculation that there may be additional NTF/CTF interactions that do not involve the stalk [1]. In the case of G1, evidence in support of this idea includes the observation shown in Chapter 2 that the presence of the NTF strongly suppresses signaling to NFAT luciferase by the G1 CTF even though this signaling is completely stalk-independent [167]. The present study provides additional evidence for NTF/CTF interactions that go beyond stalk/GAIN binding, as it is unclear how the effects of extracellular loop mutations on G1 trafficking could be dependent on the presence of the NTF unless the extracellular loops possess the capacity to interact with the NTF in some way.

In addition to the effects of the R565W & L640R mutations on receptor trafficking, we also observed that these mutations ablated G1-ΔNT-mediated signaling to SRF luciferase but not NFAT luciferase. This observation suggests that the pathways by which G1 signals to SRF versus NFAT luciferase are mechanistically distinct. Indeed, the studies described in Chapter 2 revealed that G1-ΔNT signaling to SRF luciferase is entirely dependent on the presence of the extracellular stalk, whereas signaling to NFAT luciferase is stalk-independent [167]. This previous work described in Chapter 2 also demonstrated that G1 signaling to SRF luciferase was

almost entirely blocked by inhibition of $G\alpha_{12/13}$, whereas signaling to NFAT luciferase was only partially dependent on $G\alpha_{12/13}$ and also dependent on liberation of Gβγ subunits [167]. The experiments described in this chapter provide additional insights into the pathways downstream of G1, as these data revealed that G1 signaling to NFAT luciferase does not involve $G\alpha_{q/11}$ or βarrestins but does involve stimulation of calcium channels in addition to Gβγ subunit liberation. Understanding the mechanism(s) by which G1 can stimulate calcium channel activity will require further elucidation, but it is interesting to note that studies on the *Drosophila* aGPCR lat-1 have shown this aGPCR robustly activates TRP-family calcium channels to regulate mechanosensation, perhaps via direct receptor/channel interactions [161].

Previous studies have demonstrated that NT-truncated, constitutively-active aGPCRs can robustly associate with βarrestins [91, 112, 167] but the functional effects of aGPCR interactions with βarrestins are largely unknown. In the studies described in this chapter, we found evidence that βarrestins can arrest G protein-mediated signaling by aGPCRs, as βarrestin2 over-expression dramatically inhibited G1 activation of SRF luciferase. Interestingly, though, G1 signaling to NFAT luciferase was unaffected by βarrestin over-expression, providing yet another mechanistic distinction between these two signaling pathways downstream of G1. We also studied the functional effects of mutating a previously-described [193] G1 phosphorylation site (S690). Mutation of this serine residue did not alter βarrestin association, but did increase G1-ΔNT surface expression and signaling to both SRF and NFAT luciferase. These data are important because they demonstrate that G1 signaling to both SRF and NFAT luciferase is not saturated under our assay conditions. A potentially trivial explanation for the differential effects of the R565W and L640R mutations on G1-ΔNT signaling to SRF vs. NFAT luciferase would be if one of these pathways was saturated, meaning that even a miniscule amount of activity in the mutant

receptors might provoke a maximal amount of signaling. However, the S690A signaling data demonstrate that neither signaling pathway is saturated under the conditions of our experiments, thereby further supporting the idea that the pathways downstream of G1 to SRF vs. NFAT luciferase are mechanistically distinct.

Several previous reports have assessed the trafficking and signaling properties of full-length BFPP-associated G1 mutants, including the R565W and L640R mutants studied here [152-154]. Lin and colleagues found via confocal immunofluorescence that the NTF protomer for mutants R38W (distal NT), R565W (second extracellular loop) and L640R (third extracellular loop) were sharply reduced at the cell surface [153]. In a separate study, Piao and colleagues demonstrated via a cell surface biotinylation approach that surface expression of both CTF and NTF protomers were sharply reduced for mutants R38Q, R38W, Y88C (distal NT), C91S (distal NT), C346S (GAIN domain), C349S (GAIN domain) and R565W [152]. Mixed results were obtained for the L640R mutant in this study, as the L640R CTF displayed a comparable level of surface expression to the wild-type receptor whereas the NTF protomer was reduced at the cell surface [152]. In further studies, Piao & colleagues found that the L640R mutant exhibits reduced signaling relative to WT following treatment with the G1-binding protein collagen III [154]. Our findings in the present study are consistent with the trafficking deficits that have previously been reported for the full-length R565W and L640R mutants. However, the present study also significantly extends work in this area with the surprising observation that the deleterious effects of these mutations on G1 trafficking are completely abrogated in the ΔNT form of the receptor. Additionally, we found that although the activated L640R mutant receptor is deficient in $G\alpha_{12/13}$-mediated signaling, as Piao & colleagues observed [154], this receptor still robustly binds to βarrestins and can activate NFAT luciferase via

stimulation of calcium channel activity. Thus, our data suggest that the L640R mutant receptor is not completely inactive but rather selectively deficient in certain aspects of its signaling.

In summary, the studies described in this chapter have provided novel insights into how disease-associated mutations to the G1 extracellular loops can differentially impact G1 trafficking and signaling. Going forward, it will be of interest to study further whether aGPCR extracellular loops do indeed interact with the tethered NTF regions, as suggested by the findings reported here, and to understand what the structural basis of these interactions may be. Additionally, another point of interest will be to further dissect stalk-dependent vs. stalk-independent modes of aGPCR signaling and to understand how aGPCR extracellular regions (the NTF and extracellular loops) can differentially modulate distinct aspects of receptor signaling. Finally, a major goal of fundamental studies into aGPCR signaling like those reported here is to set the stage for the future pharmacological targeting of aGPCRs with small molecule agonists, antagonists and modulators. Given the importance of this family of receptors for human health and disease [73], the members of this family may prove to be important drug targets for novel classes of therapeutics in the treatment of many different human diseases.

CHAPTER 4: Discussion and Future Directions

4.1 Dissecting two qualitatively distinct modes of ADGRG1-mediated signaling

The work compiled in this dissertation provides strong evidence that G1 is capable of at least two qualitatively distinct modes of signaling as observed when measuring receptor-mediated activation of SRF and NFAT. G1-mediated activation of SRF is achieved through the canonical $G\alpha_{12/13}$ pathway while activation of NFAT is only partially G protein-dependent. Co-expression of the RGS domain of p115RhoGEF ablated signaling to SRF by full-length and ΔNT G1 by >90% while reducing G1ΔNT activation of NFAT by only ~60%. Moreover, inhibition of the Gβγ subunits by gallein treatment partially reduced G1ΔNT activation of NFAT by ~50%. These results highlight a key difference between these two receptor-stimulated pathways: that while receptor-mediated SRF activity is wholly transduced via traditional Gα signaling, the receptor-mediated NFAT signal depends partially on Gβγ activity and also involves a significant G protein-independent component.

Additionally, in the course of testing whether the stalk (or stachel) region of G1 is a requisite agonist for the receptor, we discovered key differences in receptor structural determinants of either pathway. Deletion of the stalk abrogated receptor signaling to SRF but had no effect on NFAT activation. Similarly, introduction of human disease-associated point mutations to the 2nd and 3rd extracellular loops (R565W & L640R) of G1 ablated receptor activity toward SRF but not NFAT. Despite the lack of importance of the stalk region for G1 signaling to NFAT, the studies shown here support the idea that removal of the tethered NT is a pre-requisite for G1-mediated NFAT activation. Thus, the tethered NT must be restraining receptor activity in other ways beyond simply masking the stalk region. However more work remains to be done to provide a clearer picture of the mechanistic dynamics of this mode of signaling.

Based on the differences in G protein-dependence and structural determinants of activity for G1 signaling to SRF-luciferase vs. NFAT-luciferase, we propose that G1 is capable of at least two distinct modes of signal transduction: receptor-mediated activation of the SRF pathway through coupling to $G\alpha_{12/13}$ and involving the stalk and extracellular loop regions, and receptor-mediated activation of the NFAT pathway which partially involves $G\beta\gamma$ signaling and requires NT removal (or conformational change) but not liberation of the stalk.

4.2 The allosteric antagonist model of adhesion G protein-coupled receptor activation

Understanding the natural mechanism(s) of aGPCR activation is a critical step toward facilitating drug development efforts aimed at these receptors. For example, the elucidation of agonistic peptide sequences on the N-terminal stalks of certain aGPCRs has provided insights that may lead to the development of peptidomimetic small molecules with agonistic activity at these receptors. Similarly, the findings reported in Chapter 2 that cryptic agonist sequences on aGPCR stalks do not account for the entirety of aGPCR signaling are important because these observations suggest an additional antagonistic effect of tethered GAIN domains on aGPCR activity beyond the simple masking of the stalk region. Therefore, we propose an allosteric antagonist model of aGPCR activation (Fig. 17), in which the NTF can antagonize receptor activity in two distinct ways: (i) by masking the stalk region and (ii) by directly antagonizing the inherent stalk-independent constitutive activity of the 7TM region. The word “allosteric” in this context is meant to convey that the NTF presumably does not block agonist binding in the manner of a competitive antagonist, but rather constrains receptor activity in an allosteric fashion. This model is consistent with the data presented here as well as in previous studies [196] and furthermore is consistent with the possibility that aGPCRs may still signal even if they are not cleaved at the GAIN domain or lose their stalk following GAIN cleavage.

It is important to note that this model acknowledges that for any individual receptor the relative importance of the stalk may vary substantially. For ADGRB1, it was shown that stalk deletion had no observable effect on receptor activity across a broad panel of assays [167]. Efficiency of autoproteolysis may be one of many potential factors that determine the necessity of the stalk to any particular aGPCR in terms of signaling activity. If autoproteolysis is not efficient or does not occur altogether, then perhaps the need for increased activity following stalk exposition, which depends on the stalk retaining essential functional determinants, is unnecessary. If and when more members of the aGPCR subfamily are shown to exhibit non-reliance on their cognate stalks to mediate G protein signaling (like ADGRB1) then it would be fascinating to examine commonalities between those receptors as a starting point to determine why some aGPCRs rely on their stalks while others do not.

Our studies on a cleavage deficient mutant version of G1 (T383A) revealed that autoproteolysis was not necessary for stalk-mediated signaling. Therefore, at least for G1, conformational modulation of the stalk (through ligand binding and/or mechanical interactions with extracellular matrix proteins) may be sufficient for stalk-mediated signaling. When the NTF dissociates from the CTF, however, a greater extent of stalk-mediated activity may be realized as the stalk could stabilize a fully active conformation whereas the stalk may only be able to induce partial-activity conformations when the NTF is associated. In this way, the stalk region within the NTF/CTF heterodimer may provide a relatively low but sustained and tunable level of activity, whereas removal of the NTF may result in a large bolus of activity that may also be more transient due to receptor desensitization.

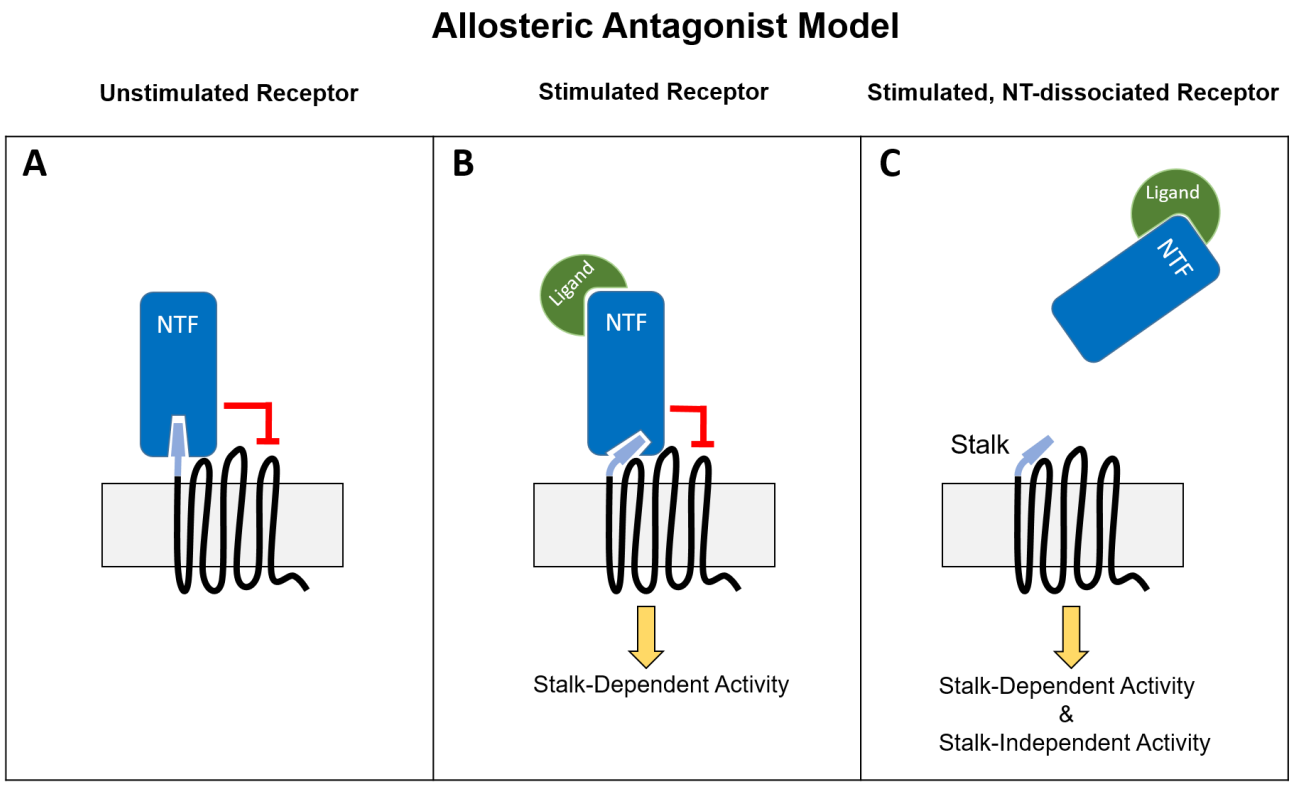
How exactly the tethered NTF can antagonize the constitutive stalk-independent activity of the CTF is not fully understood at present. A logical starting place would be to shed light on

the points of contact between the NTF and CTF. It has been well-established that the NTF and CTF are joined at the site of autoproteolysis via strong non-covalent and hydrophobic interactions, however the NTF may contact the CTF at other regions as well [76]. Relevant to this issue, our analysis of the disease-associated ECL mutations R565W & L640R revealed that the mutations conferred a trafficking deficit only for the full-length receptor but not the truncated ΔNT receptor. This unusual finding suggests that the tethered NT may normally interact with the ECLs and that disease-associated mutations to those regions corrupt NTF-CTF interactions resulting in receptor misfolding. If this is indeed true, then it would be interesting to investigate the functional implications of those interactions and furthermore to pinpoint the specific amino acid regions which mediate them, as these putative NTF-ECL interactions might plausibly mediate the antagonism of G1 stalk-independent signaling by the NTF.

Further insights into the structural determinants of the antagonistic relationship between aGPCR NTF and 7TM regions may help to facilitate discovery of distinct classes of small-molecule aGPCR modulators that either block or potentiate NTF-mediated suppression of aGPCR 7TM signaling. Additionally, a model in which aGPCRs can mediate both stalk-dependent and stalk-independent signaling has clear implications for the future development of biased agonists targeting these receptors. In many cases, it is therapeutically desirable to target some but not all pathways downstream of a given receptor. Thus, it will be of interest going forward to study the various members of the aGPCR family on a receptor-by-receptor basis in order to understand the structural determinants of receptor coupling to different downstream signaling pathways to facilitate the discovery of biased ligands possessing therapeutic potential.

Figure 17: Allosteric Antagonist Model of aGPCR Activation.

A, in this model the NTF behaves as an allosteric antagonist in two ways: (i) masking the stalk region and (ii) directly antagonizing the constitutive stalk-independent activity possessed by the 7TM region. *B*, conformational change of the NTF induced by ligand binding is sufficient to allow for enhanced stalk-dependent activity. *C*, ligand binding can also result in either NTF dissociation or a conformation change that relives the inhibitor constraint of the NTF upon the 7TM region, such that both stalk-dependent and stalk-independent pathways are activated.



4.3 Elucidating the mechanisms of stalk-independent activation of NFAT by G1ΔNT

The studies described in Chapter 2 revealed that G1 can mediate signaling to NFAT in a manner that is not dependent on the receptor's stalk. Given the difference in the structural determinants of this signaling to NFAT luciferase vs. the stalk-dependent signaling to SRF luciferase, it was a point of interest to shed light on the mechanism by which G1ΔNT activates the NFAT pathway. G1ΔNT signaling to NFAT was found to be completely blocked by treatment of SKF96365, a relatively non-specific inhibitor of several types of calcium channels at low micromolar (5-30 uM) concentrations. These include: T-, L-, N- and P/Q-type Ca^{2+} channels [194, 195], several members of the canonical transient receptor potential (TRPC) family of Ca^{2+} channels [197-199], and Ca^{2+} release-activated Ca^{2+} (CRAC) channels which include STIM and Orai1 [200]. There are precedents for GPCR modulation of Ca^{2+} channels [201-204]. Most of these instances, however, are of GPCRs that suppress channel function, often through Gβγ-dependent mechanisms [26, 205]. There are at least two different, not-mutually-exclusive ways that GPCR-Gβγ activity can modulate ion channels. The first is indirectly, via second messenger cascades. And the second is directly, via physical association of the Gβγ subunits with the ion channel. For G1ΔNT, it is currently unknown what downstream second messengers (if any) are responsible for stimulating Ca^{2+} channels, but it is clear from the studies shown here that it is not dependent on PLCβ as treatment of the inhibitor U73122 had no effect on G1ΔNT-mediated NFAT activity. Gβγ subunits are also capable of directly modulating ion channels in a membrane-delimited fashion [206]. If this applies to G1ΔNT then close proximity of the receptor and G proteins to calcium channels are likely required for direct Gβγ-channel interactions to take place.

There is also the question of the Gβγ-independent component of G1ΔNT-mediated activation of the NFAT pathway. One possibility is that G1 gains the ability to complex with and modulate calcium channels following NT removal. G1ΔNT may therefore either directly agonize calcium channels, allosterically modulate channels to lower activation thresholds, or perhaps interact with channels to block inhibitory regulators. Along these lines it is interesting to note that the *Drosophila* aGPCR lat-1 likely activates TRP channels to regulate mechanosensation, potentially through direct receptor-channel interactions [161]. Further work must be done to determine if G1 is also capable of forming complexes with and modulating calcium channels, such as TRP channels.

4.4 Future directions in studying G1 multi-modal signaling activity

Traditionally, GPCRs were viewed as bimodal switches whereby agonist stimulation caused a conformational change to shift the receptor from an inactive state to an active state from which G protein-mediated signaling would commence [207]. Now it is appreciated that GPCRs are highly dynamic proteins that can assume many different conformational states that may influence a multiplicity of signaling pathways.

As discussed in various parts of this dissertation, some GPCRs are capable of both G protein-mediated and non-G protein-mediated signaling. To add to the complexity of GPCR activity, some receptors can even couple to multiple types of G proteins. It is interesting to note that variations in splicing can bias GPCR signaling activity. For instance, for the 5-HT_4 receptor one particular splice variant (5-HT_{4b}) retains the ability to activate both $G\alpha_{i/o}$ and $G\alpha_s$, while in contrast, the 5-HT_{4a} splice variant only activates $G\alpha_s$ [208]. In another example, a single mutation to the ECL2 of the G protein-promiscuous protease-activated receptor-1 (PAR1) was

able to bias the receptor toward the $G\alpha_{q/11}$ pathway and away from the $G\alpha_{12/13}$ pathway, as opposed to the wild-type receptor which displayed the opposite preference [209].

The studies described in Chapter 3 revealed that the disease-associated mutations R565W & L640R have a biasing effect on G1 signaling by selectively disrupting stalk-dependent SRF activity but not stalk-independent NFAT activity. In future studies, it would be of interest to study other receptor mutations, both disease-associated and rationally chose, to see if mutations can be identified that bias the receptor in the opposite direction (i.e. disrupting stalk-independent activity but leaving stalk-dependent activity in tact). Such receptor mutants may serve as useful tools in dissecting the physiological relevance of the different modes of G1 signaling in future studies.

To follow-up on the idea of biased receptor signaling, high-throughput screens to identify G1 ligands, especially biased ligands, may yield invaluable research tools as well as compounds that might eventually lead to new therapeutics. The notion that aGPCRs can be modulated by small molecules is gaining traction as published reports on two aGPCRs (including G1) have provided proof of concept. As mentioned in Chapter 1, beclomethasone dipropionate was shown to be a small molecule agonist for ADGRG3 [116] and dihydromunduletone (DHM) was more recently identified as a small molecule antagonist for G1 [67]. Tall and colleagues were able to identify DHM from a 2,000 compound chemical library by taking advantage of the high constitutive activation of SRE-luciferase by G1 CTF. Compounds that were able to significantly inhibit the high baseline activity of G1 CTF were then compared against a counter screen expressing a constitutively active mutant version of $G\alpha_{13}$ to confirm receptor-specific effects.

A similar screening strategy could be utilized to identify biased G1 ligands. For example, in order to identify a biased G1 antagonist, G1 CTF expression in two separate experimental screens would be required. The first screen would examine the ability of compounds to inhibit the constitutive receptor-mediated activation of SRF/SRE-luciferase (stalk-dependent activity), and then a second screen would examine inhibition of receptor-mediated activation of NFAT-luciferase (stalk-independent activity). A biased G1 antagonist would be a compound that exhibited antagonistic activity in only one of the two screens.

It will also be of interest to gain more structural insights into the various aspects of G1 activation and signaling studied in the work presented in this dissertation. For example, it would be interesting to know how the tethered NTF antagonizes stalk-independent receptor activity and whether this regulation depends on NTF interactions with the extracellular loops. Such insights could be gained via X-ray crystallography, cryo-electron microscopy and/or other structural techniques. The past decade has seen an explosion of high-resolution structural information about GPCRs [210], and recently a crystal structure was reported for the G1 extracellular N-terminus [211], but no crystal structures are yet available for full-length adhesion GPCRs. If structures can be determined and show NTF interactions with extracellular loops, then these structures may allow insights to be gained such that peptides could be designed for the purpose of modulating NTF/CTF interactions and thereby modulating receptor signaling.

4.5 Therapeutic potential of G1 modulation

A major goal for functional studies on any human receptor is to eventually improve human health, most notably by treating diseases. This goal is especially applicable to studies on G1, a receptor that is of tremendous clinical interest due to its involvement in a number of disease states and pathologies. Developing modulators of G1 activity, whether they negatively or

positively impact receptor function, has the potential to lead to novel therapeutics that would be helpful in treating a variety of different conditions.

There are a number of cancers in which G1 is over-expressed and promotes tumor growth. For instance, G1 expression in primary human acute myeloid leukemia (AML) samples negatively correlated with overall patient survival [147]. Interestingly, in xenotransplantation experiments, mice that received leukemia cells that were pre-treated with an inhibitory antibody against G1 displayed lower leukemia cell engraftment and higher survival rates over 100 days than the control group [147]. Thus, it could be imagined that G1 antagonists or negative allosteric modulators may have value slowing down disease progression in patients with G1-positive AML.

Melanoma is another cancer in which G1 is expressed [212]. In contrast to AML, though, G1 expression and/or activation in melanoma has been shown in several studies to inhibit melanoma growth [97, 130, 131]. G1 is also overexpressed in some gliomas [92], and one report demonstrated that activating antibodies against G1 significantly inhibited the migration of a glioma cell line [99]. Thus, in melanoma & glioma, activation of G1 by agonists or positive allosteric modulators might have therapeutic benefits by reducing progression, metastasis, and/or invasion of cancerous cells.

As discussed earlier, the neurological disease BFPP stems from a lack of G1 function during development. Many BFPP mutations, including those studied here in Chapter 3, result in reduced receptor expression at the plasma membrane. Thus, increasing the trafficking and signaling of these mutant receptors could potentially counter some of the effects of the disease. This could be accomplished via the development of G1-specific pharmacological chaperones [190]. In our studies in Chapter 3 on BFPP mutations, we found that the full-length L640R

mutant receptor (FL-L640R) displayed a slight deficit in signaling to SRF but a severe surface trafficking deficit. If normalized to its reduced surface expression, FL-L640R signaling to SRF would be comparable to signaling by the wild-type G1. Moreover, as discussed in Chapter 3, the observation that only full-length BFPP mutants displayed a trafficking deficit while their ΔNT counterparts did not, suggests that corrupted interactions between the NTF and ECLs may be responsible for receptor misfolding. Perhaps then a G1-specific pharmacological chaperone could alleviate the effects of BFPP by specifically interacting with the receptor's NT and/or ECLs to 'correct' receptor misfolding and therefore boost receptor surface expression.

Another potential therapeutic application of G1-targeted drugs would be immune system modulation. G1 is expressed on immune cells, particularly on natural killer cells (NKCs) [184, 185, 213]. Interestingly, NKCs from BFPP patients displayed significantly enhanced ability to kill target cells, providing evidence that G1 plays an inhibitory role in NKC function [184]. Targeting G1 for immuno-modulatory purposes could potentially have widespread biomedical applications and would be an exciting area of G1 research.

4.6 Concluding remarks

The work presented in this dissertation has elucidated many aspects of G1 signaling and contributed to the larger debate on the mechanisms of adhesion GPCR activation. Based on the findings of this dissertation, we proposed a new model of aGPCR signaling (the allosteric antagonist model) that takes into account both stalk-dependent and stalk-independent signaling by aGPCRs. The work described in this dissertation has also shed light on the functional effects of two G1 mutations associated with human disease. Thus, this dissertation has advanced understanding of G1 signaling and contributed to the broader understanding of adhesion GPCR activity. These advances will facilitate future drug development efforts aimed at G1 and other

adhesion GPCRs, especially efforts that might seek to differentially target distinct modes of adhesion GPCR signaling.

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