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Anqi Gao April 10, 2017

Regulation of Adhesion G Protein-Coupled Receptor ADGRG1 (GPR56) by Receptor Activity-Modifying Proteins

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Abstract

Regulation of Adhesion G Protein-Coupled Receptor ADGRG1 (GPR56)
by Receptor Activity-Modifying Proteins

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G protein-coupled receptors (GPCRs) are a diverse superfamily of transmembrane proteins that transmit signals from the extracellular side of cells into the cytoplasm. Adhesion GPCRs are a family of receptors that are characterized by long N-termini containing adhesion-like motifs. ADGRG1 (also known as GPR56 or G1) is an adhesion GPCR that is involved in brain development. Mutations to this receptor cause a neurodevelopmental disorder known as bilateral frontoparietal polymicrogyria (BFPP). The purpose of this project was to examine receptor activity-modifying proteins (RAMPs) might regulate the activity of G1. We found that G1 physically associates with RAMP1 and RAMP3, and that a truncated constitutively-active version of G1 (ΔNT-G1) associates with RAMP1 and RAMP3 even more robustly. Co-expression with RAMPs had no significant effect on the signaling activity of G1 or ΔNT-G1 when receptor signaling to NFAT luciferase was assessed. Conversely, co-expression with RAMP3 sharply decreased ΔNT-G1 signaling to SRF luciferase, revealing a differential effect of RAMP3 on distinct signaling readouts. These findings represent the first description of RAMP interactions with adhesion GPCRs and provide a novel mechanism by which G1 activity may be regulated.

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Abbreviations

GPCR	G protein-coupled receptor
aGPCR	Adhesion G protein-coupled receptor
G1	G protein-coupled receptor 56
ΔNT-G1	Truncated, constitutively active version of G1
RAMP	Receptor activity-modifying proteins
BFPP	Bilateral frontoparietal polymicrogyria
GPS	G-proteolytic site
GAIN	G protein-coupled receptor autoproteolysis-inducing domain
NFAT	Nuclear factor of activated T-cells
SRF	Serum response factor
Co-IP	Co-immunoprecipitation
HEK cells	Human embryonic kidney cells

I. Introduction

Cells communicate with one another by sending and receiving chemical, electrical, or mechanical signals. One way in which cells communicate is through receptors embedded in the plasma membrane. Cell surface receptors function by binding to ligands on their extracellular faces and then transmitting this signal inside the cell. Such receptors can transmit signals from ligands that cannot cross the plasma membrane and also amplify those signals through secondary messengers. This system creates complex signal transduction mechanisms that allow cells to communicate in fine-tuned ways.

Heterotrimeric G protein-coupled receptors (GPCRs) represent the largest and most diverse class of transmembrane receptors, comprising more than 1% of the human genome (Bockaert and Pin, 1999; Marinissen and Gutkind, 2001; and Pierce et. al, 2002). Hundreds of hormones, neurotransmitters, and sensory stimuli signal through GPCRs. As such, GPCRs play important roles in an eclectic range of physiological functions, such as cell growth, cell migration, and cell effector functions. Throughout evolution, GPCR structures have been adapted to recognize a diverse range of environmental stimuli (Bockaert et al., 2002). For example, GPCRs can sense light, odorants, amino acids, peptides, lipids, nucleotides, and proteins (Bockaert et al., 2002). Odorant GPCRs represent the most abundant group of GPCRs in many organisms, constituting 90% of the total number of GPCRs in *C. elegans* and 50% in *Drosophila,* suggesting a very important biological role for these type of receptors (Bockaert et al., 2002). As a result, GPCRs regulate the actions of many enzymes—such as phospholipases, ion channels—such as Ca^{2+}, and transport vesicles (Bockaert et al., 2002). Moreover, GPCRs help control many critical features of physiological functions, such as morphological movements during

gastrulation, hormonal regulatory systems, and synaptic transmission. As such, GPCRs are often direct and indirect targets of many therapeutic drugs as well as drugs of abuse (Roush, 1996; Stadel et al., 1997). Furthermore, genetic mutations in GPCRs have been associated with many pathologies (Birnbaumer, 1995; Spiegel, 1996; Piao et. al, 2004). These mutations often disrupt normal cell to cell communication, interfering with transduction of a signal into the cell or a signal exiting the cell. Thus, developing a better understanding of GPCR signaling is key to developing more effective treatments for these disorders.

In the classic GPCR signal transduction mechanism, a ligand binds to the extracellular portion of the transmembrane receptor, inducing conformational changes transmitted to an associated heterotrimeric G protein in the cytoplasm (Iguchi et. al, 2007). In the inactive state of a GPCR, the Gα subunit of the G protein is bound to guanosine diphosphate (GDP), which dissociates upon activation. GDP is replaced by guanosine triphosphate (GTP), causing dissociation of the Gβ and Gγ subunits from Gα, which in turn leads to downstream signal amplification (Iguchi et. al, 2007). GPCRs are organized into five classes: rhodopsin-like, secretin, frizzled/taste2, glutamate, and adhesion. The rhodopsin-like family, also referred to as Family 1, encapsulates the majority of the GPCRs, including many hundreds of odorant receptors (Bockaert et al., 2002). In all the main families except the rhodopsin family, the GPCRs have long amino-termini (NT), with adhesion GPCRs being especially characteristic of this structure, and secretin, glutamate, and frizzled/taste2 receptors having long N-termini rich in cysteine residues (Fredriksson et al., 2003).

Subfamily 1a encompasses GPCRs upon which small ligands like rhodopsin, ATP, catecholamines, odorants, and smell peptides act; family 1b includes GPCRs that bind peptides at the extracellular face of the receptors, including the N terminus and extracellular loops; family

1c contains GPCRs that bind glycoproteins such as thyroid-stimulating hormone (TSH) (Bockaert et al., 2002). The secretin family receptors bind larger peptides that mostly act in a paracrine fashion (Fredriksson et al., 2003). Members of this family include the calcitonin receptor (CALCR), the corticotropin-releasing hormone receptors (CRHRs), and the glucagon receptor (GCGR). The frizzled/taste2 receptor family is divided into two subgroups: the frizzled receptors and the TAS2 receptors. Little is known about the TAS2 receptors; however, it has been shown that they are expressed in the tongue and palate epithelium, functioning primarily as bitter taste receptors (Fredriksson et al., 2003). Unlike TAS2 receptors, frizzled receptors play an important role in the regulation of cell fate, proliferation, and polarity by intercepting signals from the secreted glycoproteins called Wnts (Fredriksson et al., 2003). The glutamate receptor family contains eight metabotropic glutamate receptors (GRM), two GABA receptors, a single calcium-sensing receptor (CASR), and five taste receptors (TAS1). One unique feature about these receptors is that the N terminus folds into two distinct portions separated by a cavity in which glutamate binds, in turn prompting the portions to fold over the ligand (Fredriksson et al., 2003).

Adhesion GPCRs (aGPCRs) exhibit both adhesion and signaling functions, both of which are thought to occur via cell-cell and cell-matrix interactions (Salzman et. al, 2016). The structure of aGPCRs is characterized by a long N-terminus with adhesion-like motifs, a seven-pass transmembrane helix bundle (7TM) (Salzman et. al, 2016), and an intracellular carboxyl-terminus (CT) (Iguchi et. al, 2008). Importantly, however, aGPCRs differ from other GPCR subfamilies due to the presence of a long NT that contains a GPCR-Autoproteolysis-Inducing (GAIN) domain, at which the receptor is cleaved (Arac et. al, 2012). The N termini vary in length, but are usually between 200-2800 amino acids long. They contain many glycosylation

sites and proline residues, forming "mucin-like" stalks (Huang et al., 2008). Some of the aGPCRs appear in clusters of three or four, including the brain-specific angiogenesis-inhibitory receptors (BAIs) and EGF-like module containing receptors (EMRs) (Fredriksson et al., 2003). Members of the aGPCR family have restricted expression patterns in cell types and tissues such as leukocytes, smooth muscle cells, and the brain (Lin et al., 2012). Despite this expression specificity, aGPCRs share another common quality aside from a long N-terminus: a Cys-box located immediately upstream of the first TM domain that often contains a GPCR proteolytic site (GPS) motif for many aGPCRs (Krasnoperov et al., 2003). This cleavage event generates a heterodimeric receptor consisting of a cleaved N-termal fragment (NTF) non-covalently associated with a membrane-spanning C-terminal fragment (CTF) (Langenhan, T., & Schöneberg, T, 2016).

ADGRG1, also known as GPR56, is an aGPCR that is widely distributed throughout the body and plays significant roles in many biological functions. For example, ADGRG1 (referred to as "G1" from this point forward) helps regulate oligodendrocyte and cortex development in the brain (Piao et. al, 2004) and acts as an inhibitory receptor on T cells and NK cells (Peng et. al, 2011). Most notably, loss of function point mutations in G1 lead to bilateral frontoparietal polymicrogyria (BFPP), a genetic disorder characterized by cortical misfolding, altered cortex lamination in the frontal cortex, and reduced white matter volume (Piao et. al, 2004; Bahi-Buisson et. al, 2010). Behaviorally, individuals with BFPP display developmental delay, psychomotor slowness, language problems, and seizures (Luo et. al, 2011). Currently, 26 independent disease-associated mutations have been reported (Jin et. al, 2007). R565W and C346S are two mutations that have been shown to significantly reduce surface expression of G1 in human embryonic kidney (HEK)-293T cells (Chiang et. al, 2011; Jin et. al, 2007). The HEK-

293T cell line is derived from HEK cells and exhibits a somewhat neuronal phenotype (Shaw et. al, 2002). Since HEK cells grow quickly and are easily transfectable, they have been utilized for many experiments studying pathways related to brain development, including many studies published to date on G1 signaling (Kishore et al., 2015; Paavola et al., 2011).

In addition to altering brain development, the mutations that cause BFPP can also induce functional changes in the immune system. For example, it was shown that the R565W and C346S mutations alter G1 expression and activity in certain immune cells *in vitro* (Chang et. al, 2016). Specifically, the R565W mutation completely abolished G1 surface expression in natural killer (NK) and T cells, and the C346S mutation caused a 20-fold reduction in expression levels (Chang et. al, 2016). Importantly, the $CD56^{dim}$ NK cells in BFPP patients with the R565W mutation exhibited increased killing of K562 cells, a myelogenous leukemia line, achieved by increased degranulation and enhanced induction of apoptosis (Chang et. al, 2016).

High levels of G1 transcripts have been detected in NK cells, effector/memory T cells, and γδ T cells by Affymetrix microarrays (Peng et. al, 2011). In terms of T cells, CD8+ T cells, cytotoxic CD4+ T cells but not naïve and memory CD4+ T cells, and effector-type γδ T cells expressed GPR56. NK cells are one of the most potent innate immune cells. Their interaction with MHC class I (MHCI) molecules, which are expressed on the surfaces of almost all healthy cells and acts as an inhibitory receptor, allows NK cells to specifically target infected cells while avoiding destruction of healthy, intact cells (Vivier et. al, 2008). In humans, NK cells are divided into $CD56^{dim}$ and $CD56^{bright}$ subsets. The $CD56^{dim}$ subset represents about 90% of peripheral blood and spleen NK cells (Vivier et. al, 2008). The presence of GPR56 in $CD56^{dim}$ cells is downregulated upon cellular activation, thereby inhibiting inflammatory cytokine production and degranulation (Chang et. al, 2016). Introducing the R565W mutation in G1 in NK cells leads to

increased killing efficiency of K562 cells, further indicting that wildtype G1 regulates NK cell cytotoxicity (Chang et. al, 2016).

In addition to playing roles in BFPP and immune cells, G1 has also been implicated in cancer. Generally speaking, tumor-associated aGPCRs have been shown to affect the growth of tumor cells, angiogenesis, tumor cell migration, and also may serve as biomarkers for certain types of cancers (Lin et al, 2012). Specifically, G1 acts antagonistically on VEGF production and angiogenesis via signaling through PKCα (Yang et al., 2012). G1 is also overexpressed in glioblastomas, found on the leading edge of extending cell membranes (Shashidhar et al., 2004). In addition to its presence in glioblastomas, G1 acts as a predictor for melanoma, breast cancer, colon cancer, and pancreatic cancer (Liu et al., 2017). Moreover, G1 knockdown might decrease proliferation and invasion of epithelial ovarian cells by down-regulating RhoA-GTP levels and up-regulating E-cadherin levels, suggesting that G1 is a potentially favorable drug target (Liu et al., 2017). When considering G1 in the context of developing therapeutic drugs, it is important to keep in mind that the receptor directly interacts with other proteins.

G1 associates with a range of extracellular, intracellular, and transmembrane molecules to exert its effects on cells. These interactions allow for increased specificity in terms of G1 signaling. Intracellular scaffolding proteins containing modular domains such as PDZ and SH2 domains help organize many GPCRs into signaling complexes (Pierce et. al, 2002). The tetraspanin family of cell surface proteins is another group of scaffolding proteins that can interact with GPCRs in general, and G1 in particular, to modulate downstream signaling (Little et. al, 2004). Cross-talk between GPCRs and integrins can also be important, as exemplified by the synergistic actions of G1 and α3β1 integrin in cerebral cortical development (Jeong et. al, 2013). Heterodimerization of GPCRs to receptor channels can also be important in trafficking of

the two; for example, direct protein-protein coupling between dopamine D5, which is a Gs-coupled GPCR, and $GABA_A$ ionotropic receptors allow both to colocalize to dendritic shafts (Bockaert et al., 2002).

One group of proteins that has been found to play important roles in modulating the function of various members of the secretin-like family of GPCRs is the receptor activity-modifying proteins (RAMPs). For example, associations between the calcitonin receptor-like receptor (CRLR) and RAMP1 leads to generation of a calcitonin gene-related peptide (CGRP) receptor, whereas associations between CRLR and RAMP2 lead to creation of a receptor activated by adrenomedullin (Bockaert et al., 2002). Moreover, RAMP1 and RAMP2 are necessary for correct glycosylation and transport of CRLR to the cell surface (Born et al., 2002). RAMP3 is also important in that it helps calcitonin receptors (CTRs) interact with amylin (Born et al., 2002). It was shown that RAMPs and CRLR or CTRs associate non-covalently and form complexes at the cell surface. Recent work on these proteins has emphasized the necessity of the receptor complex as a whole rather than just the receptor or RAMP by itself in ligand-induced internalization, recycling and resensitization of secretin-like receptors (Nag et al., 2015).

There are reasons to believe that RAMPs may have broader physiological effects than just regulating a handful of secretin-like GPCRs. First, there is a substantial degree of conservation of RAMP proteins through evolution, evidenced by 37% identity across 139 sequences in 53 species, including zebrafish, mice, rats, guinea pigs, and non-human primates (Klein et al., 2016). In most species in which RAMPs are expressed, RAMPs 1-3 are encoded by single genes. In the genomes of some bony fish species, however, two genes encode *Ramp1* and *Ramp2*, whereas only one encodes RAMP3, suggesting that RAMP1 and RAMP2 are more similar to each other than either is to RAMP3 (Klein et al., 2016). Nonetheless, all RAMP

proteins are characterized by a large extracellular N-terminus, a single-pass transmembrane domain, and a cytoplasmic C-terminus (McLatchie et al., 1998). Despite this shared structure, however, RAMPs 1 and 3 are 148 amino acids long, whereas RAMP2 is 175 amino acids long (McLatche et al., 1998). It is these variations that allow RAMPs to differentially modulate GPCR functions such as ligand specificity, trafficking, signaling, and with and functional consequences resulting from these interactions.

While there exists a substantial amount of literature characterizing the relationships between RAMPs with the secretin-like family of GPCRs, no published data to date have examined the interactions between RAMPs and adhesion GPCRs. Given the importance of G1 in neurodevelopmental processes and its roles in disease pathologies such as BFPP and gliomas, this project aimed to study the effects of RAMPs 1-3 on G1 signaling and trafficking to the cell surface. Although adhesion GPCRs are now characterized as their own GPCR family, they were once classified as a sub-family of secretin-like GPCRs (Liu et al., 1999). Thus, given the sequence similarity in the transmembrane regions between secretin-like GPCRs and adhesion GPCRs, we hypothesized that RAMPs 1-3 might be able to associate with and influence the activity of adhesion GPCRs such as G1 activity to some degree. The studies described below assessed this possibility.

II. Materials and methods

Constructs

Human G1ΔNT (383–693) was 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) (Kishore et al., 2016). RAMP cDNA constructs with hemagglutinin (HA) epitope tags (HA-RAMP1, HA-RAMP2, and HA-RAMP3) were obtained from Missouri S&T cDNA Resource Center.

Cell culture and transfection

Human Embryonic Kidney (HEK-293T) cells were grown in DMEM (Invitrogen) supplemented with 10% fetal calf serum (FBS, Atlanta Biologicals) and 1% Penicillin-Streptomycin (Invitrogen) and maintained in a humidified incubator at 37°C with 5% CO_2. The cells were grown in 10 cm^2 dishes and were split either into new 10 cm^2 dishes or into 96-well plates 24 h prior to transfection in overexpression experiments.

Co-immunoprecipitation

HEK-293T cells were transfected with either 1 μg EV, G1, or ΔNT-G1, along with 1 μg EV, HA-RAMP1, HA-RAMP2, or HA-RAMP3. Twenty-four hours post-transfection, cells were lysed in a buffer containing 1% Triton X-100, 150 nM, NaCl, 25 mM HEPES, 10 mM $MgCl_2$, 1 mM EDTA solution with a protease inhibitor tablet (Roche) and dissociated membranes were cleared by centrifugation. A portion of the lysates were set aside and the remainder was

incubated end-over-end with 40 μL of A/G Agarose beads (Thermo Scientific) and anti-G1 C-terminal antibody (1:5000) for 1.5 hours at 4°C. A/G Agarose Beads were washed 3X with lysis buffer and antigens were eluted using 2x Laemmli buffer. Meanwhile, 4x Laemmli buffer was added to the soluble lysates.

SRF and NFAT luciferase gene reporter assays

HEK-293T cells were seeded in 96-well plates 24 h prior to transfection. When the cells reached 70% confluence, each well was transfected with 25 ng of SRF or NFAT firefly reporter, 1 ng of *Renilla* luciferase, and 5 ng of EV, G1, or ΔNT-G1 along with 5 ng of EV, HA-RAMP1, HA-RAMP2, or HA-RAMP3. Eight technical replicates were transfected for each condition. The SRF luciferase assay constitutes a readout of G1 coupling to $G\alpha_{12/13}$, whereas the NFAT luciferase assay represents a readout of $G\alpha_q$ activity. All reporter constructs (NFAT: pGL4.30, SRF: pGL4.34, *Renilla* pRLSV40) were acquired from Promega (Madison, WI). 48 h and 72 h post transfection, Dual-Glo luciferase assays (Promega) were performed according to the manufacturer's protocol. Cells were incubated for 10 min first with luciferase, followed by Renilla. Plates were read on a BMG Omega plate reader; a gain of 3300 was for the readout of SRF firefly values and a gain of 3800 was used for the readout of NFAT firefly values as well as SRF and NFAT *Renilla* luciferase values. Outliers for both Firefly and *Renilla* luciferase values were removed using Grubbs' test at the 5% significance level on GraphPad. Results were determined for both the SRF and NFAT assays by calculating the luminescence ratio of firefly:*Renilla* luciferase. The ratio values for HA-RAMP1, HA-RAMP2, HA-RAMP3, G1, and ΔNT-G1 were normalized to the mean of EV transfected wells. The ratio values for wells co-transfected with G1 or ΔNT-G1 along with HA-RAMP1, HA-RAMP2, or HA-RAMP3 were

normalized to the mean of respective wells transfected with EV along with the respective RAMP cDNA.

Cell surface biotinylation

Surface expression of G1, ΔNT-G1, and HA-RAMP3 was assessed in HEK-293T cells. Upon reaching 70-80% confluence in 10 cm^2 dishes, HEK-293T cells were transfected for 24 h with 1 μg empty vector (pcDNA3.2 or EV), cDNA encoding full length G1, or cDNA encoding the truncated version of G1 that lacks the GAIN domain (ΔNT-G1). Cells were then washed in PBS/Ca^{2+} and treated with the biotinylation reagent EZ-link Sulfo-NHS-SS-Biotin (Thermo Scientific) at 5 mM concentration. This reagent was allowed to thaw at room temperature for 10 minutes before being reconstituted in PBS/Ca^{2+}. Cells were incubated in EZ-link Sulfo-NHS-SS-Biotin for 30 minutes at room temperature, and then 30 minutes on ice. The set of plates that did not receive biotin were incubated in the same conditions in PBS/ Ca^{2+}. EZ-link Sulfo-NHS-SS-Biotin works by attaching a biotin group with a linker arm to all free extracellular amines, thus labeling all proteins that are expressed at the cell surface at and during the time of incubation. Biotin labelling was quenched by washing with 100 mM glycine (Sigma) in PBS/Ca^{2+}. Cells were then collected into microcentrifuge tubes in PBS and frozen at -80º C overnight. After a quick thaw in the water bath to break apart membranes, samples were spun down to pellet the membranes. The cytosolic proteins in the supernatants were removed from each sample, and the pellets were re-suspended with solublization buffer (1% Triton X-100, 150 mM NaCl, 25 mM HEPES, 1 mM EDTA, 10 mM $MgCl_2$ with 1X HALT protease/phosphatase inhibitor). Samples were mixed end-over-end at 4º C for 1 hour, during which time the proteins were extracted from the membranes. After spinning down the samples following the one-hour incubation, portions of

the soluble lysates were reserved for analysis; 4x Laemmli buffer was added to these lysates. Laemmli buffer reduces and denatures the proteins the samples. The remaining supernatants were incubated with streptavidin agarose beads (Thermo Scientific) for 1 hour at 4º C. Streptavidin binds to biotin, pulling down the biotinylated proteins. Streptavidin agarose beads were washed 3 times with solubilization buffer to reduce nonspecific binding. Samples were then spun down, and the supernatants containing non-biotinylated proteins were discarded. Biotinylated proteins were then eluted off the beads overnight in 2x Laemmli buffer. Samples were kept at room temperature until subjected to gel electrophoresis followed by Western blot to detect RAMP3 and G1 solubility and surface expression.

Western blot

Co-immunoprecipitation, streptavidin pull-down, and soluble lysate protein samples were loaded into 4-20% Tris-Glycine gels (Bio-Rad) and subjected to SDS-PAGE electrophoresis at 150 mV for 45 min. Proteins were then transferred to nitrocellulose membranes (Bio-Rad) for 10 min. Afterward, blots were blocked with a buffer containing 2% nonfat dried milk, 50 mM NaCl, 10 mM HEPES, and 0.1% Tween 20 (Sigma) for 1 h and then incubated with primary antibodies for 1 h at room temperature or overnight at 4º C. Primary antibodies used were a rabbit anti-G1 C-terminal antibody (1:2500, Oribgen, Inc.) developed by injecting rabbits with a peptide (CSNSDSARLPISSGSTSSSRI) derived from the C terminus of G1 (Paavola et al., 2011), monoclonal rat anti-HA 3F10 (1:2500, Roche), and polyclonal chicken anti-GAPDH (1:2500, EMD Millipore). Following primary antibody incubation, nitrocellulose membranes were subjected to three 7-minute washes with the 2% milk buffer to eliminate nonspecific binding. Secondary antibodies used were enhanced chemiluminscence Horse-Radish Peroxidase linked

anti-rabbit, anti-rat, and anti-chicken (1:2500). Membranes were incubated with secondary antibodies were 1 h. Blots were then subjected to three 7-min washes with the 2% milk buffer before being developed with Supersignal West Pico for 2 min (Thermo Scientific). Finally, blots were imaged on a LI-COR instrument and visualized using Image Studio. Western blots were then quantified using ImageJ by creating profile plots from rectangles drawn around each band. The profile plots represent the relative density of the band captured in the rectangle in each lane, and the area underneath the peak in each profile plot was calculated as a measure of band density.

Stripping of western blot membranes

After the western blots analyzed in the surface biotinylation assay experiments were visualized, the membranes were stripped to remove the primary and secondary antibodies with which they were initially incubated. The purpose of this procedure is to then probe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein expression, which was used as housekeeping protein. Normalization against GAPDH or other standard housekeeping proteins such as β-actin and β-tubulin is a common tool used to control for protein loading and transfer efficiency (Ferguson et al., 2005). GAPDH is an enzyme with a molecular weight of ~37 kDa. It is involved in metabolic and non-metabolic physiological functions, such as catalyzing the sixth step in glycolysis and initiation of apoptosis, vesicle shuttling from the endoplasmic reticulum to the Golgi apparatus, and transcription activation (Zala et al., 2013; Tarze et al., 2006). Since GAPDH is expressed at high levels in most cell types and tissues, it is widely used as a loading control in Western blot analysis. Furthermore, in this study, GAPDH was used to control for the number of cells collected following incubation with EZ-link Sulfo-NHS-SS-Biotin, as a visible

difference in cell pellet sizes were observed following centrifugation. Thus, after visualizing blots on the LI-COR instrument, membranes were washed with PBS with 0.1% TWEEN 20 (PBST) for 10 min. Then, the membranes were incubated in Restore™ PLUS Western Blot Stripping Buffer (Thermo Scientific) for 15 min, followed by another 10 min wash with PBST. All of these incubations were performed at room temperature. Following this step, the standard Western blot protocol was followed, starting with a 1 h blocking period with containing 2% nonfat dried milk, 50 mM NaCl, 10 mM HEPES, and 0.1% Tween 20.

Statistical analysis

Statistical analyses were performed using Prism software. One-way ANOVA was used to measure significance by comparing the means of each condition to each other. In the SRF and NFAT-luciferase signaling assays, mean values from three independent experiments were averaged. Eight technical replicates were performed in each of the three independent experiments.

III. Results

G1 and ΔNT-G1 physically interact with RAMP1 and RAMP3

G1 and ΔNT-G1 were expressed in HEK-293T cells alone or in the presence of RAMP1-3. Immunoprecipitation was performed using an anti-G1 antibody, and co-immunoprecipitation of the RAMPs was assessed. Western blot analyses showed that RAMP1 and RAMP3 associated with both the full length and constitutively active (ΔNT) versions of G1 (Fig. 3). Notably, in all three repeats of the experiment, the RAMPs appeared to co-immunoprecipitate more robustly with ΔNT-G1 than with full-length G1. Parallel Western blots of the cell lysates revealed that

expression levels of RAMPs 1 and 3 were comparable, as were expression levels of G1 and ΔNT-G1 (Fig. 3). However, RAMP2 expression (as detected in anti-HA Western blots of the cell lysates) was visibly lower than expression of RAMP1 and RAMP3.

Co-overexpression of RAMP3 with ΔNT-G1 increases signaling to NFAT luciferase

It has been previously established that both G1 and ΔNT-G1 transfection into HEK-293T cells results in significant increases in signaling through SRF, with more dramatic increases in signaling seen with transfection of ΔNT-G1 (Kishore et al., 2016). It has also been shown that G1 expression can increase signaling to NFAT luciferase, although this effect is only seen with ΔNT-G1 and not full-length G1. In the NFAT luciferase assays performed in this present study, these patterns were also observed as significant increases in signaling in ΔNT-G1 + RAMP3 compared to EV + RAMP3 were observed (cells transfected with RAMP3 alone; Figure 4). However, at 72 h, NFAT activity was comparable in ΔNT-G1 + RAMP1, RAMP2 or RAMP3 conditions compared to ΔNT-G1 alone. A modest increase in ΔNT-G1-mediated NFAT signaling was observed with RAMP3 co-transfection, but the difference was not statistically significant. Thus, moving forward, emphasis was placed on RAMP3 in experiments aimed at understanding the functional effects of RAMPs on G1. It important to note that NFAT signaling in wells transfected with RAMP1, RAMP2, or RAMP3 alone were comparable to EV. Although there is a very low level of endogenous G1 expressed in HEK-293T cells, these data show that over-expression of RAMPs does not elicit any changes in baseline signaling activity to NFAT luciferase. Furthermore, G1, G1 + RAMP1, G1 + RAMP2, and G1 + RAMP3 were all comparably active in this assay, indicating that while the co-immunoprecipitation data reveal physical interactions between full-length G1 and RAMP1 as well as full-length G1 and RAMP3,

these associations did not affect signaling of the full-length receptor at 72 h in the NFAT luciferase assay.

Co-overexpression of ΔNT-G1 and RAMP3 suppresses signaling through SRF

In addition to activating NFAT luciferase, overexpression of both full length G1 and ΔNT-G1 has been shown to increase SRF luciferase activity in HEK-293T cells (Kishore et al., 2016). Thus, we assessed SRF luciferase in HEK cells transfected with G1 or ΔNT-G1 in the absence and presence of RAMPs 1-3. Interestingly, these studies resulted in findings distinct from what was observed in the NFAT signaling experiments. At 72 h post-transfection, a significant increase in SRF activity was observed in cells transfected with full-length G1 compared to the empty vector (EV) condition (~20 fold increase at 72 h; Figure 5). Furthermore, a more dramatic significant increase in SRF signaling activation was demonstrated in cells transfected with ΔNT-G1 compared to cells transfected with either EV or G1 (~70 fold increase over EV and ~3.5 fold increase over G1 at 72 h; Figure 5). There were no significant changes in SRF G1 signaling activity in the presence of RAMPs 1, 2, or 3 at 72 h. On the other hand, co-expression of ΔNT-G1 and RAMP1 resulted in a significant decrease in SRF-luciferase activity in cells transfected with ΔNT-G1 compared to ΔNT-G1 in the presence of RAMPs 1, 2, or 3 for 72 h. Similarly, cells co-transfected with ΔNT-G1 and RAMP3 lead to a significant suppression in signaling to SRF luciferase. Thus, the effects of RAMP co-expression on G1 signaling were distinct depending on the signaling output measured.

G1 and RAMP3 interactions decrease surface expression of G1 and ΔNT-G1

To explore potential underlying mechanisms by which RAMPs might modulate G1 signaling activity, surface expression levels of both versions of the receptor in the absence and presence of RAMP3 were evaluated. After half of the samples were labeled with biotin for 1 h and subsequently pulled down with streptavidin beads, an anti-G1 C-terminal antibody was used to blot for the detection of both G1 and ΔNT-G1. An C-terminal antibody was used to allow for the recognition of ΔNT-G1, which lacks a portion of the N-terminus, but has an intact C-terminus. After these blots were visualized, they were stripped of C-terminus primary antibody and anti-rabbit secondary antibody for the purpose of re-blotting for GAPDH protein expression.

The data from this experiment showed that surface expression of full-length G1 decreased in the presence of RAMP3 after all bands on the Western blots were quantified and pull-down values were normalized to GAPDH. Levels of G1 surface expression following G1 and RAMP3 interactions were comparable to the levels of ΔNT-G1 surface expression in cells transfected with ΔNT-G1 but not the cells co-transfected with RAMP3. Interestingly, ΔNT-G1 surface expression increased ~5 fold in cells overexpressing both ΔNT-G1 and RAMP3 compared to cells transfected with ΔNT-G1 alone (Figure 7). Thus, RAMP3 had distinct effects on the full-length vs. truncated form of G1. This experiment was only performed once due to time constraints, so at least two more replicates are essential to assess whether the observed effects are statistically significant.

G1 and RAMP3 interactions increase surface expression of RAMP3

There has been substantial evidence in the literature highlighting the important roles that RAMPs play in helping G protein coupled receptors traffic to the cell surface in HEK-293T cells

(Bomberger et al., 2005; Morfis et al., 2008). Specifically, a role in the post-endocytic sorting of adrenomedullin receptors (AM-Rs) was identified for RAMP3 (Bomberger et al., 2005). In addition, RAMP3 was shown to harbor a PDZ type I domain in its C terminus that leads to protein-protein interactions determining receptor trafficking (Bomberger et al., 2005). However, in addition to RAMPs modulating GPCR trafficking, in some cases the receptors can also modulate trafficking of the RAMPs (Hay et al., 2016). Thus, the samples from the surface biotinylation assay performed in this study were also assessed in anti-RAMP3 Western blots, which revealed that co-transfection of G1 with RAMP3 had no effect on RAMP3 surface expression (Figure 8). However, co-overexpression of ΔNT-G1 with RAMP3 brought about an ~18 fold increase in RAMP3 surface expression compared to cells transfected with RAMP3 alone (Figure 8).

IV. Discussion and Conclusions

Prior to this study, there has been no published research investigating potential interactions between adhesion GPCRs and RAMPs. The results of this project show that RAMP1 and RAMP3 physically associate with both the full-length version of the adhesion G protein-coupled receptor G1 as well as the truncated version that mimics the GAIN-cleaved receptor (ΔNT-G1). In fact, the associations between the RAMPs and ΔNT-G1 appear to be stronger than the associations between the RAMPs and full-length G1. Although no interactions were observed with RAMP2, this result could be due to poor overall expression of RAMP2, as indicated by faint anti-RAMP2 bands in the lysate samples. Further studies with a different RAMP2 construct that results in better expression will be necessary to determine whether all RAMPs interact with G1 or if the interaction is specific to RAMP1 and RAMP3. In any case, the establishment of physical associations between RAMPs and G1 represent a novel finding, and this information

was used as a starting point to investigate further the functional effects resulting from G1-RAMP interactions.

Overexpression studies in HEK-293T cells revealed that co-transfection of ΔNT-G1 with either RAMP1 or RAMP3 resulted in a significant decrease in signaling in the SRF-luciferase gene reporter assay, with more dramatic suppression observed by RAMP3. SRF is a downstream target in the RhoA pathway, so it represents a readout of ΔNT-G1 coupling to $G\alpha_{12/13}$ (Cheng et al., 2010). Co-expression of RAMP1 and RAMP2 with G1 also resulted in a trend toward inhibition in the SRF-luciferase assay, although only the inhibition mediated by RAMP3 was statistically significant. In addition to activating SRF-luciferase, G1 has also been shown to activate NFAT luciferase (Kishore et al., 2016). Interestingly, unlike the results seen in the SRF-luciferase assay, overexpression of RAMPs with ΔNT-G1 did not result in decreased signaling to NFAT. Over-expression of RAMP3 with ΔNT-G1 actually resulted in somewhat increased signaling to NFAT, although in the set of experiments presented here the results were not statistically significant. Taken together, these observations demonstrate that RAMPs preferentially inhibit G1 signaling to SRF luciferase but not NFAT luciferase, which raises the possibility that RAMP association induces biased signaling by G1.

Biased signaling, also known as functional selectivity, has been well characterized in G protein coupled receptors (Kenakin, 2011). One way in which GPCRs are subjected to receptor bias is a phenomenon known as conformational selection, whereby different conformations of the receptor lead to varied intracellular downstream actions (Burgen, 1981). For example, certain conformations put GPCRs in an "active" conformation, whereas other conformations inhibit activation even in the presence of a ligand. Biased signaling can be due to differential receptor interactions with distinct signaling proteins, such as G proteins, β-arrestins, or G protein-coupled

receptor kinases (GRKs) in different cell types (Kenakin et al., 2011). Biased signaling is a very important concept in the context of drug discovery because it challenges a simpler model of drug efficacy, where one ligand confers identical effects on many different downstream pathways following receptor activation. In reality, the opposite is often observed. That is, a ligand can display multiple varying efficacies, acting as a full agonist in some physiological signaling processes but a partial agonist or even an antagonist in others. The phrases "full versus partial agonism", "constitutive activity", "inverse agonist", and "neutral antagonists" are only a few that have been incorporated into the rich vocabulary describing GPCR activity. One group of well-known ligands that act as both antagonists and agonists for distinct receptor pathways are β-blockers, such as propranolol or carvedilolo, which can function as inverse agonists on $G\alpha_s$-mediated cAMP signaling but positive agonists for extracellular receptor kinase (ERK) activation (Azzi et al., 2003; Wisler et al., 2007).

Recently, studies focusing on biased ligands in the context of aGPCRs have proposed models in which the stalk regions of G1 and another aGPCR, brain-specific angiogenesis inhibitor 1 (BAI1), might bias activation toward certain signaling pathways and away from others (Kishore et al, 2016). Specifically, Kishore et al. 2016 created stalkless (SL-G1) versions of G1 and demonstrated that this mutant receptor expressed and trafficked to the plasma membrane at a level that comparable to those of G1 and ΔNT-G1. Interestingly, when SL-G1 was overexpressed in HEK-293T cells for evaluation in several signaling assays, the removal of the stalk conferred varying effects. In the NFAT luciferase and AP-TGFα assays, G1-SL exhibited comparable signaling levels compared to G1 and ΔNT-G1; however, in the SRF-luciferase assay, removal of the stalk resulted in a loss of signaling. Relating these findings to the results presented in this report, then, it is possible that interactions between G1 with RAMP1

and RAMP3 translate into differential downstream signaling. The NFAT-luciferase assay data generated by Kishore et al., 2016 distinguishes NFAT-luciferase as a "stalk-independent" signaling pathway for G1, whereas SRF-luciferase is "stalk-dependent." Since the N terminal fragment has been characterized as an allosteric antagonist by directly inhibiting constitutive stalk-independent activity mediated by the 7TM region (Kishore et al., 2016), and since ΔNT-G1 constitutes a version of G1 that lacks most of its NTF, perhaps RAMP3 interaction with ΔNT-G1 induces a gross conformational change in the receptor that makes it more conducive to downstream activation of NFAT. Another possibility is that RAMPs may specifically antagonize interactions of the G1 stalk with the rest of the receptor. Thus, an interesting future direction to pursue would be to assess the stalkless version of G1 in these experiments and examine effects on RAMP interactions and signaling.

A potential explanation for the effects of RAMPs on G1 signaling could be that RAMPs modulate surface expression of G1 and/or ΔNT-G1. The results of G1 and RAMP surface expression analyses via biotinylation and streptavidin pull-down of surface assays followed by immunoblotting with anti-G1-CT and anti-HA antibodies in this report revealed a decrease in G1 surface expression in the presence of RAMP3 overexpression compared to the G1 alone condition. On the other hand, there was an increase in ΔNT-G1 surface expression in the presence of RAMP3 compared to ΔNT-G1 alone. These data are based only on n = 1 of the surface biotinylation assay, and therefore need to be taken with a grain of salt. In the future, the surface biotinylation assay (looking especially at RAMP3) should be repeated at least two more times and should also be performed with RAMP1 and RAMP2 in order to assess whether altered surface expression levels of the receptors and RAMPs explain the effects of the various RAMPs on G1 signaling.

In addition to further investigating the functional effects of RAMP interactions with G1, another important future direction will be to dissect the site(s) of interaction between RAMPs and G1. To achieve this, mutations can be introduced in the NT, CT, and 7TM portions of the receptor, followed by co-immunoprecipitation, surface expression, and signaling assays. Elucidating the sites of interaction between G1 and RAMPs would provide a foundation upon which manipulation of the receptor with increased control can occur. Overall, the results of this project have provided novel insights into the modulatory effects of RAMPs on G1 and ΔNT-G1 surface expression and signaling. These studies have significant implications for future drug development efforts aimed at G1, which may be an important target for therapeutics aimed at treating BFPP, glioma and demyelination disorders, amongst other diseases (Langenhan et al, 2016). Future studies strengthening the understanding of these connections as well as exploring connections between RAMPs and other adhesion G protein coupled receptors might open many doors to investigating the potential pharmacological benefits of targeting adhesion GPCR/RAMP complexes.

VI. Figures

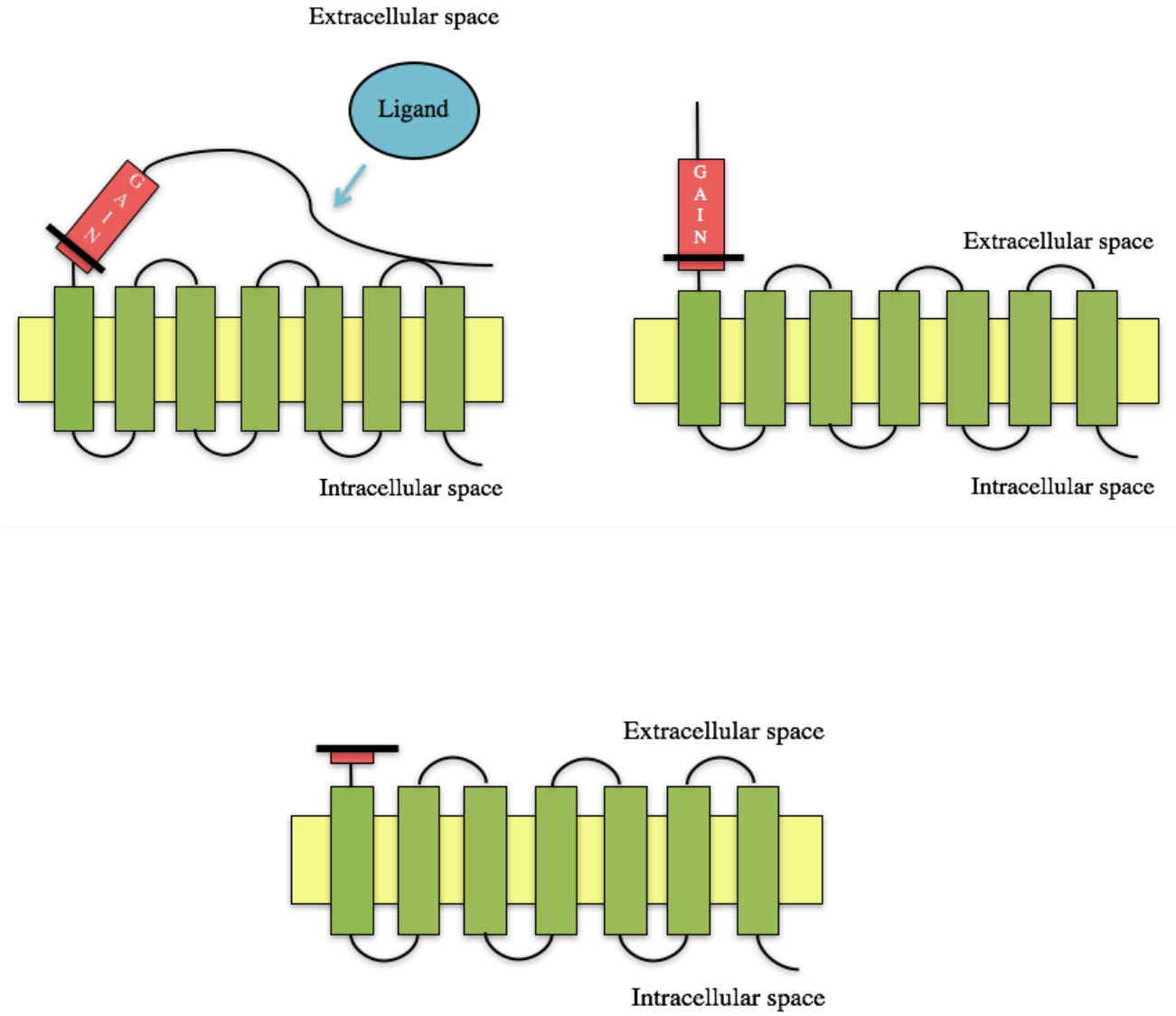


Figure 1: Schematic of G1 activation by a ligand**.** ΔNT-G1 represents a truncated version of G1 that lacks the GAIN domain and exhibits constitutive activity.

RAMP	Interacting GPCRs	Resulting functions of interactions	Molecular weight (kDa)
RAMP1	Calcitonin Calcitonin-like receptor Calcium sensing receptor $VPAC_1$* $VPAC_2$*	Terminal glycosylation of CGRP Maturation of CGRP Presentation of CGRP to cell surface	17 kDa (USCD Signaling Gateway)
RAMP2	Parathyroid hormone 1 receptor Calcitonin Calcitonin-like receptor Glucagon $VPAC_1$* $VPAC_2$* Corticotropin-releasing hormone receptor	In the presence of RAMP2, calcitonion-like receptor (CRLR) functions as an adrenomedullin receptor. RAMP2 is involved in core glycosylation. RAMP2 plays a role in transporting CRLR to the cell surface.	Monomer: 20 kDa (Santa Cruz Biotechnology, Inc.)
RAMP3	GPR30 (estrogen receptor) Calcitonin Calcitonin-like receptor Calcium sensing receptor Secretin Parathyroid hormone 1 receptor	Transports CRLR to cell surface	Monomer: 28 kDa Homodimer: 50 kDa Heterodimer: 73-75 kDa (Santa Cruz Biotechnology, Inc.)

Figure 2: Summary of RAMP functions.

***** VPAC = Vasoactive intestinal polypeptide receptor
* CGRP = calcitonin gene-related peptide receptor

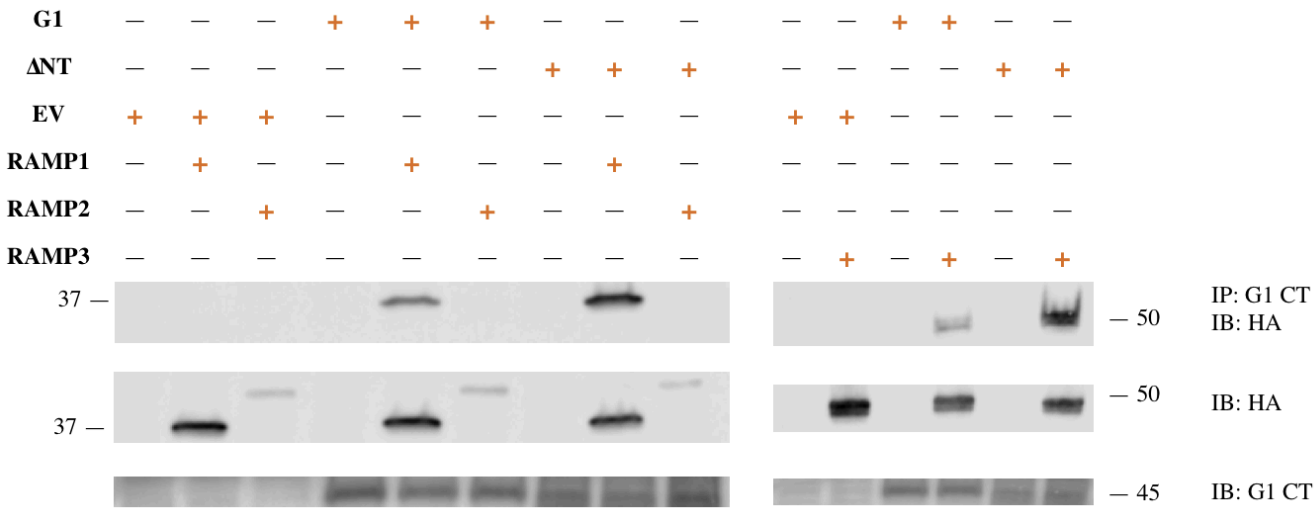


Figure 3: RAMP1 and RAMP3 interact with G1 and ΔNT-G1 in HEK293T cells following 24 h of co-transfection. After a portion of the soluble lysates were set aside for analysis, the remaining amount was incubated with A/G Agarose beads and G1 C-terminal antibody. The soluble lysate samples show equal input of RAMPs 1-3 as well as G1 and ΔNT-G1, and the co-immunoprecipitation samples demonstrate physical associations between RAMP1 and RAMP3 with G1 and ΔNT-G1. The interactions between both RAMP1 and RAMP3 with ΔNT-G1 appears to be stronger than the interactions between RAMP1 and RAMP3 with G1.

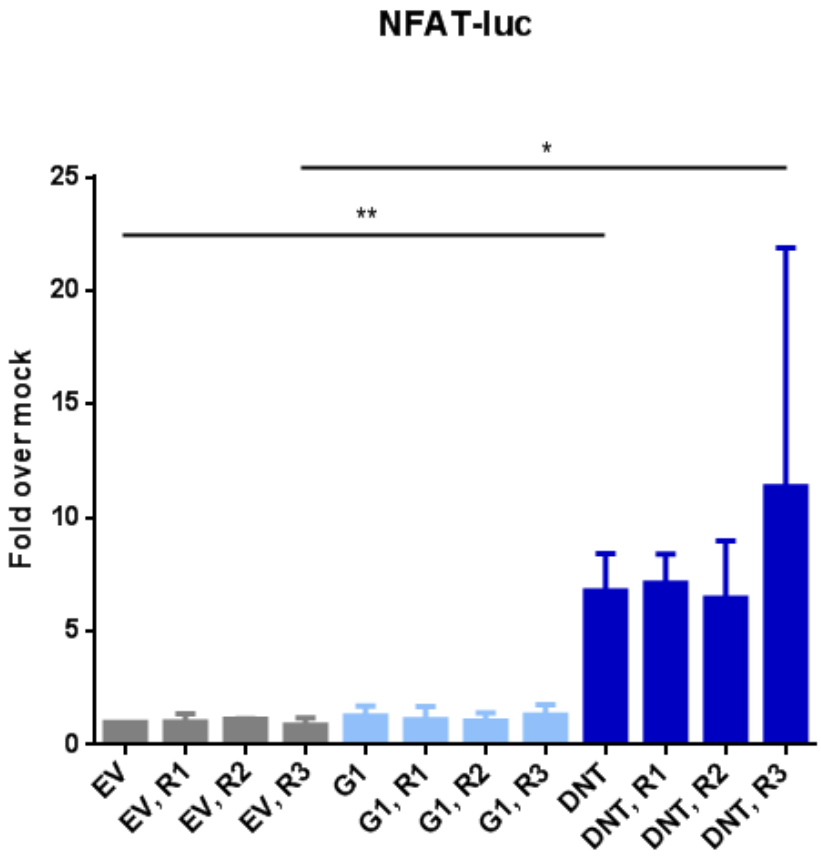


Figure 4: NFAT luciferase gene reporter assay. HEK-293T cells were transfected or co-transfected with G1 or ΔNT-G1 and RAMPs 1-3 for 72 h. RAMP1 is denoted as "R1", RAMP2 as "R2", and RAMP3 as "R3" in this graph. There is no significant change in NFAT activity in the G1 transfected cells; however, ΔNT-G1 exhibited a significant increase in NFAT signaling, which has been previously observed in literature. These results represent three independent experiments (+/- S.E. is shown, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$). EV + R1, EV + R2, EV + R3, G1, and ΔNT-G1 were compared to empty vector, denoted by EV, when calculating fold over mock. Similarly, G1 + RAMPs 1-3 and ΔNT-G1 + RAMPs 1-3 were compared to their respective empty vector controls with the appropriate RAMP added.

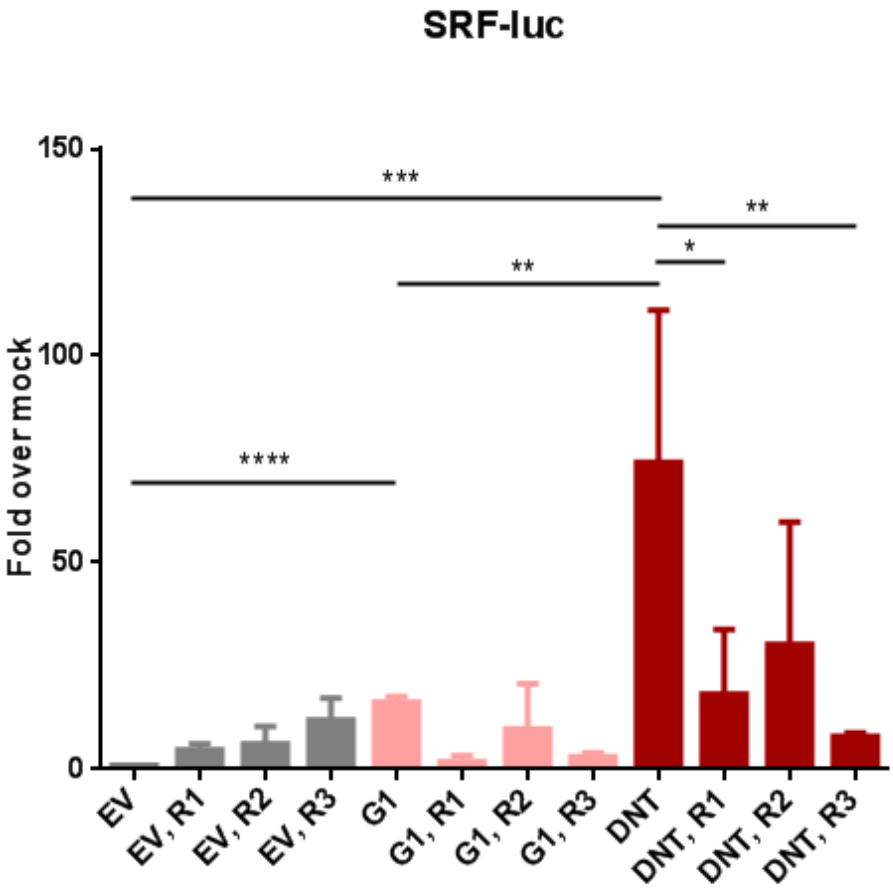


Figure 5: SRF luciferase gene reporter assay. HEK-293T cells were transfected or co-transfected with G1 or ΔNT-G1 and RAMPs 1-3 for 72 h. RAMP1 is denoted as "R1", RAMP2 as "R2", and RAMP3 as "R3" in this graph. Both G1 and ΔNT-G1 exhibited a significant increase in SRF signaling, which has been previously observed in literature. Furthermore, ΔNT-G1 is significantly more active than G1 in the SRF luciferase assay. At least in this system, the addition of RAMPs 1-3 on SRF-luciferase seems to lead to a decrease in signaling activity through SRF. These results represent three independent experiments (+/- S.E. is shown, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$). EV + R1, EV + R2, EV + R3, G1, and ΔNT-G1 were compared to empty vector, denoted by EV, when calculating fold over mock. Similarly, G1 + RAMPs 1-3 and ΔNT-G1 + RAMPs 1-3 were compared to their respective empty vector controls with the appropriate RAMP added.

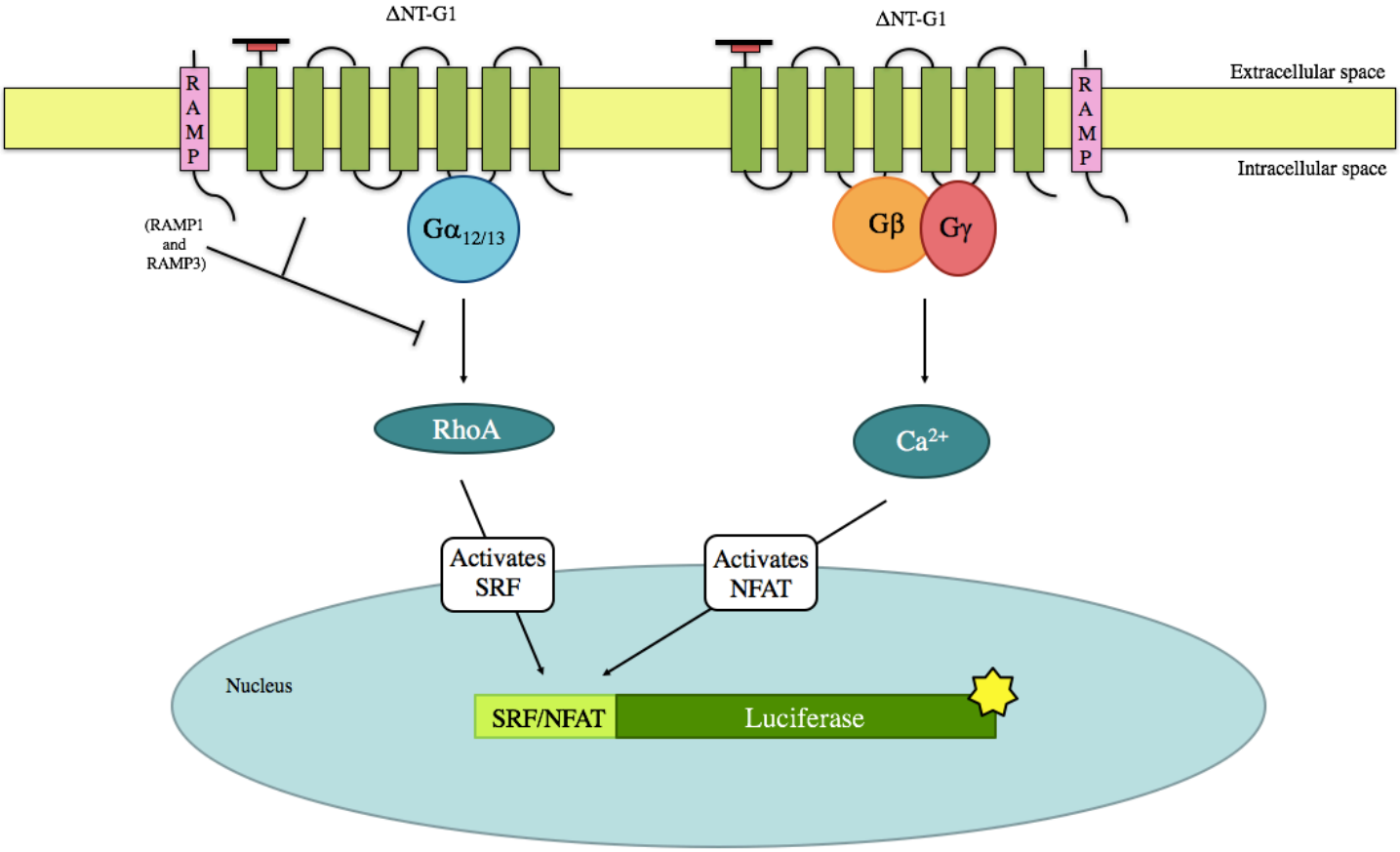


Figure 6: Schematic of SRF and NFAT luciferase assays and summary of signaling data. In experiments performed in this project, the addition of RAMPs 1-3 on SRF-luciferase seems to lead to a decrease in signaling activity through SRF at 72 h post-transfection. Co-expression of ΔNT-G1 and RAMP1 resulted in a significant decrease in SRF-luciferase activity in cells transfected with ΔNT-G1 compared to ΔNT-G1 in the presence of RAMPs 1, 2, or 3 for 72 h. Similarly, cells co-transfected with ΔNT-G1 and RAMP3 lead to a significant suppression in signaling to SRF luciferase. There is no significant change in NFAT activity when cells were co-transfected with ΔNT-G1 and RAMP proteins. These data show that the effects of RAMP co-expression on G1 signaling were distinct depending on the signaling output measured.

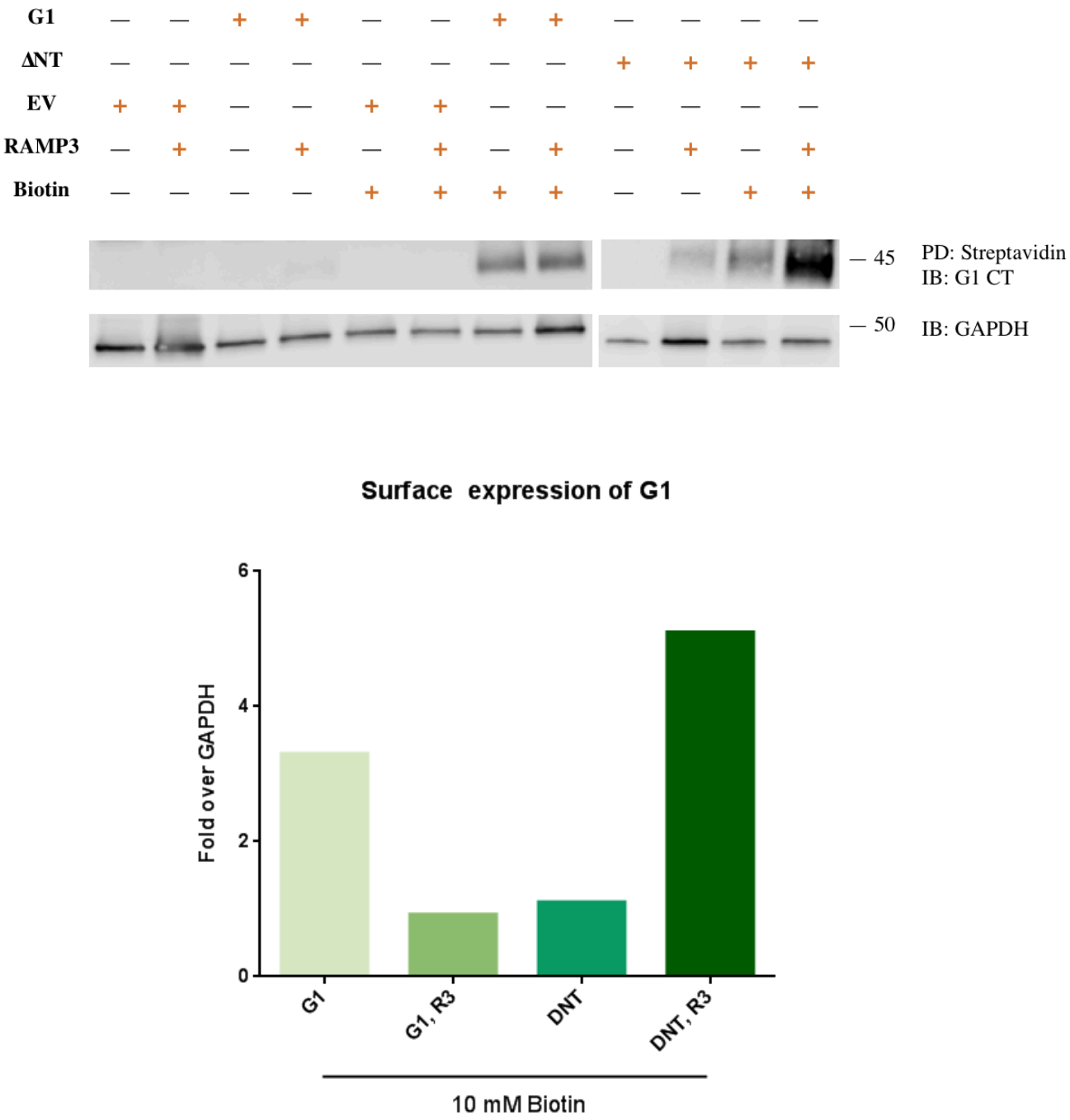
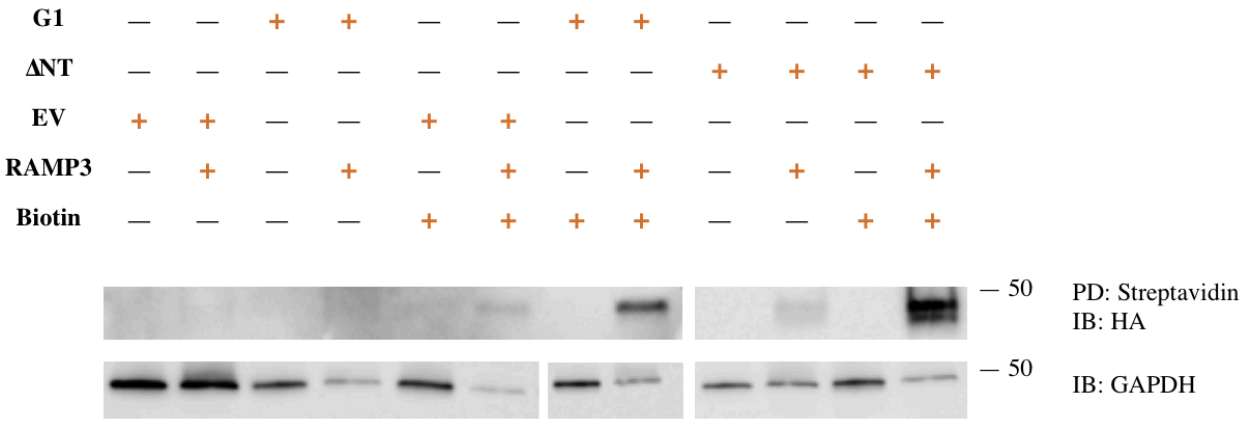


Figure 7: Co-overexpression of G1 and RAMP3 decreases surface expression of G1, but co-expression of ΔNT-G1 and RAMP3 increases surface expression of ΔNT-G1. The results above represent one experiment.



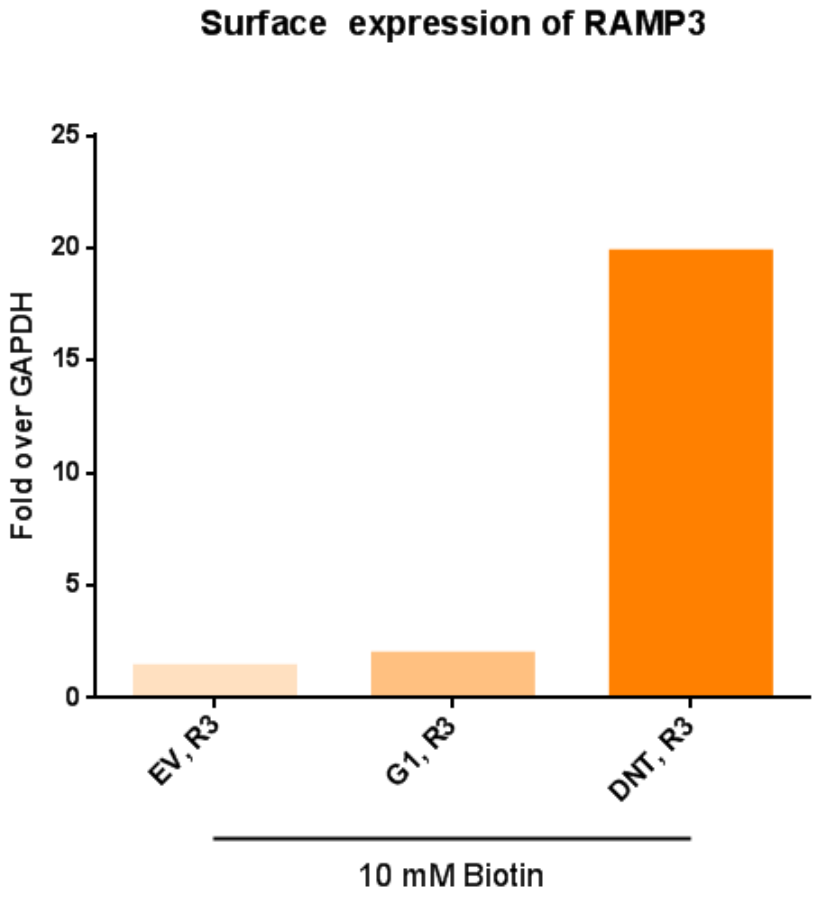


Figure 8: Co-overexpression of G1 or ΔNT-G1 along with RAMP3 or increases surface expression of RAMP3. The results above represent one experiment.

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