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Signaling and Regulation of the Polymicrogyria-associated receptor GPR56: A Model Biochemical Study of the Adhesion G Protein-coupled Receptor Family

By

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Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences

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Abstract

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The G protein-coupled receptor (GPCR) superfamily represents the largest library of pharmaceutical drug targets. This is mostly due to their large number, surface expression, signal amplification, and connection to human disease. GPR56 is a member of the adhesion GPCR subfamily and mutations to this receptor have been shown to cause cortical developmental defects leading to bilateral frontoparietal polymicrogyria. In this dissertation, we sought to biochemically decipher the G protein dependent signaling pathways of the receptor, as well as the mechanism of activation. Moreover, we searched for novel protein-protein interaction that could regulate receptor function. Our studies showed that GPR56 signals through $G\alpha_{12/13}$ to activate Rho and β-catenin. We also performed truncation studies on the large heavily glycosylated cleave N-terminus (NT) and discovered that it remains non-covalently associated to the seven transmembrane (7TM) region of the GPCR. Moreover, association to the 7TM region antagonized GPR56-dependent activation, and removal of the NT showed evidence of a constitutively active receptor—characterized by increased GPR56-stimulated signaling upon transfection of HEK293 cells with truncated GPR56, greatly enhanced binding of β-arrestins by truncated GPR56 relative to the full-length receptor, extensive ubiquitination of truncated GPR56, and cytotoxicity induced by truncated GPR56 that could be rescued by co-transfection of cells with β-arrestin2. Furthermore, we found that the GPR56-NT is capable of homophilic trans-trans interactions that enhance receptor signaling activity. Finally, we showed novel protein-protein interactions between GPR56 and Magi-3 and CFTR-associated ligand, as well as localization to the cilia in IMCD-3 cells. Based on these data, we propose a novel general mechanism of activation for the adhesion GPCR family where the NT antagonizes receptor activation and removal by large NT binding partners can alleviate this inhibitory influence. These studies offer a template for the decoding of other adhesion GPCR signaling pathways and activation mechanisms, in which all our considered orphan receptors to this point. Moreover, studies like these will aid in future drug screening to the eventual benefit of human health.

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List of Abbreviations

Alzheimer's disease……AD

Basement membrane……BM

Bilateral frontoparietal polymicrogyria…….....BFPP

Brain-specific angiogenesis inhibitor……..BAI

cAMP response element……..CREB

CFTR-associated ligand……..CAL

Chinese hamster ovary…….CHO

Co-immunoprecipitation……..CO-IP

C-terminus……CT

CV-1 in Origin…….COS-7

Cysteine-rich angiogenic inducer 61……...Cry61

Dulbecco's modified eagle's medium…….DMEM

EGF module-containing mucin-like receptor……..EMR

Endoplasmic reticulum……ER

Epidermal growth factor……..EGF

Extracellular matrix…….ECM

Extracellular signal-regulated kinase……..ERK

Fetal bovine serum……..FBS

Fibronectin leucine-rich repeat transmembrane……..FLRT

GPCR proteolytic site……..GPS

GPCR-autoproteolysis inducing……..GAIN

G-protein coupled receptor……..GPCR

G-protein receptor kinase…….GRK

Granulocyte-macrophage colony-stimulating factor……..GM-CSF

Human embryonic kidney……HEK

Human epididymal protein 6……HE6

Immunoprecipitation……IP

Intramedullary collecting duct -3……IMCD-3

Latrotoxin……LTX

Long N-terminus…….LN

Lysophosphatidic acid…….LPA

Mitogen-activated protein kinase…….MAP

Na+-H+ exchanger regulatory factor……..NHERF

Neural cell adhesion molecule…….NCAM

N-terminus……NT

Paraformaldehyde……PFA

Parkinson's disease……PD

Phosphate buffered saline……PBS

Phosphoinositol-3-kinase…….PI3K

Phospholipase C……..PLC

Post synaptic density protein, Disc large tumor…….PDZ
suppressor and zonula occludens-1 protein

Post-synaptic density protein 95…….PSD-95

Protease activated receptor……..PAR

Protein kinase B……...Akt

Regulators of G-proteins……..RGS

Rho kinase……ROCK

Seven-transmembrane……..7TM

Thyrotropin receptor……TSHR

Transglutaminase 2……..TG2

Usher's Syndrome……USH

Very large G-protein coupled Receptor – 1……VLGR1

Chapter I: Introduction

1.1 G protein-coupled receptors

The survival of every organism is dependent on the ability to react appropriately to the external environment. Similarly, at the cellular level, it is critically important for cells to respond to many different external cues, and there exist a multitude of receptors that detect various signals and transduce this information into changes in cellular physiology. This cellular phenomenon is called signal transduction. Mechanisms of signal transduction can be generally divided into two major classes—those involving intracellular receptors and those involving cell surface receptors (Spiegel, 1996). The largest and most diverse groups of cell surface receptors are the G protein-coupled receptors (GPCRs), which play a major role in cell-cell communication, detecting signals including neurotransmitters, hormones, and extracellular matrix components. Moreover, these receptors also play important roles in the perception of the environment, since they are activated by odorants, tastants and light (Pin et al., 2003). Due to the large number of different signals that are detected by G protein coupled receptors, it is not surprising that they account for over 1% of the human genome (Flower, 1999).

Structurally, all GPCRs are composed of a seven transmembrane helix (7TM) with an intracellular C-terminus and an extracellular N-terminus. In humans, GPCRs have been classified into five major families based on sequence homology in the seven transmembrane regions. These families are as follows: glutamate, rhodopsin, adhesion, frizzled/taste2, and secretin, forming what is known as the GRAFS classification system (Fredriksson et al., 2003). Although the 7TM regions are highly homologous within these families, the N-terminal regions are quite different. Other classification systems also exist, including the most frequently used system that divides GPCRs into classes A

through F. However, this system attempts to classify all GPCRs from both vertebrates and invertebrates, with several classes (D and E) that don't exist in humans. Such classification schemes can be problematic due to major differences between mammalian and invertebrate GPCRs. For example, the GPCRs in *Drosophila melanogaster* show little resemblance to those in mammals (Broeck, 2001). Moreover, different evolutionary paths have created vast differences between the numbers of GPCRs in each class between species (Fredriksson et al., 2003). In the GRAFS systems, the glutamate, rhodopsin, and secretin families are the same as in the A-F class system. The GRAFS system does include 23 receptors that are not part of the five major classes, but do not necessarily share any resemblance to each other.

The glutamate family of human GPCRs contains 15 members, consisting of eight metabotropic glutamate receptors, two GABA receptors, the calcium sensing receptor, and five taste receptors. The rhodopsin family is the largest family of GPCRs and contains 701 receptors, although only 241 are non-olfactory sensing. Most members of the rhodopsin family possess a small N-terminus, with ligand binding occurring within a cavity between the transmembrane region (Baldwin, 1994). The adhesion receptor family contains 33 receptors that are comprised of a seven transmembrane domain fused to an N-terminus containing functional domains mimicking adhesive proteins. Originally, these receptors were grouped as class B receptors, but newer analysis has shown they exist as an entirely distinct family (Harmar, 2001; Fredriksson et al., 2003). The frizzled/taste family consists of two distinct clusters, the frizzled receptors, in which there are ten members, and the taste receptors, in which there are thirteen. Finally, the secretin family contains fifteen members and bind large peptides as their endogenous ligands.

1.2 G protein-coupled receptor signaling

GPCRs are characteristically activated by binding of a ligand to the extracellular structure causing a conformational change to elicit downstream intracellular signaling cascades. The classical understanding is upon GPCR activation, a heterotrimeric guanine-nucleotide regulatory protein complex connects the receptor with various effectors to elicit second messenger signaling that eventually leads to cellular responses. The heterotimeric G-protein complex consists of three subunits: α, β, and γ. The Gα subunit binds both GTP and GDP and is principally responsible for the hydrolysis of GTP. The β and γ subunits are tightly bound together forming a complex. In the simplest model for G protein dependent signaling, when in the absence of agonist, a GPCR exists in a low affinity state for G protein binding, but upon ligand binding, undergoes a conformational change that increases the affinity of the GPCR for G proteins association, leading to a complex of agonist, receptor, and G protein. In this state, the receptor facilitates guanine nucleotide exchange or the exchange of GTP for GDP on the Gα protein, leading to the disassociation of the Gα protein from the Gβγ dimer (Pierce et al., 2002). At this juncture, the Gα and Gβγ are released to effect downstream effectors and elicit cellular response.

There is tremendous diversity of different G protein subunits, with at least sixteen α, five β, and twelve γ G proteins, discounting multiple splice variants (Cabrera-Vera et al., 2003). The heterotimeric G proteins can be divided into four families based on primary sequence homology of their Gα subunit. As a result, the heterotimeric complex is frequently identified based on what Gα protein is present. Furthermore, the G proteins are identified and classified by the downstream effector of the Gα subunit. The four families of G proteins are Gs, Gi, Gq, and G12 and their most prominent biological effects are the

stimulation of adenylyl cyclase (Gs), inhibition of adenylyl cyclase (Gi), activation of phospho lipase C (PLC) (Gq), and the regulation of RhoGEF (G12) respectively (Kristiansen, 2004). After the immediate action of the G proteins, the effected proteins (adenylyl cyclase, PLC, and RhoGEF) can lead to further signaling of downstream effectors through modulation of cAMP, influx of intracellular calcium, and protein phosphorylation, amongst other changes, leading to a variety of biological responses.

Although signaling by Gα subunit serves to classify G proteins, it is not the only subunit to serve as a signaling molecule. The role for the Gβγ subunit was first thought to simply target the α subunit to the plasma membrane (Sternweis, 1986) and later structural studies also showed that Gβα helped to position Gα in the correct orientation for receptor binding (Oldham and Hamm, 2006). However, the Gβγ complex has also been linked to several biological responses independent of Gα signaling, suggesting that it too elicits separate biological responses upon GPCR activation. For example, it was first shown that the disassociated Gβγ subunit directly binds to the inward rectifying K^+ channel in atrial monocytes to drive channel activation (Codina et al., 1987; Logothetis et al., 1987). Now there is numerous evidence supporting direct physiological responses due to Gβα signaling, such as the regulation of adenylyl cyclase, activation of ion channels, and activation of kinases (Smrcka, 2008).

While G protein dependent signaling is the classical mechanism by which GPCRs elicit their effects, there are many examples of unconventional GPCR-activated pathways independent of classical G protein stimulation. One of the most well characterized GPCR signaling cascades that is G protein-independent is receptor signal transduction by β-arrestin proteins. Conventionally, β-arrestins bind exclusively to the phosphorylated C-

terminus of the receptor to desensitize G protein-mediated signaling of an activated receptor (Kohout and Lefkowitz, 2003). Therefore, like the heterotimeric G proteins, β-arrestin binding and subsequent biological effects are dependent upon receptor activation. For example, after β-adrenergic receptor activation, β-arrestins mediate recruitment of c-Src to the receptor, thus facilitating activation of the mitogen-activated protein (MAP) kinase pathway leading to extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation (Luttrell et al., 1999). Moreover, β-arrestins contribute to antiapoptotic signaling through the insulin-like growth factor 1 receptor in a G protein-independent manner by leading to the activation of phosphatidylinositol 3-kinase (PI3K) and protein kinase B (AKT) (Povsic et al., 2003). Likewise, binding of the β2-adrenergic G protein coupled receptor to the Na^+-H^+ exchanger regulatory factor (NHERF) allows G protein independent inhibition of renal Na^+-H^+ exchangers (Hall et al., 1998). Clearly, through binding to various scaffolds and signaling proteins, GPCRs are capable of expanding their signaling capabilities outside of traditional G protein dependent pathways. Furthermore, with these examples of G protein independent signaling pathways for the G protein coupled receptors, it creates a level of complexity where certain ligands might preferentially conform the receptor to signal either through conventional G proteins or other means. Indeed, this is the case for the endogenous ligands of the chemokine receptor CCR7 in which the Epstein-Barr virus–induced receptor ligand chemokine can signal through both G protein dependent and independent manners, but the secondary lymphoid tissue chemokine cannot (Lefkowitz and Shenoy, 2005).

1.3 Clinical relevance of G protein coupled receptors

G protein-coupled receptors are important clinically because they represent almost 50% of all drug targets and continue to be the main target for new therapeutics (Drews, 2000). One of the main reasons for this clinical importance is the overall physiological importance of these receptors, as each cell can express dozens of different GPCRs that will exhibit extensive cross-talk to control many aspects of cellular physiology (Civelli et al., 2006). However, there are a variety of additional reasons why GPCRs represent ideal drug targets, one being the sheer number of these proteins in the human genome. Recent genome studies have counted 948 GPCRs (Takeda et al., 2002), accounting for nearly 5% of the entire human genome (Rubin et al., 2004). Besides the large size of GPCR superfamily, though there are also specific characteristics of these receptors that allow them to be successful drug targets.

A common property of many drug targets is surface expression. By one estimate, 60% of drug targets are on the cell surface, yet only 22% of all human proteins are considered to be surface-expressed (Overington et al., 2006). If a drug target is on the surface, drugs and ligands are able to reach their destination with much greater ease to elicit the desired response. This is because the structures of many drugs are based on the structures of naturally occurring peptides and hormones, which inherently have difficulty crossing the lipid bilayer. Moreover, many drugs are hydrophobic in nature, thereby making membrane permeation very difficult. Currently, there are efforts to use endogenous transporters as a tool to target non-surface expressed proteins and receptors, but these methods have shown limited potential (Majumdar et al., 2004). Therefore, the ability of GPCRs to transduce exoplasmic signals inside cells due to their surface

expression will likely allow GPCRs to remain as primary drug targets until better drug delivery technology is developed.

In addition to their surface expression, GPCRs are also ideal drug targets due to their second messenger signaling cascades. As explained previously, binding of a single ligand to the receptor activates G proteins which then act on effector molecules to produce many second messengers. This process is called signal amplification and is a key reason why GPCRs important drug targets. Using the odorant receptor as an example, when one odorant molecule binds to a receptor, it can in turn activate 10 G proteins, each then activating one adenylyl cyclase. These adenylyl cyclases can then each produce approximately a thousand cAMP molecules per second (Firestein, 2001) Next, these cAMP molecules are able to open calcium channels, eventually leading to neuronal action potential generation (Lynch and Barry, 1989). Thus, single ligand-receptor interactions can lead to profound influences on an entire cell's homeostasis very quickly. Of course, cells possess many ways to control these signals so that amplification does not get out of control. These methods include arrestins to desensitize GPCRs, GPCR regulated kinases (GRKs) to prevent G protein coupling and phosphodiesterases to metabolize second messenger quickly (Violin et al., 2008).

Furthermore, as described above, GPCR signals can be amplified for extremely quick bursts of signaling to control cell function with temporal precision. Thus, although GPCR activation leads to intracellular chemical changes that may directly affect the state of the cells, activation of the receptor can also lead to transcriptional regulation, thereby resulting in long-lasting changes (Civelli et al., 2006). The ability to affect cell systems and function long term through transcriptional regulation is another reason that GPCRs

are great drug targets. For example, many people suffering from asthma use albuterol inhalers in order to relax smooth muscles in their airways to release constriction and increase breathing. These drugs are agonists that target the β2-adrenergic receptor, which is a GPCR. The short term effects are G_s stimulation of cAMP leading to bronchial relaxation through potassium channel regulation (Billington and Penn, 2003). However, there is emerging evidence that treatment with β2-adrenergic agonists can lead to transcriptional modifications through the cAMP response element-binding (CREB), which regulates the expression of numerous immunomodulatory proteins implicated in asthma, including interleukin-6, CCL5, eotaxin, and Granulocyte-macrophage colony-stimulating factor (GM-CSF), to lead to positive long term effects (Hallsworth et al., 2001; Lazzeri et al., 2001; Wuyts et al., 2003). Hence, not only are GPCRs great drug targets for their ability to amplify exoplasmic signals rapidly, but also through their ability to regulate transcription for long term therapeutic benefits.

The most desired property of any potential drug is to have maximum efficacy with minimal side effects. For the most part, this is done by selectively targeting receptors in the areas where the effect is desired, while bypassing tissues and cells where it would be detrimental or undesired. Another reason that GPCRs are exceptional drug targets is that they have selective tissue distribution that allows such tissue specificity of drug action (Insel et al., 2007). Moreover, many GPCRs contain multiple subtypes with distinct tissue distributions and enough structural diversity to create highly-specific drugs to target one subtype over another, adding another level of GPCR drug selectivity. In addition, the last decade has seen the crystallization of a number of GPCRs, including the β2-adrenergic receptor (Rasmussen et al., 2007), β1-adrenergic receptor (Warne et al.,

2008), adenosine A2A receptor (Jaakola et al., 2008), the D3 dopamine receptor (Chien et al., 2010) , the CXCR4 receptor (Wu et al., 2010), the histamine H1 receptor (Shimamura et al., 2011), the sphingosine 1 phosphate receptor (Hanson et al., 2012), the M2 muscarinic receptor (Haga et al., 2012), the M3 muscarinic receptor (Kruse et al., 2012), and the mu opioid receptor (Manglik et al., 2012). Already, structure-based drug design directly influenced by these crystal structures has led to the creation of a number of marketable drugs (Shoichet and Kobilka, 2012). The recent advances in the crystallization of GPCRs should further aid in creating subtype-selective drugs, thereby making GPCRs even better drug targets.

Finally, G protein-coupled receptor signaling is not a simple linear process, but rather involves a complex interconnected web of many regulatory proteins and molecules. This is yet another reason why GPCR signaling offer great opportunities for therapeutics, as drugs can be designed that acts not on the GPCR themselves, but rather auxiliary proteins involved in G protein signaling. For example, regulators of G protein signaling (RGS) proteins are capable of quickly inactivating G protein signaling by promoting G protein hydrolysis (De Vries et al., 2000; McCoy and Hepler, 2009). These RGS proteins are widely expressed and have been implicated in numerous biological functions, from regulation of the nervous system to cardiac function. As a result, drugs are already being developed to both inhibit and potentiate RGS function, including antagonists to RGS4, although none have yet been approved for use in the clinic (Sjogren and Neubig, 2010). Moreover, post synaptic density protein, Drosophila disc large tumor suppressor, and zonula occludens-1 (PDZ) proteins are important scaffold proteins that bind to the C-terminus (CT) of GPCRs to regulate a multitude of functions

from localization, signaling, degradation, and trafficking (Romero et al., 2011). As a result, recent work has focused on creating drugs that block GPCR-PDZ protein interactions. For example, the drug sulindac sulfone has been shown to block Dishevelled-PDZ interactions leading to decreased Wnt3A β-catenin signaling and resulting in anticancer effects (Lee et al., 2009). These few examples are just a small subset of GPCR regulators that are potential targets for therapeutics aimed at modulating GPCR signaling.

1.4 Orphan G protein coupled receptors

The conventional paradigm for GPCR signaling begins with receptor interactions with ligands, which alter receptor conformation to initiate signaling pathways. Early work in the 19th century by Claude Bernard using curare demonstrated that the efficacy of a drug depends on its access to a particular location, one of the first observations that led to modern day understanding of receptor-ligand interactions. Langley was the first scientist to explicitly state the idea of "receptive substances" on reactive cells through gross manipulation of skeletal muscle stimulation (Langley, 1901). It wasn't until the era of receptor cloning that it was understood how vast the GPCR superfamily is (Lefkowitz, 2004). At this time, the idea of "orphan receptors" originated as many GPCRs of unknown function were being discovered through cloning approaches based on sequence homology to known receptors. The first orphan receptor was called G21, and, since it resembled the β2-adrenergic receptor, it was suspected to be the β1-adrenergic receptor. However, further work eventually identified it as the serotonin 5-HT_{1A} receptor, making this work the first demonstration of "deorphanizing" a GPCR (Fargin et al., 1988). Today, an orphan receptor is defined as a GPCR with no known endogenous ligand. So,

although the era of cloning identified many GPCRs, and the human genome project identified even more (Takeda et al., 2002), these efforts did not identify the ligands for all of these receptors. Presently, there still exist more than 100 orphan GPCRs that may bind endogenous ligands (Chung et al., 2008) As a result, there are potentially over 100 GPCRs—already described as ideal drug targets—that offer great opportunities for potential therapeutics due to their unknown pharmacology.

Since orphan GPCRs offer such great therapeutic potential, there has been extensive work in both academia and industry to identify ligands for these receptors. The traditional method for finding ligands for orphan GPCRs is through high-throughput screening, in which cells expressing orphan GPCRs are screened against large libraries of various molecules, peptides, and potential ligands and a signaling output (cAMP, calcium, etc.) is measured. Early efforts using such approaches were very successful, with 7-8 yearly ligand interactions with orphan GPCRs identified (Civelli et al., 2006). However, this rate has slowed dramatically for a variety of reasons. One possibility for the slowing success rate of high-throughput screening is that remaining orphan GPCRs may be more complex than traditional GPCRs in terms of their mechanisms of activation and/or signaling pathways. For example, many of the proposed GPCRs from the human genome project are only distantly related to conventional GPCRs, and may in fact represent a new sub family in the GPCRs superfamily (Takeda et al., 2002). Moreover, as mentioned, G protein-coupled receptors can couple to different G proteins, all with differing functional outputs and this complexity poses another pitfall for screening since the correct functional output must be used. Therefore, new strategies beyond simple high-

throughput screening probably need to be employed to deorphanize many of the remaining orphan GPCRs.

1.5 Adhesion family G protein-coupled receptors

The largest family of orphan GPCRs at present is the adhesion GPCR family. This family has been categorized and named a number of different ways over the past two decades as it has failed to fit into the traditional GPCR classification system. As mentioned above, the complexity and distinctiveness of this receptor family is probably the main reason all of these receptors remain orphans. This class has variously been referred to as, *i)* "EGF-TM7" to indicate the presence of epidermal growth factor domains on the N-terminus of some members (McKnight and Gordon, 1998), *ii)* "LN-TM7" to reflect the long N-termini (LN) attached to a traditional seven-transmembrane region (TM7) and *iii)* "B2/LNB-7TM" to suggest an ambiguous homology to the secretin receptors (Harmar, 2001). However, as mentioned earlier, the adhesion GPCR receptors were designated as their own family in 2003 to reflect their many differences from other GPCRs. Phylogenetic analysis has grouped the adhesion GPCRs together based of their homology in the 7TM region, while each possesses exceptionally diverse N-termini within the family. According to this categorizing, the human genome encodes 33 member of the adhesion GPCR family (Bjarnadottir et al., 2004). It is interesting to note, that certain other species have many more adhesion GPCRs than do humans: for example, the recent completion of the *Strongylocentrotus purpuratus* (sea urchin) genome showed a surprisingly large taxon-specific expansion of adhesion GPCRs, with more than 90 members (Whittaker et al., 2006). The sea urchin genome even contains an ortholog of the Very Large G Protein Receptor 1 (VLGR1) adhesion receptor, a receptor that in

humans is known to be involved in vision and hearing—although the sea urchin is blind and deaf.

This large family of orphan receptors has a wide range of tissue distribution, but it must be noted that most of the individual receptors are quite discretely localized to a small subset of cell types and tissues. For example, the adhesion GPCR known as Human epididymal protein 6 (HE6) is strictly expressed in the epididymis (Osterhoff et al., 1997). Moreover, CD97 and EGF-module containing receptors 1-4 (EMR1–4) are expressed almost exclusively in leukocytes (Yona et al., 2008). The restricted expression pattern exhibited by many adhesion GPCRs is another reason why these GPCRs in particular may make for excellent drug targets, since specificity in the tissue expression of a receptor can contribute to the specificity of action for drugs targeting that receptor. Plus, as will be discussed later, a number of adhesion GPCRs has been implicated in human disease states, adding to their appeal as potential drug targets.

1.6 Adhesion G protein coupled receptor structure

Each adhesion GPCR is comprised of a seven transmembrane region with a long, heavily glycosylated N-terminus (NT). Adhesion GPCR N-termini typically contains many different adhesion domains capable of mediating extracellular protein-protein interactions, thus leading to the "adhesion GPCR" nomenclature. Some of the common adhesive structural domains contained within the N-termini of adhesion GPCRs include thrombospondin repeats, EGF-like repeats, leucine-rich repeats, and cadherin-like repeats (Yona et al., 2008). The most unique feature of this class of receptor, however, is the presence of the GPCR proteolysis site (GPS) motif. The GPS motif is a cysteine-rich

domain of approximately 50 amino acids that is highly conserved in nearly all of the adhesion GPCRs, as well as the polycystic kidney disease proteins (Krasnoperov et al., 1997; Hughes et al., 1999). At the GPS motif, an autoproteolysis event occurs during protein processing wherein the N-terminal stalk is cleaved from the 7TM region at the consensus GPS site, (histidine$_{p-2}$ leucine$_{p-1p0}$ serine/threonine$_{p+1}$, where p = cleavage position) (Yona et al., 2008). This autoproteolysis creates two distinct subunits, an N-terminal region containing the adhesive domains and a 7TM region encompassing the transmembrane domains plus the intracellular C-terminus. Some investigators have referred to these subunits as the α and β subunits respectively (Gray et al., 1996). Proper protein folding is required for correct GPS cleavage, as deletions and mutations outside of the conserved cysteine region prevent autoproteolysis (Chang et al., 2003). Moreover, mutations within the GPS motif prevent proper cleavage in general and without cleavage there is evidence for protein misfolding and trafficking for both GPR56 (Jin et al., 2007) and Polycystin-1 (Yu et al., 2007). Biochemical studies have shown that this GPS autoproteolysis event occurs subcellularly in the ER-Golgi compartment, although it may be different for distinct receptors. For polycystin-1, for example, autoproteolysis occurs in the endoplasmic reticulum-Golgi intermediate compartment, but for EMR2 it occurs exclusively in the ER (Lin et al., 2004; Appenzeller-Herzog and Hauri, 2006). Recent structural studies suggested that the GPS motif is actually part of a much larger evolutionarily conserved domain that is referred to as the GPCR-Autoproteolysis inducing (GAIN) domain (Arac et al., 2012). Crystallographic analysis and mutagenesis studies of the adhesion GPCRs CL1 and Brain-specific angiogenesis inhibitor receptor 3 (BAI3) revealed that the GPS motifs alone are nonfunctional and need to be encompassed

in the much larger GAIN domain to produce autoproteolysis. The physiological importance of GPS motif/GAIN domain cleavage is mysterious, but mutations in this domain can cause receptor misfolding and human disease in some cases (Jin et al., 2007; Ke et al., 2008; Chiang et al., 2011)

Since the adhesion G protein coupled receptors undergo cleavage at the GPS/GAIN domain, creating two subunits consisting of the NT and 7TM region, it is important to know whether these distinct protein products form a heterodimer or act as separate entities. In fact, it has been shown for several different adhesion GPCRs, notably EMR2, latrophilin, CD97 and GPR56, that the receptors' NT and 7TM regions remain non-covalently associated for some period of time following autoproteolysis at the GPS motif (Gray et al., 1996; Krasnoperov et al., 1997; Krasnoperov et al., 2002; Kwakkenbos et al., 2002; Lin et al., 2004; Lin et al., 2010; Paavola et al., 2011). This begs the question as to what the physiological role of this complex formation between the NT and 7TM regions might be. Furthermore, although studies have shown association between the cleaved NT and 7TM regions, there is also evidence of possible roles of the cleaved NT independent from the transmembrane region. For example, the cleaved NT of EMR2 has been shown to localize separately from the 7TM in membrane raft microdomains in certain cases, and upon ligation between the two subunits there is translocation and colocalization into lipid rafts leading to receptor signaling (Huang et al., 2012). Moreover, the NT of BAI1 has been shown to reduce glioma growth *in vivo* when expressed as a fusion protein in mice, independent of the 7TM region (Kaur et al., 2005). Furthermore, there has been evidence of cross interactions between the NT of certain adhesion GPCRs and the 7TM of others. For example, in rat forebrain

homogenate, the GPR56 7TM region can be immunoprecipitated using a latrophilin NT antibody, indicating an interaction between the cleaved latrophilin NT and GPR56 (Silva et al., 2009). Undeniably, more work needs to be done in order to decipher the physiological significance of the cleaved yet associated N-terminus of the adhesion GPCRs and how this might regulate and effect G protein signaling.

1.7 Adhesion GPCR family ligands

Although all members of the adhesion GPCR family are still considered to be orphan receptors, in fact representing the largest family of orphan GPCRs, over the past few years extracellular binding partners have been identified for a number of different members of the family (Table I-1). It should be appreciated that not every adhesion GPCR binding partner must necessarily be a ligand that activates the receptors' coupling to G proteins; some of the interactions may be purely adhesive in nature, consistent with the general view of adhesion GPCRs as both adhesion molecules and cell surface receptors. For example, chondroitin sulfates have been reported as ligands for both EMR2 and CD97 (Stacey et al., 2003). These interactions have been characterized as low-affinity, calcium-dependent associations that are mediated through the receptors' EGF-like repeats, resulting in changes in cell attachment and motility. However, there is no evidence at present that these interactions with chondroitin sulfates can activate signaling by EMR2 or CD97 signaling. Similarly, CD97 was first identified as a counter-receptor on immune cells for CD55, also known as the decay accelerating factor (Hamann et al., 1996). This interaction has been extensively studied and shown to have a variety of effects on cell adhesion, cell motility and carcinoma invasiveness, but at present there is no evidence that this interaction can activate G protein-coupled signaling

Table I-1. Comprehensive list of adhesion GPCRs with reported G protein coupling and extracellular ligands. The members of the adhesion GPCR family are shown grouped by sequence similarity, according to the scheme proposed by Bjarnadottir *et al.* (2007). Additionally, for receptors that have been reported to couple to G proteins, the coupling preference is listed. Question marks indicate cases where G protein coupling has been suggested based on second messenger production but not definitively proven. Reported ligands for each receptor are also listed (TBD = "to be determined"). It is important to note that the ligands listed here are not necessarily agonists, as some ligands may mediate adhesive and/or regulatory functions without inducing receptor activation.

Sub-Family	Receptor	G-protein	Ligands	Reference
1	BAI1	TBD	Phosphatidylserine on apoptotic cells	(Park et al., 2007)
1	BAI2	TBD	TBD	
1	BAI3	TBD	C1q-like proteins	(Bolliger et al., 2011)
2	GPR56	G12/13	TG2, CD9, CD81, GPR56 NT, collagen III	(Little et al., 2004; Xu et al., 2006; Iguchi et al., 2008; Luo et al., 2011; Paavola et al., 2011)
2	GPR97	Go	beclomethasone dipropionate	(Gupte et al., 2012)
2	GPR112	TBD	TBD	
2	GPR114	Gs	TBD	(Gupte et al., 2012)
2	GPR126	Gs?	TBD	(Monk et al., 2009)
2	GPR128	TBD	TBD	
2	HE6	TBD	TBD	
2	VLGR1	TBD	TBD	
3	CD97	G12/13	Chondroitin sulfates, CD55, CD90	(Hamann et al., 1996; Stacey et al., 2003; Ward et al., 2011; Wandel et al., 2012)
3	EMR1	TBD	TBD	

3	EMR2	TBD	Chondroitin sulfates	(Stacey et al., 2003)
3	EMR3	TBD	TBD	
3	EMR4	TBD	TBD	
3	ETL	TBD	TBD	
3	LEC1 (Latrophilin -1; CIRL-1)	Gq, Go	LTX, teneurin-2, neurexin, FLRT proteins	(Lelianova et al., 1997; Rahman et al., 1999; Silva et al., 2011; Boucard et al., 2012; O'Sullivan et al., 2012)
3	LEC2 (Latrophilin -2; CIRL-2)	TBD	LTX	(Ichtchenko et al., 1999)
3	LEC3 (Latrophilin -3; CIRL-3)	TBD	FLRT proteins	(O'Sullivan et al., 2012)
4	GPR123	TBD	TBD	
4	GPR124	TBD	Integrins, Glycosaminoglycan	(Vallon and Essler, 2006)
4	GPR125	TBD	TBD	
5	CELSR1	TBD	TBD	
5	CELSR2	Gq?	Celsr2-NT	(Shima et al., 2007)
5	CELSR3	Gq?	Celsr3-NT	(Shima et al., 2007)

6	GPR133	Gs	TBD	(Bohnekamp and Schoneberg, 2011)
6	GPR144	TBD	TBD	
7	GPR110	TBD	TBD	
7	GPR111	TBD	TBD	
7	GPR113	TBD	TBD	
7	GPR115	TBD	TBD	
7	GPR116	TBD	TBD	

by CD97 (Mustafa et al., 2004; Liu et al., 2005). Moreover, Thy-1 (CD90) has recently been shown to interact with CD97 to regulate polymorphonuclear cell adhesion, but no corresponding signaling effects were reported (Wandel et al., 2012).

The adhesion GPCR latrophilin-1 has been shown to initiate G protein-dependent signaling when bound by latrotoxin, an exogenous toxin that is a component of black widow spider venom (Rahman et al., 1999). However, this is not an endogenous interaction, and there are no proteins known to exist in vertebrates that exhibit significant homology to latrotoxin. Recent studies have revealed three distinct potential endogenous ligands for latrophilin-1. One of these reported ligands is the single transmembrane glycoprotein teneurin-2 (also called Oz, tenascin-m, neurestin, and DOC4), which has been shown to bind to the latrophilin-1 NT with nanomolar affinity and form heterophilic complexes with latrophilin-1 at points of cell-cell contact (Silva et al., 2011). Moreover, treatment of cells expressing latrophilin-1 with a soluble fragment of teneurin-2 was found to induce increases in intracellular calcium, probably reflecting activation of G protein-dependent signaling (Silva et al., 2011). A second reported ligand for latrophilin-1 is the presynaptic transmembrane protein neurexin (Boucard et al., 2012). Interestingly, neurexin, like latrophilin-1, is a cellular target of latrotoxin (Davletov et al., 1995). Like teneurin-2, neurexin was shown to interact with latrophilin-1 with nanomolar affinity to form heterophilic complexes at cell-cell junctions (Boucard et al., 2012). However, it remains to be explored whether this interaction can stimulate latrophilin-1 signaling. A third identified family of ligands for latrophilin-1 is the fibronectin leucine-rich repeat transmembrane (FLRT) proteins (O'Sullivan et al., 2012). Latrophilin-1 and the related latrophilin-3 were shown to interact with FLRT proteins in a

heterophilic cell-cell manner with nanomolar affinity, and a trans-synaptic complex between FLRT3 and latrophilin-3 was found to regulate synaptic density and dendritic spine number in cultured neurons (O'Sullivan et al., 2012). It is not yet clear whether FLRT interactions with latrophilin NT regions can activate latrophilin signaling but this point will likely be clarified by future work in this area.

GPR56 is another adhesion GPCR that has been reported to bind to multiple extracellular ligands. The first identified binding partners of GPR56 were the tetraspanins CD9 and CD81, although the region of GPR56 required for these interactions and the significance for GPR56 signaling have not been fully defined (Little et al., 2004). A second ligand that has been identified for GPR56 is transglutaminase 2 (TG2), an extracellular matrix protein that enzymatically cross-links proteins together to help form adhesive complexes (Xu et al., 2006). TG2 was shown to bind to a specific domain on the GPR56 NT, and deletion of this domain was shown to lead to increased GPR56-promoted tumor growth *in vivo* (Yang et al., 2011). However, it is not yet clear if TG2 binding to the GPR56 NT can stimulate GPR56-mediated signaling. A third ligand that has been found for GPR56 is collagen III, which binds to the GPR56 NT and has been reported to stimulate GPR56-mediated signaling to Rho in NIH3T3 cells (Luo et al., 2011). Interestingly, knockout of collagen III has been shown to result in a cobblestone-like malformation of the cerebral cortex due to neuronal over-migration during brain development (Jeong et al., 2012), which is a phenotype strikingly similar to that observed upon knockout of GPR56 (Li et al., 2008).

The brain-specific angiogenesis inhibitors 1-3 (BAI1-3) are a sub-family of adhesion GPCRs that have been shown to associate with both lipids and proteins via the

multiple thrombospondin-like repeats on their large NT regions. For example, BAI1 was shown to bind to externalized phosphatidylserine on apoptotic cells to promote apoptotic cell engulfment, in a manner that involves ELMO a protein that associates with the cytoplasmic regions of BAI1, acting as a guanine nucleotide exchange factor for Rac (Park et al., 2007). However, it remains to be determined whether BAI1-mediated engulfment of apoptotic cells involves G protein-dependent signaling by BAI1 or whether any such signaling is initiated by the binding of the BAI1-NT to phosphatidylserine-rich membranes. In separate studies, the BAI3-NT has been shown to be a high-affinity binding partner for a family of complement-like secreted proteins called the C1q-like proteins (Bolliger et al., 2011). Upon addition of C1ql to cultured hippocampal neurons, a significant decrease in synaptic density was observed in a manner that could be blocked by interfering with the ability of C1ql to bind to thrombospondin-like repeats (Bolliger et al., 2011). The specificity of the C1ql proteins for different BAI1 family members and the importance of these interactions for stimulating BAI-mediated signaling are likely to be topics of significant future research interest.

Several adhesion GPCRs have been shown to undergo homophilic trans-trans interactions, meaning that they can interact with other versions of themselves on neighboring cells. Interestingly, these homophilic associations have been shown in several cases to promote adhesion GPCR signaling. For example, the adhesion GPCRs Celsr2 and Celsr3 have been shown to undergo receptor-specific NT-NT interactions that induce increases in intracellular calcium in a phospholipase-dependent (and probably G protein-dependent) manner (Shima et al., 2007). The homophilic trans-trans interactions

of Celsr2 and Celsr3 were demonstrated to be physiologically important in the regulation of neurite outgrowth in cultured neurons (Shima et al., 2007). Additionally, the *Drosophila* adhesion GPCR known as Flamingo has been shown to be capable of homophilic trans-trans associations, although it is not yet clear if these associations promote receptor signaling (Chen and Clandinin, 2008). It should be pointed out that important roles for NT-NT interactions in adhesion GPCR activation are not mutually exclusive with crucial roles for other large adhesive ligands, since NT-NT interactions might be required to create binding sites for certain ligands. Conversely, or perhaps concurrently, association with large adhesive ligands might stabilize NT-NT interactions in a manner that promotes receptor signaling.

In conclusion, there is ample evidence of N-terminal binding partners for the adhesion GPCR family. However, this should not come as any surprise as many of the N-termini contain known adhesive domains. The fact that most of these interactions fail to activate the receptors in any significant way, and have not been demonstrated to be physiologically important *in vivo* means that the members of the adhesion family of GPCRs must still be classified as orphan receptors.

1.8 G Protein dependent signaling of the adhesion GPCR family

As discussed in the previous section, although the adhesion GPCRs have been shown to interact with a variety of different N-terminal binding partners, the majority of these ligands fail to activate the receptor in any significant manner. Moreover, the members of the adhesion GPCR family have mostly been defined by their unique adhesive NT regions rather than functional G protein-coupled receptors. The fact that the

NT region are cleaved from the 7TM regions, where G protein coupling occurs, has led to questions as to whether or not these receptors even act and signal as traditional G protein-coupled receptors.

Studies on several different adhesion GPCRs have provided evidence that these receptors are in fact authentic G protein-coupled receptors. For example, over-expression of GPR56 in various cell types can lead to Rho activation through $G\alpha_{12/13}$ (Iguchi et al., 2008; Paavola et al., 2011). Moreover, GPR56 has been shown via co-immunoprecipitation to interact with $G\alpha_{q/11}$ (Little et al., 2004), which is consistent with work on other receptor types demonstrating that receptors coupling to $G\alpha_{12/13}$ can also typically couple to $G\alpha_{q/11}$ (Takashima et al., 2008). In a similar vein, over-expression of GPR133 in various cell types has been shown to stimulate $G\alpha_s$ and promote cAMP generation (Bohnekamp and Schoneberg, 2011; Gupte et al., 2012). GPR126 has also been shown to increase cAMP levels in Schwann cells, most likely via a $G\alpha_s$-dependent mechanism (Monk et al., 2009), and GPR114 has been shown to constitutively increase cAMP levels when over-expressed in HEK293 cells (Gupte et al., 2012). GPR97 has also been shown to be constitutively active upon over-expression in HEK293 cells, but only when co-expressed with a chimeric version of $G\alpha_o$ (Gupte et al., 2012).

Other studies on adhesion GPCR signaling have made use of activating antibodies or toxins. There is precedent from work on certain classical GPCRs, including adrenergic, muscarinic and angiotensin receptors, demonstrating that antibodies or other large proteins associating with the receptors' extracellular regions can sometimes cause conformational changes to stimulate receptor signaling (Lebesgue et al., 1998; Peter et al., 2004; Dragun et al., 2005; Dragun, 2007). Along these same lines, the

aforementioned $G\alpha_{12/13}$ -mediated signaling by GPR56 has been shown to be robustly promoted by treatment with antibodies directed against the receptor's NT (Iguchi et al., 2008). Moreover, regulation of neutrophil signaling by the adhesion GPCR EMR2 has been shown to be modulated by anti-EMR2-NT antibodies in a manner that probably involves receptor coupling to G proteins (Yona et al., 2008; Huang et al., 2012). The adhesion GPCR latrophilin-1 has been intensively studied because it is a key target of latrotoxin (LTX), which is derived from the venom of the black widow spider (Krasnoperov et al., 1997; Lelianova et al., 1997). LTX binds to the latrophilin-1 NT and has been demonstrated to promote latrophilin-1 coupling to $G\alpha_q$ and $G\alpha_o$ (Lelianova et al., 1997; Rahman et al., 1999). The pathological effects of LTX are complicated by the fact that the toxin can integrate into membranes to form pores, but the specific ability of LTX to bind latrophilin-1 and promote the receptor's G protein coupling has been established using a mutant version of the toxin that does not form pores but still binds to latrophilin-1 (Ichtchenko et al., 1998; Capogna et al., 2003; Volynski et al., 2003).

Clearly, there is considerable evidence to support the idea that adhesion GPCRs do in fact couple to G proteins. Moreover, the fact that signaling pathways are beginning to be elucidated for the various members of the adhesion GPCR family will facilitate the search for both endogenous ligands and synthetic drugs capable of activating adhesion GPCRs. Based on studies on the physiological importance of these receptors, as summarized in the next section, there is good reason to believe that small molecules capable of activating or blocking adhesion GPCR signaling may make for outstanding therapeutics for the treatment of a variety of different disorders.

1.9 Physiological importance of adhesion G protein-coupled receptors

Genetic studies, including analyses of gene deletions in mice and zebrafish have provided striking evidence regarding the physiological importance of various adhesion GPCRs. The manipulation of adhesion GPCR genes is a powerful tool in understanding receptor function without knowing the endogenous ligand or signaling pathways. For example, knockout studies on Gpr126 have revealed a pivotal role of this receptor in the myelination of Schwann cells (Monk et al., 2009; Monk et al., 2011) in both zebrafish and mice. Specifically, Gpr126 was play a key role in allowing Schwann cells to initiate myelination after attachment, and loss of the receptor stopped myelin at 1 to 1.5 wraps around peripheral nerves. Surprisingly, no other phenotype was observed besides and enlargement of the eye and strikingly the CNS was completely intact and fully myelinated (Monk et al., 2009). Moreover, knockout studies on He6 have demonstrated an essential role of this adhesion GPCR in spermatogenesis and fertility (Davies et al., 2004). Specifically, knockout of He6 in mice led to a dysregulation of fluid reabsorbtion within the efferent ductules, and a backup of fluid accumulation in the testis and a subsequent stagnation of sperm within the efferent ducts. These data suggest a possible role for the He6 receptor in directly monitoring and communicating the state of the luminal environment to the cellular machinery determining fluid uptake. Other gene deletion studies on adhesion GPCRs have focused on the receptor Gpr124. Knockout of Gpr124 caused complete lethality from arrest of CNS-specific angiogenesis, meaning that no blood vessels at all were formed in the forebrain or neural tube. Conversely, over-expression of Gpr124 caused hyperproliferative vascularization. (Kuhnert et al., 2010). These data suggest a pivotal role of GPR124 in the development of brain angiogenesis, though the exact function and signaling pathways for the receptor remain a mystery.

Obviously, gross knockout of the adhesion GPCRs is an important tool for learning about the basic functions of these unique receptors. However, these studies leave numerous biochemical questions unanswered, including the receptors' ligands and signaling pathways. These genetic studies also emphasize the possibilities that exist for adhesion GPCRs to be targeted by potential therapeutics. For all studies discussed, the receptors seemed to play extremely specific roles with discrete cell and tissue distribution. For example, the adhesion GPCRs described in the preceding paragraph could be ideal drug targets for demyelination disorders (GPR126), male infertility (HE6), and brain tumors (GPR124), due to its powerful regulation of brain angiogenesis.

1.10 Human diseases associated with mutations to adhesion GPCRs

When studying orphan G protein coupled receptors, one of the most useful techniques for determining a receptors' physiological importance is through analysis of human diseases that might be associated with mutations to the receptor. There are at least two members of the adhesion GPCR that have been linked to inherited human diseases: VLGR1 and GPR56

1.10.1 VLGR1 and Ushers Syndrome

Usher syndrome (USH) is the most prevalent cause of hereditary deafness-blindness in humans. Usher's syndrome accounts for 50% of all hereditary cases of deafness and blindness, and affects 1 in every 25,000 children (Kremer et al., 2006). The inner ear contains the cochlea, which is a spiral cavity that contains the Organ of Corti, the sensing organ of the ear (Lim, 1986). Within this organ are the inner ear hair cells that transduce mechanosensation into electrical signals that are transmitted to the brain and

translated into sound (Fuchs et al., 2003). Usher's syndrome is caused by improper formation of these hair cells and associated stereocilia, neurogenesis, and synaptogenesis during development. There are three subtypes of Ushers syndrome that can be caused by mutations to as many as 10 genes. Ushers Type II has been associated with mutations to the adhesion GPCR VLGR1 (Weston et al., 2004). Mutations to VLGR1 cause defects in the cochlea and retina during development. It is hypothesized that mutations to VLGR1 disrupt the stereocilia formation in the hair cells through loss of binding to harmonin, an important protein in the assembly of this sensory complex. This is because the proteins that cause Ushers syndrome type I—cadherin 23, cadherin 15, and SANS—all bind to harmonin as well, and it is speculated that these components form a complex necessary to shape the stereocilia as an organized cohesive unit (Boeda et al., 2002). Moreover, it has been speculated that mutations to VLGR1 may cause impaired ligand binding. For example, it is speculated that the matrix protein Usherin may be a ligand for VLGR1 since mutations to that protein can also lead to Ushers syndrome type 2, and thus disruption of either protein causes loss of cell adhesion or G protein signaling (Bhattacharya et al., 2002; Pearsall et al., 2002). Overall, however, the exact mechanism of how mutations to VLGR1 remain unclear, and could involve either altered G protein signaling or defective interactions between the VLGR1 ectodomain and necessary Usher proteins involved in the formation of the stereocilia (McMillan and White, 2010).

1.10.2 Bilateral frontoparietal polymicrogyria and GPR56

The most extensively studied link between human disease and an adhesion GPCR is bilateral frontoparietal polymicrogyria (BFPP). Broadly speaking, polymicrogyria is a disorder of neuronal migration during development leading to structural abnormalities of

the cerebral cortex. BFPP is a recessively inherited disease characterized by malformation of the cerebral cortex in the frontoparietal area. In normal development, neuronal precursors from the subventricular zone migrate radially outward to the pial membrane to form conserved layers (Rakic, 1988). However, the migration process of the progenitor cells is impaired in BFPP. Specifically, the disorder is a nonlissencephalic cortical dysplasia with abnormal gyration characterized by narrow and crowded gyri (Barkovich and Kjos, 1992). BFPP patients show mental retardation, gait difficulty, language impairment, and seizures, consistent with frontal lobe dysfunction (Piao et al., 2002; Chang et al., 2003). In 2004 and 2005, gene mutations to the adhesion GPCR GPR56 were linked to BFPP in a number of families. In all, eleven mutations were found in GPR56 including missense mutations and a seven base pair deletion (Piao et al., 2004; Piao et al., 2005). Specifically, these mutations were as follows: four of the eight missense mutations (R38Q, R38W, Y88C and C91S) are located at the tip of the GPR56 N-terminus, two (C346S and W349S) in the GPS domain and two (R565W and L640R) in the 7TM region (Jin et al., 2007).

The locations of the disease-causing mutations are intriguing as they provide insights into the physiological importance of the different domains of GPR56. Two independent groups have studied the biochemical effects exerted by these mutations on GPR56 biochemical properties and cellular trafficking. In both studies, GPR56 mutant constructs were created with the corresponding human mutations and transfected into HEK293 cells. Then, using immunoprecipitation (IP) techniques the groups looked at the ability of the mutant GPR56 to undergo GPS cleavage. These studies revealed that cells expressing the R38Q, R38W, Y88C, C91S, R565W mutations did not show any

impairment in GPS-mediated cleavage. However, cells expressing mutants C346S or W349S were unable to be cleaved at the GPS motif (Jin et al., 2007) Moreover, it was shown that the mutants that were unable to cleave at the GPS were also unable to leave the endoplasmic reticulum, indicating a role for GPS cleavage in proper receptor trafficking. Finally, the effects of the polymicrogyria mutations on GPR56 trafficking were tested. The results showed that mutations to the distal NT and GPS motif prevented cell surface expression, whereas mutations to the exoplasmic loops had no effect (Jin et al., 2007). All of these results were confirmed by an independent group that showed the same effect of the mutations on GPS cleavage and receptor trafficking (Ke et al., 2008). From these data, it appears that most of the mutations cause BFPP due to the mutant receptors' inability to traffic to the surface properly, rather than through defects in direct receptor signaling or ligand binding.

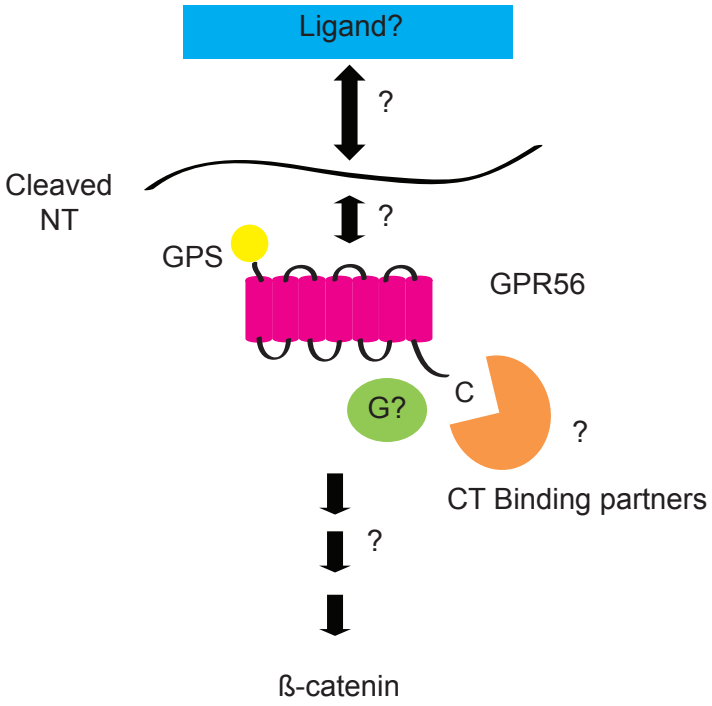
To further characterize the connection between GPR56 and bilateral frontoparietal polymicrogyria, the localization of Gpr56 in the mouse brain was characterized and a Gpr56 knockout mouse was created. These studies revealed that Gpr56 is most abundantly expressed in radial glia cells and associated neural progenitors (Li et al., 2008). Radial glia are important in forming patterns for migrating progenitor cells, suggesting a role of GPR56 in progenitor cell migration (Noctor et al., 2001). These radial glia can be considered as pathways from the ventricular zones to the pial membrane, by which neuronal progenitor cell migration and proliferation are orchestrated (Merkle et al., 2004). Since the pial basement membrane (BM) plays a pivotal role in the cortex formation during development, its integrity was examined in Gpr56 knockout mice. The results showed that at early stages the BM was intact, but at stage E12.8 there

was evidence of overmigration of neurons and a fragile pial membrane. Moreover, the radial glia end feet were also disorganized and had migrated past the BM. This work led to three main conclusions: (1) GPGpr56 is present in abundance in radial glial end feet (2) Gpr56 binds a putative ligand in the extracellular matrix (ECM) and (3) loss of Gpr56 in mice result in defective pial BM and neuronal ectopia, a cobblestone-like cortex. (Li et al., 2008). Thus, loss of Gpr56 resulted in a disorganized pial basement membrane, overmigration of radial glia end feet and subsequent overmigration of neuronal progenitor cells. In subsequent analyses of other brain regions in the Gpr56 knockout mice, it was shown that Gpr56 plays a role in regulating the adhesion of cerebellar granule cells of the perinatal rostral cerebellum (Koirala et al., 2009). Both studies on Gpr56 knockout mice reveal a cobblestone-like malformation of the cerebral cortex similar to the phenotype of BFPP human patients, further connecting the mutations of GPR56 to the human disorder. These findings, along with the aforementioned studies showing that GPR56 activation leads to the inhibition of neuronal progenitor cell migration (Iguchi et al., 2008) shed light on the role played by GPR56 in properly forming the pial basement membrane and facilitating the proper migration of neuronal progenitor cells.

1.11 Aim of Dissertation Research

The aim of my dissertation research has been is to decipher the mechanism of activation and specific signaling pathways downstream of GPR56 (Fig I-1). In this work, my colleagues and I used a heterologous overexpression system to assess activation of a variety of signaling pathways and reporter gene assays. Additionally, we used inhibitors of second messengers and G proteins to decode GPR56-activatedd signaling pathways.

Figure I-1. A schematic drawing that represents unknown aspects of GPR56 biology. Areas of GPR56 biology that will be explored in this Dissertation include *i)* the functional importance of the receptor's cleaved N-terminus, *ii)* whether GPR56 signaling to β-catenin involves G proteins, *iii)* potential ligands of GPR56 and *iv)* potential binding partners of the GPR56 C-terminus (including arrestins and PDZ scaffolds).


Ligand?
?
Cleaved
NT
?
GPS
GPR56
C
G?
?
CT Binding partners
?
ß-catenin

We found that GPR56 signals through $G\alpha_{12/13}$ to activate Rho GTPases and β-catenin.

Additionally, through truncation analysis we found that the cleaved N-terminus of GPR56 antagonizes receptor signaling to Rho, and removal of the NT region leads to a large enhancement of GPR56 constitutive activity. Moreover, we demonstrate that the N-termini of GPR56 on adjacent cells can interact with each other and that this interaction leads to a positive regulation of receptor activity. Based on these findings, we propose a potentially general mechanism of activation for adhesion GPCRs in which removal of the cleaved N-terminus results in subsequent receptor activation.

Additional aspects of my dissertation research focused on potential regulators of GPR56 signaling. Many different types of cytoplasmic proteins can interact with GPCRs to control receptor signaling, trafficking, and localization (Ritter and Hall, 2009). As stated earlier, these regulators of GPCR function offer potential drug targets independent of therapeutics that directly target GPCRs. We found a novel interaction between GPR56 and the PDZ proteins Magi-3 and CFTR-associated ligand (CAL). Moreover, we studies GPR56 interactions with arrestins and described a novel cilial localization of GPR56 in intramedullary collecting duct-3 (IMCD-3) cells, which may be dependent on arrestin interactions. Taken together, these various aspects of my dissertation research have shed significant new light on the activation and regulation of GPR56, thereby providing a basis for the future targeting of this receptor by therapeutics.

CHAPTER II: The N-terminus of GPR56 Controls Receptor Signaling Activity

2.1 Introduction

As mentioned in the preceding chapter, several adhesion GPCRs, including GPR56, exhibit considerable constitutive activation of various reporter assays when transfected into heterologous cells. Therefore, we studied the previously-reported ability of GPR56 to stimulate the tcf/β-catenin pathway upon transfection into HEK293 cells (Shashidhar et al., 2005) and deciphered the signaling pathway by which GPR56 can regulate tcf/β-catenin. Additionally, we explored the role of the cleaved GPR56 N-terminus in receptor activation. Our initial hypothesis was that removing the receptor's N-terminus should result in a receptor that lacks signaling activity since the NT regions are involved in ligand binding for many GPCRs, including the Class B family of GPCRs, the family that is most closely related to GPCRs (Hjorth and Schwartz, 1996; Al-Sabah and Donnelly, 2003). Surprisingly, however, we instead found evidence that truncation of the GPR56 N-terminus results in enhanced constitutive activity of the receptor.

2.2 Experimental Procedures

Cell Culture: For all cell based assays, HEK293 cells (ATTC) were cultured and maintained in DMEM containing 10% FBS and 1% penicillin/streptomycin at 37 °C with 5% CO_2. Transfections were performed by incubating cells with Lipofectamine 2000 (Invitrogen) and cDNA for 4 hours in serum free DMEM, then stopping transfection with complete media. Experiments were performed 24-48 hours post transfection.

Antibodies: Antibodies against HA (Roche), FLAG (Sigma-Aldrich), β-arrestin2 (Sigma-Aldrich), Actin (Sigma-Aldrich), c-myc (Sigma-Aldrich), Cyr61 (AbCam) and biotinylated GPR56 N-terminus (R & D Systems) were purchased from manufacturer. The anti-GPR56 C-terminal antibody was developed by Orbigen via injection of rabbits with a peptide (CSNSDSARLPISSGSTSSSRI) derived from the GPR56 C-terminus, followed by affinity purification using the same peptide that was used as the immunogen.

Plasmids*:* N-terminal Flag-GPR56 was a gift from Christopher Stipp (University of Iowa). Untagged human GPR56 wild-type was subcloned into pcDNA 3.1. The Flag-tagged ΔNT mutant was cloned into pcDNA 3.1 by creating primers starting with an N-terminal Flag epitope followed by the human GPR56 sequence starting at amino acid 343. Similarly, an untagged GPR56ΔNT mutant was made by creating primers using the human. The HA-Ubiquitin construct was a gift from Keqiang Ye (Emory University) and the GFP-β-arrestin2 construct was a gift from Jeffrey Benovic (Thomas Jefferson University). GST-RBD (Addgene), HA-β-arrestin2 (Addgene), HA-Rho (Missouri S&T cDNA Resource Center), and dominant negative $G\alpha_{12/13}$ (Missouri S&T cDNA Resource Center) were all commercially obtained.

Western Blotting: Samples were resolved by SDS−PAGE on 4 to 20% Tris-glycine gels, followed by transfer to nitrocellulose membranes. The membranes were incubated in blocking buffer (2% nonfat dry milk, 50 mM NaCl, 20 mM HEPES, and 0.1% Tween 20) for 30 min and then incubated with primary antibody for 1 hour at room temperature. Next, the membranes were washed three times in blocking buffer and incubated with HRP-conjugated secondary antibody for 30 min, washed three times more, and finally visualized via ECL reagent followed by exposure to film. When using the biotinylated GPR56 N-terminal primary antibody, the ABC kit (Vectastain) was used to visualize immunoreactive bands in lieu of secondary antibody.

β-catenin Activation Assay: HEK293 cells were transfected with Top Flash TCF-reporter plasmid and either pcDNA 3.1, GPR56 wild-type, Flag-GPR56 or Flag-GPR56 truncations. Or other plasmids mentioned in the dissertation. Cells were incubated at 37°C for 24 hours. After 24 hours, cells were lysed with Promega Lysis Reagent (Promega), put on ice, and probed for luciferase activity using the Luciferase Assay System (Promega).

Rho Activation Assay: HEK293 cells were transfected with HA-Rho (Addgene) and either pcDNA 3.1, GPR56 wild-type, or GPR56ΔNT. After 24 hours, cells were scraped and resuspended in 500 μL Lysis Buffer [1% Triton X-100, 150 mM NaCl, 25 mM Hepes, 10 mM $MgCl_2$, 1 mM EDTA, and 2% glycerol]. Cells were incubated in lysis buffer for 30 minutes at 4°C then cleared by high-speed centrifugation. Soluble lysates were incubated for 30 minutes with 30 μL of GST-Rhotekin Binding Domain (GST-RBD) coupled to glutathione agarose beads. Beads were washed 2x with Lysis Buffer,

resuspended in 60 μL 2x sample buffer, and boiled for 10 minutes. Active Rho was detected by standard Western blot procedure, probing for HA.

Co-immunoprecipitation: HEK293 cells were transfected with various constructs to be assessed for ability to co-immunoprecipitate. After 24 hours, cells were scraped and resuspended in 500 μL Lysis Buffer [1% Triton X-100, 150 mM NaCl, 25 mM Hepes, 10 mM $MgCl_2$, 1 mM EDTA, and 2% glycerol]. Cells were incubated in lysis buffer for 30 minutes at 4°C then cleared by high-speed centrifugation. Soluble lysates were incubated for 60 minutes with 30 μL of protein A/G beads with corresponding antibody (to protein being immunoprecipitated). Beads were washed 3x with lysis buffer then resuspended in 60 μL 2x sample buffer, and boiled for 10 minutes. Co-immunoprecipitation was detected by standard Western blot procedure. Rat kidney was substituted for transfected HEK293 cells using same protocol. For ubiquitination studies, HEK293 cells were transfected with HA-Ubiquitin and either empty vector, GPR56 wild-type, or GPR56ΔNT mutant. Cells were lysed as described and immunoprecipitated with protein A/G beads coupled to HA-antibody. Western blot procedure was used to detect GPR56 using the GPR56-CT antibody.

Cytotoxicity: HEK293 cells were transfected with pcDNA 3.1, GPR56 wild-type, or GPR56ΔNT in the presence or absence of HA-β-arrestin2. After transfection, a media sample was taken from each plate of cells every 24 hours for 72 hours. Cytotoxicity was assessed by measuring LDH levels in the media samples using the CytoTox 96 Cytotoxicity Assay kit (Promega).

Confocal Microscopy: HEK293 cells transiently-transfected with GFP-β-arrestin2, plus or minus wild-type GPR56 or GPR56ΔNT, were plated onto poly-D-lysine coated chamber slides (Bio coat), allowed to attach overnight, and fixed at room temperature in 2% paraformaldehyde. The cells were incubated with the anti-GPR56-CT antibody (1:500) for two hours at 37°C, washed extensively, and then incubated with AlexaFluor goat anti-rabbit 546 secondary (1:250) for one hour at 37°C. Slides were viewed using the x63 objective of an LSM 510 META confocal microscope (Carl Zeiss, Inc., Thornwood, NY) and images were acquired with a constant setting for comparison across conditions using Zeiss LSM software.

Deglycosylation: HEK293 cells were transfected with GPR56 wild-type or Flag-NT constructs. After 24 hours, cells were scraped and resuspended in 500 μL lysis buffer [1% Triton X-100, 150 mM NaCl, 25 mM Hepes, 10 mM $MgCl_2$, 1 mM EDTA, and 2% glycerol]. Cells were incubated in lysis buffer for 30 minutes at 4°C with end-over-end agitation and then cleared by high-speed centrifugation. Soluble lysates were then immunoprecipitated (GPR56 wild-type with anti-GPR56-CT antibody plus protein A/G and Flag-NT with anti-Flag antibody beads) for 1 hour. After immunoprecipitation, beads were washed three times and resuspended in PBS with or without PNGase F (5 U, Sigma-Aldrich). This reaction mixture was incubated for 4 hours at 37 °C. After incubation, the reaction was stopped by the addition of sample buffer, and the samples were analyzed by Western blotting for GPR56-NT or Flag-NT.

Cell Surface Biotinylation: HEK293 cells were transfected with GPR56 wild-type or GPR56ΔNT constructs. After 24 hours, cells were washed and incubated for 2 hr at 4°C

in 2 mM Sulfo-NHS-LC-Biotin (Thermo Scientific) in PBS to biotinylate surface proteins. After biotinylation, cells were washed, scraped, and resuspended in lysis buffer [1% Triton X-100, 150 mM NaCl, 25 mM Hepes, 10 mM $MgCl_2$, 1 mM EDTA, and 2% glycerol]. Cells were incubated in lysis buffer for 30 minutes at 4°C with end-over-end agitation and then cleared by high-speed centrifugation. Soluble lysates were incubated with Streptavidin beads (Pierce) for 2 hours at 4°C to pull down surface biotinylated proteins. Beads were washed three times and resuspended in sample buffer. Surface expression of GPR56 and GPR56ΔNT was assessed by analyzing these samples via Western blotting.

Statistical Analysis: All statistical analysis was performed on single comparisons with student's t-test using GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

2.3 Results

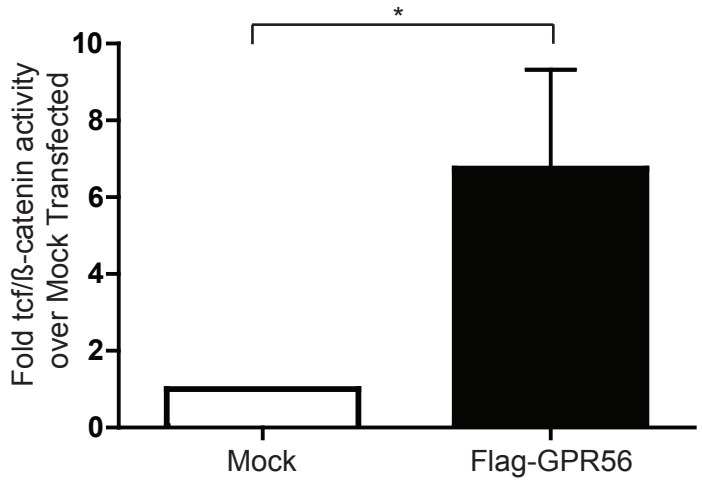
2.3.1 GPR56 couples to $G\alpha_{12/13}$ to activate tcf/β-catenin through Rho

Since GPR56 has been reported to activate tcf/βcatenin when transfected into HEK293 cells (Shashidhar et al., 2005), we chose HEK293 cells as our model system and transfected HEK293 cells with Top Flash reporter plasmid and either empty vector or full length Flag-GPR56. After 24 hr post transfection, cells were lysed and assessed for tcf/β-catenin activity using the Promega Luciferase kit. The results show clear activation of tcf/β-catenin in GPR56 transfected cells relative to mock-transfected cells (Fig II-1A).

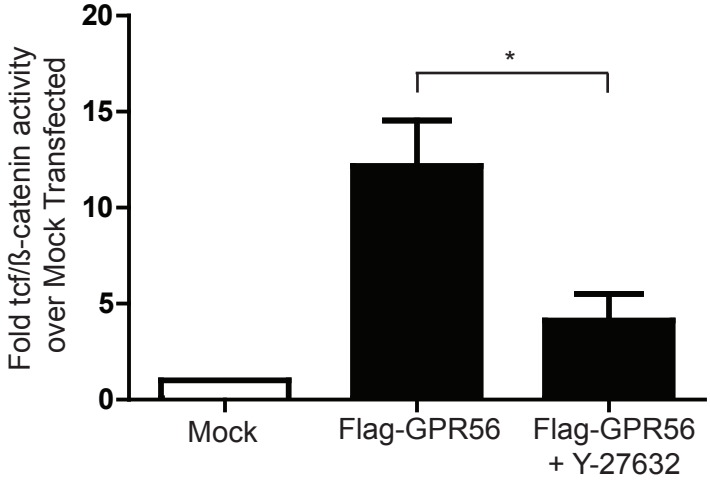
Next, using the reporter assay to measure receptor activity, second messenger and G protein inhibition studies were performed to decipher the specific signaling pathway of GPR56. Many other GPCRs that are known to activate β-catenin do this via coupling to $G\alpha_{12/13}$ and Rho (Siehler, 2009), so we explored this possibility for GPR56. β-catenin activation can occur though a pathway where $G\alpha_{12/13}$ activates Rho, which then stimulates Rho kinases (ROCK) to signal further downstream (Nelson and Nusse, 2004). Therefore, we sought to individually inhibit both ROCK and $G\alpha_{12/13}$ to see if this would in turn block GPR56-dependent activation of β-catenin. Specifically, HEK293 cells were transfected with Top Flash reporter plasmid and either empty vector or Flag-GPR56. After 24 hr post transfection, cells were treated over night with 20 μM Y-27632, a potent inhibitor of Rho kinases. Cells were then assessed for tcf/β-catenin activation using the Promega Luciferase kit. As shown in Fig II-1B, inhibition of Rho kinases led to a significant decrease in GPR56-depednent activation of β-catenin, suggesting that Rho and ROCK are essential components of this pathway. Furthermore, in parallel experiments,

Figure II-1. Overexpression of GPR56 in HEK293 cells activates β-catenin through a $G\alpha_{12/13}$ and Rho-dependent pathway. Top Flash tcf/β-catenin luciferase reporter plasmid was transfected into HEK293 cells with either empty vector ("mock") or Flag-GPR56 constructs. (A) GPR56 transfection resulted in a significant increase in luciferase activity over mock-transfected cells, indicating robust β-catenin activation. (B) After transfection, cells transfected with Flag-GPR56 were treated over night with 20 μM Y-27632, an inhibitor of Rho kinase. This treatment resulted in a significant decrease in luciferase production upon Y-27632 treatment, indicating Rho kinase-dependent β-catenin activation. (C) HEK293 cells were transfected with Top Flash reporter plasmid and Flag-GPR56. Corresponding cells were also transfected with a dominant negative $G\alpha_{12/13}$ plasmid. There was a significant decrease in luciferase production in cells expressing the dominant negative $G\alpha_{12/13}$ indicating $G\alpha_{12/13}$ dependent β-catenin activation. Unpaired t-tests were used for statistical analysis (n = 3; *, $p<0.005$).

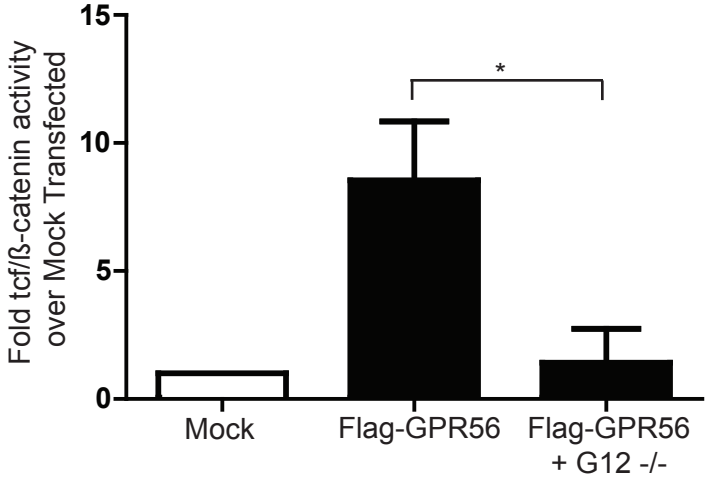
A


*
10
8
6
4
2
0
Fold tcf/ß-catenin activity over Mock Transfected
Mock
Flag-GPR56

B


*
20
15
10
5
0
Fold tcf/ß-catenin activity over Mock Transfected
Mock
Flag-GPR56
Flag-GPR56 + Y-27632

C


*
15
10
5
0
Fold tcf/ß-catenin activity over Mock Transfected
Mock
Flag-GPR56
Flag-GPR56 + G12 -/-

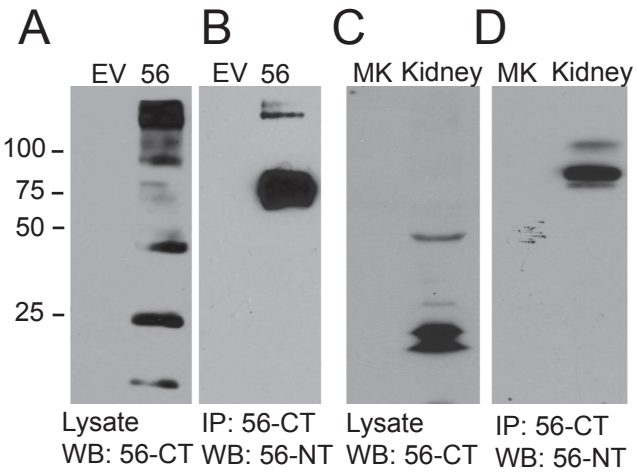
HEK293 cells were transfected with Top Flash reporter plasmid and either empty vector or Flag-GPR56. In addition, HEK293 cells were transfected in the same manner, but with a dominant negative $G\alpha_{12/13}$ protein (G12 -/-) that has been used successfully in other studies (Iftinca et al., 2007). As shown in Fig II-1C, the cotransfection of a dominant negative $G\alpha_{12/13}$ protein led to a significant decrease in GPR56-depednent activation of β-catenin. Taken together, these data show that GPR56 couples to the $G\alpha_{12/13}$ protein and signals to β-catenin through Rho and ROCK. These findings have been independently confirmed by an independent group who also reported that GPR56 activates Rho through coupling to $G\alpha_{12/13}$. Specifically, Itoh and colleagues showed that transfection of GPR56 into various cell lines led to increases in active Rho through a Rhotekin Rho binding domain pull down assay (Iguchi et al., 2008). Moreover, they created an N-terminal antibody that could further stimulate GPR56-dependent Rho activity. Thus, the tcf/β-catenin inhibition studies performed by our lab and the Rho activation assays performed by Itoh & colleagues are in agreement that GPR56 signals predominantly through the $G\alpha_{12/13}$/Rho pathway.

2.3.2 GPR56 is processed into two fragments that remain associated at the cell surface.

We developed a polyclonal anti-GPR56 antibody to visualize the receptor's C-terminus. A representative Western blot utilizing this antibody to detect GPR56 in transfected HEK293 cells is shown in Figure II-2A. The specificity of the antibody is evident from the fact that no immunoreactive bands are detectable in untransfected

HEK293 cells (first lane). In cells transfected with GPR56, several different processed forms of the receptor are evident. The prominent band at 45 kDa represents the 7TM region following GPS domain cleavage to remove the large N-terminus, as this 45 kDa band was observed in cell surface biotinylation experiments to be the main surface-expressed fragment of the receptor (Fig II-2E) and is precisely the predicted size of the 7TM region following cleavage at the GPS domain. The handful of lower molecular weight bands are presumably derived from additional cleavage events, and the higher molecular weight bands probably represent GPR56 not yet processed at the GPS domain and/or unresolved receptor oligomers; none of these species were found in the plasma membrane, as determined by cell surface biotinylation (Fig II-2E). GPR56 from these transfected cells was solubilized in 1% Triton X-100, immunoprecipitated with the C-terminal antibody and visualized on Western blots using a commercially-available antibody to detect the GPR56-NT. The GPR56-NT was visualized in these Western blots as a ~75 kDa band, which upon deglycosylation decreased in size to 37 kDa (Fig II-2F), consistent with past reports (Jin et al., 2007; Ke et al., 2008). As shown in Figure II-2B, robust co-immunoprecipitation of the cleaved GPR56-NT was observed with the receptor's 7TM region, suggesting that the two fragments of the receptor remain associated in cells even following cleavage at the GPS domain. Similar experiments were performed on endogenous GPR56 in rat kidney, a tissue where GPR56 is highly expressed (Huang et al., 2008). Figure II-2C shows the expression of GPR56 in rat kidney using the GPR56 C-terminal antibody. The main band at just under 50 kDa and the processed forms at smaller sizes were very similar to the pattern of bands observed in the GPR56-transfected HEK293 cells. Immunoprecipitation with the C-terminal antibody

Figure II-2. The cleaved N-terminus of GPR56 associates with the receptor's seven-transmembrane (7TM) region. (A) Lysates from HEK293 cells transfected with empty vector (EV) or GPR56 were probed with anti-GPR56 C-terminal antibody. (B) The same antibody was used to immunoprecipitate (IP) the GPR56 7TM region from solubilized lysates. The IP samples were then probed with the GPR56 N-terminal antibody (n=3). (C) Rat kidney tissue was solubilized and probed for GPR56 expression using the GPR56 C-terminal antibody. The mock lane (MK) was loaded with just buffer. (D) IP was performed with the GPR56 C-terminal antibody, and IP samples were probed with the GPR56 N-terminal antibody. Mock (MK) in this case represents the IP protocol performed with beads, but no antibody (n=3). (E) Western blot analysis of the surface biotinylation of GPR56 and GPR56ΔNT. As shown in the panel on the right, both the wild-type and truncated mutant were expressed at roughly comparable levels. As shown on the left, surface biotinylation of GPR56 and GPR56ΔNT was similar, revealing that both receptors were found equally at the plasma membrane. (F) Deglycosylation of GPR56. Immunoprecipitation of the GPR56 C-terminus from cells transfected with full-length GPR56 revealed that the cleaved N-terminus remains associated with the 7TM region and is approximately 75 kDa. Upon deglycosylation with PNGase F, the associated N-terminus resolves at approximately 37 kDa, which is the predicted molecular weight of the unmodified NT.


A
B
C
D
EV 56
EV 56
MK Kidney
MK Kidney
100
75
50
25
Lysate
WB: 56-CT
IP: 56-CT
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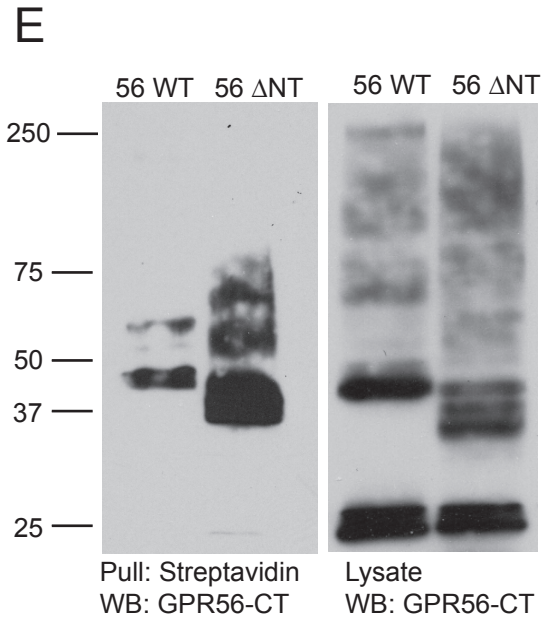
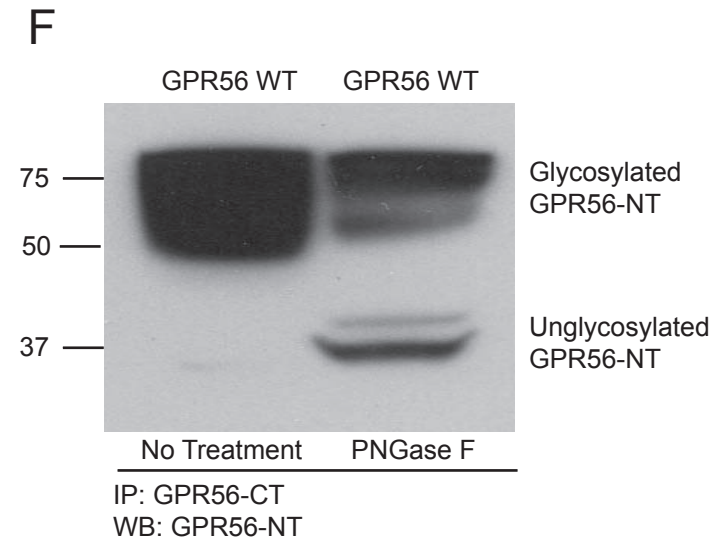
IP: 56-CT
WB: 56-NT


E
56 WT
56 ΔNT
56 WT
56 ΔNT
250
75
50
37
25
Pull: Streptavidin
WB: GPR56-CT
Lysate
WB: GPR56-CT

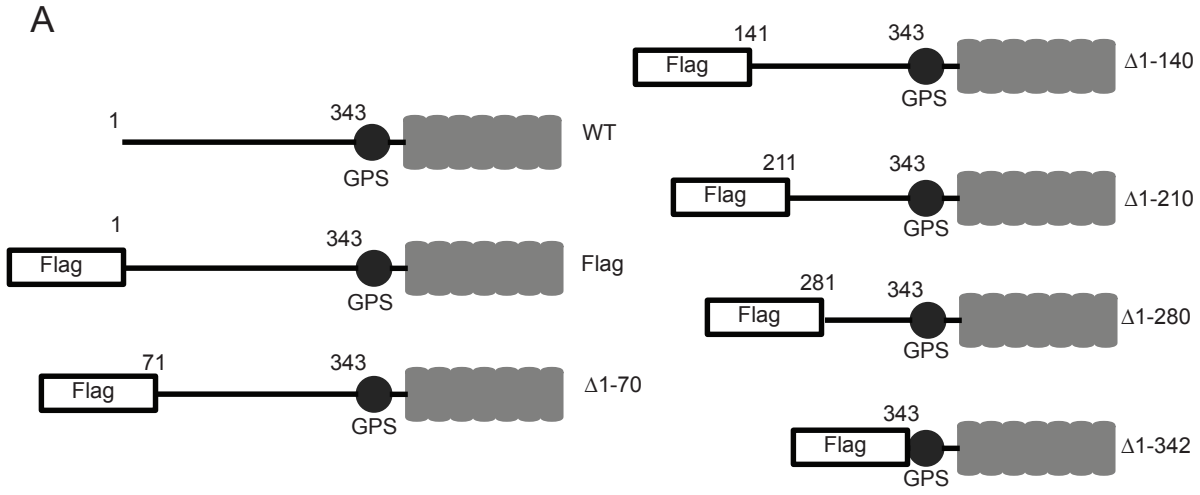

F
GPR56 WT
GPR56 WT
75
50
37
Glycosylated
GPR56-NT
Unglycosylated
GPR56-NT
No Treatment
PNGase F
IP: GPR56-CT
WB: GPR56-NT

resulted in robust co-immunoprecipitation (CO-IP) of the endogenous N-terminal fragment (Fig II-2D). These data show, like the CD97, EMR2 and latrophilin receptors, that GPR56 exists at the cell surface as a heterodimer between the heavily glycosylated cleaved N-terminus and 7TM region.

2.3.3 Sequential truncations of the GPR56 N-terminus results in reduction of total Rho levels.

To investigate regions of the GPR56 N-terminus that mediate signaling, we created a series of N-terminal truncations. Our hypothesis was that sequential truncation of the N-terminus would eventually lead to loss of GPR56 signaling (Rho and β-catenin) due to loss of ligand binding. As a result, we could map the region of ligand-GPR56 binding to get a better sense of functional domains in the large GPR56 N-termini. Thus, we created sequential 70 amino acid truncations starting at the first N-terminal amino acid and ending at number 342. These constructs were also N-terminally Flag-tagged as shown in Fig II-3A. The last truncation, designated Δ1-342, is missing almost the entire N-terminus, excluding the GPS domain, thus mimicking the physiologically cleaved GPR56 receptor. We next wanted to see how the N-terminus effected GPR56-dependent signaling. To do this, we transfected HEK293 cells with an HA-Rho construct and either empty vector, GPR56 wild type, Flag-GPR56 full length, and each of the N-terminal truncations. After 24 hrs post transfection, the cells were lysed and assayed for Rho activation through pull down with Rhotekin Rho binding domain. As the results show in Fig II-3B/C, GPR56 full length was able to stimulate Rho as expected, however

Figure II-3. Sequential truncation of the GPR56 N-terminus leads to sequential loss of both active and total Rho. Sequential 70 amino acid truncations were made to the GPR56 N-terminus, starting at the distal end. (A) Illustration of the truncations as well as the corresponding nomenclature for each construct. (B) Quantification of active RhoA via pull-down with GST-RBD. (C) *Top Panel:* Western blot analysis of active RhoA pull-down with GST-RBD beads from HEK293 cells transfected with mock vector, wild-type GPR56, Flag-GPR56 full length or the truncated mutants. *Middle Panel:* Western blot analysis of total RhoA levels from HEK293 cells transfected with empty vector ("mock"), GPR56, Flag-GPR56 full length and each truncated mutant. Statistical analysis was performed using unpaired t-tests. (n=3; *, $p<0.005$)


A
1
343
GPS
WT
1
Flag
343
GPS
Flag
71
Flag
343
GPS
Δ1-70
141
343
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GPS
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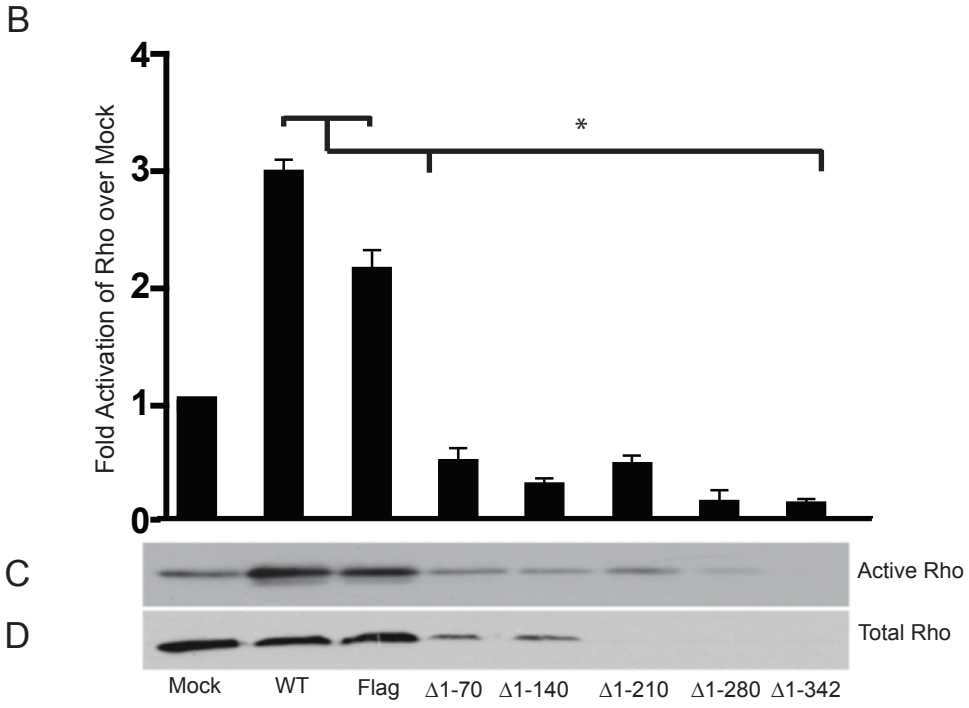
Flag
GPS
Δ1-342

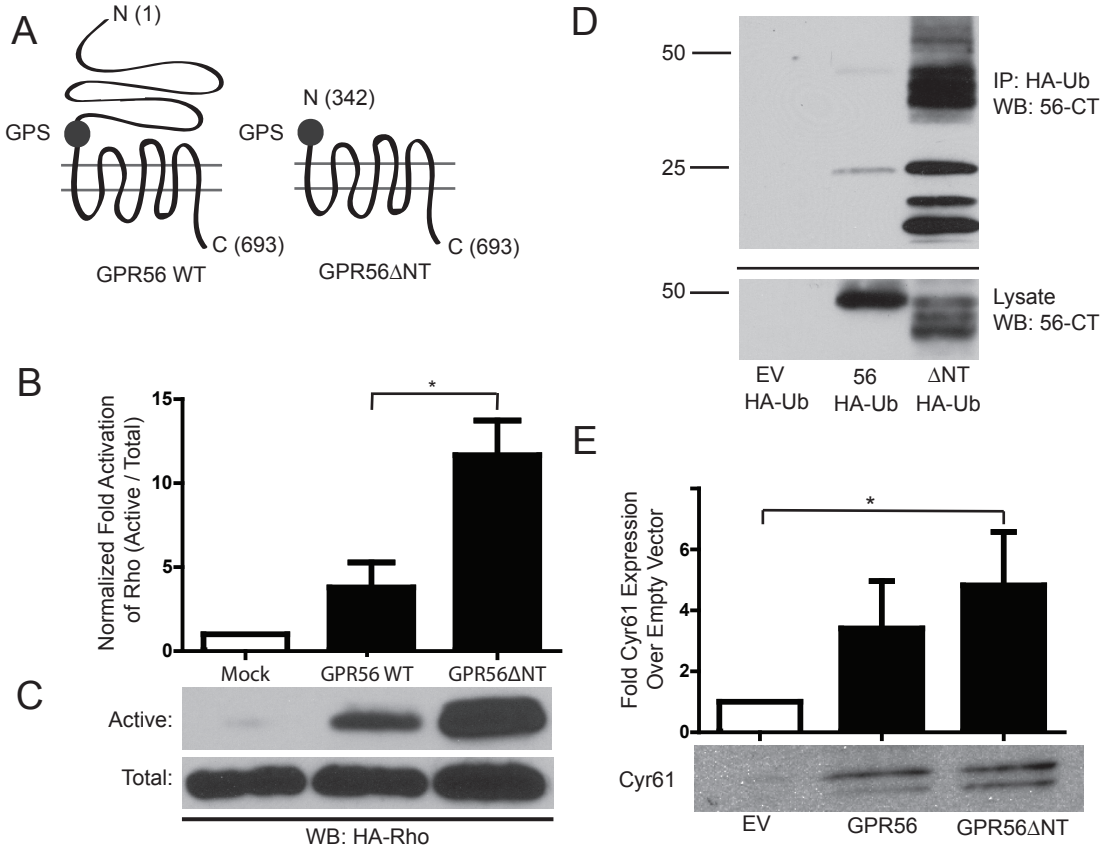

B
Fold Activation of Rho over Mock
0
1
2
3
4
*
C
Active Rho
D
Total Rho
Mock
WT
Flag
Δ1-70
Δ1-140
Δ1-210
Δ1-280
Δ1-342

sequential truncations of the N-terminus showed sequential loss of active Rho. At first glance, this result was consistent with our initial hypothesis that truncation of the GPR56 N-terminus would cause loss of receptor-dependent Rho activation. However, as Fig II-3D shows, there was a corresponding loss of total Rho along with active Rho. It is known that over-activation of signaling pathways can lead to profound desensitization of signaling components (Fujimori et al., 1993). Moreover, it has been reported that Rho can be degraded in response to over-stimulation of the Rho pathway (Wang et al., 2003). Thus, we speculated that sequential losses of the GPR56 N-terminus led to profound desensitization of the Rho pathway do to over-activity of the receptor.

2.3.4 Removal of the GPR56-NT enhances GPR56-mediated stimulation of Rho activity and induces receptor ubiquitination and Cyr61 expression.

Besides our aforementioned data, previous reports have shown that transfection of GPR56 into HEK293 cells results in stimulation of $G\alpha_{12/13}$ to activate downstream Rho and β-catenin signaling (Shashidhar et al., 2005; Iguchi et al., 2008). To further explore the importance of the GPR56-NT for the receptor's signaling activity, and decipher the appearance of Rho down regulation, we created an untagged truncated GPR56 construct lacking the N-terminus up to the GPS domain (Fig II-4A). This truncated receptor ("ΔNT") is a better representation of the physiologically cleaved GPR56, as it lacks the Flag-tag which could have affected receptor signaling. In addition to down regulation of signaling pathways, another explanation to the observed decrease in Rho activation of our Flag-tagged truncation mutants could be altered surface expression. For example,

Figure II-4. Further analysis of the effect of N-terminal truncation on GPR56-mediated signaling, receptor ubiquitination, and Cyr61 expression indicates a constitutively active receptor. (A) Schematic drawing showing the location of the N-terminal GPR56 truncation. The numbers in parenthesis indicate the amino acid positions of the N-terminus, GPS domain starting position, and C-terminus for each construct. (B) Quantification of active RhoA via pull-down with GST-RBD. Active Rho for each construct was first normalized to total Rho before quantification and comparison to empty vector (*, $p<0.05$ n=10). (C) *Top Panel:* Western blot analysis of active RhoA pull-down with GST-RBD beads from HEK293 cells transfected with empty vector (EV), wild-type GPR56, or the ΔNT mutant. *Middle Panel:* Western blot analysis of total RhoA levels from HEK293 cells transfected with empty vector (EV), GPR56 and GPR56ΔNT. *Bottom Panel:* Western blot analysis of actin as a loading control for the same samples shown in the middle panel. (D) Ubiquitination of wild-type versus truncated GPR56. Full-length GPR56 or GPR56ΔNT were transfected into HEK293 cells with HA-Ubiquitin (HA-Ub). Immunoprecipitation was performed with anti-HA antibodies and immunoprecipitates were probed via Western blot with anti-GPR56-CT antibodies to visualize ubiquitinated GPR56. The data shown are representative of three independent experiments. (E) Quantification of Western blot analysis of changes in Cyr61 expression induced by transfection of HEK293 cells with empty vector (EV), GPR56 full-length, and GPR56ΔNT constructs. Statistical analysis was performed using unpaired t-tests. (n=12; *, $p<0.05$).


A
N (1)
GPS
C (693)
GPR56 WT
N (342)
GPS
C (693)
GPR56ΔNT
B
Normalized Fold Activation of Rho (Active / Total)
15
10
5
0
*
Mock
GPR56 WT
GPR56ΔNT
C
Active:
Total:
WB: HA-Rho
D
50
25
50
IP: HA-Ub
WB: 56-CT
Lysate
WB: 56-CT
EV
HA-Ub
56
HA-Ub
ΔNT
HA-Ub
E
Fold Cyr61 Expression Over Empty Vector
6
4
2
0
*
Cyr61
EV
GPR56
GPR56ΔNT

Dunham et al. reported that deletion of the GPR37 N-terminus resulted in increased surface expression of the receptor (Dunham et al., 2009). Moreover, many GPCRs contain cleavable signal sequences on their proximal N-terminus that assist in plasma membrane insertion, so deletion of this sequence could disrupt normal receptor trafficking (Walter et al., 1984; Singer, 1990; Spiess, 1995). However, as mentioned earlier, we performed cell surface biotinylation experiments (Fig II-2B) which revealed that wild-type GPR56 and the ΔNT mutant were found in the plasma membrane at roughly comparable levels.

The truncated receptor was next expressed in HEK293 cells and assessed for its ability to stimulate Rho activity relative to wild-type. Unlike in previously described Rho experiments for the Flag-truncations, total Rho was first normalized before active Rho was assayed, to insure an accurate comparison of the activation state between receptors. As shown in Fig II-4C and Fig II-4D, transfection of the cells with wild-type GPR56 resulted in significant increases in Rho activity, consistent with our previous findings. Strikingly, transfection with the ΔNT mutant enhanced Rho signaling to an even greater extent than transfection with the wild-type GPR56. These findings further and more directly suggest that the ΔNT mutant exhibits enhanced constitutive activity. To further decipher the activity levels of wild-type versus truncated mutant GPR56, we examined the ubiquitination state of each receptor, since many GPCRs undergo extensive ubiquitination upon prolonged activation (Shenoy et al., 2001; Marchese et al., 2003; Martin et al., 2003; Shenoy, 2007). As shown in Fig II-4E, GPR56ΔNT was found to be heavily ubiquitinated, whereas ubiquitination of the wild-type receptor was barely detectable.

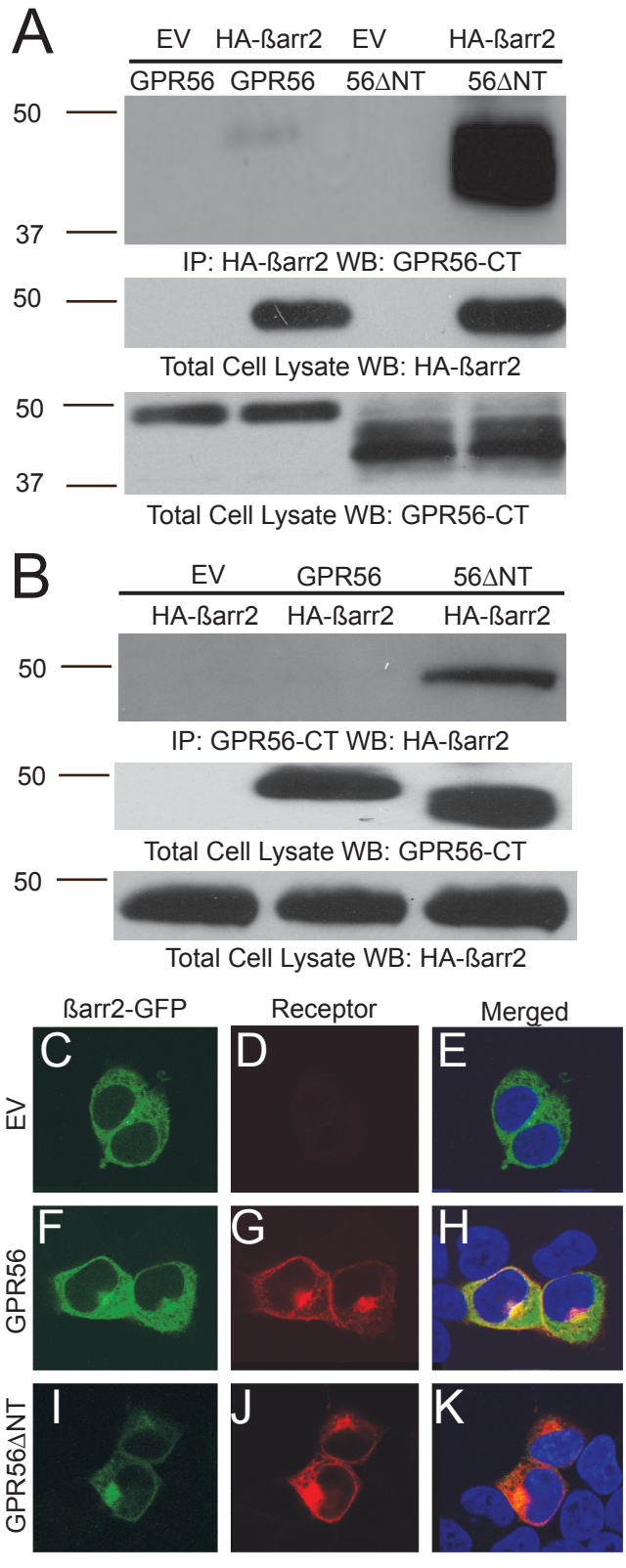
To further explore the activity state between GPR56 wild type and the ΔNT truncated mutant, we looked at production of the matricellular protein cysteine-rich angiogenic inducer 61 (Cyr61). Cyr61 is a highly regulated matricellular protein that has been shown to signal through integrins (Perbal, 2001). More importantly, it has been established that activation of GPCRs that signal through Rho, particularly the protease activates receptor-1 (PAR-1) and lysophosphatidic acid (LPA) receptors, cause a significant increase in Cyr61 production (Pendurthi et al., 2002; Sakamoto et al., 2004). Furthermore, increases in Cyr61expression have also been shown to be less sensitive to desensitization than Rho, making it an excellent readout for GPR56ΔNT dependent Rho activation (Walsh et al., 2008). For this reason, HEK293 cells were transfected with mock vector, GPR56 wild-type, or GPR56ΔNT and incubated for 48 hrs. The cells were then lysed and Western blotted for Cyr61 expression. As seen in Fig II-4F, both GPR56 wild-type and GPR56ΔNT showed significant increases in Cyr61 expression over mock transfected. Although GPR56ΔNT did not show a statistical significance in Cyr61 expression verse wild-type, the trend suggested a greater degree of Rho activation. Therefore, these data indicate that removal of the GPR56 N-terminus causes an increase in receptor-dependent Rho activation, an intriguing finding that ran contrary to our original hypothesis.

2.3.5 Truncation of the GPR56-NT enhances receptor interactions with β-arrestin2.

The signaling and ubiquitination studies suggested that GPR56ΔNT may be a constitutively-active receptor. If this were the case, then one additional prediction would be that this truncated receptor should exhibit enhanced interactions with β-arrestins, a

family of regulatory proteins that are known to interact with active GPCRs to tone down G protein-mediated signaling (Reiter and Lefkowitz, 2006) and exhibit especially robust associations with constitutively-active receptors (Mhaouty-Kodja et al., 1999; Ferrari and Bisello, 2001). As shown in Figure II-5A, wild-type GPR56 could be detected in complex with β-arrestin2 in co-immunoprecipitation experiments, which is consistent with observations that this receptor has some level of activity when transfected into HEK293 cells. Strikingly, however, the ΔNT mutant exhibited a massive increase in co-immunoprecipitation with β-arrestin2 relative to the wild-type receptor (Fig II-5A, last lane; more than 10-fold increase in β-arrestin2 associations was observed for the ΔNT mutant relative to wild-type in 6 independent experiments). Reciprocal co-immunoprecipitation experiments in which anti-GPR56 immunoprecipitates were probed for β-arrestin2 also showed significant increases in β-arrestin2 associations with the ΔNT mutant over wild-type GPR56 (Fig II-5B, last lane). The enhanced interaction of the ΔNT mutant with β-arrestin2 was also observed in confocal microscopy experiments (Fig II-5C-K). β-arrestin2-GFP was found to be evenly distributed throughout the cell when transfected into HEK293 cells by itself (Fig II-5C). However, co-transfection with GPR56 resulted in enrichment of β-arrestin2-GFP in a perinuclear compartment (Fig II-5F), and co-transfection with the ΔNT mutant resulted in an even more dramatic targeting of β-arrestin2 to the perinuclear region (Fig II-5I), where it exhibited strong co-localization with internalized receptor (Fig II-5K). Such targeting of arrestins and internalized receptors to perinuclear endosomes has been observed for many GPCRs upon prolonged periods of receptor activation (Zhang et al., 1999; Innamorati et al.,

Figure II-5. β-arrestin2 binds avidly to GPR56ΔNT. (A) HEK293 cells were transfected with GPR56 or GPR56ΔNT in the absence or presence of HA-β-arrestin2. Immunoprecipitation was performed with HA-antibody coupled to agarose beads. Co-immunoprecipitation of GPR56 was detected by Western blotting with the custom GPR56 C-terminal antibody. (B) HEK293 cells were transfected with HA-β-arrestin2 and either empty vector (EV), GPR56 or GPR56ΔNT. Immunoprecipitation was performed with the anti-GPR56-CT antibody and Protein A/G agarose. Co-immunoprecipitation of β-arrestin2 was detected by Western blotting with anti-HA antibody. (C-K) Wild-type GPR56 and GPR56ΔNT promote β-arrestin2 cellular redistribution and perinuclear aggregation. β-arrestin2-GFP expressed alone was distributed evenly throughout HEK-293 cells (panels C, E), but co-expression with GPR56 promoted translocation of β-arrestin2 to the perinuclear region, where it co-localized with the receptor (panel H). Translocation of β-arrestin2 to the perinuclear region was even more dramatic upon co-expression with GPR56ΔNT (panel K). DAPI staining is shown in panels E, H, and K. These data are representative of 4 independent experiments.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GPR56
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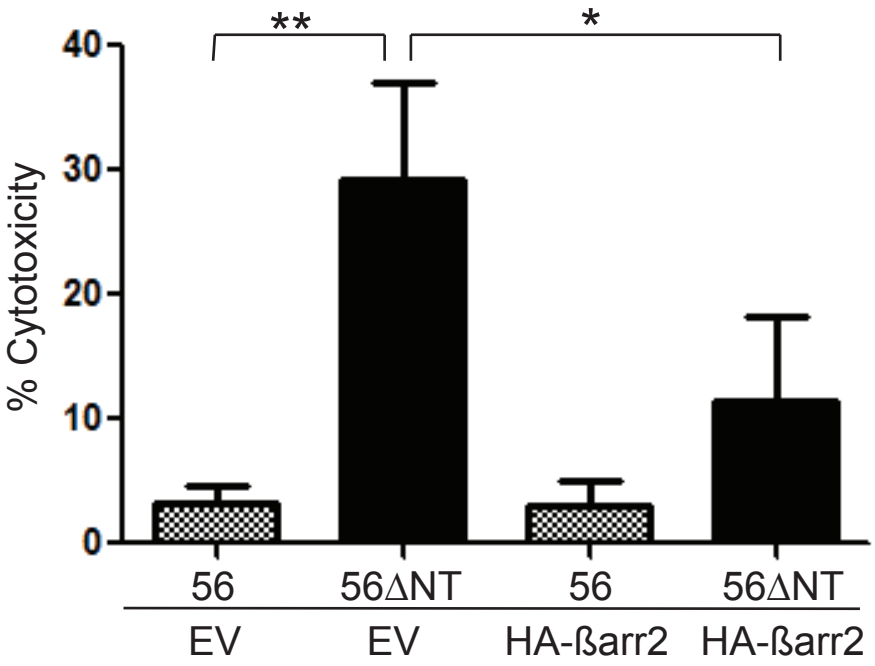
50
Total Cell Lysate WB: HA-ßarr2
50
37
Total Cell Lysate WB: GPR56-CT
B
EV
GPR56
56ΔNT
HA-ßarr2
HA-ßarr2
HA-ßarr2
50
IP: GPR56-CT WB: HA-ßarr2
50
Total Cell Lysate WB: GPR56-CT
50
Total Cell Lysate WB: HA-ßarr2
ßarr2-GFP
Receptor
Merged
EV
GPR56
GPR56ΔNT
C
D
E
F
G
H
I
J
K

2001; Lelouvier et al., 2008). Thus, these data provide further evidence for the idea that removal of the N-terminus induces constitutive activation of GPR56.

2.3.6 Over-expression of GPR56ΔNT induces cell death that can be rescued by co-expression of β-arrestin2

In addition to enhancing signaling, undergoing extensive ubiquitination and interacting robustly with β-arrestins, another hallmark of constitutively-active GPCRs is causing toxicity in the cells in which they are expressed (Dale et al., 2000; Miura and Karnik, 2000). Thus, we performed cytotoxicity tests on HEK293 cells transfected with wild-type GPR56 versus the ΔNT mutant. Over-expression of wild-type GPR56 failed to induce any toxicity at any of the time points examined (Fig II-6). Transfection of the ΔNT mutant also did not result in any evident cytotoxicity at 24 hours post-transfection, the time point at which all of the signaling studies described above were performed. However, over-expression of the ΔNT mutant did cause a significant increase in cell death at 48 hours and an even larger increase in cell death at 72 hours. Furthermore, this cytotoxicity induced by over-expression of the ΔNT mutant could be greatly attenuated by co-expression of β-arrestin2, which would be expected to boost the arrestin-to-receptor ratio in the cells and thereby drive arrestin associations with the overactive receptors to dampen down their activity. Taken together with the signaling data, ubiquitination data and β-arrestin2 interaction data, these findings suggest that truncation of the GPR56-NT results in constitutive receptor activation.

Figure II-6. β-arrestin2 attenuates GPR56ΔNT-stimulated cytotoxicity. Cytotoxicity induced by GPR56 or GPR56ΔNT expression in HEK293 cells was determined by LDH secretion in the media at 72 hours post-transfection. A significant difference was observed between GPR56 and GPR56ΔNT (**, $p<0.01$, n=6). Co-transfection of HA-β-arrestin2 resulted in a significant reduction in GPR56ΔNT-dependent cytotoxicity (n=3; *, $p<0.03$), but had no effect on toxicity in cells transfected with GPR56 wild-type. Statistical analysis was performed using unpaired t-tests.


**
*
40
30
20
10
0
% Cytotoxicity
56
56ΔNT
56
56ΔNT
EV
EV
HA-ßarr2
HA-ßarr2

2.4 Discussion

In the studies described in this chapter, we have shown definitively that GPR56 signals through coupling to G proteins to activate Rho GTPases and tcf/β-catenin. Upon overexpression of wild type GPR56 in HEK293 cells, we observed significant increases in activated Rho and β-catenin. We then used inhibitors of $G\alpha_{12/13}$ and ROCK to block activation. These data suggest that GPR56-dependent activation of the tcf/β-catenin pathway is dependent on coupling to $G\alpha_{12/13}$ and Rho. In truncation studies to determine functional domains that might control GPR56 ligand binding, we surprisingly discovered that loss of the N-terminus results in a profound increase in the constitutive activity of GPR56.

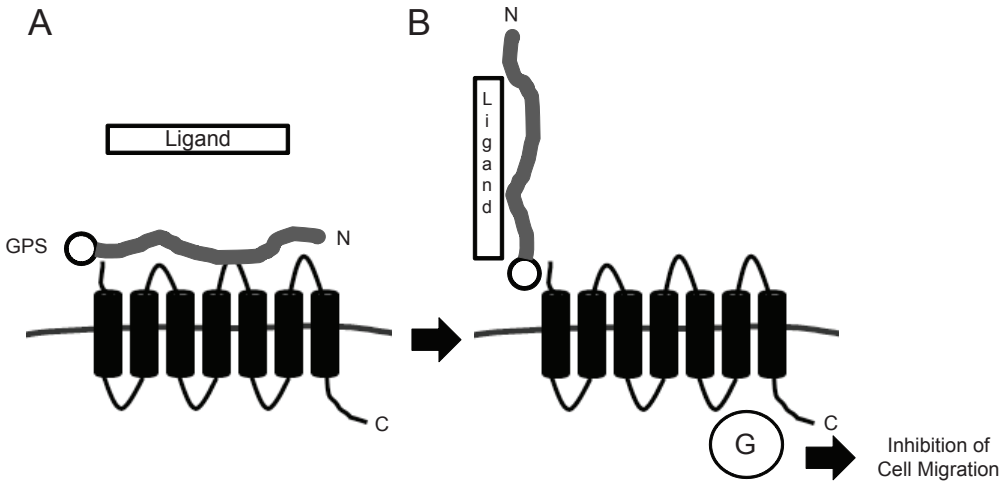
GPR56 regulates the migration of neural precursor cells *in vitro* (Iguchi et al., 2008) and *in vivo* (Li et al., 2008; Koirala et al., 2009), but little is known about the mechanism of activation for GPR56. Based on our findings described in this chapter, we propose that removal of the GPR56 N-terminus results in receptor activation. This idea is based on four lines of evidence: *i)* transfection of cells with the GPR56 ΔNT mutant results in significantly enhanced activation of Rho signaling, relative to wild-type GPR56, *ii)* the ΔNT mutant is much more heavily ubiquitinated than wild-type GPR56, *iii)* the ΔNT mutant associates much more avidly than wild-type GPR56 with β-arrestins, which preferentially bind to active receptors, and *iv)* expression of the ΔNT mutant is toxic for cells in a manner that is rescued by co-expression of β-arrestins. All of these phenomena – enhanced signaling activity, increased ubiquitination, enhanced binding of β-arrestins, and toxicity to cells – are characteristic of constitutively-active GPCRs.

Thus, we propose that removal of the GPR56 N-terminus results in greatly enhanced constitutive signaling activity of the receptor.

N-terminal truncations do not typically result in constitutive activation of GPCRs. For example, truncations to the N-termini of β_2-adrenergic (Dixon et al., 1987), α_{1D}-adrenergic (Hague et al., 2004), CB1 cannabinoid (Andersson et al., 2003), GPR37 orphan (Dunham et al., 2009) or μ opioid (Muller et al., 2009) receptors can cause alterations in receptor trafficking and/or ligand binding properties, but do not result in constitutive receptor activation. The only GPCRs that are known to be activated by N-terminal removal are the four members of the protease-activated receptor (PAR) family (Traynelis and Trejo, 2007) and the thyrotropin receptor (TSHR) (Zhang et al., 2000; Quellari et al., 2003). PAR and TSHR cleavage by extracellular proteases is believed to be a key step in the natural mechanism of activation for these receptors. Similarly, we propose that relief of the inhibitory influence of the GPR56-NT on the receptor's signaling activity may be a key step in the mechanism of activation for GPR56. Since GPS domain cleavage is believed to be autoproteolytic (Lin et al., 2004), GPR56 does not require the action of an exogenous protease to achieve separation of its N-terminal region from the rest of the receptor. However, the two halves of GPR56 remain non-covalently associated for some period of time following GPS domain cleavage, and our data suggest that this association constrains the signaling activity of the receptor's 7TM region. We propose that interaction of the GPR56-NT with an extracellular ligand results in either the release of the GPR56-NT from the 7TM region or a conformational change in the GPR56-NT that relieves the inhibitory influence of the GPR56-NT and thereby allows for receptor activation (Fig II-7).

Figure II-7. Schematic diagram of the proposed mechanism of activation for GPR56

(A) The GPR56 N-terminus (NT) is cleaved from the receptors' seven transmembrane (7TM) region, but the two halves of the receptor remain non-covalently associated and the receptor is largely inactive. (B) The GPR56 N-terminus engages an adhesive ligand to induce receptor activation. GPR56-NT interactions with the adhesive ligand might physically disrupt NT associations with the GPR56 7TM region or simply alter NT conformation to enhance the functional activity of the 7TM region.


A
B
Ligand
GPS
N
C
N
Ligand
C
G
Inhibition of
Cell Migration

CHAPTER III: GPR56 N-termini Undergo Homophilic Trans-interactions to Positively Regulate Receptor-Dependent Signaling.

3.1 Introduction

In the previous chapter, a novel mechanism of activation for GPR56 was presented wherein removal of the GPR56 N-terminus leads to receptor activation. Therefore, it might reasonably be hypothesized that GPR56 may be activated by extracellular interactions that either remove the NT or alter its conformation. GPR56 has been shown to associate via its N-terminal region with the extracellular matrix protein transglutaminase 2 (Xu et al., 2006), and also shown to form complexes with the tetraspanins CD9 and CD81 via undetermined domains (Little et al., 2004), but these associations have not been shown to have any effect on receptor activity. The only two proteins that are known to enhance GPR56-mediated signaling are anti-GPR56 antibodies that bind to the receptor's N-terminus (Iguchi et al., 2008) and the matrix protein collagen type III (Luo et al., 2011). Similarly, N-terminal antibodies have been shown to activate the adhesion GPCR EMR2 (Yona et al., 2008) and an N-terminal-binding toxin (latrotoxin) has been shown to activate the adhesion GPCR latrophilin-1 (Lelianova et al., 1997). Despite the artificial nature of these antibody and toxin treatments, these studies are of significant interest in that they reveal the importance of N-terminal binding partners for receptor activation. A less-artificial example of N-terminal engagement leading to adhesion GPCR activation comes from work on Celsr2 and Celsr3, which have been shown to be activated via homophilic N-terminal interactions in a trans-trans fashion (Shima et al., 2007). Similarly, Flamingo, the Celsr homolog in *Drosophila*, has been shown to undergo homophilic trans-trans interactions that are critical for the development of planar cell polarity (Usui et al., 1999; Carreira-Barbosa et al., 2009). Therefore, we attempted to determine if GPR56 was also capable of homophilic

interactions with its N-terminus. In our studies, we found that the N-terminus of GPR56 is capable of homophilic trans-trans interactions, and that these associations promote receptor activation. We also showed functional effects of these interactions in controlling cell migration. It remains to be determined whether these GPR56/GPR56 associations truly activate the receptor or simply make the receptor permissive for signaling (for example, by creating a binding site for an as-yet-unidentified ligand). It also remains to be determined whether NT/NT interactions are a common feature for other members of the adhesion GPCR family beyond Celsr2/3 and GPR56.

3.2 Experimental Procedure

Cell Culture: For all cell based assays, HEK293 and U118-mg cells (ATTC) were cultured and maintained in DMEM containing 10% FBS and 1% penicillin/streptomycin at 37 °C with 5% CO_2. Transfections were performed by incubating cells with Lipofectamine 2000 (Invitrogen) and cDNA for 4 hours in serum free DMEM, then stopping transfection with complete media. Experiments were performed 24-48 hours post transfection.

Antibodies: Antibodies against HA (Roche), FLAG (Sigma-Aldrich), β-arrestin2 (Sigma-Aldrich), c-myc (Sigma-Aldrich), acetylated tubulin (Sigma Aldrich) and biotinylated GPR56 N-terminus (R & D Systems) were purchased from manufacturer. The anti-GPR56 C-terminal antibody was developed by Orbigen via injection of rabbits with a peptide (CSNSDSARLPISSGSTSSSRI) derived from the GPR56 C-terminus, followed by affinity purification using the same peptide that was used as the immunogen.

Plasmids: N-terminal Flag-GPR56 was a gift from Christopher Stipp (University of Iowa). Untagged human GPR56 wild-type was subcloned into pcDNA 3.1. Flag-NT GPR56 and c-myc-NT GPR56 constructs were created in the pCMV2B plasmid and correspond to human GPR56 amino acids 1-342. Other Myc-NT truncations were made with appropriate primers to amino acid regions for the desired listed truncations. GST-RBD (Addgene) and HA-Rho (Missouri S&T cDNA Resource Center) were all commercially obtained.

Western Blotting*:* Samples were resolved by SDS−PAGE on 4 to 20% Tris-glycine gels, followed by transfer to nitrocellulose membranes. The membranes were incubated

in blocking buffer (2% nonfat dry milk, 50 mM NaCl, 20 mM HEPES, and 0.1% Tween 20) for 30 min and then incubated with primary antibody for 1 hour at room temperature. Next, the membranes were washed three times in blocking buffer and incubated with HRP-conjugated secondary antibody for 30 min, washed three times more, and finally visualized via ECL reagent followed by exposure to film. When using the biotinylated GPR56 N-terminal primary antibody, the ABC kit (Vectastain) was used to visualize immunoreactive bands in lieu of secondary antibody.

Rho Activation Assay: HEK293 cells were transfected with HA-Rho (Addgene) and either pcDNA 3.1, GPR56 wild-type, or GPR56ΔNT. Or used with through co-culture experimental set-up. After 24 hours, cells were scraped and resuspended in 500 μL Lysis Buffer [1% Triton X-100, 150 mM NaCl, 25 mM Hepes, 10 mM $MgCl_2$, 1 mM EDTA, and 2% glycerol]. Cells were incubated in lysis buffer for 30 minutes at 4°C then cleared by high-speed centrifugation. Soluble lysates were incubated for 30 minutes with 30 μL of GST-Rhotekin Binding Domain (GST-RBD) coupled to glutathione agarose beads. Beads were washed 2x with Lysis Buffer, resuspended in 60 μL 2x sample buffer, and boiled for 10 minutes. Total Rho levels were first determined through Western blotting of cell lysates with anti-HA antibody to normalize levels before determining active Rho levels. Active Rho was visualized by standard Western blot procedure, probing the normalized GST-RBD samples for HA.

Co-immunoprecipitation: HEK293 cells were transfected with various constructs to be assessed for ability to co-immunoprecipitate. After 24 hours, cells were scraped and resuspended in 500 μL Lysis Buffer [1% Triton X-100, 150 mM NaCl, 25 mM Hepes, 10

mM $MgCl_2$, 1 mM EDTA, and 2% glycerol]. Cells were incubated in lysis buffer for 30 minutes at 4°C then cleared by high-speed centrifugation. Soluble lysates were incubated for 60 minutes with 30 μL of protein A/G beads with corresponding antibody (to protein being immunoprecipitated). Beads were washed 3x with lysis buffer then resuspended in 60 μL 2x sample buffer, and boiled for 10 minutes

Co-culturing Experiments: HEK-293 cells were transfected with desired plasmids and incubated at 37°C (called "base cells"). After 24 hours, different plates of HEK-293 cells (transfected depending on experiment) were resuspended using 0.25% Trypsin and complete media and plated on top of "base cells" and incubated at 37°C. After 24 hours, cells were lysed and used for co-immunoprecipitation or Rho activation experiments.

Cell Surface Luminometry: HEK293 cells were transfected with pcDNA3.1, Flag-GPR56 or Flag-NT and incubated at 37°C for 24 hours. After 24 hours, each HEK293 plate was split into triplicate 35 mm dishes and incubated at 37°C for 12 hours. After 12 hours, cells were washed and fixed with 4% paraformaldehyde (PFA). Fixed cells were then blocked with 2% dry non-fat milk in PBS for 30 minutes. Cells were then incubated for 2 hours at room temperature with Flag-horseradish peroxidase-linked (HRP) primary antibody (1:1000). After washing three times (2 ml each wash), luminescence was measured using a TD 20/20 luminometer (Turner Designs).

Cell Migration Assay: U118-mg cells were seeded in the top chamber of the Boyden Chamber Migration Assay (Millipore) in serum free DMEM. The lower chamber was filled with complete DMEM. Cells were treated with HEK cell membranes transfected with pcDNA3.1 or Flag-NT. These membranes were prepared by scraping plate of cells

into PBS. The harvested cells were then briefly sonicated, spun down for 1 minute at 3000 rpm, measured, and resuspended in 500 uL PBS. After treatment, cells were allowed to incubate and migrate overnight in normal culture conditions

Statistical Analysis: All statistical analysis was performed on single comparisons with student's t-test using GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

3.3 Results

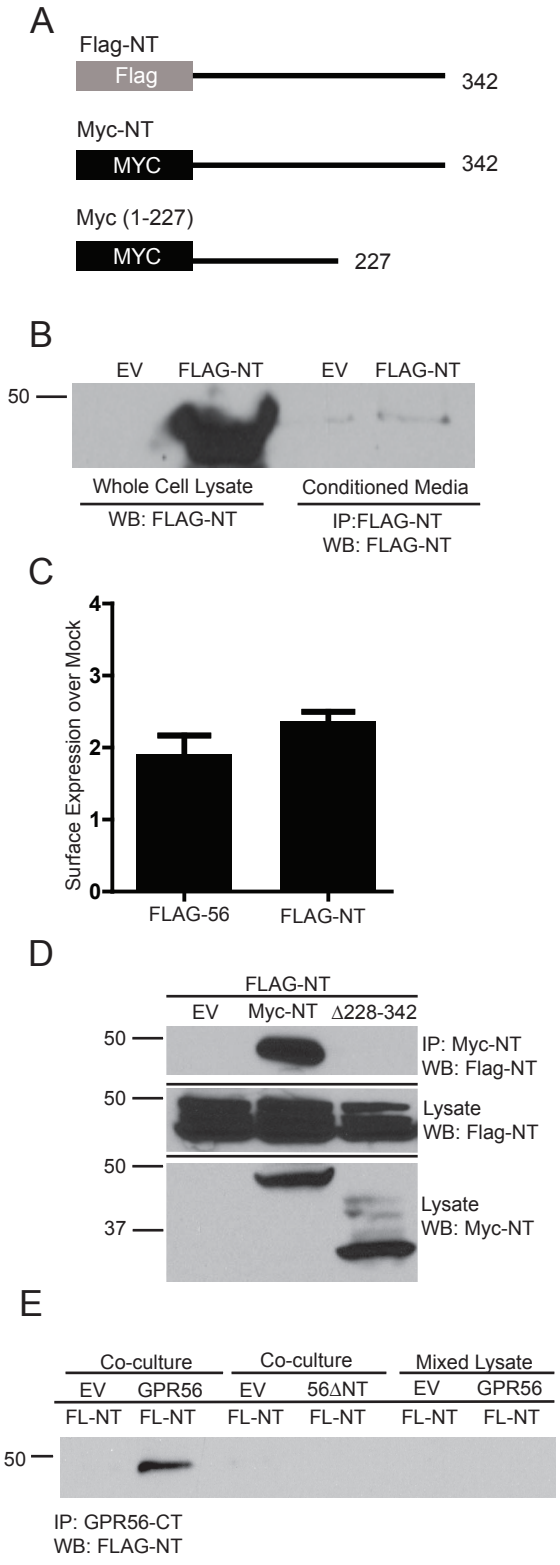
3.3.1 The N-termini of GPR56 are capable of homophilic trans-trans interactions

If it is true that the GPR56-NT associates with the receptor's 7TM region to constrain receptor activity, then GPR56-NT binding partners might conceivably alter the conformation of the GPR56-NT to alleviate this inhibitory influence on receptor signaling. In this regard, it is of interest to reiterate that the adhesion GPCRs Celsr2 and Celsr3 have been reported to undergo homophilic N-terminal interactions in a trans-trans fashion (e.g., interactions of the same receptor type on adjacent cells), with these interactions strongly influencing receptor activity (Shima et al., 2007). It is not known if such trans-trans interactions represent a general mechanism for controlling the activity of adhesion GPCRs or if instead this mechanism is unique to Celsr2/3. Since our above-described data in chapter 2 revealed the importance of the GPR56 N-terminus in controlling receptor activity, we examined whether GPR56 might be capable of trans-trans interactions via its N-terminal domain via a co-culture approach.

A Flag-tagged GPR56-NT construct (lacking the seven-transmembrane and intracellular domains) was created and transfected into HEK293 cells. A similar GPR56NT construct was also created containing a Myc tag in place of the Flag-tag (Fig III-1A). Both Flag- and Myc-GPR56-NT expressed well in cells with only minimal amounts of the expressed GPR56-NT protein secreted into the medium (Fig III-1B). Moreover, cell surface luminometry experiments showed that the secreted GPR56-NT was mainly found associated with the extracellular face of the plasma membrane, explaining its absence from the media (Fig III-1C). The GPR56-NT may be tethered to

Figure III-1. GPR56 N-termini can engage in homophilic N-terminal interactions. To determine if GPR56 N-termini can interact, two differentially tagged constructs were made that represent the full-length NT. (A) Schematic diagram representing these constructs is shown, including a Myc-tagged truncated NT missing the last 114 amino acids. (B) HEK293 cells were transfected with the GPR56 Flag-NT construct and incubated for 24 hours. The next day, the conditioned medium was collected and immunoprecipitation was performed with anti-Flag antibodies in order to concentrate any Flag-NT in the medium (last 2 lanes). Also, a whole cell lysate sample from the transfected cells was prepared (first two lanes). Western blot analysis revealed that very little of the Flag-GPR56-NT fragment was secreted into the medium, but rather the vast majority of the Flag-NT remained associated with the cells. (C) Surface luminometer assays revealed that Flag-GPR56 wild-type and Flag-GPR56-NT are expressed at relatively equal levels (per microgram of plasmid transfected) on the surface of transfected HEK293 cells. (D) Co-immunoprecipitation between Flag-GPR56-NT and Myc-GPR56-NT. HEK293 cells were separately transfected with Flag-NT, empty vector, Myc-NT, or Myc-NTΔ228-342. After 24 hr, Flag-NT transfected cells were co-cultured with empty vector (Lane 1), Myc-NT (Lane 2), or Myc-NTΔ228-342 (Lane 3) transfected cells. The next day, cells were lysed and immunoprecipitation was performed with Protein A/G beads coupled to c-myc antibody, followed by Western blotting for Flag-NT. (E) HEK293 cells transfected with Flag-NT (FL-NT) were co-cultured with HEK293 cells transfected with empty vector (Lanes 1 and 3), GPR56 wild-type (Lane 2), or GPR56ΔNT mutant (Lane 4). After co-culturing for 24 hr, immunoprecipitation was performed with Protein A/G beads coupled to GPR56-CT antibody, followed by Western

blotting to visualize Flag-NT. In parallel experiments, Flag-NT-expressing cells were grown separately, harvested and then mixed with lysates from cells transfected with empty vector (Lane 5) or GPR56 wild-type (Lane 6) prior to immunoprecipitation with anti-GPR56-CT antibodies. The data shown are representative of 3-5 independent experiments for the various conditions


A
Flag-NT
Flag
342
Myc-NT
MYC
342
Myc (1-227)
MYC
227
B
EV
FLAG-NT
EV
FLAG-NT
50
Whole Cell Lysate
WB: FLAG-NT
Conditioned Media
IP:FLAG-NT
WB: FLAG-NT
C
Surface Expression over Mock
4
3
2
1
0
FLAG-56
FLAG-NT
D
FLAG-NT
EV
Myc-NT
Δ228-342
50
IP: Myc-NT
WB: Flag-NT
50
Lysate
WB: Flag-NT
50
37
Lysate
WB: Myc-NT
E
Co-culture
Co-culture
Mixed Lysate
EV
GPR56
EV
56ΔNT
EV
GPR56
FL-NT
FL-NT
FL-NT
FL-NT
FL-NT
FL-NT
50
IP: GPR56-CT
WB: FLAG-NT

the cell membrane through interaction with known GPR56-binding partners such as the matricellular protein transglutaminase 2 and/or the transmembrane proteins CD9 and CD81 (Little et al., 2004; Xu et al., 2006). To investigate GPR56 N-terminal interactions, the two differentially-tagged GPR56-NT constructs were separately transfected into HEK293 cells, which were co-cultured for 24 hr before co-immunoprecipitation experiments were performed. As shown in Figure III-1D, the two GPR56-NT proteins were found to robustly interact with each other, even though they were expressed in different sets of cells. Interestingly, removal of the C-terminal third of the Myc-GPR56-NT (Fig III-1A) (114 amino acids removed) resulted in complete abrogation of the interaction with Flag-GPR56-NT, thereby mapping the essential region for GPR56 NT-NT association as between amino acids 228 and 342 (Fig III-1D, Lane 3).

Since we found that the N-terminus of GPR56 could interact with other GPR56 N-termini, we further examined whether such NT-NT interactions might occur in the context of the full-length receptor. Cells expressing Flag-GPR56-NT were co-cultured with cells expressing untagged full-length GPR56, and potential GPR56 trans-trans interactions were examined via a co-immunoprecipitation approach. As shown in Figure III-1E, immunoprecipitation of the full-length GPR56 (from the "base cells" in this experiment) using our GPR56-CT antibody resulted in robust co-immunoprecipitation of the Flag-GPR56-NT from the co-cultured cells. Interestingly, no co-immunoprecipitation was observed between the GPR56 ΔNT mutant and Flag-GPR56-NT upon co-culturing of cells transfected with these two constructs (Fig III-1E, Lane 4), revealing the importance of the N-terminal domain for GPR56 trans-trans interactions. Additionally, no co-immunoprecipitation was observed when GPR56 and Flag-GPR56-NT transfected

cells were cultured separately and then solubilized and mixed together (Fig III-1E, last two lanes), demonstrating that the NT-NT interactions are not artifacts of post-solubilization aggregation. These data reveal that GPR56 is capable of trans-trans interactions via its N-terminal domain.

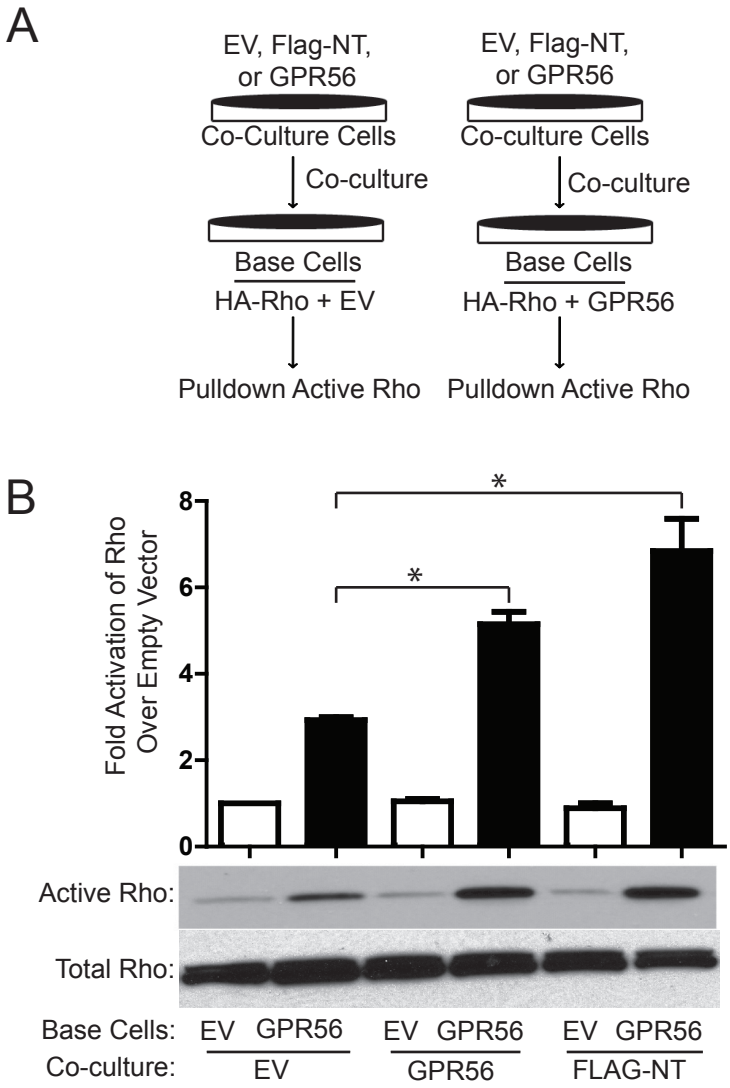
3.3.2 GPR56 trans-trans N-terminal interactions enhance receptor signaling.

We next examined whether the GPR56 trans-trans interactions that we observed in the co-culturing experiments might influence GPR56-mediated Rho activation. HEK293 cells transfected with either empty vector, full-length GPR56 or Flag-GPR56-NT were co-cultured with base cells transfected with either HA-Rho alone or HA-Rho/full-length GPR56, and the activation state of HA-Rho in the base cells was assessed (Fig III-2A). Co-culturing with any of the differentially-transfected cells had no effect on the activity of HA-Rho in base cells transfected with HA-Rho alone. However, in base cells transfected with HA-Rho/full-length GPR56, co-culturing with cells expressing GPR56 or Flag-GPR56-NT resulted in a significant potentiation in GPR56-mediated HA-Rho activation in the base cells (Fig III-2B). These data provide evidence that GPR56 trans-trans N-terminal interactions can enhance GPR56-mediated Rho signaling.

3.3.3 A region between amino acids 258 – 288 is necessary to mediate interactions between GPR56 N-termini.

We have shown that the N-termini of GPR56 can undergo homophilic trans-interactions, and that these interactions positively regulate GPR56-dependent Rho activation. Moreover, we originally mapped the last 114 amino acids of the N-terminus as the region mediating binding. Although NT-NT interactions may only facilitate GPR56

Figure III-2. GPR56 NT-NT interactions enhance GPR56-mediated signaling. (A) Schematic drawing of co-culturing technique. HEK293 cells were transfected with HA-RhoA plus empty vector (EV) or GPR56 wild-type. These "base cells" were then co-cultured for 24 hr with cells expressing EV, GPR56, or Flag-NT. The activity of HA-Rho in the base cells was then measured and expressed as fold over EV/EV cells. (B) Quantification of active Rho in co-culturing experiments. Also included is Western blot analysis of active and total Rho. A significant difference in GPR56-mediated RhoA activation was seen for "base cells" co-cultured with cells expressing GPR56 (*, $p<0.05$ n=3) and Flag-NT (*, $p<0.04$, n=3).

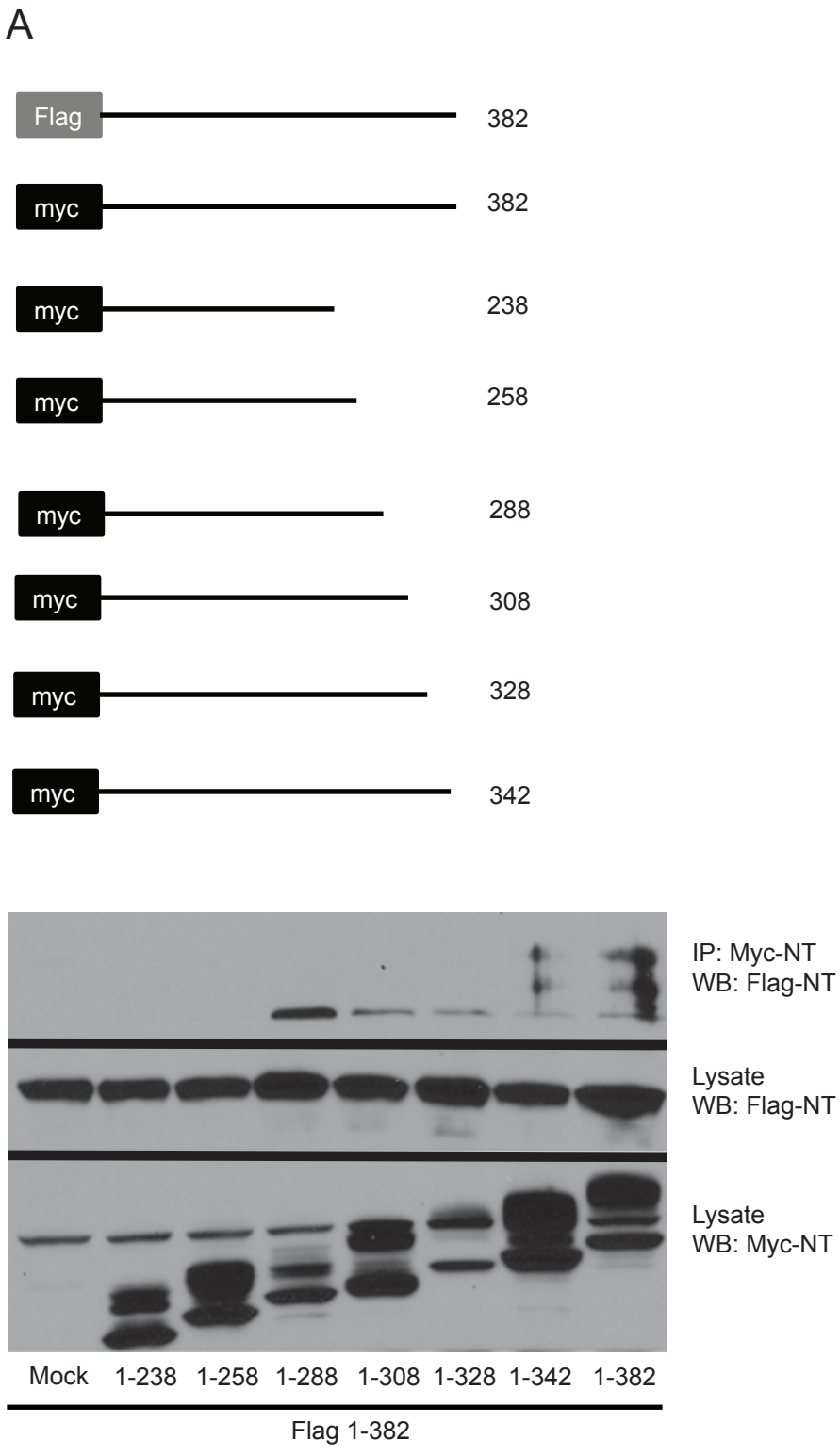

A
EV, Flag-NT, or GPR56
Co-Culture Cells
Co-culture
Base Cells
HA-Rho + EV
Pulldown Active Rho
EV, Flag-NT, or GPR56
Co-culture Cells
Co-culture
Base Cells
HA-Rho + GPR56
Pulldown Active Rho
B
Fold Activation of Rho Over Empty Vector
0
2
4
6
8
*
*
Active Rho:
Total Rho:
Base Cells: EV GPR56 EV GPR56 EV GPR56
Co-culture: EV GPR56 FLAG-NT

activation through allosteric modulation, locating the exact region of the N-terminus that is necessary for these interactions could potentially lead to the development of small peptides that could be utilized to regulate receptor activity. Therefore, we created sequential truncations of the GPR56 N-terminus (Fig III-3A) and performed co-immunoprecipitation experiments to determine when NT-NT binding ceased. Transfection of both the Flag-NT (Fig III-3C) and Myc-NT truncations was successful (Fig III-3D). As Figure III-3B (last lane) shows, there was robust co-immunoprecipitation between the two differentially tagged GPR56 N-termini, as has been illustrated in section 3.3.1. However, co-immunoprecipitation between the full length Flag-N-terminus and the myc-tagged truncations ceased between truncation 1-258 and 1-288. This indicates that NT-NT binding is mediated through this region of the GPR56 N-terminus. As a result, we have more finally mapped the region necessary for GPR56 homophilic trans-interactions and can use this information to create peptides capable of modulating receptor activity by altering or mimicking NT-NT interactions.

3.3.4 GPR56 N-terminal trans-interactions functionally inhibit glioma cell migration

Activation of GPR56-dependent Rho pathways have been shown to inhibit neuronal progenitor cell migration both in vitro (Iguchi et al., 2008) through activating antibody treatment, and in vivo (Li et al., 2008) through GPR56 gene knockout. We wanted to determine whether the GPR56 N-terminal trans- interactions that we have shown can positively regulate GPR56-dependent Rho activation could also affect cellular migration. To measure cellular migration, using the Boyden chamber assay, we transfected HEK293 cells with myc-tagged GPR56 NT, than harvested the cells 48 hrs post transfection into a 50 mM NaCl buffered solution. These membranes were then

Figure III-3. A region between amino acids 258 – 288 is necessary to mediate interactions between GPR56 N-termini. To further map the region necessary to mediate GPR56 NT-NT interactions, another series of sequential N-terminal truncations were created. (A) A schematic diagram of the truncations is shown. Numbers indicate the terminal amino acid of each construct. All truncations were Myc-tagged to differentiate from the full-length Flag-NT. (B) Co-immunoprecipitation between Flag-GPR56-NT and the Myc-GPR56-NT truncations. HEK293 cells were separately transfected with full-length Flag-NT and either empty vector or one of the truncated Myc-NT mutants. After 24 hr, Flag-NT-transfected cells were co-cultured with cells transfected with either empty vector (Lane 1), Myc-NT 1-238 (Lane 2), Myc-NT 1-258 (Lane 3), Myc-NT 1-288 (Lane 4), Myc-NT 1-308 (Lane 5), Myc-NT 1-328 (Lane 6), Myc-NT 1-342 (Lane 7), or Myc-NT 1-382 (Lane 8). The next day, cells were lysed and immunoprecipitation was performed with Protein A/G beads coupled to anti-Myc antibodies, followed by Western blotting for Flag-NT to detect NT-NT associations.

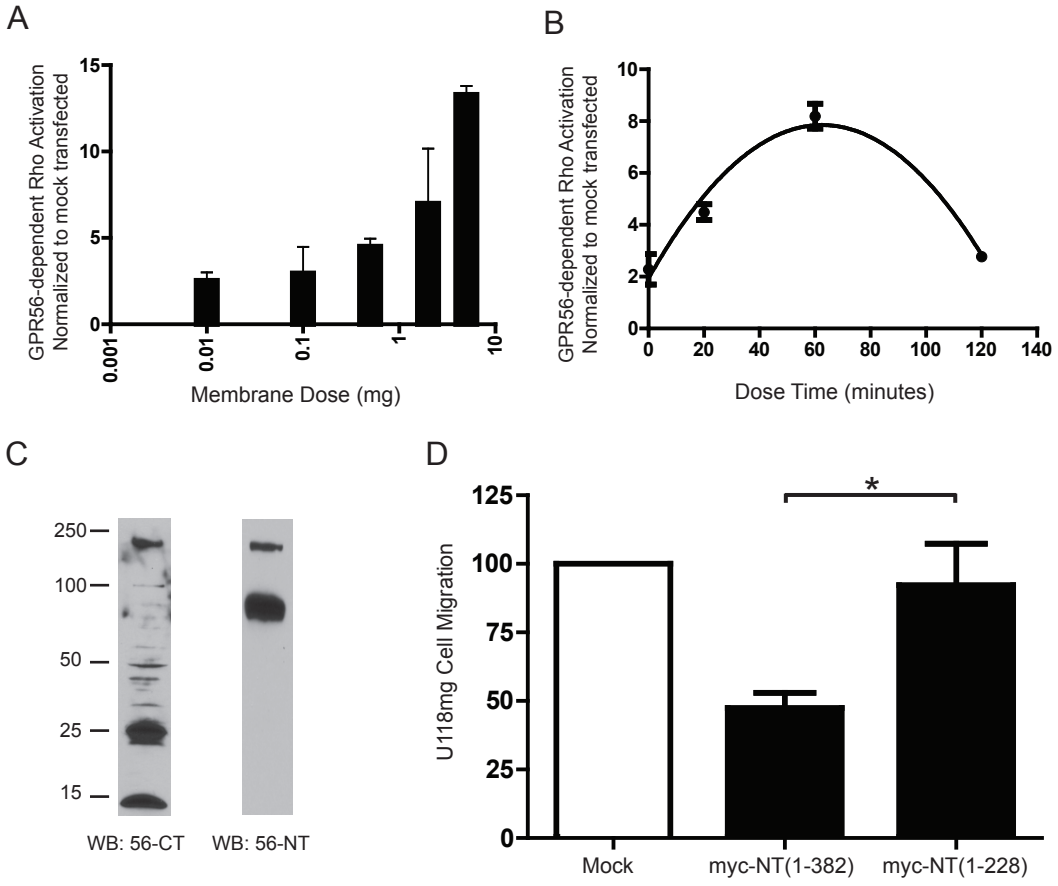

A
Flag
382
myc
382
myc
238
myc
258
myc
288
myc
308
myc
328
myc
342
B
IP: Myc-NT
WB: Flag-NT
C
Lysate
WB: Flag-NT
D
Lysate
WB: Myc-NT
Mock
1-238
1-258
1-288
1-308
1-328
1-342
1-382
Flag 1-382

sonicated briefly and used to treat HEK293 cells transfected with full-length GPR56. Comparable to our co-culture signaling assays, treatment of GPR56-expressing HEK293 cells with the Myc-GPR56-NT expressing membrane preps caused receptor Rho activation up to 12 fold over mock transfected in both a dose (Fig III-4A) and time (Fig III-4B) dependent manner.

Since HEK293 cells do not express significant levels of endogenous GPR56, we probed a variety of human glioblastoma cell lines for their GPR56 expression profiles. GPR56 has been widely associated with cancer progression (Xu, 2010; Xu et al., 2010; Yang and Xu, 2012) and also exhibits increased expression in human glioblastoma (Shashidhar et al., 2005). Thus, we explored glioblastoma cells as potential model system to look at functional effects of endogenous GPR56 activation. Figure III-4C shows that we detected substantial GPR56 expression in the human glioblastoma cell line U118-mg.

We examined the ability of GPR56 N-terminal trans-interactions to regulate glioma cell migration with the Boyden chamber assay, Briefly, U118mg cells were seeded into the top chamber at 1.0×10^6 cells per well in serum free media. Concurrently, HEK293 cell membrane preps were added exogenously. These preps were generated by transfecting HEK293 cells with mock vector, myc-NT (1-382) or myc-NT (1-228), and then suspended in buffered saline solution and briefly sonicated. After addition, cells were allowed to incubate overnight. The following day, migrated cells were counted using the Millipore calorimetric migration assay kit. The results were normalized to cells treated with mock transfected membrane preps. Figure III-4D (middle lane) shows that indeed, treatment of U118mg glioma cells with membranes expressing the full length GPR56 N-terminus showed a significant inhibition of migration of approximately 50%.

Figure III-4. GPR56 N-terminal trans-interactions functionally inhibit glioma cell migration. HEK293 cells were transfected with HA-Rho and either empty vector or GPR56 wild type. After 24 hr, cells were treated with lysed membranes derived from cells that had been transfected with Myc-NT. Treated cells were then harvested, lysed and assayed for active Rho using the GST-RBD pull down method. (A) Quantification of the dose-dependent effect of membrane treatment on Rho activation in the U118 glioma cells. (B) Quantification of the time course of membrane treatment on Rho activation in the U118 glioma cells. (C) Western blot analysis of U118-mg cell lysates probed with the GPR56-CT antibody (left lane) and GPR56-NT antibody (right lane). (D) GPR56 NT-NT trans-interactions inhibit glioma cell migration. U118-mg cells were seeded in serum free media in the top chamber of a Boyden Chamber and allowed to migrate overnight. Lane 1 shows the migration of U118 glioma cells treated with membranes derived from mock-transfected HEK293 cells. Lane 2 shows the reduced migration of U118 cells treated with membranes derived from HEK293 cells transfected with Myc-NT. Lane 3 shows migration of U118 cells treated with membranes derived from HEK293 transfected with Myc-NT (1-228). Statistical analyses were performed using unpaired t-tests. (n=3; *, $p<0.005$).


A
GPR56-dependent Rho Activation
Normalized to mock transfected
15
10
5
0
0.001
0.01
0.1
1
10
Membrane Dose (mg)
B
GPR56-dependent Rho Activation
Normalized to mock transfected
10
8
6
4
2
0
0
20
40
60
80
100
120
140
Dose Time (minutes)
C
250
100
50
25
15
WB: 56-CT
WB: 56-NT
D
U118mg Cell Migration
125
100
75
50
25
0
*
Mock
myc-NT(1-382)
myc-NT(1-228)

These data are consistent with the fact that activated GPR56 *in vitro* and *in vivo* controls cellular migration (Iguchi et al., 2008; Li et al., 2008), although this is the first report of this phenomenon in glioma cells. Interestingly, the migration of cells treated with membranes expressing myc-NT (1-228) (Fig III-4D last lane) was not inhibited. This is important because myc-NT (1-228) lacks the necessary region that mediates GPR56 trans-interactions, indicating that these interactions regulate migration, presumably through their ability to activate receptor signaling.

3.4 Discussion

The most striking diversity between different receptors in the GPCR superfamily is probably the sites and modes of ligand binding (Ji et al., 1998). Some GPCR ligands bind exclusively to the N-terminus (large glycoproteins), to both the exoloops and N-terminus (small polypeptides), to the 7TM core and exoloops (small peptides) and to solely the core (photons, amines, etc.) (Ji et al., 1998). Nonetheless, though the modes of ligand binding are diverse, the majority of GPCRs are activated by three conserved steps: signal generation, transmembrane signal transduction, and signal transfer to cytoplasmic signal molecules (Ji et al., 1995), where signal generation is typically considered indistinguishable from ligand binding. Signal generation by the Celsr receptors has been suggested to involve homophilic trans-interaction between receptor N-termini on adjacent cells (Shima et al., 2007). Therefore, we examined whether if GPR56 might also be capable of a similar process of signal generation.

We found that GPR56 signaling can be activated by homophilic trans-interactions between GPR56 N-termini. Moreover, we mapped the region necessary for GPR56 N-terminal interaction to a segment between amino acid 258 and 288. Finally, we demonstrated an inhibition of cellular migration due to GPR56-dependent Rho activation mediated by NT-NT interactions. It remains to be determined whether these GPR56/GPR56 associations truly activate the receptor or simply make the receptor permissive for signaling (for example, by creating a binding site for an as-yet-unidentified ligand). It also remains to be determined whether NT/NT interactions are a common feature for other members of the adhesion GPCR family beyond Celsr2/3 and GPR56.

Although this proposed mode of signal generation is unusual amongst GPCRs, trans-interactions initiating signaling events are not uncommon outside of the GPCR superfamily. For example, neural recognition molecules undergo homophilic interactions to relay extracellular spatial and contact information to the cell. Specifically, it has been shown that Neural Cell Adhesion Molecule (NCAM) and L1 activate a variety of signaling pathways, including G protein dependent channel activation, through homophilic trans-interactions (Maness and Schachner, 2007). This signal activation has been linked to regulation of neuronal spine growth and migration (Beggs et al., 1994). Similarly, cadherins are a superfamily of adhesion receptors that undergo homophilic interactions to signal through β-catenin to regulate many biological processes (Buckley et al., 1998). Interestingly, many of the adhesion domains present on the N-termini of adhesion GPCRs are shared by other adhesion proteins that are known to undergo homophilic trans-interactions as a mechanism of activation. For example, the Celsr isoforms exhibit both EGF and cadherin repeat domains (Bjarnadottir et al., 2007), two domains that are also found in the adhesion proteins described above (NCAMs and cadherins). Therefore, since the adhesion GPCRs are composed of a traditional GPCR 7TM domain attached to N-terminal domains that are analogous to adhesion proteins known to undergo homophilic trans-interactions, it is not too much of a stretch to envision that homophilic trans-interactions may be a conserved mechanism of activation within the adhesion GPCR family.

CHAPTER IV: Protein-Protein Interactions Regulate GPR56 Biology

4.1 Introduction

GPCRs can interact with many different types of proteins beyond just G proteins. There are at least four distinct roles that GPCR-associated proteins can play: *a)* directly mediating signaling, similar to G proteins, *b)* controlling receptor localization or trafficking, *c)* acting as an allosteric modulator to alter receptor pharmacology or receptor function, and *d)* serving as a scaffold to physically link receptors to downstream effectors (Hall and Lefkowitz, 2002). The proceeding chapters have presented a model in which the adhesion G protein-coupled receptor GPR56 signals through $G\alpha_{12/13}$ to activate Rho and β-catenin. Moreover, a novel mechanism of action where the N-termini of adjacent GPR56 can interact to positively regulate receptor activation was suggested in which the N-termini of adjacent GPR56 can interact to positively regulate receptor activation through removal of the antagonistic N-terminus. In further studies, described in this chapter, my colleagues and I assessed the ability of GPR56-interacting proteins to regulate receptor trafficking, signaling, and localization, with the goal of shedding further light on the fundamental biology of GPR56 and adhesion GPCRs in general.

We first determined if GPR56 might interact with PDZ-domain containing scaffold proteins, as such proteins have been shown to regulate a variety of GPCRs in many different manners (Ritter and Hall, 2009). PDZ domains are conserved module of about 80-90 amino acids that were named after the first three proteins in which the domain was discovered: the post-synaptic density protein of 95 kDa (PSD95), Drosophila discs large (Dlg), and zonula occludens-1 (zo-1)(Kennedy, 1995). PDZ domains bind to the C-terminus of specific proteins to regulate a variety of functions. For example, the β2-adrenergic receptor can bind to the PDZ domain containing protein NHERF to regulate

Na^+/H^+ exchange in an agonist dependent manner, independent of G proteins (Hall et al., 1998). Moreover, NHERF proteins have also been shown to interact with a variety of GPCRs besides the β2-adrenergic receptor, including the kappa-opioid and P2Y1 purinergic receptor, as well as tyrosine kinase receptors to regulate receptor trafficking and activity (Weinman et al., 2006). Moreover, PDZ containing proteins can bind to receptors and tether these receptors to downstream effectors, therefore creating multi-protein complexes to expedite signaling activity (Ranganathan and Ross, 1997). Interaction with PDZ scaffold proteins can have pronounced effects on a diverse variety of receptor functions. Therefore, we examined whether GPR56 could interact with PDZ proteins and, if so, what effects these interactions might have.

As mentioned previously, GPR56 has been shown to have multiple binding partners, particularly at the N-terminus. These include tetraspanins CD9 and CD81 (Little et al., 2004) and transglutaminase 2 (Xu et al., 2006). Recently, collagen type-III has been discovered as another possible ligand for GPR56; specifically, collagen III was shown to bind to the receptor N-terminus and activate receptor dependent Rho activity. (Luo et al., 2011). Moreover, knockout of collagen type-III in mice exhibit a brain development phenotype that resembles that of Gpr56 knockout, suggesting a connection between collagen and GPR56 function (Jeong et al., 2012). We sought to connect these findings to our data showing that the N-termini of GPR56 exhibit trans-interactions that enhance receptor signaling.

The subcellular localization of GPCRs is an extremely important determinant of receptor functional activity. During the early stages of our work on GPR56, it was reported by another group that disruption of cilia function via a hypomorphic allele for

the gene encoding the cilial protein Ift88 induces a cobblestone-like malformation of the cerebral cortex that is strikingly similar to the cobblestone-like cortical malformation exhibited by GPR56 knockout mice (Willaredt et al., 2008). Moreover, an essential role for primary cilia is the regulation of neural precursor cells migration was also first reported around that same time (Breunig et al., 2008). Additionally, two other adhesion GPCR family members, HE6 (Kirchhoff et al., 2008) and VLGR1 (Yagi et al., 2007), which are two of the closest relatives of GPR56 (Fredriksson et al., 2003), were also shown at that time to traffic to the cilia. Therefore, using confocal microscopy, we assessed the localization of GPR56 in ciliated cells.

4.2 Experimental Procedure

Cell Culture: For all cell based assays, HEK293 and NIH3T3 cells (ATTC) were cultured and maintained in DMEM containing 10% FBS and 1% penicillin/streptomycin at 37 °C with 5% CO_2. IMCD-3 cells (ATTC) were cultured and maintained in F10:DMEM containing 10% FBS and 1% penicillin/streptomycin at 37 °C with 5% CO_2. Transfections were performed by incubating cells with Lipofectamine 2000 (Invitrogen) and cDNA for 4 hours in serum free DMEM, then stopping transfection with complete media. Experiments were performed 24-48 hours post transfection.

Antibodies: Antibodies against HA (Roche), FLAG (Sigma-Aldrich), β-arrestin2 (Sigma-Aldrich), c-myc (Sigma-Aldrich), acetylated tubulin (Sigma Aldrich), HIS (Santa Cruz) and biotinylated GPR56 N-terminus (R & D Systems) were purchased from manufacturer. The anti-GPR56 C-terminal antibody was developed by Orbigen via injection of rabbits with a peptide (CSNSDSARLPISSGSTSSSRI) derived from the GPR56 C-terminus, followed by affinity purification using the same peptide that was used as the immunogen.

Plasmids: N-terminal Flag-GPR56 was a gift from Christopher Stipp (University of Iowa). Untagged human GPR56 wild-type was subcloned into pcDNA 3.1. The Flag-tagged ΔNT mutant was cloned into pcDNA 3.1 by creating primers starting with an N-terminal Flag epitope followed by the human GPR56 sequence starting at amino acid 343. Similarly, an untagged GPR56ΔNT mutant was made by creating primers using the human GPR56 sequence starting at amino acid 343. Flag-NT GPR56 and c-myc-NT GPR56 constructs were created in the pCMV 2B plasmid and correspond to human

GPR56 amino acids 1-342. The HA-Ubiquitin construct was a gift from Keqiang Ye (Emory University) and the GFP-β-arrestin2 construct was a gift from Jeffrey Benovic (Thomas Jefferson University). GST-RBD (Addgene), HA-β-arrestin2 (Addgene), and HA-Rho (Missouri S&T cDNA Resource Center) were all commercially obtained.

Western Blotting*:* Samples were resolved by SDS−PAGE on 4 to 20% Tris-glycine gels, followed by transfer to nitrocellulose membranes. The membranes were incubated in blocking buffer (2% nonfat dry milk, 50 mM NaCl, 20 mM HEPES, and 0.1% Tween 20) for 30 min and then incubated with primary antibody for 1 hour at room temperature. Next, the membranes were washed three times in blocking buffer and incubated with HRP-conjugated secondary antibody for 30 min, washed three times more, and finally visualized via ECL reagent followed by exposure to film. When using the biotinylated GPR56 N-terminal primary antibody, the ABC kit (Vectastain) was used to visualize immunoreactive bands in lieu of secondary antibody.

Rho Activation Assay: HEK293 cells were transfected with HA-Rho (Addgene) and either pcDNA 3.1, GPR56 wild-type, or GPR56ΔNT. After 24 hours, cells were scraped and resuspended in 500 μL Lysis Buffer [1% Triton X-100, 150 mM NaCl, 25 mM Hepes, 10 mM $MgCl_2$, 1 mM EDTA, and 2% glycerol]. Cells were incubated in lysis buffer for 30 minutes at 4°C then cleared by high-speed centrifugation. Soluble lysates were incubated for 30 minutes with 30 μL of GST-Rhotekin Binding Domain (GST-RBD) coupled to glutathione agarose beads. Beads were washed 2x with Lysis Buffer, resuspended in 60 μL 2x sample buffer, and boiled for 10 minutes. Total Rho levels were first determined through Western blotting of cell lysates with anti-HA antibody to

normalize levels before determining active Rho levels. Active Rho was visualized by standard Western blot procedure, probing the normalized GST-RBD samples for HA.

Co-immunoprecipitation: HEK293 cells were transfected with various constructs to be assessed for ability to co-immunoprecipitate. After 24 hours, cells were scraped and resuspended in 500 μL Lysis Buffer [1% Triton X-100, 150 mM NaCl, 25 mM Hepes, 10 mM $MgCl_2$, 1 mM EDTA, and 2% glycerol]. Cells were incubated in lysis buffer for 30 minutes at 4°C then cleared by high-speed centrifugation. Soluble lysates were incubated for 60 minutes with 30 μL of protein A/G beads with corresponding antibody (to protein being immunoprecipitated). Beads were washed 3x with lysis buffer then resuspended in 60 μL 2x sample buffer, and boiled for 10 minutes. Co-immunoprecipitation was detected by standard Western blot procedure. Rat kidney was substituted for transfected HEK-293 cells using same protocol. For ubiquitination studies, HEK293 cells were transfected with HA-Ubiquitin and either empty vector, GPR56 wild-type, or GPR56ΔNT mutant. Cells were lysed as described and immunoprecipitated with protein A/G beads coupled to HA-antibody. Western blot procedure was used to detect GPR56 using the GPR56-CT antibody.

Cell Surface Biotinylation: HEK293 cells were transfected with GPR56 wild-type and either HA-CAL or His-Magi 3 constructs. After 24 hours, cells were washed and incubated for 2 hr at 4°C in 2 mM Sulfo-NHS-LC-Biotin (Thermo Scientific) in PBS to biotinylate surface proteins. After biotinylation, cells were washed, scraped, and resuspended in lysis buffer [1% Triton X-100, 150 mM NaCl, 25 mM Hepes, 10 mM $MgCl_2$, 1 mM EDTA, and 2% glycerol]. Cells were incubated in lysis buffer for 30

minutes at 4°C with end-over-end agitation and then cleared by high-speed centrifugation. Soluble lysates were incubated with Streptavidin beads (Pierce) for 2 hours at 4°C to pull down surface biotinylated proteins. Beads were washed three times and resuspended in sample buffer. Surface expression of GPR56 was assessed by analyzing these samples via Western blotting.

Pull down Assay: GST-56-CT fusion proteins were made by making primers corresponding to last 25 amino acids of GPR56 C-terminus. PCR product was created and ligated into pGEX4 vector (GE). GST and GST-56-CT fusion proteins were purified on glutathione agarose beads. Specifically, a HEK293 cell plate was transfected with HA-CAL or His-MAGI-3 and incubated 24 hrs then lysed. The lysate was then split equally between two tubes containing equal amounts of either GST-agarose beads or GST-56-CT-agarose beads. The tubes and lysate were incubated at 4 degrees Celsius for 1 hr, than ran on SDS page. Western blot analysis with an HA or His antibody was performed to measure level of pull down.

PDZ Array: To assay for potential PDZ interactions 1 μg of His- and S-tagged PDZ domain fusion proteins were spotted onto 96 well nitrocellulose, dried overnight, and overlaid with GST-alone (control) or GST-56-CT. Membranes were washed and incubated with an HRP-coupled anti-GST monoclonal antibody (Amersham Pharmacia Biotech) and binding was visualized using enhanced chemiluminescence.

β-catenin Activation Assay: HEK293 cells were transfected with Top Flash TCF-reporter plasmid and either pcDNA 3.1, GPR56 wild-type, Flag-GPR56 or Flag-GPR56 truncations. Or other plasmids mentioned in the dissertation. Cells were incubated at

37°C for 24 hours. After 24 hours, cells were lysed with Promega Lysis Reagent (Promega), put on ice, and probed for luciferase activity using the Luciferase Assay System (Promega). For collagen III assays, we first transfected HEK293cells with GPR56 and Top Flash reporter, incubated 24 hrs, serum starved another 24 hrs and then treated the cells with 84 nM collagen type III as instructed.

Statistical Analysis: All statistical analysis was performed on single comparisons with student's t-test using GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

4.3 Results

4.3.1 GPR56 interacts with PDZ proteins MAGI-3 and CFTR-associated ligand

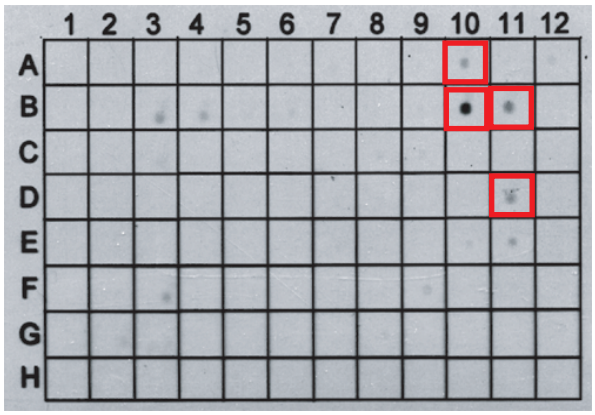
Since GPR56 contains a consensus Class I PDZ binding motif (S-S-R-I) (Songyang et al., 1997), and PDZ interactions can powerfully regulate GPCR function (Fam et al., 2005; Chen et al., 2006; Balasubramanian et al., 2007), we sought to examine the ability of GPR56 to bind PDZ domain-containing proteins. Thus, we created a GPR56-CT GST fusion protein and screened it against a proteomic array consisting of 96 distinct purified PDZ proteins (Fam et al., 2005; He et al., 2006). Figure IV-1A shows each purified PDZ protein and its corresponding position on the array. This proteomic screen revealed that the GPR56-CT interacted with multiple PDZ proteins; the strongest interactions are highlighted in red in Figure IV-1B. These PDZ domains were MAGI-3 PDZ1, CAL PDZ, nNOS PDZ, and Densin-180 PDZ. We chose to focus our studies on Magi-3 and CAL as they appeared to be the strongest interactions. To further confirm the interactions, we performed GST fusion protein pull down assays between our purified GPR56 C-terminus and HEK293 cell lysates transfected with either HA-tagged CAL PDZ or His-tagged-MAGI-3 PDZ proteins. As shown in Figure-IV-1C, robust pull-down was observed between our GPR56-CT fusion protein and HA-CAL, confirming the interaction seen on the array. Likewise, Figure-IV-1D illustrates the pull down that was observed between the GPR56 CT fusion protein and His-MAGI-3, also confirming the interaction seen on the array. However, the interaction between MAGI-3 and GPR56 appeared to be less robust than the interaction with CAL.

Figure IV-1. The C-terminus of GPR56 interacts with Magi-3 and CFTR-associated ligand (CAL). (A) The ability of the GPR56 C-terminus to interact with PDZ proteins was assessed using a proteomic array of 96 distinct PDZ proteins. (B) A GST fusion protein corresponding to the last 50 amino acids of the GPR56-CT was overlayed at 100 nM and shown to selectively bind to PDZ domains from Magi-3, nNOS, PDZ1, and CAL, as indicated in red. (C) HA-CAL transfected HEK293 cell lysates were incubated with GST or GST-56-CT fusion protein adsorbed to glutathione agarose beads. Pull-down of HA-CAL was assessed via Western blotting with anti-HA (D) His-Magi-3-transfected HEK293 cell lysates were incubated with GST or GST-GPR56-CT fusion protein. Pull-down of His-Magi-3 was assessed via Western blot with anti-His antibody. (E) Cell surface expression of GPR56 in the presence or absence of overexpressed CAL was assayed via surface biotinylation. Surface expression was normalized to total expression, as determined by Western blot. (F) Cell surface expression of GPR56 in the presence or absence of overexpressed Magi-3 was assayed via surface biotinylation. Surface expression was normalized to total expression, as determined by Western blot.

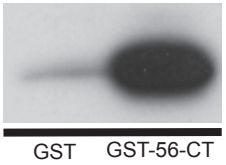
A

1. MAGI-1 PDZ1	25. INADL PDZ6	49. Rhophilin PDZ2	73. PDZK1 PDZ4
2. MAGI-1 PDZ2	26. SAP97 PDZ1+2	50. Harmonin PDZ1	74. PDZK2 PDZ1
3. MAGI-1 PDZ3	27. SAP97 PDZ3	51. Harmonin PDZ2	75. PDZK2 PDZ2
4. MAGI-1 PDZ4+5	28. SAP102 PDZ1+2	52. Neurabin PDZ	76. PDZK2 PDZ3
5. MAGI-2 PDZ1	29. SAP102 PDZ3	53. Spinophilin PDZ	77. PDZK2 PDZ4
6. MAGI-2 PDZ2	30. Chapsyn110 PDZ1+2	54. α1 syntrophin PDZ	78. LNX1 PDZ1
7. MAGI-2 PDZ3	31. Chapsyn110 PDZ3	55. β1 syntrophin PDZ	79. LNX1 PDZ2
8. MAGI-2 PDZ4	32. E6TP1 PDZ	56. β2 syntrophin PDZ	80. LNX1 PDZ3
9. MAGI-2 PDZ5	33. ERBIN PDZ	57. γ1 syntrophin PDZ	81. LNX1 PDZ4
10. MAGI-3 PDZ1	34. ZO-1 PDZ1	58. γ2 syntrophin PDZ	82. LNX2 PDZ1
11. MAGI-3 PDZ2	35. ZO-1 PDZ2	59. PAPIN PDZ1	83. LNX2 PDZ2
12. MAGI-3 PDZ3	36. ZO-1 PDZ3	60. MUPP1 PDZ1	84. LNX2 PDZ4
13. MAGI-3 PDZ4	37. ZO-2 PDZ1	61. MUPP1 PDZ6	85. PTPN4 PDZ
14. MAGI-3 PDZ5	38. ZO-2 PDZ2	62. MUPP1 PDZ7	86. RHO-GEF PDZ
15. NHERF-1 PDZ1	39. ZO-2 PDZ3	63. MUPP1 PDZ8	87. RA-GEF PDZ
16. NHERF-1 PDZ2	40. ZO-3 PDZ1	64. MUPP1 PDZ10	88. Enigma PDZ
17. NHERF-2 PDZ1	41. ZO-3 PDZ2	65. MUPP1 PDZ12	89. LARG PDZ
18. NHERF-2 PDZ2	42. ZO-3 PDZ3	66. MUPP1 PDZ13	90. MAST-205 PDZ
19. PSD-95 PDZ1+2	43. C2PA PDZ	67. PTPN13 PDZ1	91. PTPN3 PDZ
20. PSD-95 PDZ3	44. GIPC PDZ	68. PTPN13 PDZ3	92. Shank1 PDZ
21. PDZ-GEF-1 PDZ	45. MALS-1 PDZ	69. PTPN13 PDZ4+5	93. Tamalin PDZ
22. CAL PDZ	46. MALS-3 PDZ	70. PDZK1 PDZ1	94. PAR-3 PDZ1
23. nNOS PDZ	47. Densin-180 PDZ	71. PDZK1 PDZ2	95. PAR-3 PDZ2
24. INADL PDZ5	48. Rhophilin PDZ1	72. PDZK1 PDZ3	96. PAR-3 PDZ3

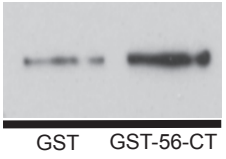
B



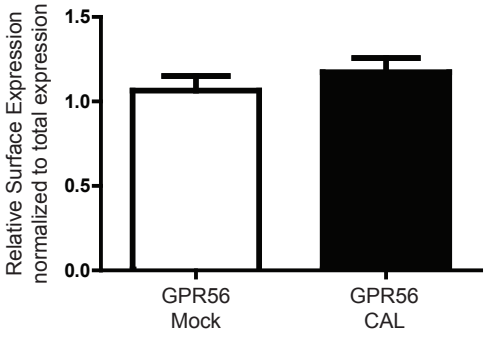
C



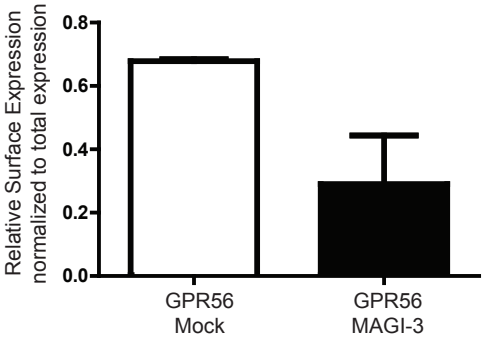
D



E



F



We next studied the effects of both CAL and MAGI-3 on GPR56 surface expression. CAL is predominantly found at the Golgi apparatus and has been shown to retain CFTR within the cell and decrease its surface expression and chloride currents (Cheng et al., 2002). Moreover, studies on the β1-adrenergic receptor also showed that associations with CAL negatively regulated receptor surface expression (He et al., 2004). Therefore, we explored whether CAL might inhibit GPR56 surface expression using a cell surface biotinylation approach. As seen in Figure IV-1E, CAL co-expression had no effect on GPR56 surface expression. In similar experiments, we looked at the effect of MAGI-3 on GPR56 surface expression. As shown in Figure IV-1F MAGI-3 co-expression resulted in a slight decrease in receptor surface expression, although the effect was not statistically significant. Thus, neither CAL nor MAGI-3 exerted a significant effect on the surface expression of GPR56

4.3.2 Collagen III antagonizes GPR56 signaling in transfected HEK293 cells

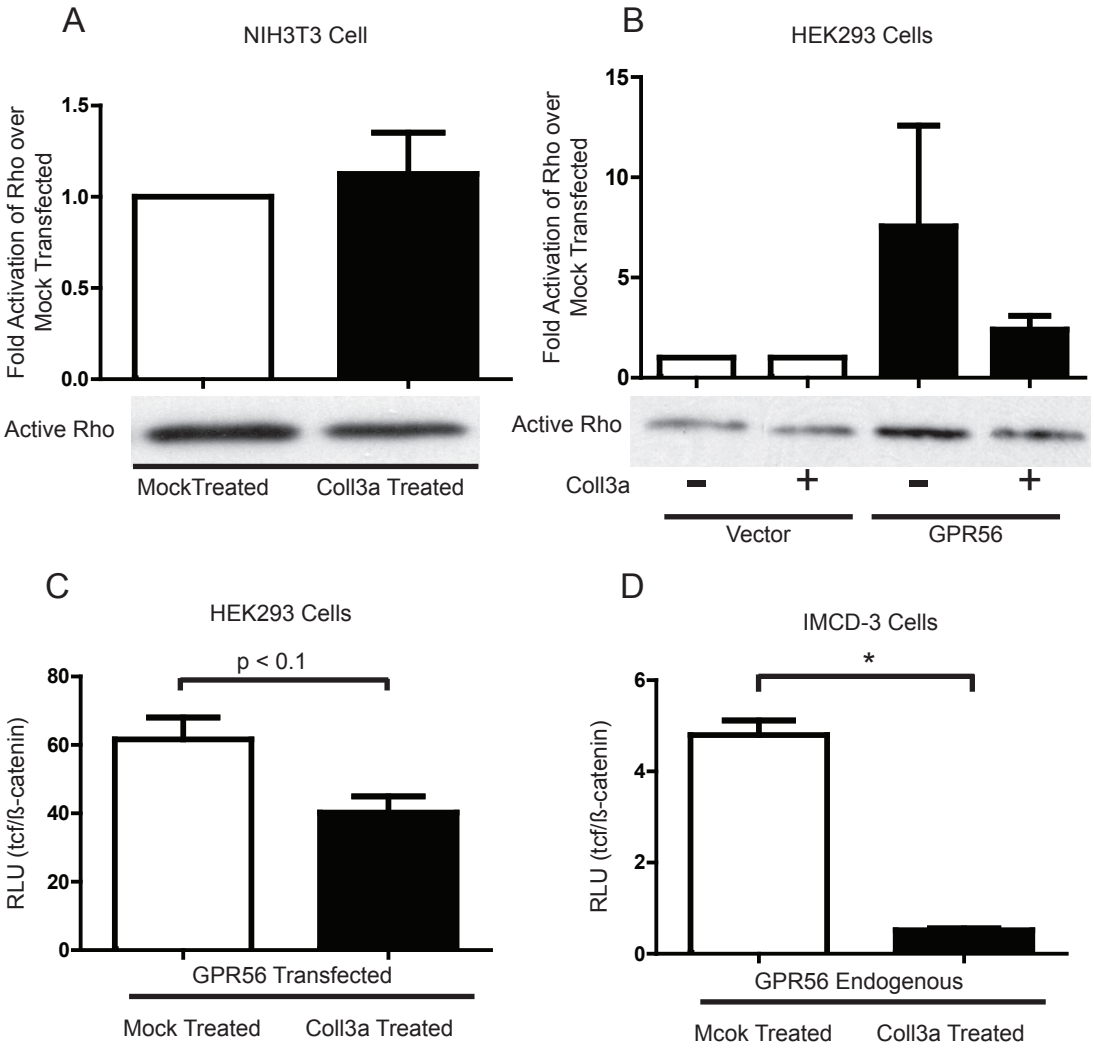
During the course of our studies on signaling by GPR56, Piao and colleagues reported that collagen type III binds to and activates GPR56 in NIH3T3 cells to inhibit cellular migration (Luo et al., 2011). This is an interesting finding because collagen III is known to associate with transglutaminase 2 (TG2), another reported GPR56-interacting partner (Xu et al., 2006), with the TG2/collagen interaction resulting in the crosslinking of collagen to the extracellular matrix during tissue repair (Chau et al., 2005). Therefore, we sought to determine if collagen type III might regulate GPR56 activation by facilitating NT-NT interactions, possibly in a manner involving TG2-dependent crosslinking. First, we attempted to replicate studies of Piao et al. by treating NIH3T3 cells with 84 nm collagen type III for 5 minutes per the published protocol. As seen in

Figure-IV-2A, no collagen-induced Rho activation was observed in these experiments. However, Western blot analysis revealed that the NIH3T3 cells used in these studies had extremely low GPR56 expression (data not shown), so therefore we attempted the same experiments in HEK293 cells transfected with GPR56. As shown in Figure-IV-2B, collagen III treatment of GPR56-transfected cells (last lane) resulted in a significant decrease in Rho activity, which was the opposite of the expected result. No effect of collagen III treatment was observed on mock transfected cells (lane 2). In complementary studies, we also assessed the effect of collagen III on GPR56 signaling in HEK293 cells using the tcf/β-catenin luciferase assay. Figure-IV-2C reveals that collagen III treatment inhibited GPR56 activation in the reporter gene assay, although the effect was not statistically significant. Finally, we performed similar experiments using IMCD-3 cells, which Western blot studies revealed to express high levels of endogenous GPR56 (data not shown). Using the same treatment protocol described above (84 nM collagen III for 5 minutes), observed that the collagen III treatment resulted in a significant decrease in β-catenin activation (Figure-IV-2D), although we do not know if this effect is via engagement of GPR56 or through some other effects of the exogenously-added collagen III.

4.3.3 GPR56 localizes to primary cilia & interacts with the cilial protein β-arrestin2

To assess the subcellular localization of GPR56, we examined IMCD-3 cells, a ciliated cell type that, as mentioned earlier, expresses significant levels of endogenous GPR56 (Fig-IV-3B). Interestingly, the immunostaining observed in these cells with the anti-GPR56 antibody appeared to be preferentially localized to discrete subcellular structures resembling cilia. To explore this further, we performed double-labeling studies

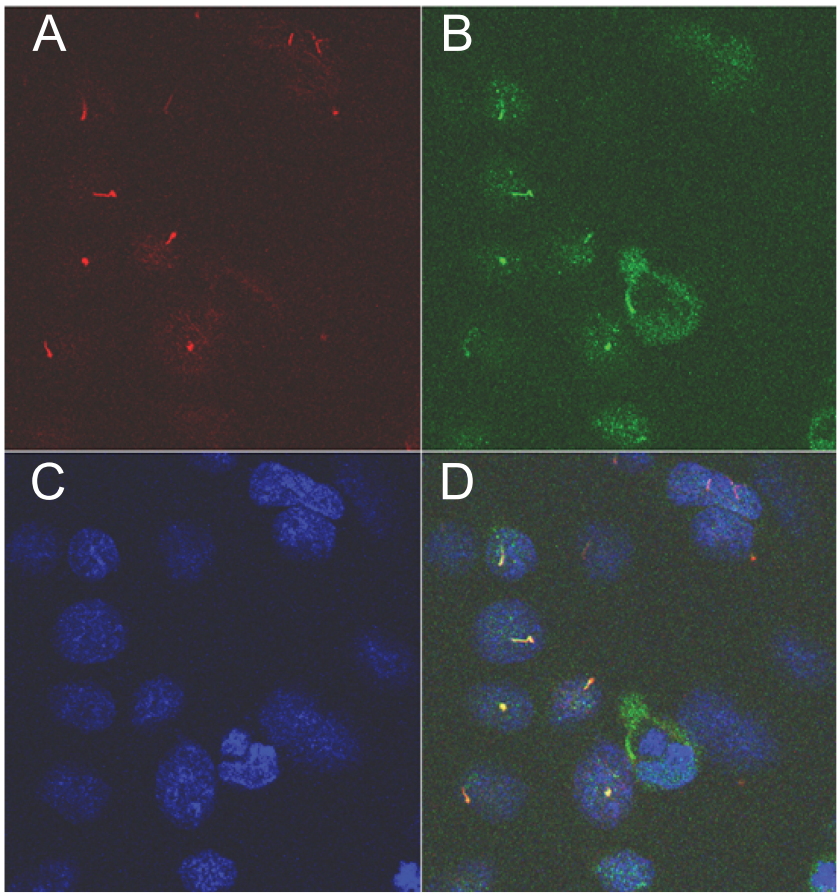
Figure IV-2. Collagen type III treatment has variable effects on Rho activation depending on cellular context. The ability of collagen type III to activate GPR56 was assayed using multiple cell types and two signaling readouts. (A) NIH3T3 cells were transfected with HA-Rho, incubated 24 hrs then serum starved another 24 hrs. Cells were treated with 10 mM acetic acid or 84 nM collagen type III for 5 minutes, lysed and assayed for active Rho using GST-RBD pull down. Activation was normalized to mock treated cells. (B) HEK293 cells were transfected with HA-Rho and either empty vector or GPR56 incubated 24 hrs then serum starved another 24 hrs. Cells were treated with 10 mM acetic acid (mock) or collagen type III at 84 nM for 5 minutes. Cells were lysed and assayed for active Rho using GST-RBD pull down. Active Rho in the treated samples was normalized to mock-transfected. (C) HEK293 cells were transfected with Top Flash reporter plasmid and GPR56. After 24 hr incubation followed by 24 hr serum starvation, cell were treated with 10 mM acetic acid (mock) or 84 nM collagen type III for 5 minutes and assayed for β-catenin activation using Promega luciferase assay (n=3; $p < 0.010$). (D) IMCD-3 cells were transfected with Top Flash reporter plasmid, incubated 24 hrs, and then serum starved another 24 hrs. Cell were treated with 10 mM acetic acid (mock) or 84 nM collagen type III for 5 minutes, then lysed and assayed for β-catenin activation using Promega luciferase assay (n=3; *, $p < 0.010$).

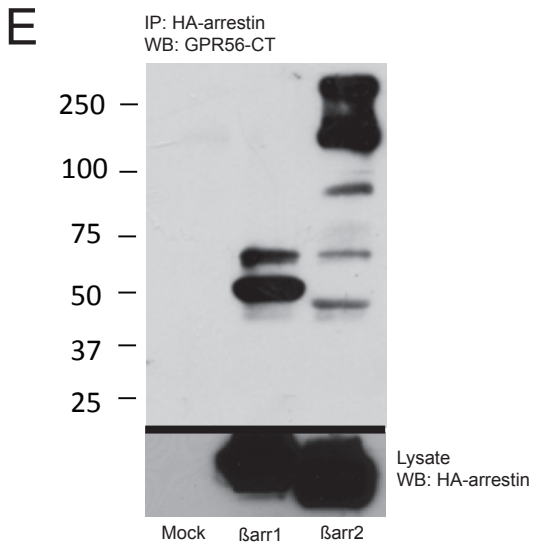

A
NIH3T3 Cell
Fold Activation of Rho over Mock Transfected
1.5
1.0
0.5
0.0
Active Rho
MockTreated
Coll3a Treated
B
HEK293 Cells
Fold Activation of Rho over Mock Transfected
15
10
5
0
Active Rho
Coll3a
-
+
-
+
Vector
GPR56
C
HEK293 Cells
p < 0.1
RLU (tcf/ß-catenin)
80
60
40
20
0
GPR56 Transfected
Mock Treated
Coll3a Treated
D
IMCD-3 Cells
*
RLU (tcf/ß-catenin)
6
4
2
0
GPR56 Endogenous
Mcok Treated
Coll3a Treated

in the IMCD-3 cells to assess co-localization of GPR56 with acetylated tubulin (Fig-IV-3A), a commonly-used marker for primary cilia. As shown in Figure-IV-3D we found substantial co-localization between GPR56 and acetylated tubulin in the IMCD-3 cells, indicating that GPR56 preferentially localizes to the cilia in these cells.

In addition to the aforementioned adhesion GPCRs that have been shown to localize to cilia (HE6 and VLGR1), a handful of other GPCRs have been shown to exhibit cilial localization, including the SSTR3 somatostatin receptor (Berbari et al., 2008), 5-HT6 serotonin receptor (Berbari et al., 2008), and Smoothened (Kovacs et al., 2008). Several of these receptors possess specific motifs, which are absent in GPR56, controlling the cilial trafficking of the receptors (Berbari et al., 2008). However, Smoothened has been shown to localize to cilia through interaction with β-arrestin2 (Kovacs et al., 2008), and β-arrestin2 has been shown to preferentially localize to cilia in certain ciliated cell types (Molla-Herman et al., 2008). Therefore, we sought to further explore GPR56 interactions with arrestins. As seen in Figure-IV-3E, GPR56 can be co-immunoprecipitated with both β-arrestin 1 (Lane 2) and β-arrestin 2 (Lane 3). Interestingly, though the different arrestin subtypes appear to interact with distinctly-processed forms of GPR56. For example, β-arrestin 1 preferentially interacts with the 50 kDa cleaved form of GPR56 (Lane 2), whereas β-arrestin 2 preferentially binds to the higher order full-length and oligomerized GPR56 (Lane 3). Further work will be required to elucidate the significance of this difference, but it is tempting to speculate that the preferential association of β-arrestin 2 with the full length, unprocessed form of GPR56 may reflect a key role of β-arrestin2 in controlling the trafficking of GPR56.

Figure IV-3. GPR56 localizes to the primary cilia in IMCD-3 cells and differentially binds to β-arrestin proteins. The subcellular location of GPR56 was assayed for possible cilia localization. (A-D) IMCD-3 cells were probed with an anti-acetylated tubulin antibody (A) and the GPR56 C-terminal antibody (B). DAPI staining is shown in panel (C), and the merged images are shown in panel (D). (E) HEK293 cells were transfected with GPR56 and either empty vector, HA-β-arrestin1, or HA-β-arrestin2. Cells were incubated for 24 hrs then lysed and subjected to immunoprecipitation with an anti-HA antibody. Western blot analysis with anti-GPR56-CT antibodies was used to visualize co-immunoprecipitation of GPR56-CT.


A
B
C
D


E
IP: HA-arrestin
WB: GPR56-CT
250
100
75
50
37
25
Lysate
WB: HA-arrestin
Mock
ßarr1
ßarr2

4.4 Discussion

GPCR-interacting proteins can regulate GPCR function in a variety of ways, from scaffolding GPCRs into signaling complexes to controlling receptor trafficking to dictating receptor localization (Ritter and Hall, 2009). As a result, studying these protein-protein interactions is of the utmost importance, since studies of this type can shed light on the fundamental biology of a given receptor and also offer novel therapeutic targets beyond the receptor itself. As shown in this chapter, my colleagues and I explored several interacting partners of GPR56 and attempted to understand how these associations might regulate receptor signaling activity and localization.

We screened the GPR56 C-terminus against an array of 96 PDZ proteins and determined that GPR56 can bind to both MAGI-3 and CAL. These interactions were confirmed din pull-down assays from cell lysates. Many PDZ-GPCR interactions control receptor trafficking (Dunham et al., 2009; Ritter and Hall, 2009), so we explored the hypothesis that MAGI-3 and/or CAL might alter GPR56 trafficking to the plasma membrane. However, co-expression of GPR56 with either PDZ protein resulted in no significant differences in receptor surface expression. Some PDZ proteins, like sorting nexin 27 (SNX27), control recycling and sorting of GPCRs only following long periods of agonist stimulation, and thus surface expression assays under non-stimulated conditions might not detect much effect of such PDZ-mediated regulation (Lauffer et al., 2010). Furthermore, many PDZ-GPCR interactions do not appear to alter receptor trafficking at all, but rather create a signaling complex at the cell surface. For example, MAGI-3 has been shown to interact with Frizzled receptors to promote JNK signaling cascades (Yao et al., 2004). Usually, Frizzled receptors are involved in Wnt signaling to

Rho, similar to GPR56, and thus binding to MAGI-3 alters typical receptor signaling (Li et al., 2005). This may be the case for MAGI-3 interactions with GPR56, an idea that could be explored by studying of the ability of GPR56 to couple to signaling pathways other than Rho. Similarly, CAL has been shown to regulate Rho signaling (Cheng et al., 2005) and associate with the Rho effector Rhotekin (Ito et al., 2006; Ito et al., 2006). Thus, instead of CAL controlling GPR56 trafficking, as has been reported for certain other GPCRs (He et al., 2004), CAL may instead link GPR56 to Rho effectors to create cell signaling complexes in a cell-specific manner. GPR56 interactions with PDZ proteins offer many further avenues for study and add more layers of complexity to understanding the regulation of GPR56 signaling. .

Collagen III has been reported to stimulate GPR56 signaling to Rho in NIH3T3 cells (Luo et al., 2011). We attempted to replicate this finding and connect it to our observations shown in Chapter 3, that NT-NT interactions enhance GPR56 signaling activity. However, our attempts to replicate the findings in NIH3T3 cells were derailed by the fact that 3T3 cells, under the conditions in which they are grown in our laboratory, do not express detectable levels of GPR56. It is well known that gene expression profiles for a given cell type can vary significantly under different growth conditions, with the type of serum used being a particularly important variable, and the expression level of GPR56 in NIH3T3 cells must be heavily dependent on some aspect of the growth conditions. Since we were not able to observe GPR56 expression in NIH3T3 cells, we explored the effects of collagen III on GPR56-induced Rho signaling in transfected HEK293 cells as well as IMCD-3 cells, which we found to express significant levels of endogenous GPR56. In these cell types, however, collagen III treatment did not enhance

GPR56 signaling to Rho or β-catenin. Indeed, collagen treatment of these cell types resulted in modest decreases in Rho & β-catenin signaling. These data are consistent with the findings of Piao et al. in terms of demonstrating the ability of collagen III to regulate GPR56 signaling. However, the directionality of the regulation seems to be highly dependent on cellular context. Further work will be needed to understand the importance of cellular context in determining the physiological effects of the interaction between collagen III and GPR56.

The discovery that GPR56 can localize to cilia, at least in IMCD-3 cells, may be highly relevant to understanding the function of GPR56 *in vivo*. During neurogenesis, cilia pay a vital role in regulating progenitor cell migration from the subventricular zone (Duan et al., 2008). Also, as mentioned, disruption of the cilial protein *ift88* results in a cobblestone-like malformation of the cerebral cortex (Willaredt et al., 2008) strikingly similar to the phenotype observed upon knockout of *Gpr56* (Li et al., 2008). Given the fact that the two closest relatives of GPR56, HE6 (Kirchhoff et al., 2008) and VLGR1 (Yagi et al., 2007) are both known to localize to cilia, it may be the case that cilial localization is an important aspect of the fundamental biology of all members of the GPR56 sub-family of adhesion GPCRs.

CHAPTER V: Further Discussion and Future Directions

5.1 Signaling and mechanism of activation for GPR56

The work compiled in this dissertation has shed light on the signaling and regulation of the polymicrogyria-associated orphan G protein-coupled receptor GPR56. For example, this work demonstrated that GPR56 does in fact signal through coupling to G proteins, specifically through coupling to $G\alpha_{12/13}$ to activate Rho and β-catenin. As mentioned earlier, similar findings have been reported by an independent group (Iguchi et al., 2008), thereby confirming the validity of these data. We also showed that the cleaved N-terminus of GPR56 stays associated at the cell surface with the receptor's 7TM region. This is consistent with past reports about other adhesion GPCRs for which it has been shown that the receptors' NT and 7TM regions (sometimes referred to as the receptors' α and β subunits, respectively) remain non-covalently associated for some period of time following autoproteolysis at the GPS motif (Gray et al., 1996; Krasnoperov et al., 1997; Krasnoperov et al., 2002; Kwakkenbos et al., 2002; Lin et al., 2004; Lin et al., 2010; Paavola et al., 2011). Since our N-terminal truncated mutant traffics well to the plasma membrane (Fig. II-2E), these findings imply that the association between the cleaved N-terminus and 7TM region is not necessary for receptor surface expression, but does have important implications for receptor signaling activity.

This point about the importance of the GPR56 NT in controlling receptor signaling was elucidated in our GPR56 truncation studies, which revealed that truncation of the GPR56 N-terminus leads to dramatically enhanced constitutive activity of the receptor. This conclusion was drawn based on four lines of evidence: *i)* transfection of cells with the GPR56 ΔNT mutant results in significantly enhanced activation of Rho signaling, relative to wild-type GPR56, *ii)* the ΔNT mutant is much more heavily

ubiquitinated than wild-type GPR56, *iii)* the ΔNT mutant associates much more avidly than wild-type GPR56 with β-arrestins, which preferentially bind to active receptors, and *iv)* expression of the ΔNT mutant is toxic for cells in a manner that is rescued by co-expression of β-arrestins. Based on these results, we propose a mechanism of GPR56 activation wherein the cleaved N-terminus remains non-covalently associated with the 7TM region to antagonize receptor signaling, and once removal of the NT occurs, allows for GPR56 coupling to $G\alpha_{12/13}$ and signaling to Rho and β-catenin (Fig II-7).

5.2 A possible conserved mechanism of activation for the adhesion GPCR family

Since all adhesion GPCRs are believed to undergo autocatalytic cleavage at the GPS motif, and as mentioned it has been shown for a number of these receptors that the cleaved N-termini can remain associated with the 7TM regions, it raises the question as to whether antagonism of receptor activity by NT association is a common feature of the entire adhesion GPCR family or whether this phenomenon is unique to GPR56. Interestingly, similar findings demonstrating that NT truncations can induce enhanced constitutive activity of adhesion GPCRs have been made for several other receptors beyond GPR56. For example, the brain-specific angiogenesis inhibitor 2 (BAI2) was shown to activate NFAT signaling upon over-expression in HEK293 cells, possibly via a G protein-dependent pathway, while over-expression of an NT-truncated mutant resulted in dramatically increased NFAT activation compared to the wild-type receptor (Okajima et al., 2010). Furthermore, transfection of the adhesion GPCR CD97 into COS-7 cells was shown to stimulate Rho and SRE through a $G\alpha_{12/13}$-dependent mechanism, and transfection of an NT-truncated mutant version of CD97 resulted in stimulation of signaling to SRE that was 10-fold stronger than that induced by the wild-type receptor

(Ward et al., 2011). Taken together, these data from work on GPR56, BAI2 and CD97 paint a picture of a potentially general mechanism of activation for adhesion GPCRs, in which the NT regions are cleaved by autoproteolysis, but remain associated with the receptors' 7TM regions to exert an inhibitory influence on receptor signaling. In this model, engagement of the NT by a large protein, whether an antibody, toxin or endogenous adhesive ligand, can result in either the removal of the NT or a gross conformational rearrangement that alleviates the inhibitory constraint of the NT on signaling by the 7TM region, thereby allowing for the initiation of G protein-mediated signaling. Consequently, our demonstration of the effect of the N-terminus on GPR56 signaling, along with similar findings by other groups working on distinct adhesion GPCRs, may provide insight into the mechanism of activation for the entire family. Moreover, these results shed light on why these receptors have GPS motifs and undergo such complex and unusual processing.

By way of comparison with other GPCR sub-families, it should be pointed out that removal of NT regions does not typically lead to activation of GPCRs. In fact, the only examples of this phenomenon beyond the adhesion GPCRs are the members of the protease-activated receptor family (PAR1-4) (Macfarlane et al., 2001) and the thyrotropin receptor (Van Sande et al., 1996; Zhang et al., 2000). In the case of the well-studied PAR family, cleavage by an exogenous protease (such as thrombin) is required for receptor activation, and the PAR NT regions do not seem to remain associated with the receptors' 7TM regions for any period of time following cleavage (Traynelis and Trejo, 2007). Thus, this mechanism of activation for the PAR family is quite distinct from that proposed here for adhesion GPCR activation, which, as discussed above, seems to

involve autoproteolysis followed by sustained association between the cleaved portions of the receptor, until engagement of the NT by a ligand results in a conformational rearrangement to the NT/7TM complex, allowing for signaling by the 7TM region.

5.3 Complexity and multiplicity of adhesion GPCR biology

We found that GPR56 homophilic NT-NT interactions positively regulate GPR56 signaling, similar to findings made for the adhesion GPCRs Celsr 2/3 (Shima et al., 2007). In addition to observing receptor activation via these trans-interactions, we also observed the NT-NT interactions to inhibit glioma cell migration, a physiological effect consistent with activation of Rho. It should be pointed out that a role for NT-NT interactions in adhesion GPCR activation are not mutually exclusive with crucial roles for other large adhesive ligands, since NT-NT interactions might be required to create binding sites for certain ligands. Conversely, or perhaps concurrently, association with large adhesive ligands might stabilize NT-NT interactions in a manner that promotes receptor signaling.

Further complexity in the realm of adhesion GPCR signaling comes from the fact that the NT regions of these receptors can exert physiological effects that may be independent of the 7TM regions. For example, a fragment of the BAI1 NT has been shown to suppress tumor growth *in vivo*, independent of the BAI1 7TM, in a manner that is dependent on association of the released BAI1-NT with CD36 and integrins (Koh et al., 2004; Kaur et al., 2009). Thus, the NT regions of adhesion GPCRs may serve multiple biological functions, including *i)* inhibiting receptor signaling activity for as long as they are in complex with the receptors' 7TM regions, *ii)* mediating cell adhesion,

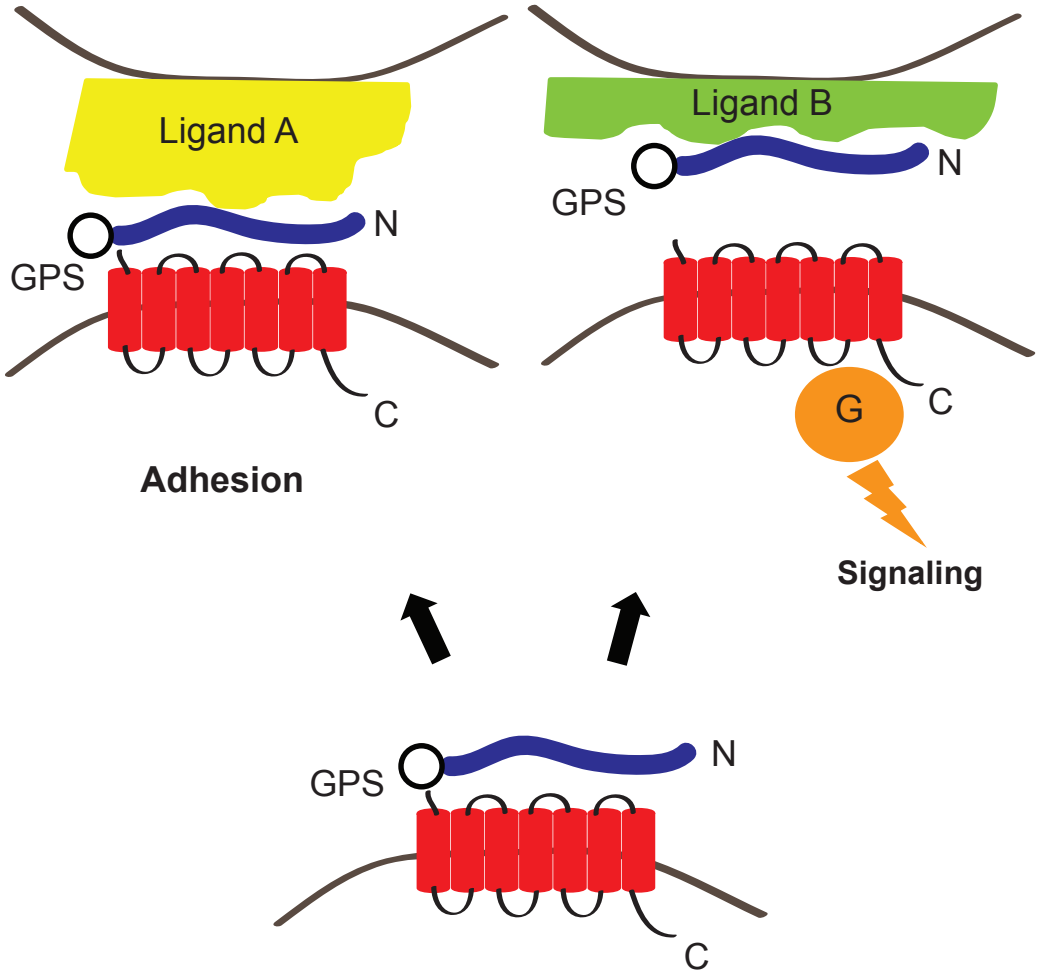
iii) allowing signaling by the 7TM regions to occur after engagement by particular endogenous ligands, and *iv)* exerting additional effects as extracellular secreted proteins following their disengagement from the 7TM regions (Fig V-1).

5.4 Potential drug development strategies for targeting GPR56

The study of GPR56 and the other adhesion GPCRs signaling is an emerging area that is highly relevant to drug development. GPCRs are outstanding drug targets in general, and the adhesion family of GPCRs is particularly intriguing targets for therapeutics since several members of the adhesion GPCR sub-family are human disease genes. Moreover, almost all members of the adhesion GPCR sub-family exhibit very discrete patterns of distributions (Schioth et al., 2010), which is appealing in terms of the possibilities for the development of therapeutics with tissue-specific and cell-specific actions. Thus, understanding the mechanisms of activation, diversity of potential ligands, and multi-faceted physiological functions of adhesion GPCRs may offer tremendous future opportunities for pharmacological intervention in a number of different disease states.

There are a number of ways that GPR56 and other adhesion GPCRs might be targeted therapeutically. One of the goals of the work described in this dissertation has been to shed light on the signaling outputs and G protein dependent pathways activated by GPR56, as such information can be utilized in high-throughput screening approaches to look for novel small molecules that modulate receptor activity. Such high-throughput screening studies have already resulted in the identification of a small molecule agonist (beclomethasone dipropionate) for the adhesion GPCR GPR97 (Gupte et al., 2012). This finding represents proof of principle that adhesion GPCR activity can be regulated by

Figure V-1: Differential ligand binding to adhesion GPCRs can result in distinct physiological responses. An unliganded adhesion GPCR is shown in the lower part of the figure, with its large N-terminal region cleaved at the GPS motif but remaining associated with the receptor's seven-transmembrane region. Ligands for adhesion GPCRs are often large secreted glycoproteins and/or components of the extracellular matrix. Some ligands (illustrated here by "Ligand A") can interact with adhesion GPCRs to facilitate cell adhesion without stimulating downstream receptor signaling. Conversely, other ligands (illustrated here by "Ligand B") induce either removal of the receptor's N-terminus or large-scale N-terminal conformational changes to promote receptor coupling to intracellular G proteins and activation of G protein-mediated signaling pathways.


Ligand A
N
GPS
C
Adhesion
Ligand B
N
GPS
G
C
Signaling
GPS
N
C

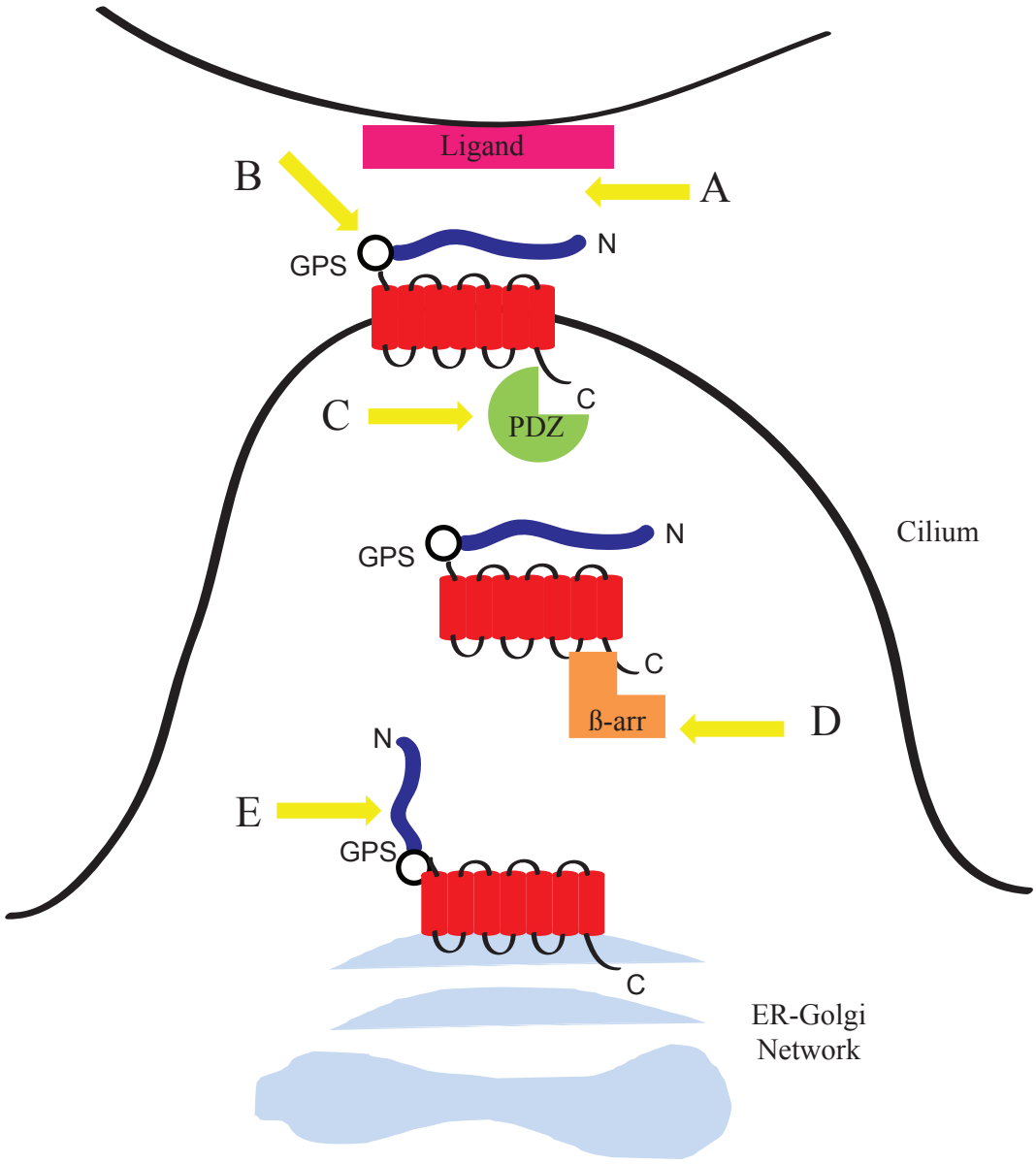
small molecules. It is not yet known how beclomethasone dipropionate regulates GPR97 activity, but there are a number of possibilities as to how small molecules might influence the activity of adhesion GPCRs. Figure V-2 summarize potential drug target sites on GPR56. Therapeutic targeting GPR56 could lead to novel treatments for cortical developmental disorders, neurodegenerative diseases, traumatic brain injury, and cancer. The work presented in this dissertation has laid the groundwork for the potential development of GPR56-taretedd therapeutics by providing insights into fundamental aspects of the activation and regulation of GPR56.

5.5 Future directions: From GPR56-dependent Rho activation to the inhibition of cellular migration

The work presented in this dissertation, as well as published studies by other labs, has demonstrated that GPR56 activates the Rho pathway to inhibit cellular migration. However, there is a large gap of knowledge as to how this specific GPR56-dependent signaling pathway can lead to the inhibition of migration

Cellular migration is a complex process that involves the dynamic reorganization of the actin cytoskeleton, directing protrusion at the front of the cell and retraction at the rear. Moreover, there must also be a coordinated orchestration of biochemical signals to direct secretion (to provide new membrane components), turnover cell–matrix interactions (to control adhesion), and change in gene transcription (to provide autocrine/ paracrine signals) (Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996; Martin, 1997). Of particular interest in the context of GPR56 research, Rho GTPases have been shown to control the formation of focal adhesion complexes in fibroblast cells

Figure V-2. Schematic diagram of potential modulation of GPR56 function by therapeutics. The illustration shows how drugs might potentially target GPR56 for therapeutic benefit. (A) Small molecules could target the GPR56-ligand interaction to either agonize receptor activity by mimicking endogenous ligands or blocking interactions with endogenous ligands to antagonize receptor activation. (B) GPR56 exists at the cell surface as a non-covalent dimer between the N-terminus and 7TM regions. Drugs that disrupt this interaction should, according to our findings, potentiate receptor activity. (C) Drugs that block GPR56 interactions with PDZ proteins might regulate receptor activity by altering receptor-activated signaling pathways and/or perturbing receptor localization. (D) Modulation of GPR56 targeting to cilia, for example by small molecules targeting the GPR56/arrestin interface, might alter receptor function by perturbing receptor localization. (E) GPS cleavage is necessary for GPR56 surface expression. Thus, drugs targeting the autoproteolytic activity of the GPS motif could potentially inhibit GPR56 activity by preventing GPR56 from reaching the cell surface.


Ligand
B
A
GPS
N
C
C
PDZ
Cilium
GPS
N
C
ß-arr
D
N
E
GPS
C
ER-Golgi
Network

by regulating actin stress fibers (Ridley and Hall, 1992). More importantly, work done on wound healing has shown that Rho activation is not required for cell movement, but instead leads to focal adhesion formation and the inhibition of cellular migration (Nobes and Hall, 1999). Thus, it may be of interest for future studies to focus on the specific manner in which GPR56-dependent Rho activation regulates the actin cytoskeleton and regulates the formation of focal adhesion complexes to stop neuronal progenitor cells in their proper positions at the pial basement membrane.

Itoh and colleagues showed that overexpression of GPR56 caused reorganization of F-actin in a Rho dependent manner in NIH3T3 cells, which is a biochemical change consistent with the inhibition of cellular migration (Iguchi et al., 2008). It may be interesting to determine how known GPR56-interacting partners such as collagen III, CD9, CD81, and transglutaminase 2 might be involved in GPR56-dependent actin cytoskeleton rearrangement. Additionally, since Rho regulation of focal adhesions typically involves regulation of integrins (Petit and Thiery, 2000), and β1 integrin knockout mice exhibit severe cortical malformations due to disorganization of radial end feet (Graus-Porta et al., 2001), a phenotype that bears a passing similarity to the phenotype of *Gpr56* knockout mice (Li et al., 2008), it may be a point of future interest to study cross-talk between GPR56 and integrins.

5.6 Future Directions: GPR56 as a target for anti-cancer therapeutics

Early studies on GPR56 expression showed highly upregulated levels of GPR56 in glioblastoma multiforme tumors, as well as in a majority of glioblastoma/astrocytoma tumor samples (Shashidhar et al., 2005). This is consistent with the view that glioma and

many other tumors primarily arise from “cancer stem cells” that re-activate patterns of gene expression seen during development and behave functionally as stem or progenitor cells (Pantic, 2011). Since GPR56 is highly-expressed in neuronal progenitor cells, it makes intuitive sense that GPR56 expression may be re-activated in glioma cells. The high expression of GPR56 in glioma cells, and lack of GPR56 expression in mature neurons and glia, makes GPR56 an attractive target for anti-glioma therapeutics.

The signaling pathways downstream of GPR56 are already major targets for anti-cancer therapies. Rho GTPases are known to play important roles in tumor development and progression (Mardilovich et al., 2012) and powerfully control the migration of cancer cells (Tatsuta et al., 2005; Wang et al., 2009). Drug development efforts in this area include the use of farnesyltransferase and geranylgeranyl transferase inhibitors, which block the addition of isoprenoid lipids to Rho GTPases and impair GTPase membrane anchorage and subsequent activation (Lobell et al., 2001; Sebti and Adjei, 2004). However, drugs of this type have failed thus far to advance pass the early stages of clinical trials due to toxicity issues. Furthermore, ROCK inhibitors like fasudil are currently being used for the treatment of cerebral vasospasm in Japanese patients without any serious adverse reactions (Olson, 2008), but have yet been approved for use elsewhere. Targeting GPR56, rather than the pathways downstream of the receptor, could potentially make for anti-glioma therapeutics with reduced toxicity, given the more limited expression pattern of GPR56 relative to the widespread expression patterns of Rho, ROCK, and farnesyltransferases. GPR56 could be targeted by small molecule agonists or modulators, and/or targeted via the attachment of toxins to anti-GPR56 antibodies in order to specifically kill glioblastoma cells with minimal collateral damage

to surrounding normal cells and tissues. Recent advances in targeting tumor cells with antibody-directed toxins speak to the plausibility of such an approach (Marcucci and Lefoulon, 2004).

5.7 Future Directions: Targeting GPR56 in treating neurodegenerative diseases

Neurodegenerative diseases represent a broad description of disorders that are characterized by losses in the function and/or structure of neurons. The two most commonly studied neurodegenerative diseases are Parkinson's disease (PD) and Alzheimer's disease (AD). PD is marked by the loss of dopaminergic neurons in the substantia nigra region of the brain, leading to motor difficulties and later progressing to cognitive defects and dementia (Lang and Lozano, 1998; Lang and Lozano, 1998). Current therapies aim mainly just to ease symptoms by elevating dopamine levels in the brain. AD is the most common form of dementia and, although the cause is not yet fully known, this disease is correlated with the accumulation of neuritic plaques and tangles leading to neuronal death (Tiraboschi et al., 2004). Current therapies aim mainly just to treat symptoms by inhibiting acetylcholinesterase to increase acetylcholine in the brain, thus balancing the loss of cholinergic neurons loss (Stahl, 2000; Stahl, 2000). It is widely hoped that future treatments for PD and AD will be able to stop the neurodegeneration process and/or facilitate addition of new neurons.

Targeting GPR56 could be a viable approach for novel PD and AD therapies if the receptor can be targeted in such a way that the migration of neural progenitor cells into degenerative zones can be facilitated. There exist populations of neuronal progenitor cells in the subventricular zone and other brain areas all throughout adulthood

(Beukelaers et al., 2012). However, no current therapeutic approaches are currently available to promote the proliferation and migration of these progenitor cells. It is known from *Gpr56* knockout studies that the receptor inhibits the migration of neural progenitor cells during development (Li et al., 2008; Koirala et al., 2009). GPR56 antagonists might encourage the proliferation and migration of adult neural progenitor cells in order to help replenish neurons that have been lost due to neurodegenerative disease. Proper homing of the progenitor cells to affected areas could be an issue, although it is conceivable that a cocktail of chemokines and/or other chemoattractants could be employed to help in this regard (Gazitt, 2004).

5.8 Concluding thoughts

The work presented in this Dissertation has shed light on many new aspects of GPR56 signaling. Moreover, our findings concerning the activation of GPR56 may provide insights into the mechanisms of activation for other members of the adhesion GPCR family. Prior to the work shown in this Dissertation, the sum total of knowledge about GPR56 was that i*)* it was a member of the adhesion GPCR family, *ii)* mutations to the receptor in humans can cause the inherited cortical developmental disorder bilateral frontoparietal polymicrogyria, *iii)* over-expression of the receptor can lead to increases in β-catenin signaling and *iv)* the receptor can physically interact with TG2, CD9, and CD81. The work shown in this Dissertation has greatly extended the body of knowledge about GPR56 signaling be demonstrating that GPR56 signals through $G\alpha_{12/13}$ to activate Rho GTPases and control cellular migration. Additionally, we showed that the cleaved N-terminus of GPR56 stays associated with the 7TM region of the receptor in a manner that

antagonizes receptor signaling activity. When the NT is removed, G protein-dependent signaling by the receptor is greatly enhanced. In the time since we made these observations, multiple groups have mirrored our truncation studies to show similar results for a variety of adhesion GPCRs including GPR56 (Yang et al., 2011), CD97 (Ward et al., 2011), and BAI2 (Okajima et al., 2010). Thus, the model of activation we have proposed for GPR56 may represent a conserved mechanism of activation for the entire adhesion GPCR family, wherein the cleaved N-terminus stays associated with the 7TM region until removal of the NT by ligand binding allows for receptor activation. In terms of ligands, we have also demonstrated that the N-terminus of GPR56 can interact with other GPR56 N-termini in a homophilic trans-fashion to positively regulate receptor signaling. Regulation of receptor signaling by such homophilic trans-interactions may represent a general phenomenon for the adhesion GPCR family, since similar data have been shown for Celsr2/3 (Shima et al., 2007).

In conclusion, in addition to specifically shedding light on the biology of the human disease gene product GPR56, the work shown in this Dissertation also represents a model study for understanding adhesion GPCR signaling pathways and mechanisms of activation. The findings and concepts presented din this Dissertation may help to guide future studies on all members of the adhesion GPCR family. Furthermore, this work may facilitate drug development efforts aimed at GPR56 in particular and adhesion GPCRs in general.

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