PDZ-RhoGEF Drives Gastrin Releasing Peptide

Stimulated Colon Cancer Cell Migration

BY

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THESIS

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DEDICATION

I dedicate this work to Purna Purushottam Narayan Swaminarayan Bhagwan and Mul-Aksharbrahma Gunatitanand Swami Maharaj. I also dedicate this work to my guru Pragat Brahmaswarup Pramukh Swami Maharaj whose love, guidance, and inspiration has made this work possible.

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CONTRIBUTION OF AUTHORS

Chapter 1 introduces my dissertation question. Chapter 2 is a literature review, where I summarize the current understanding in the field and highlight the significance of my dissertation question. Chapter 2 includes a portion of a published review, (Patel M & Karginov AV (2014) Phosphorylation-mediated regulation of GEFs for RhoA. *Cell Adhesion & Migration* 8(1):11-18), of which I was the first author that was responsible for writing the review after a thorough literature search. Chapter 3, 4, and 5 includes a published manuscript (Patel M*, et al.* (2014) Gα13/PDZ-RhoGEF/RhoA Signaling Is Essential for Gastrin-Releasing Peptide Receptor–Mediated Colon Cancer Cell Migration. *Molecular Pharmacology* 86(3):252-262.), of which I was the primary author and the major driver of research. In Chapter 4, Figure 9A, the experiment was conducted and the data obtained by Takeharu Kawano. My research mentors, Dr. Tohru Kozasa and Dr. Andrei Karginov, guided the direction of the research project, and were instrumental with the planning and editing of the manuscript. I have generated all the other figures found in Chapter 4. Chapter 5 repersents the discussion portion of my dissertation, where I address and interpret the data in the larger context. Chapter 6 contains a discussion on the potential future directions for this project.

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LIST OF ABBREVIATIONS

ABCA1	ATP-binding cassette transporter-1
AC	Adenylyl cyclase
Ang-II	Angiotensin II
ANOVA	Analysis of variance
AT_1	Angiotensin-II receptor
BB2	Bombesin 2 receptor
cAMP	cyclic adenosine monophosphate
CCK2R	Cholecystokinin 2 receptor
CNVs	Copy number variations
COSMIC	Catalogue of Somatic Mutations in Cancer
Cox-2	Cyclooxygenase-2
CXCR4	C-X-C motif receptor 4
DCM	Distal colonic mucosa
DH	Dbl homology
DLBCL	Diffuse large B-cell lymphoma
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EP_{1-4}	Prostaglandin E_2 receptors
ERK	Extracellular signal related kinase
ET_{A-B}	Endothelin receptors

LIST OF ABBREVIATIONS (continued)

FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FPG	Fasting plasma glucose
GAP	GTPase activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBM	Glioblastoma multiforme
GCB	Germinal center B-cell
GDP	Guanosine-5’-diphosphate
GFP	Green fluorescent protein
G protein	Guanine nucleotide binding protein
GPCR	G protein coupled receptor
GRK	G protein coupled receptor kinase
GRP	Gastrin releasing peptide
GRPR	Gastrin releasing peptide-receptor
GST	Glutathione-s-transferase
GTP	Guanosine-5’-triphosphate
HCoEpiC	Human colonic epithelial cells
HFD	High fat diet
IGF	Insulin growth factor
IGT	Impaired glucose tolerance
IL	Interleukin
JAK2	Janus kinase 2

LIST OF ABBREVIATIONS (continued)

KO	Knockout
LPA	Lysophosphatidic acid
LPA_{1-4}	Lysophosphatidic acid receptors
LARG	Leukemia associated RhoGEF
MAPK	Mitogen activated protein kinase
MDCK	Madin-Darby canine kidney
NMBR	Neuromedin B receptor
OSCC	Oral squamous cell carcinoma
p115	p115RhoGEF
PAR1	Protease activated receptor 1
PBS	Phosphate buffered saline
PDZ	Postsynaptic density 95, Disk large, Zona occludens
PGE_2	Prostaglandin E_2
PH	Pleckstrin homology
PKA	protein kinase A
PKC	protein kinase C
PLCβ	phospholipase Cβ
PRG	PDZ-RhoGEF
Ptch	Patched receptor
Pyk2	Proline rich tyrosine kinase 2
RBD	Rho binding domain
RH	Regulator of G protein signaling homology

LIST OF ABBREVIATIONS (continued)

RGS	Regulator of G protein signaling
RhoGEF	Rho guanine nucleotide exchange factor
ROCK	Rho associated kinase
RTKs	Receptor tyrosine kinases
S1P	Sphingosine-1-phosphate
$S1P_{1-4}$	Sphingosine-1-phosphate receptors
SCLC	Small cell lung cancer
S.E.M	Standard error of the mean
Sema4D	Semaphorin 4D
Shh	Sonic hedgehog
siRNA	Small interfering RNA
Smo	Smoothened receptor
SNP	Single nucleotide polymorphisms
SRE	Serum response element
TP	Thromboxane A_2 receptor
TSGs	Tumor suppressor genes
WT	Wild type

SUMMARY

Colorectal cancer is a heterogeneous disease that is estimated to impact 5% of all Americans during their lifetime. Current evidence indicates that colorectal cancer develops as normal colonic epithelium acquires genetic and epigenetic alterations that contribute to development and progression of the colorectal tumor. The concerted effort of the scientific community has identified several key oncogenes and tumor suppressor genes that play a role in development and progression of colorectal cancer. At the same time, evidence also implicates over-expression and activation of signaling pathways regulated by RTKs (receptor tyrosine kinases) and GPCRs (G protein coupled receptors) to contribute to development and progression of colorectal cancer. One such GPCR, GRPR (gastrin releasing peptide receptor), has been implicated in progression of many different solid tumors including colon cancer. GRPR signaling through Gq heterotrimeric G protein contributes to cancer cell proliferation in variety of solid tumors. GRPR activation in colon cancer cells has also been implicated to regulate colon cancer cell motility and invasion. However, the specific signaling pathway(s) initiated by GRPR activation that regulates colon cancer cell migration have not been identified. Thus, we set out to molecularly dissect the signaling pathway(s) initiated by GRPR that contributes to colon cancer cell migration.

Utilizing cell-based and biochemical techniques with colon cancer cell lines that endogenously express functional GRPR, we were able to identify G13 heterotrimeric G-protein to predominantly regulate GRP stimulated RhoA activation. More importantly, we have identified PRG (Postsynaptic density 95, Disk large, Zona occludens-1-RhoGEF), a member of RH-RhoGEF (RGS-homology domain containing guanine nucleotide exchange factors) family, to be the predominant activator of RhoA downstream of GRPR. Our data provides evidence that

PRG-RhoA signaling through ROCK (Rho-associated kinase) regulates GRP stimulated colon cancer cell migration and Cox-2 (cycoloxygenase-2) expression. Overall, these data suggests that GRPR mediated $G\alpha_{13}$-PRG-RhoA-ROCK axis regulates colon cancer cell migration.

I. Introduction

GRPR has been found to be ectopically expressed or over-expressed in SCLC (small cell lung cancer), breast cancer, prostate cancer, and colon cancer (reviewed in (1)). In colon cancer, several studies support the role of GRPR in increasing tumor cell proliferation (2,3), and morphogenic transformation leading to increased tumor cell differentiation (4). GRPR has also been shown to stimulate colon cancer cell motility (5). However, the molecular mechanisms by which GRPR activation leads to colon cancer cell migration are not well understood.

GRPR is a seven transmembrane GPCR that couples to members of the Gq/11 and G12/13 families of heterotrimeric G-proteins (reviewed in (1)). GRPR-mediated cancer cell proliferation is thought to be primarily regulated through activation of $G\alpha_q$ canonical signaling pathway ((6) and reviewed in (1,7)). In comparison to $G\alpha_q$ signaling, relatively little is known about $G\alpha_{12/13}$–mediated pathways downstream of GRPR and their contributions to colon cancer progression. Receptors coupled to $G\alpha_{13}$ are known to activate small GTPase RhoA that controls cell migration (8,9). This is accomplished by direct interaction of activated $G\alpha_{13}$ with family of guanine nucleotide exchange factors for RhoA known as RH-RhoGEFs. The RH-RhoGEF subfamily consists of p115 (p115RhoGEF), PRG, and LARG (Leukemia-associated RhoGEF) (10-15). GTP-(guanosine-5'-triphosphate) bound $G\alpha_{13}$ interacts with the RH domain of these large multi-domain containing GEFs. This interaction stimulates their GEF activity leading to exchange of GDP (guanosine-5'-diphosphate) to GTP on RhoA (11,12,16). Thus, RH-RhoGEFs are primary candidates that may link GRPR stimulation to RhoA activation.

Activation of RhoA is known to contribute to tumorigenesis by playing a role in cellular transformation, proliferation, migration and invasion (reviewed in (17)). Several studies have shown that RhoA is over-expressed and highly activated in many solid tumors, including colon

cancer ((18-20) reviewed in (17)). Increased RhoA activation and signaling through its downstream effectors, such as ROCK, contributes to cancer progression. Activation of the RhoA-ROCK signaling axis initiates cytoskeletal changes that are essential for cancer cell motility and invasion, initiates gene transcription, and promotes cancer cell proliferation (21-23).

A. Statement of Hypothesis

GPCRs coupled to $G\alpha_{13,}$ signaling through RhoA, have been implicated in regulation of breast and prostate cancer cell migration and invasion (24,25). However, these reports fail to identify the critical elements, the RhoGEF(s), that regulate RhoA activation in these cancers-which subsequently regulates diverse sets of cellular processes that promote cancer progression. Currently, the signaling events downstream of GRPR that regulate colon cancer cell migration are not completely understood. Based on the available evidence, we hypothesize that GRPR regulates colon cancer cell migration through $G\alpha_{13}$ mediated regulation of RhoA activation. We also set out to identify the RH-RhoGEF(s) that are activated downstream of $G\alpha_{13,}$ serving as the molecular link between $G\alpha_{13}$ and RhoA activation, in order to provide a complete picture of GRPR signaling events that govern colon cancer cell migration. To address this hypothesis we utilized colon cancer cell lines that endogenously express functional GRPR for cell based and biochemical assays that interrogated GRPR signaling to identify the critical elements within the signaling pathway(s) that regulate colon cancer cell migration.

B. Significance of Study

Here we have identified key molecular players downstream of GRPR that regulates colon cancer cell migration. Our data shows that GRP stimulation of colon cancer cell lines leads predominantly to $G\alpha_{13}$ mediated RhoA activation. More importantly we have determined PRG as

the dominant RH-RhoGEF mediating GRP stimulated RhoA activation, and have provided evidence that PRG-RhoA-ROCK signaling axis regulates GRP stimulated colon cancer cell migration. Furthermore, our data suggests that PRG-RhoA-ROCK axis leads to GRP mediated Cox-2 expression, and find that Cox-2 activity contributes to the overall GRP stimulated colon cancer cell migration. We have also discovered that PRG is over-expressed in these cancer cell lines in comparison to primary HCoEpiC (human colonic epithelial cells) and human colonic mucosal samples. This observation is further supported by data which shows that in human colon cancer samples, PRG gene (ARHGEF11), have increased gene copy number. Overall, our data demonstrates that GRPR signaling through $G\alpha_{13}$-PRG-RhoA-ROCK signaling axis is critical for colon cancer cell migration. More importantly, it has implicated PRG as a central player in regulation of colon cancer cell motility. This finding provides sound rationale for future studies utilizing recently developed small molecule inhibitor specific for RH-RhoGEFs in an *in vivo* colon cancer model system to test PRG and other RH-RhoGEFs role in colon cancer cell migration and metastatic spread (26). Based on our findings, it would be interesting to see if this signaling pathway can be generalized to other solid tumors (i.e., SCLC) that express GRPR and are known to have a high metastatic potential.

II. Literature Review

A. Colorectal cancer

Colorectal cancer is the 3rd leading cause of cancer and the 3rd leading cause of cancer deaths in both men and women. It is estimated that approximately 5% of all Americans will be diagnosed with colorectal cancer within their lifetime (27). Risk factors associated with development of colorectal cancer include; genetic predisposition (family history of colorectal cancer), dietary habits (high intake in red meats and unsaturated fats), excessive alcohol intake, obesity, diabetes, and inflammatory bowel disease ((27) reviewed in (28)). The etiology for sporadic colorectal cancer is multifactorial as evidenced by the diverse genetic, lifestyle, and dietary factors that increase a person's risk for colorectal cancer.

1. Molecular Pathogenesis (Adenoma-Carcinoma Sequence)

The classical model of colorectal tumorigenesis, initially proposed by Fearon and Vogelstein, indicates that colorectal cancer develops in a stepwise progression, as normal glandular colonic epithelial cells acquire selected genetic and epigenetic alterations, as it progresses from normal colonic epithelial cells to an adenoma and then to carcinoma over a time scale of years to decades (Figure 1) ((29) reviewed in(30)). It is widely accepted that adenomas are the important precursor lesions to colon cancer as studies have shown that patients who undergo polypectomy, removal of adenomatous polyps, significantly decrease their risk for development of colon cancer (31). The progression through this sequence has been correlated with gain in mutations of certain oncogenes along with loss of tumor suppressor genes (TSGs). For complete review of the most prevalent genetic alterations in colorectal cancer please access the recent review by Fearon (30).

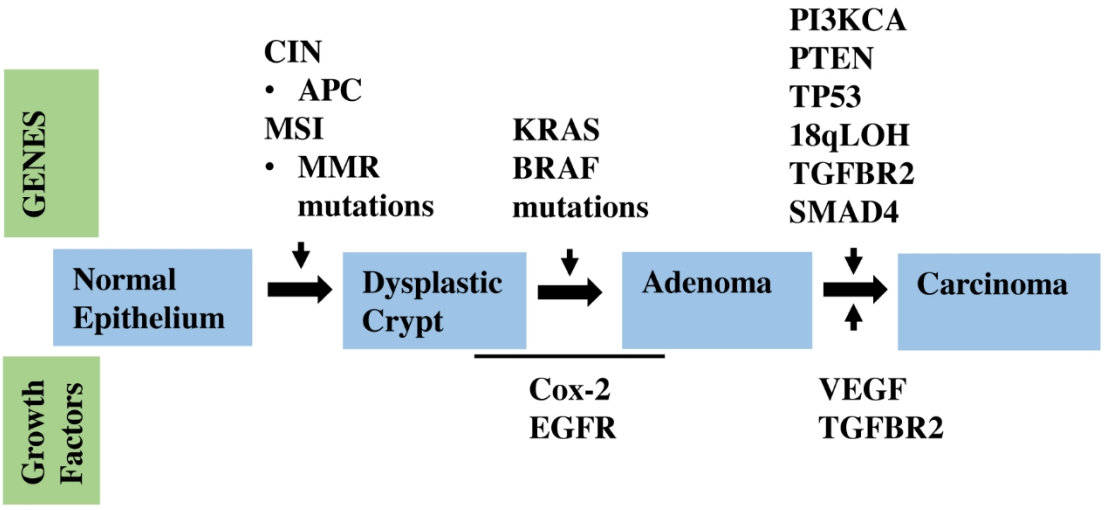


Figure 1: Genetic mutational landscape of colon cancer contributing to progression of adenoma-carcinoma sequence: Colon cancer development is initiated by genomic instability. There are two more common types of genomic instability that drives colon cancer development. Chromosomal instability (CIN) and microsatalite instability (MSI). It is well accepted that loss of adenomatous polyposis coli (APC) gene is one of the earliest events in development of colon cancers and it has been implicated to contribute to CIN. Germline mutations or somatic inactivation of DNA mismatch repair genes (MMR) have been shown to be critical events leading to MSI which initiates the sequence of events that leads to colon cancer. Here, the most frequent genetic alterations that drive tumor development and the particular tumorigenesis stage with which they have strong association are indicated. However, it is important to note that these genetic defects, within a particular stage of tumorigenesis, do not always arise in a set order. Here, we also include the role of growth factor receptors and Cox-2 in contributing the adenoma-carcinoma sequence.

2. Non-Mutational Drivers of Colorectal Cancer

It is well established that certain oncogenes along with loss of TSGs drive colon cancer development and progression. However, it is also important to remember that within this context, growth factors signaling thorough RTKs and GPCRs have also been implicated in colorectal cancer pathobiology. Up-regulation of EGFR (epidermal growth factor receptor) and of its cognate ligands have been observed in certain subsets of colon cancer (reviewed in (32)), and this may serve as an early event in the adenoma-carcinoma sequence. Another early event in development of not only colon cancer, but also breast, prostate, and lung cancers is the increased expression of Cox-2 and subsequent PGE_2 signaling ((33) and reviewed in (32,34)). Several cytokines and growth factors have been reported to increase Cox-2 expression. Indeed, GRPR signaling has also been implicated in up-regulation of Cox-2 expression in variety of cell types, including colon cancer cell lines, via signaling pathways that are not yet clearly understood (35-37). Non-the-less, what is clear is that the increased Cox-2-PGE_2 signaling has been shown to regulate colon cancer progression through increased angiogenesis, cancer cell proliferation, migration and invasion (reviewed in (34)).

3. GPCRs in Colorectal Cancer

Current evidence indicates that many GPCRs play a critical role in colon cancer progression (Table 1) (reviewed in (38-40)). However, it is unknown where in the adenoma-carcinoma sequence does aberrant GPCR signaling fit-in, as majority of the studies were conducted in cancer models utilizing cancer cell lines, and so for now the evidence only implicates GPCR signaling in colon cancer progression.

Impact of GPCRs on different aspects of cancer biology	**Receptors**
Tumor cell proliferation	EP_2, EP_4, $LPA_{1,2}$, ET_{A-B}, CCK2R, PAR1, Frizzled, **GRPR**
GPCR link inflammation to cancer	PAR1, EP_2, EP_4
Angiogenesis	EP_2, EP_4, LPA, S1P, PAR1
Cell migration & Invasion	LPA, PAR1, EP_2, **GRPR?**

Table 1: GPCRs implicated in colon cancer progression. Abbreviated list of GPCRs that have been reported to be either ectopically or over-expressed in colon cancer and that effect various aspects of cancer biology. GRPR has been shown to contribute to tumor cell proliferation in variety of solid tumors. However, the contribution and the mechanism by which GRPR signaling promotes colon cancer cell migration and invasion are not currently defined (as denoted by the '?' after GRPR).

B. GPCRs

1. Overview of GPCRs

GPCRs are integral membrane proteins which have 7TM spanning alpha-helices, with an extracellular N-terminal region, an intracellular C-terminal region, along with three intracellular and extracellular loops. Structural data also has uncovered a fourth intracellular loop created by the eighth intracellular alpha-helix at its C-terminus which contain palmitoylation sites (reviewed in (41)). GPCRs are one of the largest group of proteins encoded by the genome with ~800 genes that encode for receptors whose ligands range from neurotransmitters, hormones, to sensory stimuli such as odorants and photons of light. GPCRs represent an ideal target for treatment of human diseases as exemplified by the fact that ~30% of current pharmaceutical agents on the market are targeted for GPCRs (reviewed in (42).

2. Heterotrimeric G-Proteins

Heterotrimeric G proteins are made up of Gα subunits interacting with the tightly associated Gβ and Gγ subunits. There are 16 genes which encode for 21 Gα subunits in humans, whereas there are 5 genes encoding 6 Gβ and 12 Gγ subunits (reviewed in (43)). The heterotrimeric G proteins are divided into four major classes based on primary sequence similarity of the Gα subunit. Most well characterized Gα subunits are $G\alpha_s$, $G\alpha_q$, $G\alpha_i$, and $G\alpha_{12/13}$. The Gα subunits contain a GTPase domain and a helical domain which are conserved among small monomeric G proteins. The GTPase domain is responsible for GTP hydrolysis and also provides the interface for interactions with GPCRs, Gβγ dimers, and downstream effector proteins. Gα subunit GTPase domain contains three flexible regions termed switch I, II, and III, which enables Gα subunit to adopt different conformations depending if GDP or GTP is bound. The helical domain is composed of six alpha helices that bury the bound nucleotide into the hydrophobic core of the Gα subunits.

All Gα subunits are known to undergo palmitoylation at their N-terminus which is critical for membrane localization and function. Gα subunits bound to GTP can activate variety of signaling pathways, through interaction with their cognate downstream effectors, to bring about a cellular response (reviewed in (44)). Gα protein signaling is terminated by the hydrolysis of GTP to GDP, which can be promoted by RGS (regulators of G protein signaling) proteins that function as GAPs (GTPase activating proteins). Gα-GDP then re-associates with Gβγ dimers to prepare for another round of signaling (Figure 2).

Gβ and Gγ subunits make extensive contacts with each other forming a functional unit that can only dissociate under denaturing conditions. Structural data of heterotrimeric G proteins reveal that much of the interactions with Gα and Gβγ subunits occur primarily through contacts made between Gα and Gβ subunits. Much like Gα subunit, Gγ also undergoes lipid post-translational modifications at its C-terminus with either a farnesyl or geranylgeranyl moiety, which also plays a role in membrane localization (reviewed in (41)). Gβγ subunits can also activate distinct signaling pathways upon GPCR activation to bring about variety of cellular responses (Figure 2).

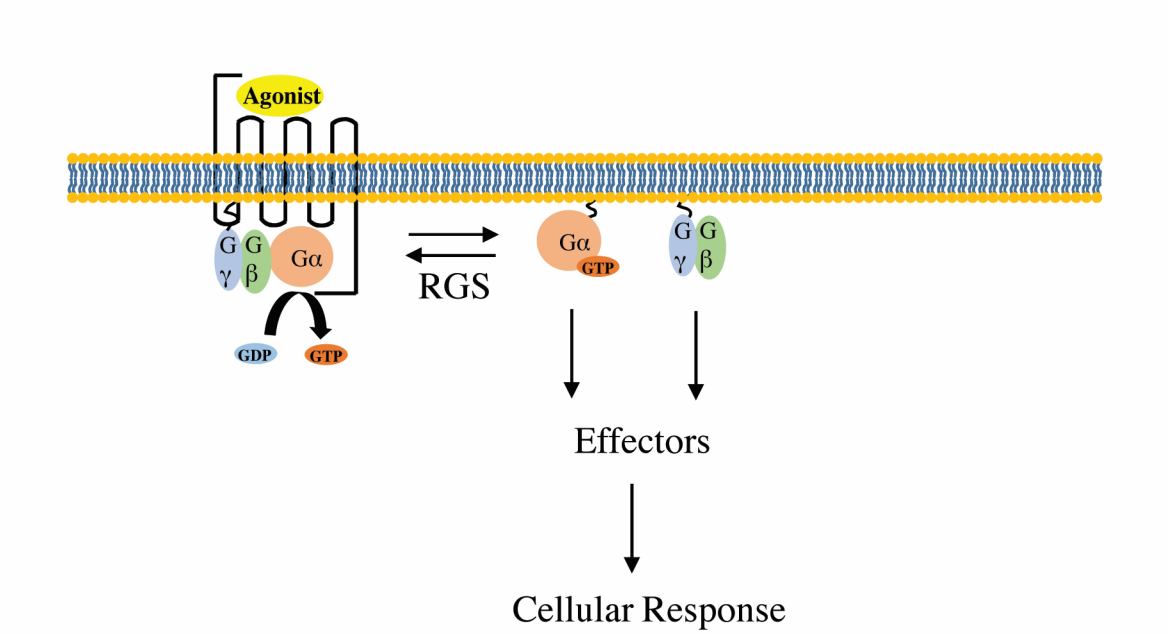


Figure 2: General schematic of GPCR signaling. Under resting conditions, Gα-GDP is bound to Gβγ subunits forming an inactive heterotrimer. Upon agonist binding, the receptor undergoes a conformational change resulting in interaction with the heterotrimeric G-proteins which stabilizes the receptor conformation in the active state. This interaction cajoles a conformational change within the Gα subunit leading to exchange of GDP for GTP. Gα-GTP subunit undergoes functional dissociation from Gβγ heterodimer allowing Gα-GTP and Gβγ to engage with their effectors and initiate signaling cascades that produce a cellular response. Also depicted is the termination of G-protein signaling, which can be regulated by various RGS proteins that accelerate the hydrolysis of GTP to GDP on Gα subunits. Thus, promoting the formation of the inactive heterotrimer.

3. GPCR Signal Transduction

Regardless of the diversity of the GPCR superfamily, it is interesting that these signal receivers interact with a relatively small subset of effector proteins that transduce the extracellular signal into a cellular response. Signal transduction occurs upon agonist binding to the GPCR, resulting in a conformational change stabilized by engagement of G proteins with the agonist bound receptor. This interaction subsequently leads to a conformational change within the Gα subunit resulting in the release the GDP and loading of GTP, which is found at higher concentrations within the cytosol. The mechanism by which activated GPCR facilitates the guanine nucleotide exchange on the Gα subunit is just now being elucidated. Data from high resolution crystal structures of GPCRs in an active and in-active states along with molecular dynamic studies revealed that G proteins play a critical role in stabilization of the active conformation of GPCR. It is postulated that the C-terminus of Gα subunit interacting with the agonist bound receptor, further facilitates interaction of receptor with the N-terminus of Gα subunit, resulting in conformational change in the P-loop. This leads to loss of coordination with the β-phosphate of GDP with subsequent release of GDP and loading of GTP ((45,46) and reviewed in (47)). Gα-GTP subunit adopts a conformation that has decreased affinity for Gβγ, leading to functional dissociation from the Gβγ subunits. Both Gα-GTP and Gβγ subunits then interact with several downstream effectors to initiate a cellular response (Figure 3).

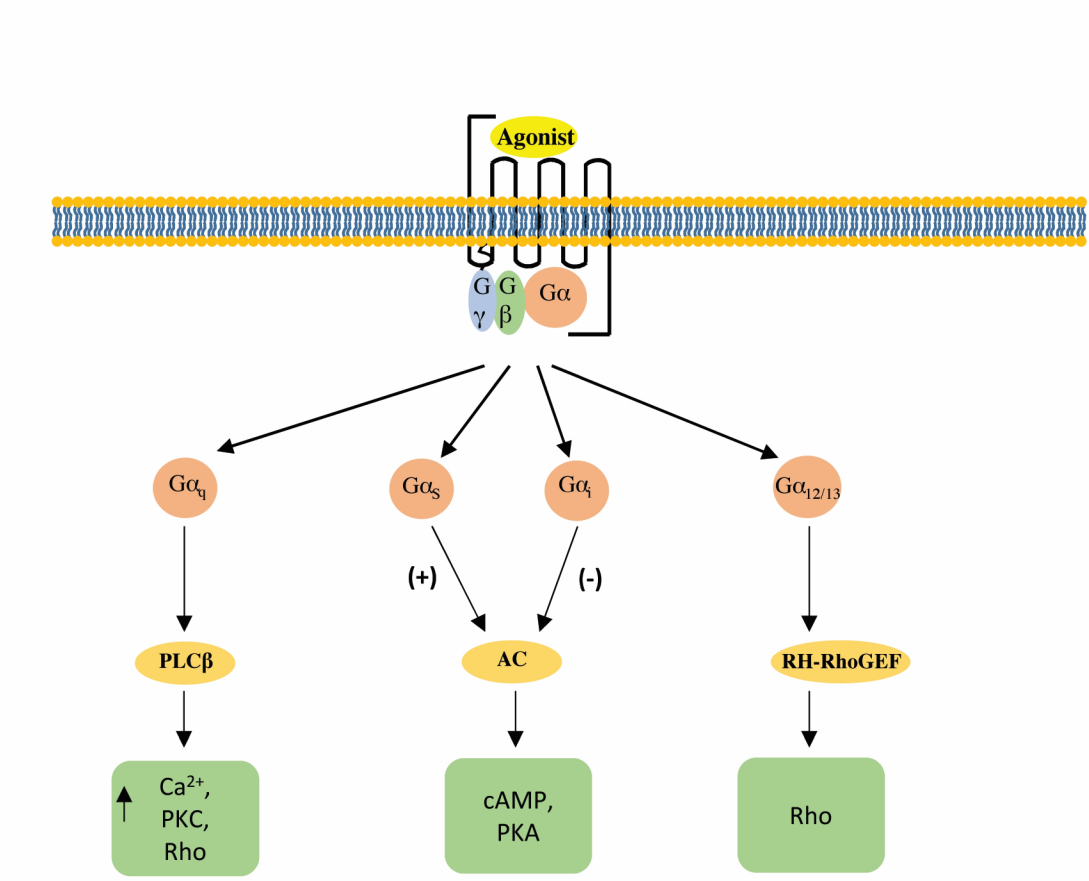


Figure 3: General schematic of GPCR signaling via Gα subunits. Gα-GTP, in an activation dependent manner, interacts with its downstream effectors leading to activation of variety of signaling pathways. Here, depicted are the most well characterized downstream effectors of the four most well studied Gα subunits. Interaction of these Gα subunits with their cognate effectors (colored gold) leads to formation of specific second messengers and activation of specific signaling pathways (as identified in green boxes). The (-) on $G\alpha_i$ denotes inhibitory effect of $G\alpha_i$-GTP on AC activity leading to decreased cAMP levels. AC (adenylyl cyclase), cAMP (cyclic adenosine monophosphate), Ca^{2+} (calcium), PKA (protein kinase A), PKC (protein kinase C), and PLCβ (phospholipase Cβ).

C. Gastrin Releasing Peptide Receptor

1. Classification-Pharmacological and Biochemical Characteristics

GRPR belongs to 7TM G protein coupled receptor class A superfamily. Within this superfamily, GRPR belongs to a small subfamily of receptors known as bombesin receptors. There are currently three family members within the bombesin receptor subfamily, which include BB1 (bombesin receptor 1) also known as NMBR (neuromedin B receptor), BB2 (bombesin receptor 2) also known as GRPR, and BB3 (bombesin receptor 3). These receptors are known as bombesin receptors since the first natural agonist found to activate these receptors was called bombesin as it was isolated from the skin of the frog *Bombina bombina*. Bombesin is an amidated tetrapeptide that is known to bind with high affinity to both BB1 and BB2 (48,49). It wasn't until much later that the natural mammalian agonist for bombesin receptors termed GRP (gastrin releasing peptide) and neuromedin B were identified (50,51). Further studies after the cloning of the BB2 receptor revealed that these endogenous agonists have higher affinity for one bombesin receptor over the other. Thus, these receptors were also named based on the preferential binding to the endogenous ligands (e.g., GRP has greater the 400 fold higher affinity for GRPR then it does for NMBR) (52).

The gene for GRPR is located on chromosome Xp22, and it encodes for a 384 amino acid GPCR that is N-linked glycosylated (53,54). Giving the mature human GRPR the apparent molecular mass of 60 $\pm$ 1 kDa. After deglycosylation of the receptor the molecular mass is 43 kDa (52,55,56). Experimental evidence supports the role of N-linked glycosylation of murine GRPR, which shares 90% homology with human GRPR, in proper intracellular sorting and membrane localization (56). Further studies utilizing murine GRPR, provide evidence that Asn^{191} glycosylation may be necessary for high affinity agonist binding and G protein coupling (57).

Though not yet tested, it is presumed that N-linked glycosylation also plays a similar role for human GRPR expression and function.

2. GRPR - Signaling and Function

In humans GRPR is expressed in variety of tissues including; GI tract (gastrointestinal) -promotes GI motility and hormone secretion, and CNS (central nervous system) - regulates satiety, memory consolidation, adult hippocampal neurogenesis, thermoregulation, and regulation of itch sensation ((58-62) and reviewed in (1)). GRPR is also expressed on immune cells where it plays a role in chemotaxis and lymphocyte function (63). In pathological condition, GRPR has also been shown to be ectopically expressed or over-expressed in variety of solid tumors including; neuroblastoma, breast, lung, prostate, and colon cancers. It is well accepted that GRPR signaling in these solid tumors promotes cancer cell proliferation ((64) and reviewed in (1,65)). However, some studies in colon cancer also implicate GRPR signaling contributing to a morphogenic phenotype (66). GRPR couples to Gq/11 heterotrimeric G proteins. GRPR activation leads to the canonical $G\alpha_q$ signaling pathway, where $G\alpha_q$ mediated activation of PLCβ (phospholipase Cβ) leads to formation of phosphoinositides and diacylglycerol with subsequent mobilization of intracellular Ca^{2+} and the activation of PKC (protein kinase C), resulting in stimulation of MAPK (mitogen activated protein kinases) signaling pathways ((52,67,68) and reviewed in (7)). Current evidence implicates $G\alpha_q$ arm of GRPR in facilitating promitogenic signaling in various solid tumors. However, certain reports indicate that MAPK signaling due to GRPR is possibly a result of GRPR mediated transactivation of EGFR (69,70). Nevertheless, it is currently not known through which mechanism(s) does GRPR activation leads to EGFR transactivation in these solid tumors? It is possible that $G\alpha_q$ arm of GRPR may be responsible for EGFR transactivation as previously suggested ((71) and reviewed in (38)). Conversely, not much is known about $G\alpha_{12/13}$

signaling downstream of GRPR in many of these malignancies. Only one report supports the role of $G\alpha_{13}$ signaling through RhoA in regulation of prostate cancer cell migration (72). Although, this study did not directly identify the RH-RhoGEF(s) involved in regulation of GRPR mediated prostate cancer cell migration.

D. <u>G12/13 family of heterotrimeric G proteins:</u>

$G\alpha_{12}$ and $G\alpha_{13}$ are ubiquitously expressed G proteins that share 67% sequence homology (reviewed in (73)). Both $G\alpha_{12}$ and $G\alpha_{13}$ undergo N-terminal palmitoylation. It is predicted that $G\alpha_{12}$ undergoes palmitoylation at the Cys^{11}, and $G\alpha_{13}$ is palmitoylated at Cys^{14} and Cys^{18} (74,75). Palmitoylation of $G\alpha_{13}$ is required for proper membrane localization and activation of RhoA signaling via p115 membrane recruitment (75). Studies have shown that many GPCRs couple to and activate more than one family of heterotrimeric G proteins. In fact, many GPCRs that couple to $G\alpha_{12/13}$ have also been shown to couple with $G\alpha_q$ (reviewed in (76,77)). Activated $G\alpha_{12/13}$ have been reported to interact with diverse set of downstream effectors such as; cadherin, radixin, Pyk2 (Proline rich tyrosine kinase 2), Btk (Burton's tyrosine kinase), PP5 (Protein phosphatase 5), AKAP-Lbc (A-kinase anchoring protein-Lbc), Hsp90 (heat shock protein 90), and integrins to bring about a variety of cellular responses as summarized in (Figure 4) ((78) and reviewed in (79,80).

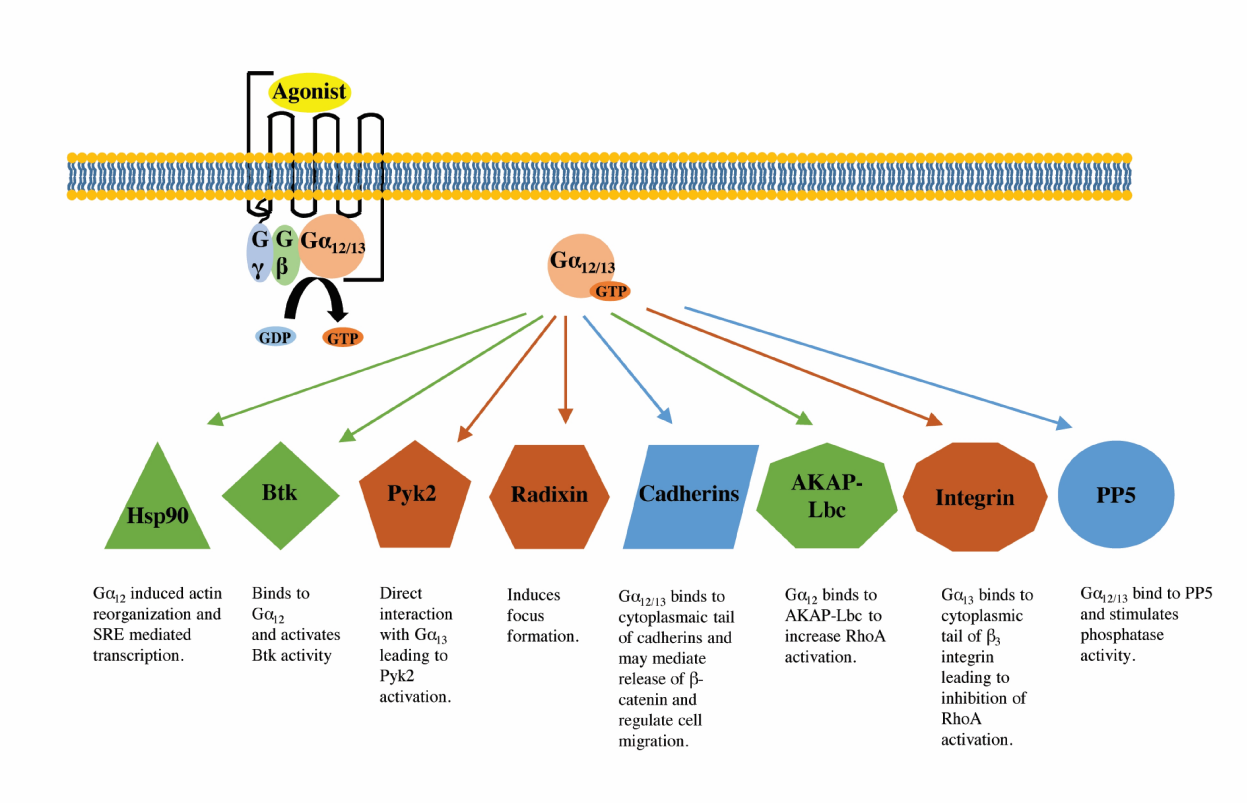


Figure 4: Interacting partners of $G\alpha_{12/13}$ subunits. $G\alpha_{12}$ interacting partners are shown by the green arrow. $G\alpha_{13}$ interacting partners are shown by Orange arrow. Interacting partners for both $G\alpha_{12/13}$ are indicated with blue arrows. See text for detail.

1. Physiological Role of $G\alpha_{12/13}$ Signaling

The signaling pathways initiated by $G\alpha_{12/13}$ have been reported to be critical for various physiological processes such as embryonic development, cell polarity and cell migration, cell growth, platelet activation, immune function, neuronal cell migration, neurite retraction, and regulation of vascular tone. The role $G\alpha_{12/13}$ play in these physiological processes is extensively reviewed by Suzuki et al. (reviewed in (81)). Here we focus primarily on the role of $G\alpha_{12/13}$ in embryonic development, cancer development and progression.

a. $G\alpha_{12/13}$ Signaling in Embryonic Development

Importance of $G\alpha_{12/13}$ signaling in embryonic development has been demonstrated in multiple model organisms. From the current evidence, it can be inferred that $G\alpha_{12/13}$-Rho signaling axis is evolutionarily conserved. In *Drosophila melanogaster*, embryos lacking expression of DRhoGEF2, a putative RH-RhoGEF, or expression of dominant negative mutant of Rho1, a homolog of RhoA, results in defective invagination and cell shape changes during gastrulation (82). It is also known that Drosophila gene, concertina, which share ~70% sequence identity with $G\alpha_{13}$, is critical for proper gastrulation, as loss of concertina expression leads to defective ventral furrow formation and embryonic lethality (83,84).

Our lab has also provided evidence for the existence $G\alpha_{12/13}$-RH-RhoGEF-Rho signaling axis in *Caenorhabditis elegans*. The $G\alpha_{12/13}$ homolog in *C. elegans*, GPA-12, was shown to interact with an RH-RhoGEF homolog, CeRhoGEF, in GPA-12 activation-dependent manner (85). Furthermore, loss of expression of GPA-12 or CeRhoGEF in *C. elegans*, results in egg laying defect and embryonic lethality (85).

In mice, $G\alpha_{13}$ gene ablation also results in embryonic lethality, as $G\alpha_{13}$ -/- mice die at E9.5. The embryonic lethality of $G\alpha_{13}$ -/- mice was primarily attributed to defect in

angiogenesis, as these embryos failed to develop a functional vascular network (86). This defect was not attributed to a defective EC (endothelial cell) differentiation, but rather postulated to be the result of defective endothelial cell migration, which is required for proper sprouting and vascular branching to form a mature vascular network. Indeed, a study utilizing EC specific $G\alpha_{13}$ -/- mice also reported to have embryonic lethality due to defective vasculature formation with mice dying between E.9.5 to E.11.5. Furthermore, these mice can be rescued with re-introduction of $G\alpha_{13}$. The data demonstrates that $G\alpha_{13}$ -/- ECs fail to undergo tubulogenesis when grown in matrigel as compared to WT ECs, indicating some form of cell migratory defect (87). Indeed, cultured mouse embryonic fibroblasts of E.8.5 embryos from $G\alpha_{13}$ -/- mice were shown to have a severe defect in cell migration in response to thrombin stimulation (86).

It is interesting to point out that $G\alpha_{12}$ -/- mice develop normally with no overt morphological or behavioral phenotype. However, $G\alpha_{12}$ -/- $G\alpha_{13}$ -/- mice seem to die earlier at E8.5 with different morphological defects from $G\alpha_{13}$ -/- mice, demonstrating that $G\alpha_{12}$ has a specific function during embryonic development. Furthermore, mice with one $G\alpha_{13}$ allele, $G\alpha_{13}$ -/+, requires at least one copy of $G\alpha_{12}$ -/+ for proper development. Indicating that $G\alpha_{12}$ does have some distinct and some over-lapping functions with $G\alpha_{13}$ in mouse embryonic development (88).

Overall, these reports provide evidence for that G12/13-Rho axis is conserved in these model systems and is critical for normal embryonic development in-part by regulation of cell shape changes and coordinated cell migration.

b. $G\alpha_{12/13}$ in Cancer

Evidence from multiple laboratories have demonstrated that over-expression of WT $G\alpha_{12/13}$ and GTP-ase deficient mutants of $G\alpha_{12}$ ($G\alpha_{12}$ Q229L) or $G\alpha_{13}$ ($G\alpha_{13}$ Q226L) promotes potent cellular transformation as observed by focus formation assays in NIH 3T3 cells ((89-91)

and reviewed in (92)). These early studies indicated that $G\alpha_{12/13}$ may play a role in cancer biology. Indeed, several GPCRs known to couple to $G\alpha_{12/13,}$ although not exclusively, have been reported to be over-expressed in various malignancies. The contributions to cancer progression made by these GPCRs, through $G\alpha_{12/13}$ signaling have been reported. Ample evidence indicates that PAR1(protease activated receptor-1), which is found to be over-expressed in invasive breast and prostate cancers, signals through $G\alpha_{12/13}$ mediated Rho activation to regulate breast and prostate cancer cell migration and invasion ((24,25,93) and reviewed in (94)). In prostate cancer, ET_{A-B} (endothelin receptors), BB_2, and TP receptor (thromboxane A_2 receptor) signal through $G\alpha_{12/13}$ to increases prostate cancer cell motility and invasion (24,72). Along with the above mentioned receptors, other GPCRs such as LPA_{1-3} (lysophosphatidic acid receptors), $S1P_{1,3}$ (sphingosine 1-phosphate receptors), and CXCR4 (C-X-C chemokine motif receptor 4) have also been shown to contribute to cancer cell migration and invasion of solid tumors (i.e., pancreatic, breast, lung, prostate, colon and ovarian cancers) ((23,95-97) and reviewed in (38,39)). Thus, overwhelming evidence supports the hypothesis that many $G\alpha_{12/13}$ coupled GPCRs signal via $G\alpha_{12/13}$-Rho axis to regulate cancer cell migration and invasion (Figure 5).

However, it should be noted that not all $G\alpha_{12/13}$ coupled GPCRs positively regulate cancer cell migration. As $S1P_2$ mediated activation of $G\alpha_{12/13}$-Rho axis results in inhibition of melanoma and glioma cancer cell migration and invasion (98-100). Thus, the role of GPCR-$G\alpha_{12/13}$-Rho axis in regulation of cancer cell migration maybe context specific, depending upon factors such as cell type and receptor sub-types.

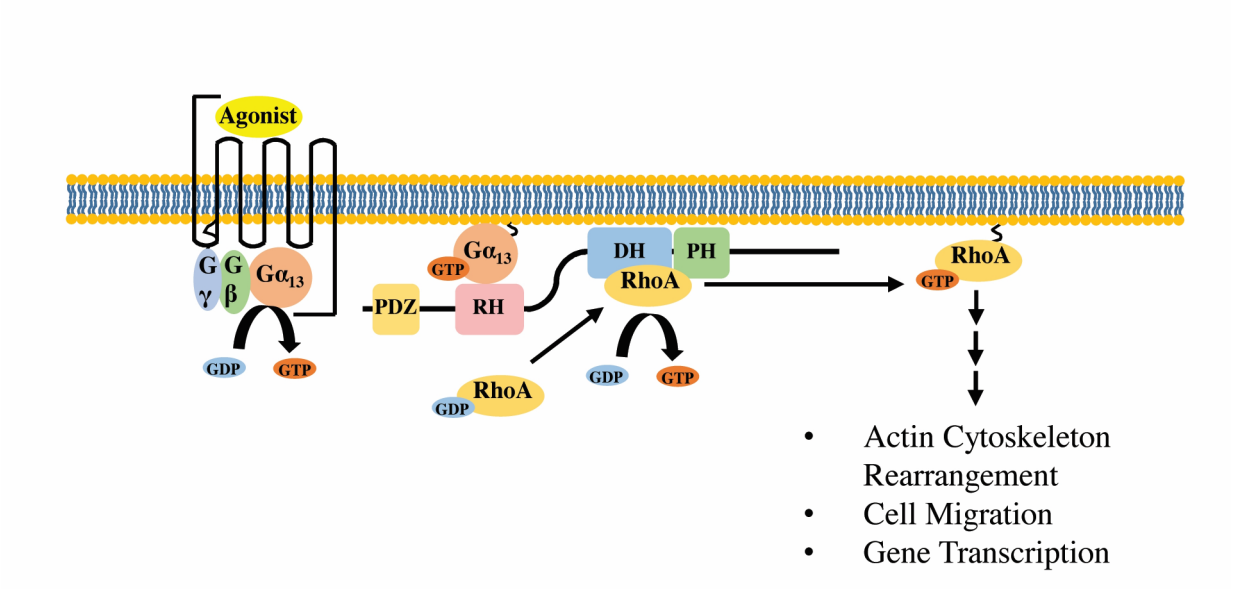


Figure 5: Regulation of RhoA signaling downstream of GPCRs coupled to $G\alpha_{13}$. Upon agonist binding and receptor activation, $G\alpha_{13}$-GTP interacts with RH domain of RH-RhoGEFs (depicted here is PRG). This interaction along with membrane localization of RH-RhoGEFs leads its GEF activity towards Rho proteins, which is accomplished by the DH-PH domains of RH-RhoGEFs. RhoA-GTP is then able to interact with its effectors to bring about a cellular response. See text for detail.

c. $G\alpha_{12/13}$ Rho-Independent Pathways in Cancer

In comparison to our current understanding of $G\alpha_{12/13}$-Rho mediated pathways regulating cancer cell migration and invasion, not much is known about $G\alpha_{12/13}$ signaling via the other effectors and their role in cancer cell proliferation or invasion. The growth-promoting signaling nodes regulated by $G\alpha_{12/13}$ are just now being identified and reported. In SCLC, $G\alpha_{12/13}$ have been implicated in regulation of cancer cell proliferation. The data demonstrates that silencing $G\alpha_{12/13}$ subunits independently or concomitantly in H69 and H209 SCLC cell lines leads to decreased cancer cell proliferation. Indeed, knockdown of both $G\alpha_{12/13}$ subunits in H69 cells nearly abolished tumor growth in s.c (subcutaneous) tumor xenograft mouse model (101). However, the mechanism(s) by which $G\alpha_{12/13}$ regulates SCLC cell proliferation and survival was not elucidated in this study. It is possible that this defect in tumor growth can be explained by a recent report that implicates $G\alpha_q$ and $G\alpha_{12/13}$ signaling downstream of GRPR, to activate Shh (Sonic hedgehog) signaling pathway. It is demonstrated that increased Shh production, in response to GRPR activation, acts through an autocrine/paracrine signaling mechanism to increase SCLC cell proliferation. Additionally, other $G\alpha_{12/13}$ coupled GPCRs were also shown to up-regulate Shh production and increase activation of Gli transcription factors that promotes SCLC cell proliferation (102). Based on these data, it is tempting to hypothesize that GPCRs coupled to $G\alpha_{12/13}$ may contribute to increased SCLC cell proliferation in-part by regulation of Shh pathway, and thus may explain the near complete loss of SCLC tumor growth when $G\alpha_{12/13}$ expression is silenced. It would be of interest to verify if GRPR-$G\alpha_{12/13}$ signaling up-regulates Shh pathway in other solid malignancies promoting increased cancer cell proliferation.

In ovarian cancer, $G\alpha_{12}$ was shown to promote ovarian cancer cell proliferation in response to LPA stimulation (103). The data suggests that LPA, through yet unidentified LPA

receptor(s), signals through $G\alpha_{12}$-Ras-Erk (extra cellular-signal regulated kinase) pathway to stimulate phosphorylation of CREB (cAMP-response element binding) protein at position 133, leading to transcriptional activation of genes governing cell survival and proliferation (104). Thus, current evidence indicates that LPA signaling in ovarian cancer may promote cancer development and progression through both $G\alpha_{12/13}$-Rho dependent and independent mechanisms (95,103).

In OSCC (oral squamous cell carcinoma), $G\alpha_{12}$ has been shown to be over-expressed and is correlated with increased invasive phenotype and poorer patient prognosis (105). It is proposed that $G\alpha_{12}$ regulates OSCC invasive phenotype through transcriptional regulation of proinflammatory cytokines, IL-6 and IL-8 (interleukin), both of which have been found to be elevated in serum, tumor, and saliva of OSCC patients ((106-108) and reviewed in (109)). IL-6 and IL-8, along with other proinflammtory cytokines, have been implicated in OSCC development. However, we currently do not know which $G\alpha_{12/13}$ coupled receptors or the signaling pathways operated by $G\alpha_{12}$ are involved in up-regulation of IL-6 and IL-8 expression. Non-the-less, based on these reports, it is clear that $G\alpha_{12/13}$ are involved not only in cancer progression through increased cell migration and invasion, but also may play a role in tumor development by transcriptional regulation of diverse set of genes that contribute to tumorigenesis. Thus, it is imperative to identify these pathways for possible discovery of novel therapeutic targets.

d. Somatic Mutations of $G\alpha_{12/13}$ in Cancer

Mutations in $G\alpha_{12/13}$ subunits have not yet been reported to occur at high frequency in solid tumors. In-contrast, ~15% of lymphomas have been reported to carry predominantly inactivating mutations in the $G\alpha_{13}$ gene, GNA13 (110). Specifically, mutations in GNA13 occur with frequency of (~20%) in DLBCL (diffuse large B-cell lymphoma), and occurring at a greater frequency in a molecular subtype of DLBCL, GCB (germinal center B-cell)

lymphoma subtype, in which 33% of samples were reported to have GNA13 mutations (111). Although, still required to be confirmed in a larger study, this report also finds GNAI2, gene for $G\alpha_{i2}$ subunit, and S1PR2 ($S1P_2$) genes to be mutated with higher frequency in the GCB subtype. This is an interesting observation, as $S1P_2$ is known to signal through $G\alpha_i$ and $G\alpha_{12/13}$ in B-cells to regulate Rho-mediated B-cell homing, which is required for proper GC (germinal center) formation with-in the lymphoid follicle (reviewed in (112)). Furthermore, $S1P_2$ signaling through $G\alpha_{13}$-Rho axis, is also implicated in inhibition of PI3K/AKT signaling to regulate B-cell proliferation. It is proposed that $S1P_2$-$G\alpha_{13}$-Rho signaling acts to dampen pro-survival signaling inputs received by B-cells in GC from B-cell receptors and chemokine receptors that are coupled to $G\alpha_i$ to regulate B-cell proliferation ((113) and reviewed in (112)). Thus, the inactivating mutations in GNA13 or acquisition of gain of function mutation in GNAI2 in DLBCL and particularly in GCB subtype, would result in overall increased AKT signaling and promoting increased B-cell survival and proliferation (111).

E. Somatic Mutations of $G\alpha_{q/11}$ and $G\alpha_s$ Subunits in Cancer

It is now becoming evident that Gα subunits are often mutated in various solid malignancies. Unlike $G\alpha_{12/13}$ subunits, $G\alpha_s$ and $G\alpha_{q/11}$ are often mutated in colon cancer, pituitary tumors, thyroid adenomas, pancreatic tumors, ocular melanomas, and subset of cutaneous melanomas. Mutations in GNAS (gene for $G\alpha_s$) occur most frequently in pituitary tumors, thyroid adenomas, subset of pancreatic carcinomas, and hepatocellular carcinomas. Most common mutation hotspots for GNAS occur at R201 and Q227, which results in decrease of the intrinsic GTP hydrolysis leading to prolonged $G\alpha_s$ signaling (reviewed in (40)).

Mutations in GNAQ (gene for Ga_q) and GNA11 (gene for $G\alpha_{11}$) occur most frequently in ocular melanomas (~66%). It should be noted that $G\alpha_q$ and $G\alpha_{11}$ in these cancers have shown to

regulate the same signaling pathways and these mutations are mutually exclusive. Mutations in GNAQ and GNA11 have been classified as driver mutations in uveal melanomas where up to 83% of tested samples carried mutations in one of the two genes (114). *In vivo* mouse models revealed that these mutations increased the metastatic potential of these tumors resulting from constitutive activation of pathways regulated by $G\alpha_{q/11}$. The mutations in GNAQ and GNA11 most commonly occurred at Q209 or R183, which resulted in impaired GTP hydrolysis (reviewed in (40)). For more information regarding mutations in Gα subunits and GPCR signaling in cancer please review ((40,115)).

F. <u>RhoGTPases</u>

RhoGTPases belong to a large Ras superfamily of small monomeric GTPases. Within RhoGTPase family there are three well characterized members with multiple isoforms; Rho (RhoA, RhoB, and RhoC), Rac (Rac1, Rac2, and Rac3), and cdc42 (Cdc42hs and G25K). These small monomeric GTPases function as bi-molecular switches, which are activated when GTP bound and inactivated in GDP bound form. All RhoGTPase family members contain an N-terminal domain, consisting of switch I and switch II regions, an effector binding domain, and C-terminal CAAX box which undergoes isoprenylation (reviewed in (116,117)). The activation of RhoGTPases is catalyzed by Dbl family of GEFs that catalyze the exchange of GDP for GTP. RhoGTPases are inactivated by RhoGAPs, which accelerate the slow intrinsic GTPase activity. Another form of negative regulation of RhoGTPases is the one afforded by family of proteins known as RhoGDIs (Rho guanine nucleotide disassociation inhibitors). RhoGDIs interact with Rho-GDP bound forms of RhoGTPases to sequester them in an inactive state within the cytosol, in-part by interacting with the isoprenylated C-terminal tail of RhoGTPases. The interaction with

RhoGDIs and RhoGTPases also have been shown to inhibit GDP disassociation and GEF mediated nucleotide exchange ((118) and reviewed in (119,120)).

The N-terminal region on RhoGTPases is the site of GDP/GTP exchange. Significant conformational rearrangement occurs within the switch I and switch II regions of RhoGTPaes during this process ((121,122) and reviewed in (119)). The nucleotide exchange occurs as the DH domain of RhoGEFs interact with the switch regions of RhoGTPases, leading disruption of interactions with GDP and Mg^{2+}, resulting in a transient exposure of nucleotide binding site on RhoGTPase. This solvent exposed surface is readily occupied by GTP-Mg^{2+}, which is highly abundant in the intracellular milieu, and followed by release of GTP bound RhoGTPase. The activated RhoGTPase in-turn interacts with its downstream effectors to bring about a variety of cellular responses such as, regulation of actin cytoskeleton, gene transcription, and cell cycle progression. RhoA has been implicated in regulation of numerous downstream effectors such as mDia (mammalian diaphanous homolog), ROCK (ROCK I and ROCK II isoforms), PKN (protein kinase N) and citron kinase (117).

One of the most well characterized downstream effector of RhoA is a serine/threonine kinase, ROCK. It has been proposed that RhoA-GTP interacts with RBD (Rho binding domain) of ROCK, resulting in disruption of autoinhibitory interactions within ROCK and leading to the exposure of the kinase domain. Specifically, RhoA-GTP interacts with ROCK via switch I and switch II regions of RhoA. It is proposed that the RhoA amino acids 23-40 and 75-92 are critical for RhoA interaction with and activation of ROCK ((123,124) and reviewed in (117)). It has been demonstrated that mutations of the amino acids Phe^{39} and Glu^{40} on RhoA leads to loss of interaction with ROCKI (123,125). Whereas, the secondary interactions with amino acids Asp^{87} and Asp^{90} within the loop 6 of RhoA were found to be critical for both interaction and activation

of ROCK (123). Once ROCK is activated, it initiate a signaling cascade, via phosphorylation of variety of downstream effectors, that regulates cell migration and invasion, and gene transcription ((126,127) and reviewed in (17)).

G. RhoA and Colon Cancer

RhoGTPases have been shown to have transforming potential as demonstrated in NIH 3T3 cells. RhoA is over-expressed in colon, breast, and lung cancers (18). Gain of function mutations resulting in increased RhoA activity have not yet been identified to occur at high frequency in solid tumors, with one exception being in diffuse type-gastric carcinoma (128). However, the components of the signaling pathways that regulate RhoA activity downstream of GPCRs (such as $LPA_{1\text{-}2}$, S1PR, ET_A, GRPR) and RTKs (EGFR) have been reported to be over-expressed and over-activated in many if not all of these malignancies. Thus, it may not be necessary for cancer cells to acquire activating mutations in RhoA, as over-activation of pathways regulating its activity may suffice for cancer progression. Activation of RhoA functions as a focal event, from which signaling through its downstream effectors, leads to various cellular responses important for cancer progression such as increased cancer cell migration, invasion, proliferation, and angiogenesis (reviewed in (17,129,130)). It is important to note that RhoA acts in coordination with other RhoGTPases to regulate these complex cellular processes, such as cell migration, as indicated by recent studies (131,132).

H. RH-RhoGEFs

As previously stated, $G\alpha_{12}$ and Ga_{13} have been shown to regulate actin polymerization, stress fiber and focal adhesion formation (133-135). However, the complete signaling mechanisms by which $G\alpha_{12/13}$-Rho signaling axis was able to regulate the actin cytoskeleton was unknown for some time. Identification of p115 provided the missing link between G12 family and regulation of

actin cytoskeleton through RhoA activation (10-12). These early studies showed that G12 family can bind to p115 via its RH-domain. It was reported that even though *in vitro* both $G\alpha_{12}$ and $G\alpha_{13}$ can bind to p115's RH domain, only $G\alpha_{13}$ has thus far been shown to positively regulate p115 activity *in vitro* (11). However, p115 can act as a negative regulator for both $G\alpha_{12}$ and $G\alpha_{13}$ by accelerating the intrinsic hydrolysis of GTP to GDP, and thus terminating $G\alpha_{12/13}$ signaling (12). Shortly after identification of p115, two other RH-RhoGEFs were identified; PRG and LARG. These three RhoGEFs make up the small subfamily of RH-RhoGEFs that belong to a larger Dbl family of RhoGEFs. This large Dbl family of proteins, which has 70 members, share a common DH (Dbl homology) domain and an adjacent PH (Pleckstrin homology) domain that catalyze the exchange of GDP for GTP on small monomeric RhoGTPases (reviewed in (119)). All three RH-RhoGEF family members have been shown to be specific GEFs for the three isoforms of Rho (RhoA, RhoB, and RhoC) and have no reported activity for Rac1 or CDC42 (136). Indeed, *in vitro* data demonstrates that PRG and LARG, and to lesser extent p115, are very efficient GEFs for RhoC (136). However, it is at this time unclear if these RH-RhoGEFs activate these other Rho isoforms, particularly RhoC, in physiological context ((136,137) and reviewed in (138)). Although, it has been suggested that ErbB-2 over-expressing breast cancers and breast cancer cell lines, which signals through plexin-B1, leads to RhoA and RhoC activation presumably via PRG and/or LARG (139).

Both PRG and LARG have been shown to activate RhoA downstream of $G\alpha_{12/13}$ coupled receptors in cell based assays (8,9,13,14). However, only LARG and p115 activity has been shown to be regulated by $G\alpha_{13}$ *in vitro*. The RH domains of LARG and p115 have also been shown to be specific GAPs for both $G\alpha_{12}$ and $G\alpha_{13}$ (12,140). Furthermore, only LARG's GEF activity has been shown to be regulated by $G\alpha_{12}$ *in vitro*. In order for LARG to be receptive to $G\alpha_{12}$ mediated GEF

activation, LARG must be phosphorylated by Tec kinase (140). The common domain structures shared by RH-RhoGEFs are shown in (Figure 6).

I. PDZ-RhoGEF

1. Identification of PRG

The first report characterizing PRG came from Fukuhara et al. PRG was identified as a candidate RhoGEF after searching the DNA database for novel proteins that had high degree of sequence similarity with DH domains of GEFs for Rho-GTPases. This search identified a novel protein which contained a DH domain, that had a high degree of sequence homology with DH domains of p115 (53% identity and 74% similarity) and DRhoGEF2 (39% identity and 64% similarity). This candidate protein also contained an RH domain like p115, and an N-terminal PDZ domain that is also found in DRhoGEF2. Since p115 was previously shown to be a specific GEF for Rho, the protein was termed PDZ-RhoGEF. PRG was found to be widely expressed in human tissues, with highest expression occurring in the brain, testis, heart, placenta, and spleen with lower level of expression observed in prostate, lung and colon tissues. Cell based and biochemical assays revealed that PRG activates Rho signaling through DH-PH domains. It was also discovered that much like p115, PRG can also interact with $G\alpha_{12}$ and $G\alpha_{13}$ in cells via the RH domain. This interaction was shown to positively regulate PRG activity and thus lead the authors to propose that PRG, along with other RH-RhoGEFs, may serve as a critical molecular link between activated GPCRs coupled to $G\alpha_{12/13}$ to Rho signaling (13).

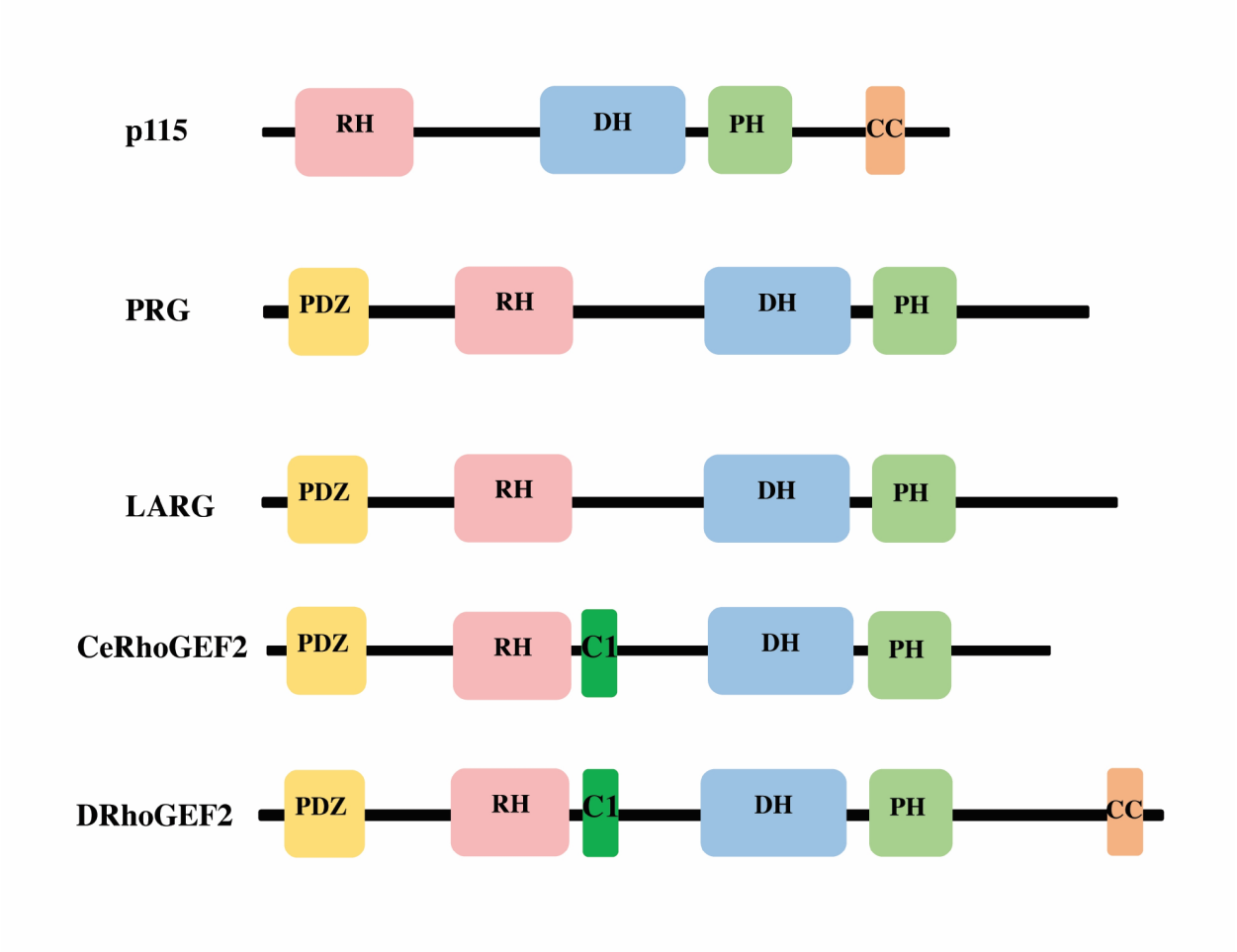


Figure 6: Domain structures of RH-RhoGEFs. Depicted here are the domain structures of three most well characterized mammalian RH-RhoGEFs (p115, PRG, and LARG). Also shown are the domain structures of *C. elegans* homolog CeRhoGEF2, and *D. melanogaster* homolog DRhoGEF2. All the proteins within this family contain the RH domain which is able to interact with $G\alpha_{12/13}$-GTP bound subunits and the interactions has been shown to positively regulate the RhoGEF activity via the DH-PH domains. C1 (protein kinase C conserved region 1) domains in CeRhoGEF2 and DRhoGEF2 have been reported to interact with diacylglycerol and phorbol esters. See text for details.

2. Structure and Function of PRG

a. PDZ Domain

The N-terminal PDZ domain of PRG and LARG share ~75% sequence identity (141). The PDZ domain of PRG and LARG is known to interact with the type-I-PDZ- binding motifs (S/T-X-ϕ-COOH where ϕ is any hydrophobic amino acid) on membrane bound proteins. One of the most well characterized binding partner for the PDZ domain of PRG is plexin-B1. Several studies have provided evidence that PRG constitutively binds to the C-terminal tail of plexin-B1, and that activation of plexin-B1 by Sema4D (Semaphorin 4D), leads to RhoA activation in neuronal cells, cancer cells, and ECs (142-144). PRG mediated RhoA activation downstream of Sema4D ligated plexin-B1 is implicated in neuronal growth cone collapse, increased cancer cell migration, and to promote a proangiogenic response through regulation of EC cell migration and tube formation (143,144). Current evidence indicates that activation of plexin-B1/ErbB-2 receptor complex leads to tyrosine phosphorylation of plexin-B1, serving as a docking site for the SH2 domain of PLCγ (phospholipase Cγ). Once recruited into the plexin-B1 receptor complex, PLCγ via its SH3 domain interacts with the C-terminal proline rich region of PRG, releasing inhibitory intra- and/or intermolecular interactions culminating in PRG activation (142,143). Additional membrane bound interacting partners for the PDZ domains of PRG and LARG have been identified and are summarized in (Table 2) (145-147).

b. RH Domain

C-terminal to the PDZ domain on PRG and LARG is the RH domain. Crystal structures of the RH domain of p115 and PRG in complex with $G\alpha_{13}$-GDP-AlF_4^- and $G\alpha_{13}$-GTPγS have been solved (16,148). These structures reveal that $G\alpha_{13}$ makes bivalent interactions with RH domains of these two RH-RhoGEFs. The first of these interactions occurs outside the RGS box,

involving the N-terminal acidic motif (EEDY in PRG and EDEDF in p115), which is responsible for the GAP activity of p115 towards $G\alpha_{13}$, and the preceding IIG^{308} motif making direct contact with $G\alpha_{13}$ alpha helical domain. The second binding interface of PRG with $G\alpha_{13}$ occurs with the C-terminal extension of the RH domain with classical effector binding site on $G\alpha_{13}$. Structural studies have shown that the RH domain of PRG is able to maintain $G\alpha_{13}$-GDP in an active conformation, which indicates that perhaps hydrolysis of $G\alpha_{13}$-GTP to GDP may not be enough to terminate $G\alpha_{13}$ signaling and so possibly other factors, such as sequestration of $G\alpha_{13}$ by $G\beta\gamma$ subunits, may be required to terminate this signaling pathway. It is currently unknown exactly how the interaction between $G\alpha_{13}$ and the RH domain of PRG leads to its activation. It is possible that $G\alpha_{13}$ may also make additional contacts, outside the RH domain of these RH-RhoGEFs. A recent study by Chen et al., suggests that $G\alpha_{13}$ makes contacts with the RH domain of p115, and also interacts with the DH domain opposite to the RhoA binding interface. It is proposed that the interaction between $G\alpha_{13}$ and RH domain of p115 facilitates the subsequent interaction of $G\alpha_{13}$ with DH domain, culminating in $G\alpha_{13}$ mediated p115 activation (149). Our lab has also shown that $G\alpha_{13}$ makes multiple contacts with LARG through its RH, DH-PH domains and C-terminus. Thermodynamic studies show that LARG undergoes significant conformational rearrangement as the RH domain interacts with $G\alpha_{13}$, leading to activation of the RhoGEF (81). Thus, it is most likely that activation of PRG by $G\alpha_{13}$ may involve multiple interactions between $G\alpha_{13}$-PRG (facilitated by the RH domain), PRG with plasma membrane, and PRG with other currently un-identified binding partners, culminating in the loss of inhibition and RhoGEF activation.

Interacting Partners	Mechanism of Interaction	Functional Impact	Reference
Plexin-B1	PDZ domain interaction with C-terminus of Plexin-B1 (T-D-L-COOH). This meets the requirements of Type-I PDZ domain binding motif with consensus sequence (S/T-X-ϕ-COOH).	Plexin-B1 is found to be stably interacting with receptor tyrosine kinase ErbB-2 in over-expressed system with HEK293 cells, primary hippocampal neurons, and breast cancer cell line MCF-7. Sema4D binding to plexin-B1 activates tyrosine kinase activity of ErbB-2 leading to phosphorylation of both plexin-B1 and ErbB-2. Tyrosine phosphorylation of plexin-B1 at pY1708 and pY1732 is required for PLCγ binding to plexin-B1 via its SH2 domain. PLCγ interacts with PRG potentially via its SH3 domain, leading PRG activation. Evidence of this mechanism comes from studies conducted in MCF-7 cells, HEK293 cells, and primary hippocampal neurons. Plexin-B1/PRG mediated RhoA activation is reported to be critical for axonal growth cone collapse, MCF-7 cell migration, and has proangiogenic effect by promoting endothelial cell migration and tubulogenisis.	142-144
IGF-1 Receptor	PDZ domain interacts with C-terminus of IGF-1 receptor (S-T-C-COOH).	IGF-1R through its interactions with LARG and PRG regulates IGF-1 mediated RhoA signaling in MDCKII cells. The mechanism by which IGF ligation activates RhoGEF is unknown.	145
ABCA1	PDZ domain interacts with C-terminus of ABCA1 (S-Y-V-COOH).	ABCA1-PRG interaction mediates ApoA-I stimulated RhoA activation leading to stabilization of ABCA1 at plasma membrane and in regulation of cholesterol efflux. Thus, ABCA1-PRG complex may play an important role in reverse cholesterol transport. The mechanism by which ApoA-I binding to ABCA1 stimulates RhoGEF activation is unkown.	146
LPA_{1-2} Receptor	PDZ domain interacts with C-terminus of LPA_1 (S-V-V-COOH), and C-terminus of LPA_2 (S-T-L-COOH).	This study utilizing an over-expressed system in HEK293 cells suggests that $LPA_{1,2}$ interaction with PRG and LARG seem to be required for maximal RhoA activation in response to LPA stimulation.	147

Table 2: Interacting partners of PDZ domain of PRG. List of membrane bound proteins that interact via their type-I-PDZ binding motif with the PDZ domain of PRG. Summarized here are the proposed functional and physiological impact of this interaction.

c. DH-PH Domains of PRG

The crystal structure of the DH-PH domains of PRG (referred as DH-PH^{PRG}) in complex with nucleotide free RhoA has been solved, and subsequent biochemical experiments have identified the regions critical for effector interaction, enzymatic activity, and residues important for RhoA selectivity. The DH-PH^{PRG} are similar to the DH-PH domains of the other solved structures for Dbl family members such as Dbs, TIAM1, and Intersectin. The DH domain of PRG is an elongated α-helical bundle, whereas the PH domain is in an anti-parallel β-sandwich with a short C-terminal α-helix (121). The interaction between RhoA and DH-PH^{PRG} leads to conformational changes within the two functionally important switch regions of RhoA. RH-RhoGEFs are known to be selective GEFs for Rho. The structural basis for the selectivity of PRG for RhoA was revealed by multiple structural and biochemical experiments. The crystal structure reveals multiple interactions that are unique to RhoA and may serve as selectivity determinant of PRG for RhoA. Indeed follow-up biochemical studies have shown that a cluster of solvent accessible amino acids on the face of RhoA- Arg^{5}, Asp^{45}, Glu^{54}, and Asp^{76}, intimately interact with several charged residues within the DH domain just C-terminal to CR3 (conserved region 3) - Arg^{867}, Arg^{868}, Arg^{872}, and Asp^{873}. These residues have been shown through mutagenesis studies to be critical selectivity determinants for RhoA by PRG (137).

i. Mechanism of DH-PH^{PRG} Catalyzed Nucleotide Exchange on RhoA

DH-PH^{PRG} catalyze nucleotide exchange by participating in highly specific interactions with RhoA. In a study utilizing NMR spectroscopy to measure GEF mediated nucleotide exchange, it was revealed that Arg^{868} near the CR3 of DH domain, which is one of the RhoA selectivity determinant residues, is also required for efficient GEF activity. Along with Arg^{868}, Glu^{741} found within the CR1 of DH domain also plays a critical role in GEF catalysis, as

the mutant E741A of DH-PH^{PRG} had nearly complete loss of GEF activity (121,137,150). These biochemical studies revealed three very interesting observations regarding PRG mediated guanine nucleotide exchange. First observation is that PRG's PH domain is involved in not only interaction with RhoA, via RhoA-Glu^{97} with Ser^{1065} and Asn^{1068} of PH domain, but also this relatively minor interaction is important for efficient nucleotide exchange. As the RhoA E97A mutant was observed to have 10 fold decrease in RhoA activation by DH-PH^{PRG}, in comparison to WT RhoA. This observation is made more interesting as the structure for DH-PH^{LARG} in complex with RhoA also reveals this interaction to be conserved with LARG-Ser^{1118} residue interacting with RhoA-Glu^{97} (151). However, the E97A mutation on RhoA did not substantially affect DH-PH^{LARG} ability to activate the mutant RhoA. This is consistent with other observations that the PH domain of LARG does not make great contributions to RhoA activation at least in *in vitro* studies (150,151). Second interesting observation is that PRG PH domain can also interact with activated RhoA bound to GTPγS. Structural and biochemical studies provide evidence that the hydrophobic patch on the PH domain interacts with switch regions on RhoA-GTPγS. The physiological significance of this interaction is not yet clear. Current evidence suggests that RhoA-GTP interaction with PH domain of PRG acts as a positive feedback mechanism, but not by regulating the intrinsic catalytic GEF activity. But rather, it is proposed that RhoA-GTP interaction with PH domain of PRG, helps localize PRG to the plasma membrane, allowing for efficient interaction with substrates and further enhancing sensitivity to other stimuli (i.e., activated $G\alpha_{12/13}$ subunits). This may contribute to a robust and localized activation of RhoA that is required for well-coordinated cellular functions like cell migration. It is interesting to note that the other RH-RhoGEF family members PH domains have also been demonstrated to interact with RhoA-GTP (152,153). The third interesting

observation is that PRG acts as a positive regulator that drives the equilibrium towards activation of RhoA by preferentially catalyzing exchange from GDP to GTP (150).

d. C-terminal Region

PRG C-terminal region spans from amino acids 1080 to 1522. Although, the C-terminal region has no predicted secondary structure, current evidence indicates that it still is an important region by which PRG activity may be regulated. More specifically the C-terminal region of PRG, encompassing amino acids 1181-1522, has been found to be both necessary and sufficient to promote homo- and hetero-oligomeric interactions with its self and LARG *in vivo*. LARG has also been shown to mediate homo- and hetero-oligomeric interactions with PRG via its C-terminal region. In contrast to PRG and LARG, p115 has been reported to only form homo-oligomeric interactions via its C-terminal region (154). It is interesting to note that only p115 and its murine ortholog Lsc have a predicted coiled-coiled domain located within its C-terminal region that is responsible for the homo-oligomeric interactions (155). Number of biochemical and cell based studies were carried out to examine if the oligomerization of these GEFs, via their C-terminal region, has a functional impact on their GEF activity. These studies revealed that the C-terminal truncation of PRG had no effect on its GEF activity *in vitro*. However, these mutants of PRG, LARG, and p115 when expressed in cells resulted in an increased RhoA activation as measured by SRE (serum response element) transcriptional activity and elevated accumulation of endogenous RhoA-GTP, as measured by GST-RBD-RhoA pulldown assay (154,156). Furthermore, over-expression of these C-termini truncated mutants had increased focus forming activity in NIH 3T3 cells when compared to over-expression of WT GEFs (154). These data suggest that C-termini mediated oligomerization may be inhibitory in nature. However, the mechanism by which the oligomerization has an inhibitory impact *in vivo* is not yet clear. It is

possible that there are other yet to be identified inhibitory molecules, whose interaction with these GEFs is mediated by oligomerization, resulting in restriction or dampening of the basal RhoGEF activity until a specific signal activates it. The physiological significance of homo- or hetero-oligomerization of PRG with LARG is not yet completely clear. A recent study, utilizing *in vitro* organ cultured blood vessels showed that stimulation with agonists, TXA_2 (thromboxane A_2) and ET-1 (endothelin-1), resulted in co-recruitment of LARG and PRG to the plasma membrane. The co-recruitment and co-activation of these two GEFs was essential for full activation of Ca^{2+} sensitized force that mediates smooth muscle cell contraction leading to vascular constriction (157). This study along with previous studies indicates that perhaps oligomerization functions to inhibit basal GEF activity by retaining them in the cytosol, and that upon a sufficient signal, these GEFs are translocated to the plasma membrane where possibly through interactions with other regulatory molecules, relieves the autoinhibitory interactions of the C-terminus. However, it is not yet clear if PRG oligomerization occurs and plays a physiological role in other tissue types.

3. PRG in Physiology

a. Embryonic Development

The first evidence for developmental role for PRG came from studies conducted in *Drosophila melanogaster* model system. These studies identified a RhoGEF in *Drosophila*, DRhoGEF2, which contained all the conserved domains and shared high sequence similarity within these domain structures with both human ARHGEF11 and zebra fish ortholog arhgef11. These early studies found that DRhoGEF2 is involved in regulation of cell shape changes during gastrulation by regulating actinomyosin contraction in epithelia of developing embryos (82,158,159). Similarly, loss of function studies carried out by Panizzi et al., also revealed an important role for this RhoGEF in zebra fish embryonic development. It was observed that

inhibiting arhgef11 expression and function lead to complex defects in embryonic development such as ventral body curvature, enlargement of brain ventricles, development of pericardial edema, and distention of pronephros at various time points after fertilization which ultimately lead to embryonic lethality around 4 days post fertilization (160). Some of these complex developmental defects were attributed to the loss of arhgef11 in ciliated epithelia leading to the defect in establishment of left-to-right asymmetry, and development of cysts in pronephric ducts. It is an interesting observation that the use of dominant negative form of arhgef11 lead to defect in establishment of left-to-right asymmetry and resulted in cardia bifida. This is interesting because embryos with loss of arhgef11 expression also had defect in left-to-right asymmetry, but they did not develop cardia bifida. However, other reports looking downstream of arhgef11, utilizing loss of function studies with Rho and ROCK in zebra fish, also reported to cause cardia bifida (161,162). Furthermore, a recent report that implicates $S1P_2$-$G\alpha_{13}$ signaling through an un-identified RH-RhoGEF, possibly combinations of RH-RhoGEF(s), is required for proper cardiac development (163). Overall, these studies indicate that Gα13-RH-RhoGEF-Rho signaling downstream of GPCRs plays a critical role in embryonic development in these model systems.

In contrast to the embryonic lethality observed with loss of arhgef11 expression in zebra fish, the ablation of PRG gene in mice did not result in any overt phenotypes. Similarly LARG KO (knockout) mice also do not have any overt phenotypes even though they are less viable, as small percentage of them make it to full term. The reasons as to why not all LARG KO mice make it to full term are not completely understood. However, mice with combined PRG and LARG KO have complex developmental defects resulting in early embryonic lethality around E10.5 (9). The double KO embryos are smaller, less developed, with enlarged pericardial sac. Early embryonic lethality was attributed to defective vasculature development as PECAM-1 whole

mount staining revealed partial branching failure in cranial vessels, and less developed vascular plexus within the yolk sac. Furthermore, vascular staining for CD34, clearly revealed decreased vessels in the labyrinth within the double KO placenta, indicating that the defect in formation of vascular network may not meet the nutrient demands of the developing embryo. It is interesting to note that the double KO mice die at E10.5, one day later than $G\alpha_{13}$ KO mice (at E9.5), which also die due to defective angiogenesis (86). Taken together, these studies demonstrate that the $G\alpha_{13}$-RH-RhoGEF-Rho axis is critical for early embryonic development in variety of organisms.

b. <u>Regulation of PRG Activity by Phosphorylation</u>

PRG was the first RH-RhoGEF reported to be regulated by tyrosine phosphorylation. Both PRG and LARG are tyrosine phosphorylated by FAK upon PAR1 activation in HEK293T cells. The report provides evidence that the tyrosine phosphorylation occurs in the C-terminus of PRG, and that this phosphorylation positively regulates GEF activity (164). Furthermore, existing data demonstrate that PRG and LARG tyrosine phosphorylation is sufficient for positive regulation of its GEF activity independently of $G\alpha_{12}$ or $G\alpha_{13}$ (164). Current evidence suggests that C-terminal domains of LARG and PRG mediate their homo- and hetero-dimerization leading to inhibition of GEF activity (157,165). Thus, it is possible that phosphorylation of the C-terminal portion of LARG and PRG disrupts inhibitory dimerization, and leads to their activation (157,165). Importantly, p115 does not have the similar sequence homology in its C-terminal fragment suggesting that this mode of regulation is unique for PRG and LARG. In the initial study, the impact of GEF phosphorylation on cell migration was not assessed. However, study by Iwanicki and colleagues showed that indeed the interaction between FAK and PRG at focal adhesions is critical for trailing-edge retraction in fibroblasts upon LPA stimulation (166). PRG phosphorylation has also been implicated in cancer cell migration. In prostate cancer

cells, circumstantial evidence implicated FAK mediated phosphorylation of PRG downstream of GRP-R receptor in regulating prostate cancer cell migration (72). Thus, collective evidence suggests that FAK-mediated regulation of PRG and potentially LARG could represent an alternative mechanism for regulation of cell migration via RH-RhoGEFs downstream of GPCR activation.

Several other tyrosine kinases have been shown to phosphorylate PRG and LARG. Pyk2 phosphorylates PRG providing positive regulation of its GEF activity (167,168). Furthermore, Pyk2-mediated tyrosine phosphorylation of PRG downstream of AT_1 (Angiotensin-II receptor) regulates Rho-ROCK cascade in VSMCs (vascular smooth muscle cells) leading to increased migration (168). Given the homology between Pyk2 and FAK, it is possible that Pyk2 regulates PRG via the same mechanism as FAK. However, the site(s) of phosphorylation on PRG by Pyk2 have not been mapped.

c. Role of PRG in Physiology and Pathophysiology

i. Role of PRG and RH-RhoGEFs in Vascular Physiology

Primary determinant of arterial blood pressure is vascular tone, which is regulated by variety of distinct mechanisms that control the contraction and relaxation of VSMCs. Many of the humoral mediators that regulate VSMCs contraction, such as Ang-II (angiotensin-II), ET-1, epinephrine, TXA_2, and vasopressin regulate vascular tone through signaling via their cognate receptors expressed on these VSMCs. Many of these mediators, such as Ang-II and ET-1, are potent vasoconstrictors and their role in development of vascular disease such as hypertension and pulmonary hypertension have been well established. Both Ang-II and ET-1 signal through their receptors AT_{1A-B} and ET_{A-B} respectively, that to couple to $G\alpha_q$ and $G\alpha_{12/13}$, to activate RhoA-ROCK signaling. The vasoconstrictor response is achieved by dual regulation of

myosin light chain (MLC) phosphorylation. $G\alpha_q$ regulated Ca^{2+} dependent activation of MLC-kinase (MLCK) leads to phosphorylation of MLC. Furthermore, $G\alpha_{12/13}$ mediated activation of RhoA-ROCK signaling leads inhibition of myosin phosphatase thus maintaining the increased phosphorylation level of MLC. The phosphorylated MLC interacts with actin to bring about a contractile response (169).

Current evidence has implicated all three RH-RhoGEF family members to regulate vascular tone. Initial studies utilizing rat animal model and rat VSMCs had identified PRG as the molecular link downstream of AT_1 to activate RhoA-ROCK signaling axis leading to vascular contraction and VSMC migration (167,168,170). However, these studies did not directly address the role of $G\alpha_{13}$ mediated activation of PRG, but instead implicated Pyk2 in phosphorylation of PRG, and provided circumstantial evidence supporting the hypothesis that PRG phosphorylation is sufficient to activate its GEF function (167,168). At the same time, another study utilizing a mouse model provided evidence for p115's role in regulation of vascular tone downstream of AT_1. This study utilizing mice with SMC-specific-KO of p115, demonstrates that p115 is responsible for the constrictor response upon Ang-II stimulation (171). Furthermore, a recent report utilizing portal vein and cerebral arteries from PRG KO mice, which then were subjected to LARG knockdown, revealed that indeed both PRG and LARG are required for efficient constrictor response upon TXA_2 and ET-1 stimulation (157). Thus, it is clear that distinct RH-RhoGEFs are utilized downstream of different GPCRs coupled to $G\alpha_q$ and $G\alpha_{12/13,}$ and potentially may involve combinations of RH-RhoGEFs that contribute to maximal vascular constrictor response and development of vascular disease.

i. Contribution of ARHGEF11 Genetic Variation to Type 2 Diabetes (T2D) Risk in Humans

The increase in the prevalence of T2D is a major worldwide health issue. The increase in T2D will not only impose great burden on health care systems, but more importantly the complications associated with T2D will result in significant morbidity and mortality worldwide. Etiology of T2D is multifactorial, with both genetic and environmental factors most likely contributing to development and progression of disease. The search for genetic linkage to T2D has revealed the chromosomal region 1q21-q25 to possibly be associated to development of the disease in various ethnic backgrounds (reviewed in (172)).

ARHGEF11is found within this genomic region and indeed several studies in multiple ethnic groups have found SNPs (single nucleotide polymorphisms) variants that are statistically more associated with development either IGT (impaired glucose tolerance) or T2D and IGT (173-176). Study conducted by Fu et al., in old order Amish patients looking at ARHGEF11 variants found two SNPs, (rs6427340) in intron 2 and (rs12136088) in intron 8, to be significantly associated with IGT and T2D (173). However, it is not clear how these variations in the intronic region impacts ARHGEF11 expression or function in a manner that may increase susceptibility for T2D development. Other studies conducted in Pima Indians, Korean, Chinese, and German populations have however identified ARHGEF11 variant, PRG R1467H, that nominally affects metabolic parameters associated with T2D development and progression (173-176). However, under stringent statistical conditions the R1467H variant alone in linkage with T2D is not significant. This suggests that R1467H may not be a functional variant, but instead it probably is in linkage disequilibrium with other SNPs not only in ARHGEF11 but also in neighboring genes constituting a haplotype block (173,174,176). This is in line with current

thought that there are most likely multiple T2D susceptibility genes within this rich gene cluster on 1q21-q25.

In order to truly address the role of PRG in T2D, it will be important to utilize animal models. There is some evidence that PRG may signal downstream of IR (Insulin Receptor) and IGF-1R (145,177). In-fact, there is provocative evidence for the role of PRG in regulation of mammalian white adipose tissue development that is currently not published, except in thesis format. Work conducted by Jang et. al., in PRG -/- mice showed that these mice are viable and have no observable phenotypic defect. However, they did notice that PRG -/- mice as they aged were smaller in size in comparison to their WT littermates. Upon further analysis it was discovered that PRG -/- mice were smaller in size due to reduced adipose tissue mass, and not due decreased skeletal muscle mass. Looking at specific metabolic parameters in male mice subjected to HFD (high fat diet) such as, glucose clearance, FPG (fasting plasma glucose), triglycerides, and fasting plasma insulin levels, clearly revealed that PRG -/- mice were protected against HFD induced derangements in these metabolic parameters. With PRG -/- mice having lower FPG, higher glucose clearance, lower triglycerides, higher adiponectin, and lower fasting plasma insulin levels in comparison to WT mice on HFD. Furthermore, data from these studies also indicates that PRG -/- mice were protected from hepatic steatosis due to HFD. The authors speculate that these effects are due to PRG's role in regulation of adipogenesis. The data shows that PRG signaling downstream of IR and IGF-1R is required for maximal proliferation of MEFs *in vitro* and adipose tissue expansion *in vivo*. It was found that PRG -/- adipose tissue, but not skeletal muscles or hepatic tissues, have reduced response to insulin signaling as indicated by AKT phosphorylation at S473 and IRS (insulin receptor substrate) phosphorylation at S632/635. It is proposed that WT mice fed HFD, have maximal insulin signaling, for which PRG expression seems to be required,

which when chronically active results in an increase in adipose tissue mass. Whereas, in PRG -/- mice fed HFD, the diminished insulin signaling within the adipose tissue may contribute to the limited adipose tissue hypertrophy observed in these mice. The mechanism by which PRG -/- mice fed HFD, have limited adipose tissue hypertrophy and conversely have adipose tissue expansion, primarily having smaller sized adipocytes, that seemed to protect PRG -/- mice from diet induced insulin resistance and T2D is not clear from these studies (177). Non-the-less the phenotypic data from PRG -/- mice fed HFD, provides initial evidence for the role of PRG in pathogenesis of T2D and encourages further studies utilizing these PRG -/- mice for mechanistic explanation for the role of PRG in adipose tissue biology.

4. PRG and RH-RhoGEFs in Cancer

a. Overview of RhoGTPases in Cancer

Current evidence has established the role of small RhoGTPases, RhoA, Rac, and Cdc42, to contribute to cancer development and progression (reviewed in (17)). Initial studies provided evidence that over-expression of GTPase deficient mutant of RhoA (RhoAQ63L), WT RhoA, WT RH-RhoGEFs, and C-termini truncated form of RH-RhoGEFs leads to NIH 3T3 cell transformation as measured by focus forming assay (89,154,178). Later-on, over-expression of these small GTPases, including RhoA, have been identified in human tumors and correlated with cancer progression ((18-20) and reviewed in (130,179)). The activation of RhoA regulated pathways can be achieved by the increased GPCR or RTK signaling that is observed in many of these solid tumors. Thus, it is imperative to identify the positive regulators (RhoGEFs) of this critical signaling molecule, RhoA, which is imbedded in various cell-signaling circuits that are essential for cancer development and progression.

b. Role of p115 and LARG in Cancer

In recent years, this question has been the focus of investigations for many laboratories as the role of RH-RhoGEFs in cancer biology of many solid tumors is currently not defined. Only a few studies have directly addressed the role of p115 in cancer biology. One study has identified that both p115 and $G\alpha_{12}$ are over-expressed in more tumorigenic and invasive prostate cancer cells and prostate tumor. The data presented implicates activation of a GPCR, CaR (Ca^{2+} sensing receptor), signaling through $G\alpha_{12}$-p115-Rho axis to stimulate activation of ChoK (Choline kinase), which was shown to contribute to prostate cancer cell proliferations (180). The same group also implicates CaR signaling through $G\alpha_{12}$-Rho to regulate ChoK activity in breast cancer cells, regulating cell proliferation (180).

The role of LARG in solid tumor biology is also not well studied. A single report has implicated LARG in regulation of HNSCC (head and neck squamous cell carcinoma) cell proliferation and invasion. This study proposes that LARG interacts with CD44, possibly via its PDZ domain, and that binding of hyaluronan (HA) to CD44 leads to LARG mediated RhoA activation and also recruitment of EGFR into CD44-LARG complex. This HA mediated formation of CD44-LARG-EGFR complex results in co-activation of RhoA and Ras signaling pathways that contributes to HNSCC cell proliferation and invasion (181). However, the generalizability of this observed signaling pathway to majority of HNSCC tumors is limited primarily by the fact that this study was conducted in only one HNSCC cell line. In-contrast to the previous study implicating a possible role for LARG in regulation of HNSCC tumor cell proliferation and migration, a comprehensive study by Ong et al., provides evidence which makes a compelling case for LARG as a candidate TSG. Previous studies have indicated that loss of chromosomal region 11q23-q24 occurs frequently in variety of tumors including in breast and colorectal cancers (182-184). Thus,

the authors set out to identify a candidate TSG within this region utilizing breast and colon cancer cell lines, along with the use of breast and colon cancer tumor samples. Their analysis with these samples revealed that expression of LARG is often significantly decreased or silenced in primary breast and colorectal tumors and in their cell lines. Furthermore, the authors demonstrated that forced expression of LARG in breast cancer cell line (MCF7) and colon cancer cell line (SW620), both of which were shown to have minimal endogenous LARG expression, results in decreased cell migration and colony formation (185). Thus, supporting their hypothesis that LARG is a candidate TSG in breast and colorectal cancers. It is interesting that the authors observed that the under-expression of LARG was significantly associated with genomic loss. As other independent data, accessible on COSMIC database, also report high percentage (47%) from the total 852 samples of human breast cancer tissues tested, reported to have loss of LARG gene copy number. These data provide a strong case for LARG as a candidate TSG in breast cancer. Further studies utilizing animal models designed to validate if LARG does function as a TSG in breast cancer are warranted. It would also be useful to see if loss of LARG expression in breast and colorectal cancer is associated with clinical parameters (i.e., advanced cancer staging, increased regional invasiveness and distant metastasis, and treatment response).

c. <u>PRG's Role in Solid Tumor Biology</u>

Much like p115 and LARG, PRG's role in solid tumor biology is just now being studied. One of the earliest reports looking at the role of RH-RhoGEFs downstream of ET_{A-B} and BB_2 receptors in PC-3 prostate cancer cells provides circumstantial evidence for PRG's role in regulating PC-3 cell migration (72). Other studies in breast cancer established the significance of other GPCRs, PAR1 and CXCR4, signaling through $G\alpha_{12/13}$-Rho axis to contribute to breast cancer cell migration and invasion (23,25). Recent report by Struckhoff et al., identified PRG as the

missing molecular link in CXCR4-Gα_{13}-RhoA signaling axis to regulate breast cancer cell migration and invasion (23,97). The importance of PRG in regulation of breast cancer cell migration and invasion was further supported by the findings in primary breast tumors, which revealed that PRG expression is increased at the invasive fronts of primary tumors and in tumor cells that have undergone lymphatic invasion in comparison to PRG expression *in-situ*. Thus, providing evidence that PRG activity and expression contributes to the invasive phenotype in breast cancer (97). The observation that increased PRG expression and activity contributes to an invasive cancer phenotype, was also observed in PC-3 cells grown in 3D organotypic culture. The data indicates that PRG expression is increased in the invasive PC-3 cells grown in 3D organotypic culture in comparison to non-invasive cells grown in organotypic cultures and cells grown as a monolayer (186).

However, increased cancer cell motility and invasive phenotype may not be the only advantage afforded by increased PRG expression to these solid tumors. Indeed, ARHGEF11 has also been implicated to be a candidate cell survival gene in glioblastoma multiforme (GBM) tumors, which are highly aggressive tumors arising from glial cells (187). In a study set out to identify GBM cell survival genes utilizing un-biased high-throughput large-scale siRNA screen, found ARHGEF11 to be one of 55 survival genes whose loss-of-expression lead to significant decrease in cell viability of T98G glioma cell line. Indeed, ARHGEF11 knockdown resulted in only ~24% cell viability (187). Furthermore, a recent study utilizing cancer genomic data from The Cancer Genome Atlas (TCGA) project, also found that ARHGEF11 is over-expressed in GBM tumors. The study found significant correlation between somatic mutation status of certain genes, such as IDH1, MAPK9, SYNE1, FBXW7, FURIN, and TRPM3, with over-expression of ARHGEF11 (188). Another report utilizing DNA microarray to identify differential expression of

genes in gallbladder cancer samples in comparison to normal control tissues, found that ARHGEF11 was significantly over-expressed in these human gallbladder cancer samples. The over-expression of ARHGEF11 in gallbladder cancer samples was confirmed with RT-PCR (189). However, the major limitation of this study was the small sample size of only 12 human cancer tissues. Furthermore, the study did not address how the increased ARHGEF11 expression may contribute to the disease process of this highly invasive and metastatic cancer.

None-the-less, these studies provide important evidence that PRG, through participating in signaling circuitry that is currently not well understood, contributes to regulation of cancer cell motility, invasion, and cell survival pathways in different tumors. However, the role of PRG in two of the most common solid cancers, colon and lung cancer, have not been studied.

Portions of the text in this chapter and the following chapters were reprinted with permission from *Molecular Pharmacology*. Patel M, *et al.* (2014) Gα13/PDZ-RhoGEF/RhoA Signaling Is Essential for Gastrin-Releasing Peptide Receptor–Mediated Colon Cancer Cell Migration. *Molecular Pharmacology* 86(3):252-262. Appendix A.

AND from Patel M & Karginov AV (2014) Phosphorylation-mediated regulation of GEFs for RhoA. *Cell Adhesion & Migration* 8(1):11-18. Appendix B.

III. Experimental Procedures

A. Materials

Gastrin Releasing Peptide-human was purchased from Sigma Aldrich (St. Louis, MO), Celecoxib and Y-27632 were purchased from Tocris (Bristol, UK), Primary Human Colonic Epithelial Cells (HCoEpiC) lysate was purchased from ScienCell (Carlsbad, CA). Normal human distal colon mucosal sample (male sample) was kind gift from Pradeep Dudeja, University of Illinois at Chicago.

B. Cell Culture and Transfection

Caco-2 and HT-29 cells (gift from Richard Benya, Loyola Medicine Chicago, IL.) were maintained in base medium consisting of Dulbecco's modified Eagle's medium (DMEM) with high glucose, glutamine, and sodium pyruvate, along with Ham's F12 medium with glutamine. Caco-2 cells were cultured in base medium supplemented with 20% fetal bovine serum (FBS), and HT-29 supplemented with 10% FBS. HEK293T cells were cultured in DMEM supplemented with 10% FBS. All cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Caco-2 cells were transfected with Silencer select PRG siRNA (s19005) with the sequence 5'-GAGAUGAAACGGUCUCGAAtt-3', Silencer select PRG siRNA (s19006) with sequence 5'-GCGAAACCCUAUCCUCAAtt-3', and Silencer select Negative control #1 siRNA (AM4611) purchased from Invitrogen. $G\alpha_{13}$ knockdown was achieved by siGENOME human GNA13 siRNA –smart pool (M-009948-00-0005) consisting of (4)GNA13 specific siRNA sequences 5'-GAGAUAAGAUGAUGUCGUU-3', 5'-CCAAGGAGAUCGACAAAUG-3', 5'-GAGAGAAGCUUCAUAUUCC-3', 5'-GAAGAUCGACUGACCAAUC-3' purchased from GE Healthcare-Dharmacon (Pittsburg, PA). siRNA transfection was done with Lipofectamine RNAiMAX per manufacturer's protocol from Invitrogen. All experiments

utilizing cells with siRNA knockdown were conducted 48 hours after siRNA transfection, and post serum starvation for 16 hours. All cells were between 50% to 70% confluent when experiments were carried out.

C. Western Blotting

Cells were lysed in 20 mM HEPES (pH 7.6), 1% Triton-X-100, 150 mM NaCl, 5 mM $MgCl_2$, 2 mM Na_3VO_4, 1 mM β-Glycerophosphate, aprotinin (16 μg/mL), and leupeptin (3.2 μg/mL). Cell lysates were then clarified by centrifugation at 14,500 RPM for 10 minutes at 4°C. Protein concentration of the lysate was then verified by BradfordDC purchased from Bio-Rad (Hercules, CA). SDS-PAGE sample buffer was then added to the lysate and the samples were boiled for 3 minutes and resolved by SDS-PAGE. Protein was then transferred to nitrocellulose membrane (GE Healthcare) and blocked with 5% milk in (T-BST) for one hour at room temperature. Membranes were then incubated with one of the following antibody at 4°C: anti-RhoA monoclonal, anti-PDZ-RhoGEF polyclonal, anti-LARG polyclonal (Kind gift from Takao Hamakubo University of Tokyo, 1:1000), anti-p115RhoGEF polyclonal, anti-GAPDH monoclonal, anti-GFP polyclonal, anti-Cox-2 polyclonal from Cell Signaling (Danvers, MA), anti-Gα13 polyclonal, anti-Gα13 polyclonal B860 (1:1000) (190), anti-$G\alpha_{q/11}$ polyclonal, anti-Gα12 polyclonal, and anti-beta-actin monoclonal from Sigma Aldrich. All other antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Membranes were then probed with horseradish peroxidase conjugated secondary antibodies from Amersham GE (Piscataway, NJ). Western blots were developed with SuperSignal West Pico Chemiluminescent Substrate from Thermo Scientific (Rockford, IL). Densitometry was performed with ImageJ software.

D. RhoA GTPase Pull-down Assay

Rho activity in cultured cells was assessed utilizing manufacturers (Cytoskeleton) protocol. Briefly, colon cancer cells were serum starved for 16 hours. After stimulation, the cells were lysed at 4^{o}C in buffer containing 50 mM Tris-HCl pH (7.5), 300 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 2mM Na_3VO_4, aprotinin (16 µg/mL), and leupeptin (3.2 µg/mL). The lysates were then incubated with glutathione *S*-transferase (GST)-rhotekin-Rho binding domain bound to glutathione Sepharose beads purchased from Cytoskeleton (Denver, CO). The samples were washed 3 times with wash buffer (per manufacturer's instructions), and then resuspended in SDS-PAGE sample buffer. Samples were then analyzed by Western blot with monoclonal RhoA antibody.

E. Purification of GST-RhoA^{G17A} Recombinant Protein

Plasmid construct for the prokaryotic expression of GST-RhoA^{G17A} was kindly provided by K. Burridge (University of North Carolina). Purification of GST-RhoA^{G17A} was carried out as previously described (191). Briefly, expression of GST-RhoA^{G17A} in BL21-CodonPlus (DE3)-RP purchased from Stratagene (Santa Clara, CA) was induced with 200 µM isopropyl-β-D-thiogalactoside (IPTG) for 16 hours at 18^{o}C. Bacterial cells were then lysed with 20 mM HEPES (pH 7.6), 1% Triton-X-100, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), aprotinin (16 µg/mL), and leupeptin (3.2 µg/mL). Protein was purified by incubating glutathione-Sepharose 4B beads, purchased from GE Healthcare, at 4^{o}C for 45 minutes. Sepharose beads were then washed with lysis buffer twice and twice with 20 mM HEPES (pH 7.6), 150 mMNaCl, 5 mM $MgCl_2$, and 1 mM dithiothreitol (DTT). Protein concentration was estimated with Coomassie Plus protein reagent (Thermo

Scientific). The beads were then aliquoted and snap frozen with liquid nitrogen and stored at -80^{o}C.

F. GST-RhoA^{G17A} Pull-down Assay

Activation of RH-RhoGEFs was monitored with GST-RhoA^{G17A} pull-down assay as previously described (191). Briefly, Caco-2 cells were stimulated with GRP 100 nM for indicated time(s). After which, cells were then lysed with 20 mM HEPES (pH 7.6), 1% Triton-X-100, 150 mM NaCl, 5 mM $MgCl_2$, 2mM Na_3VO_4 , aprotinin (16 µg/mL), and leupeptin (3.2 µg/mL) at 4^{o}C. Protein concentration of lysates was verified with Bradford DC (BioRad), and equal protein and volume of lysate was incubated with 30 µg of purified GST-RhoA^{G17A} bound glutathione-sepharose beads for 45 minutes at 4^{o}C. Samples were then washed 3 times with lysis buffer without 1% Triton-X-100, and the beads were resuspended in SDS-PAGE sample buffer. Samples were then analyzed by Western blot with RH-RhoGEF specific antibodies.

G. Generation of Lentivirus

The cDNAs encoding GFP, GFP-RH-GRK2 (1-178aa of bovine GRK2), and GFP-RH-RGS3 (378-519aa of human RGS3) were subcloned under EF-1a promoter of lentivirus transfer vector pLVTH (Cambridge, MA). Lentivirus was generated as previously described (192). In short, pLVTH (transfer vector) encoding GFP, GFP-RH-RGS3 or GFP-RH-GRK2 were transfected into HEK293T cells together with pMD2.G (envelope) and pCMVDR8.74 (packaging vector) by the calcium phosphate precipitation method. Lentivirus produced (packaged) by HEK293T cells were harvested from cell medium 48 hours later.

H. Co-immunoprecipitation Assay

HEK293T cells were infected with lentivirus for GFP, GFP conjugated RH-RGS3 or RH-GRK2. After 48 hours the cells were harvested on ice and the lysates were utilized for pulldown assay with anti-$G\alpha_{q/11}$ antibody (SantaCruz) as previously described (193).

I. Intracellular Calcium Measurement

Agonist induced intracellular calcium mobilization was performed in serum free condition with GFP, RH-RGS3, and RH-GRK2 expressing Caco-2 cells with GFP certified FluoForte calcium assay kit for microplates per manufacturers' protocol (Enzo Life Sciences Farmingdale, NY). Intracellular calcium mobilization was monitored by Molecular Devices (Sunnyvale, CA) FlexStation System. Fluorescence was monitored at Ex=530 nm/Em=570 nm. Data obtained as ratio of fold increase after stimulation over basal.

J. PGE_2 Enzyme Linked Immunosorbent Assay

Cell culture media was collected at 4^oC after incubating with GRP (100 nM) for indicated time(s). Cell culture media was then centrifuged at 8,000 RPM, to clear cellular debris. Culture media was then either assayed or stored at -80^oC for no longer than 7 days. Concentration of PGE_2 in the culture media was obtained using PGE_2 express EIA kit following manufacturers' protocol (Cayman Chemical, Ann Arbor, Michigan).

K. Cell Migration Assays

Cells were serum starved for 16 hours prior to the assay. Caco-2 and HT-29 cells were plated on the upper chamber of 6-well 8.0µm pore polycarbonate membrane insert (Corning, Tewksbury, MA) at a density of $5X10^5$ cells/well. The inserts were placed in 1% FBS containing media with or without GRP (100 nM) added to the lower chamber. The

plate was then placed in the incubator at 37^{o}C supplemented with 5% CO_2. Cells were allowed to migrate for 8 hours. After which, the cells on the top of the chamber were mechanically removed and the inserts were washed with PBS. The cells were fixed with 4% para-formaldehyde for 10 minutes (Electron Microscopy Sciences, Hatfield, PA) and stained with 2% crystal violet (Sigma Aldrich) for 5 minutes. Migrated cells on the lower chamber were visualized with microscope and counted.

L. Data Analysis and Statistics

Statistical and graphical analysis was conducted with GraphPad Prism 5 (La Jolla, CA). Data are represented as mean $\pm$ S.E.M of at least n=3 independent experiments. Statistical analysis was performed with One-way ANOVA followed by Bonferroni's multiple comparison test.

IV. Results

A. GRP Stimulation Increases RhoA Activation in Colon Cancer Cells

GRPR expression is absent in normal colonic epithelial cells (4). However, its ectopic expression on colon cancer cells contributes to tumorigenesis by stimulating cell proliferation and migration (1,3). Previous studies indicate that GRPR can promote tumorigenicity through activation of the small GTPase RhoA in prostate cancer (72). However, the role of RhoA signaling downstream of GRPR in colon cancer has not been well studied. Thus, we first sought to determine whether activation of GRPR leads to activation of RhoA in colon cancer cells. As a model we used Caco-2 and HT-29 colon cancer cell lines, which express functional GRP receptor and form moderately well-differentiated adenocarcinoma in nude mice (194). To determine RhoA activation, we conducted a time-course experiment, stimulating Caco-2 and HT-29 cells in serum free conditions with concentration of GRP (100 nM) which has been utilized for previous colon cancer studies (5,195).The level of RhoA activation was assessed using RhoA pulldown assay (196) (Fig 7 A-B). Stimulating colon cancer cells with GRP increased the fraction of RhoA in the active GTP-bound state. The activation of RhoA reaches maximum at about 10 minutes and decreases over time out to 60 minutes after GRP addition in both Caco-2 and HT-29 cells. These data indicate that GRPR activation on colon cancer cells initiates signaling pathway(s) that leads to RhoA activation.

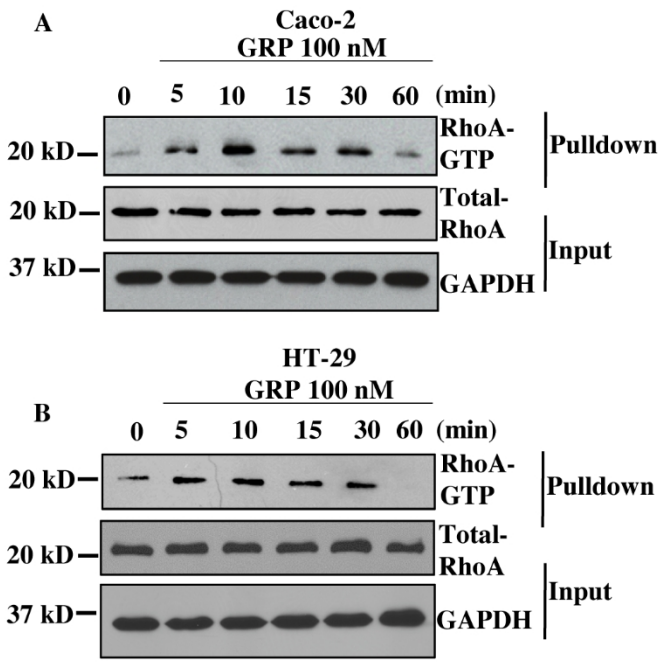


Figure 7: GRP stimulation increases RhoA activation in colon cancer cell lines. Time course of RhoA activation in colon cancer cell lines in response to GRP stimulation. Caco-2 **(A)** and HT-29 cells **(B)** serum starved overnight and then incubated with GRP for indicated time(s). Cell lysates were utilized for GST-RBD pulldown (see methods). The precipitate and lysates samples were then used for Western blot to detect RhoA and GAPDH. GAPDH is used as loading control. Shown are representative images from 3 independent experiments.

B. $G\alpha_{13}$ is the Principal Mediator of RhoA Activation Downstream of GRPR

GRPR signaling is in-part conducted through activation of the alpha subunits of Gq and G12/13 heterotrimeric G-proteins in colon cancers (1). However, the contribution of each G-protein to GRPR-mediated activation of RhoA in colon cancer cells has not been established. To address this question, we utilized siRNA to downregulate endogenous $G\alpha_{13}$ expression in Caco-2 cells. $G\alpha_{13}$ siRNA efficiently and specifically decreased $G\alpha_{13}$ expression in Caco-2 cells (Fig 8A), without affecting expression of its close structural homologue $G\alpha_{12}$ (Fig 8B). Downregulation of $G\alpha_{13}$ expression led to a significant decrease in GRP-stimulated RhoA activation indicating that in Caco-2 cells RhoA activation predominantly occurs through $G\alpha_{13}$ (Fig. 8C-D).

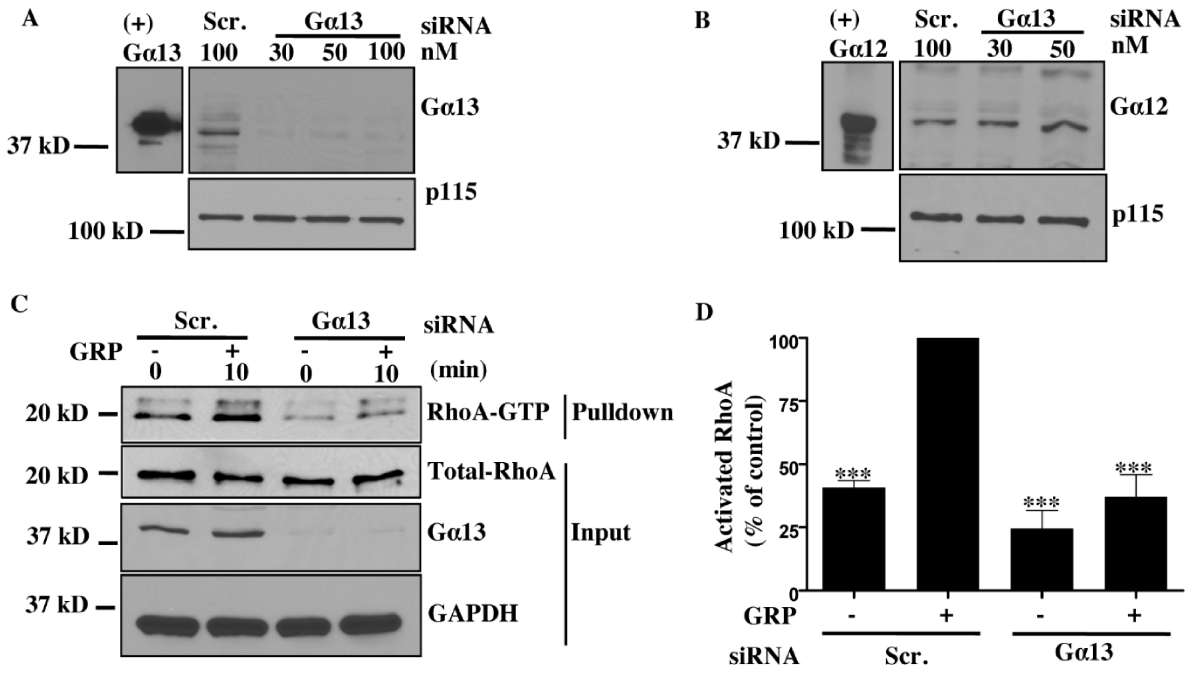
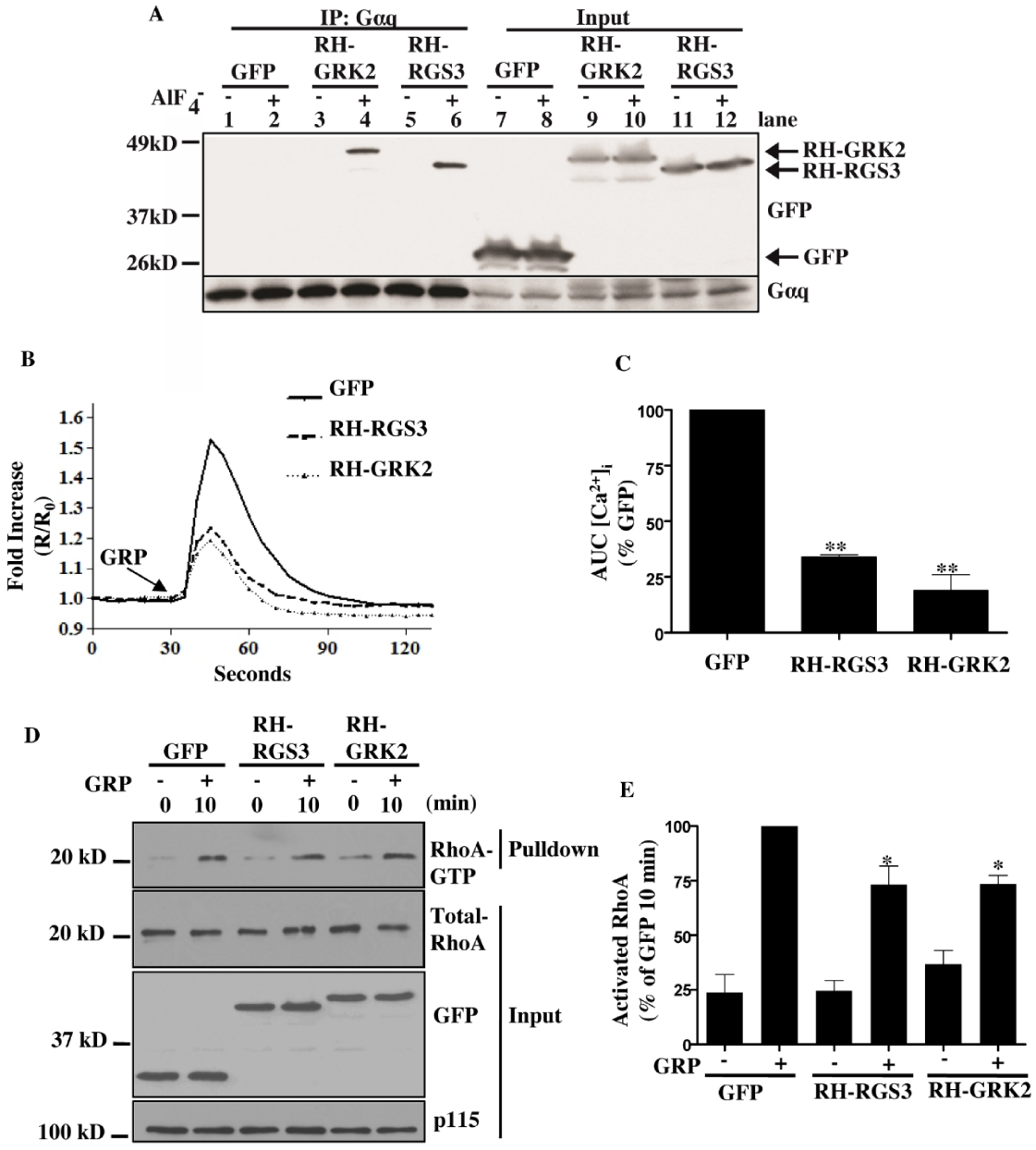


Figure 8. $G\alpha_{13}$ is the principal mediator of RhoA activation downstream of GRPR. A-B. Caco-2 cells transfected with Scrambled or $G\alpha_{13}$ smartpool siRNA for 48 hours to obtain $G\alpha_{13}$ specific knockdown without affecting $G\alpha_{12}$ expression. (+) $G\alpha_{12/13}$ lanes contain purified recombinant full-length $G\alpha_{12}$ or $G\alpha_{13}$ subunits used as positive control **C.** Caco-2 cells were serum starved over-night and then stimulated with GRP for 10 minutes and subsequently utilized for GST-RBD pulldown (see methods). Precipitate and lysate samples were then immunoblotted to detect RhoA, $G\alpha_{13}$, and GAPDH. GAPDH used as loading control. **D.** Statistical densitometric analysis of n=4. Shown are mean values $\pm$ SEM; (***, $p<0.001$).

C. $G\alpha_q$ Makes Small Contribution to Total RhoA Activation Downstream of GRPR

$G\alpha_q$ can also activate RhoA through direct interaction with RhoGEFs such as p63RhoGEF, Trio, and Kalirin (197). To determine the role of $G\alpha_q$ in mediating RhoA activation in Caco-2 cells, we transduced Caco-2 cells with lentivirus expressing GFP fused to the RH domain of RGS3 or GRK2. Both of these proteins specifically bind to activated $G\alpha_q$ and inhibit $G\alpha_q$-mediated signaling (198,199). As shown in Fig 9A, both RH-RGS3 and RH-GRK2 co-immunoprecipitated with AlF_4^- activated endogenous $G\alpha_q$. Caco-2 cells expressing RH-RGS3 and RH-GRK2 also had a defect in GRP-stimulated rise in intracellular Ca^{2+}, a known indicator of GRPR mediated $G\alpha_q$ signaling, in comparison to GFP expressing cells (Fig 9B-C). These cells were then stimulated with GRP and lysates were utilized for RhoA pulldown. Expression of RH-RGS3 or RH-GRK2 led to a small reduction in RhoA activation in response to GRP stimulation (Fig 9D-E). This indicates that $G\alpha_q$ makes a minor contribution to total RhoA activation downstream of GRPR, and that $G\alpha_{13}$ is the predominant mediator of RhoA signaling.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RH-RGS3
RH-GRK2
Fold Increase (R/R0)
GRP
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D
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(min)
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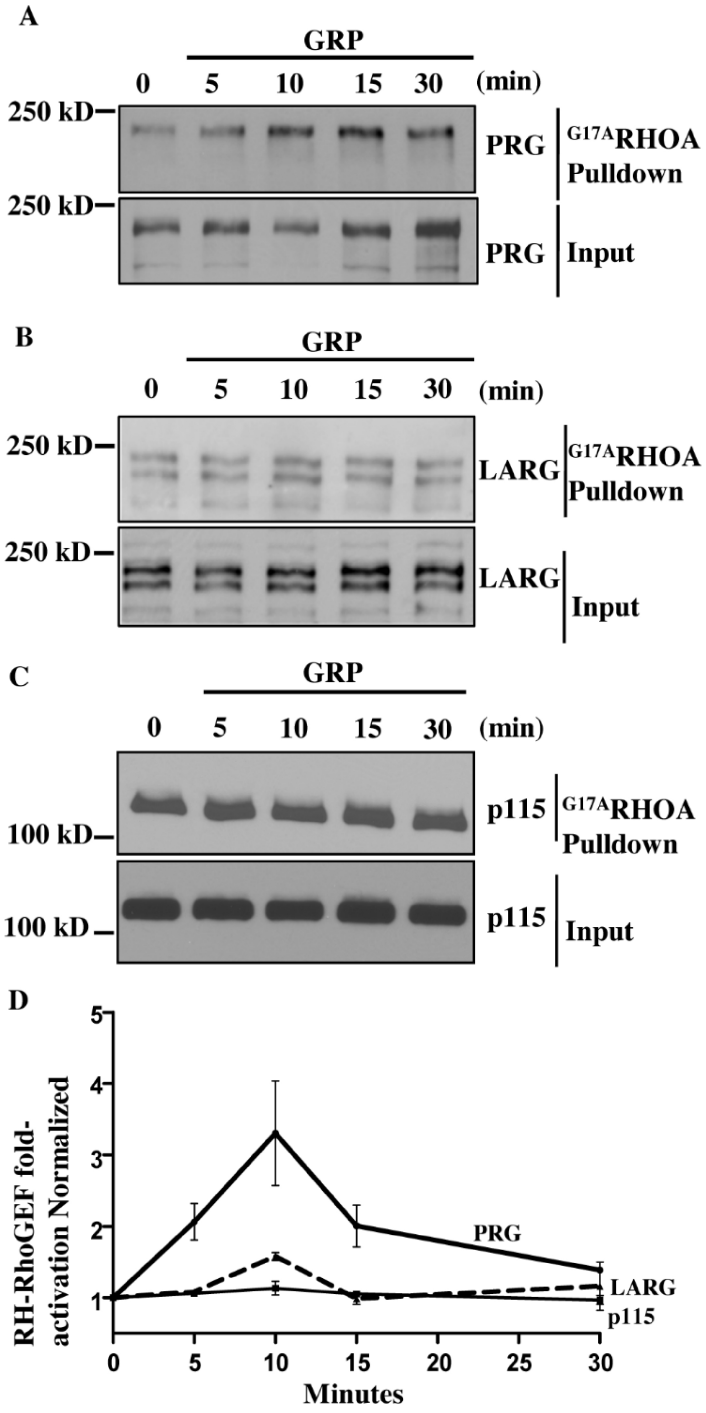
RhoA-GTP
Pulldown
Total-RhoA
37 kD
Input
100 kD
p115
E
Activated RhoA (% of GFP 10 min)

Figure 9: $G\alpha_q$ makes small contribution to total RhoA activation downstream of GRPR. **A.** HEK293T cells were infected with lentivirus for GFP, GFP conjugated RH-RGS3 or RH-GRK2. After 48 hours, cells were harvested and lysed in the buffer containing either GDP or GDP-AlF_4^-. Lysates were subjected to Western blotting in order to confirm protein expression of $G\alpha_{q/11}$ (lower panel, lanes 7-12) and GFP, RH-RGS3, or RH-GRK2 (upper panel, lanes 7-12). Immuno-precipitation was carried out using anti-$G\alpha_q$ antibody (lanes 1-6). RH-GRK2 (lane 4, upper) and RH-RGS3 (lane 6, upper) were co-precipitated with endogenous $G\alpha_q$ activated by GDP-AlF_4^-. **B-C.** Caco-2 cells stably expressing GFP, RH-RGS3, and RH-GRK2 were utilized to monitor GRP induced calcium mobilization (see methods). Shown are representative traces of at least 3 independent experiments, from which area under the curve (AUC) was quantitated and plotted (**, $p<0.01$). **D.** Caco-2 cells stably expressing GFP, RH-RGS3, and RH-GRK2 were serum starved over-night and then stimulated with GRP for 10 minutes. Cell lysates were then utilized for GST-RBD pulldown (see methods). Precipitate and lysate samples were then immunoblotted to detect RhoA, GFP, and p115. Expression of GFP, RH-RGS3, and RH-GRK2 in the lysate was confirmed with anti-GFP antibody. p115 was used as loading control. **E.** Statistical densitometric analysis of n=5. Shown are mean values $\pm$ SEM; (*, $p<0.05$, ***, $p<0.001$).

D. PRG is the Primary RH-RhoGEF Activated Downstream of GRPR

GPCRs coupled to G12/13 family of heterotrimeric G-proteins can initiate RhoA signaling by physically interacting with and activating RH-RhoGEFs. Previous studies have suggested that GPCRs coupled to $G\alpha_{12/13}$ utilize distinct RH-RhoGEFs to activate RhoA signaling (8). In Caco-2 cells all three RH-RhoGEF family members (p115, PRG, and LARG) are expressed (Fig 10A-C). Thus, in order to identify which RH-RhoGEF(s) are activated in response to GRP stimulation, we utilized GST-$RhoA^{G17A}$ fusion protein as an affinity reagent to isolate activated GEFs for RhoA. The glycine to alanine mutation in the recombinant RhoA protein mimics the nucleotide free state of RhoA which binds with high affinity to activated GEFs (191). Employing this biochemical approach, we isolated activated GEFs from Caco-2 cells treated with GRP in a time course experiment. Our data reveal that GRP stimulation resulted in strong activation of PRG as indicated by increased PRG pulldown throughout our time-course (Fig. 10A). The maximum activity was detected at 10 min after addition of GRP, consistent with the peak of RhoA activity that we observed. In contrast, GRP treatment of Caco-2 cells did not affect activation of LARG and p115 (Fig 10B-D). Thus, our data demonstrates that GRPR stimulation predominantly activates PRG in colon cancer cells.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Input
C
GRP
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100 kD
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p115
p115
G17ARHOA Pulldown
Input
D
RH-RhoGEF fold-activation Normalized
1 2 3 4 5
0 5 10 15 20 25 30
Minutes
PRG
LARG
p115

Figure 10: PRG is the primary RH-RhoGEF activated downstream of GRPR. A-C. Caco-2 cells were serum starved over-night and then stimulated with GRP for indicated time(s). The lysates were subsequently utilized for $RhoA^{G17A}$pulldown, where GST-$RhoA^{G17A}$ protein is used to pulldown activated RhoGEFs from total cell lysate (see methods). Precipitates and the lysate samples were then immunoblotted for PRG, LARG, and p115RhoGEF. Shown are representative images of 3 independent experiments. **D.** Densitometric analysis of activation states of three RH-RhoGEFs normalized to endogenous RH-RhoGEF levels and expressed as fold activation over 0 minute time point. Shown are mean values $\pm$ SEM.

E. PRG is the Primary Activator of RhoA Downstream of GRPR

Our $RhoA^{G17A}$pulldown data reveals that GRP stimulation predominantly activates PRG. This suggests that PRG should be the predominant activator of RhoA downstream of GRPR in colon cancer cells. To confirm this hypothesis, we downregulated expression of PRG using two different siRNA reagents (Fig 11A). Importantly, treatment with these siRNAs did not affect expression of the two related RH-RhoGEFs, LARG and p115 (Fig 11A). We then performed a RhoA pulldown with siRNA-transfected cells to determine the role of PRG in RhoA activation in response to GRP stimulation. As shown in figures 11B-C and 12A-B, PRG knockdown significantly decreased GRP-stimulated RhoA activation in Caco-2 and HT-29 cells. Similar decrease in RhoA activation was also observed with the PRG siRNA-2 reagent. Importantly, the decrease in RhoA activation was similar to the effect achieved by downregulation of $G\alpha_{13}$ expression (Fig. 8C). Thus, these data suggest that GRP-mediated RhoA activation in colon cancer cells occurs primarily through the $G\alpha_{13}$-PRG signaling axis.

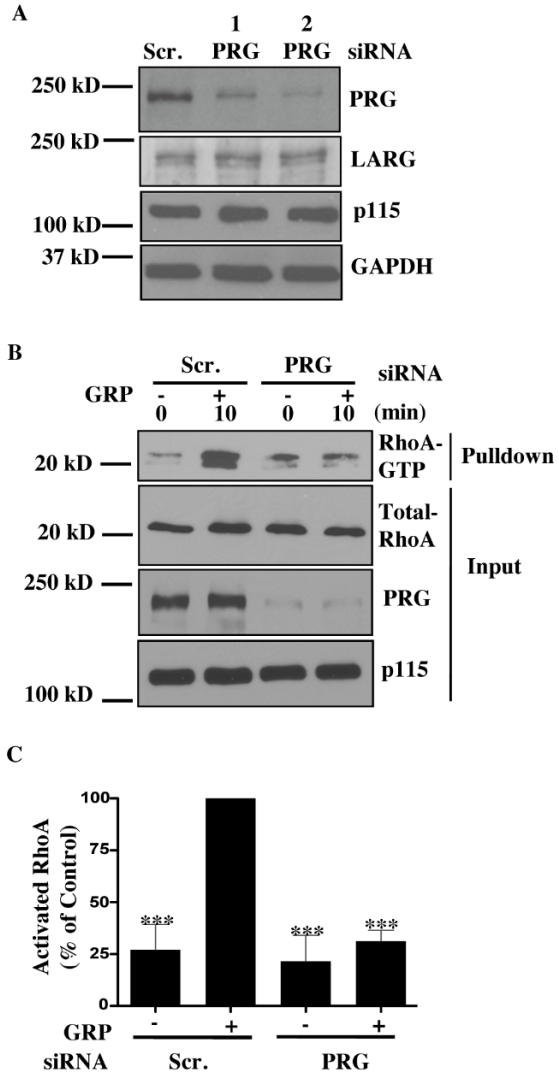

Figure 11. PRG knockdown decreases RhoA activation upon GRP stimulation in Caco-2 cells. **A.** PRG knockdown was confirmed by utilizing two different siRNA. Specific knockdown of PRG was verified by immunoblotting for p115 and LARG. **B.** Caco-2 cells were transfected with Scrambled or PRG siRNA for 48 hours. Cells were serum starved over-night and the following day were stimulated with GRP for 10 minutes. Cell lysates were then utilized for GST-RBD pulldown (see methods) and samples were then subjected to Western blotting. **C.** Statistical densitometric analysis of at least three independent experiments. Shown are mean values $\pm$ SEM (***, $p<0.001$).

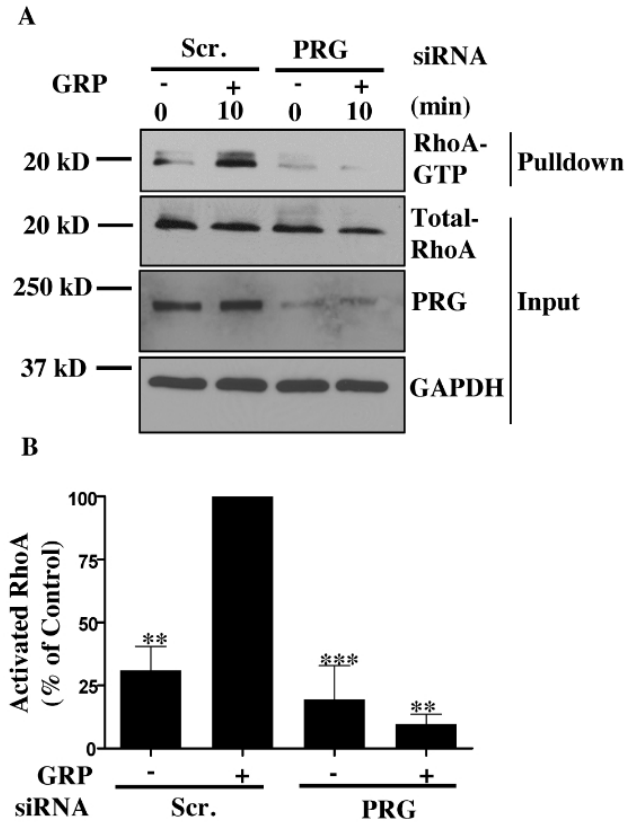

Figure 12. PRG knockdown decreases RhoA activation upon GRP stimulation in HT-29 cells. **A.** HT-29 cells were transfected with Scrambled or PRG siRNA for 48 hours. Cells were serum starved over-night and the following day were stimulated with GRP for 10 minutes. Cell lysates were then utilized for GST-RBD pulldown (see methods) and samples were then subjected to Western blotting. **B.** Statistical densitometric analysis of at least three independent experiments. Shown are mean values $\pm$ SEM (**, $p<0.01$, ***, $p<0.001$).

F. The PRG-RhoA-ROCK Axis Mediates GRP-stimulated Colon Cancer Cell Migration

Cancer cell motility is an essential process of cancer progression and invasion. RhoA is known to play a critical role in regulation of focal adhesions and stress fiber formation leading to cell migration (23,200,201). RhoA has been shown to be overexpressed in colon cancers (18). Here we have shown that PRG is the predominant activator of RhoA downstream of GRPR in colon cancer cells. This evidence suggests that PRG should regulate colon cancer cell migration downstream of GRPR. To test this hypothesis we conducted a transwell cell migration assay using Caco-2 and HT-29 cells transfected with scrambled siRNA or PRG siRNA. As shown in figures 13A-B, PRG knockdown resulted in a dramatic reduction in GRP-stimulated colon cancer cell migration almost to a level equivalent to unstimulated scrambled siRNA treated cells. This demonstrates that PRG is a critical mediator of colon cancer cell migration downstream of GRPR.

ROCK is one of the key downstream effectors of RhoA and is known to contribute to RhoA-mediated regulation of cancer cell migration and invasion (23,126). Therefore, to determine the role of ROCK in GRP-stimulated colon cancer cell migration, we conducted the transwell assay with Caco-2 cells treated with or without ROCK inhibitor Y-27632 (20 μM) in presence or absence of GRP. As shown in figure 13C, ROCK inhibition arrested GRP-stimulated Caco-2 cell migration. In agreement with previous reports, unstimulated Caco-2 cells treated with Y-27632 did have a slight increase in basal cell migration in comparison to cells with no treatment (201,202). None-the-less, our results indicate that ROCK is required for efficient GRP-stimulated colon cancer cell migration. Overall, our data demonstrate that in colon cancer cells, GRP-stimulated migration is regulated via the PRG-RhoA-ROCK pathway.

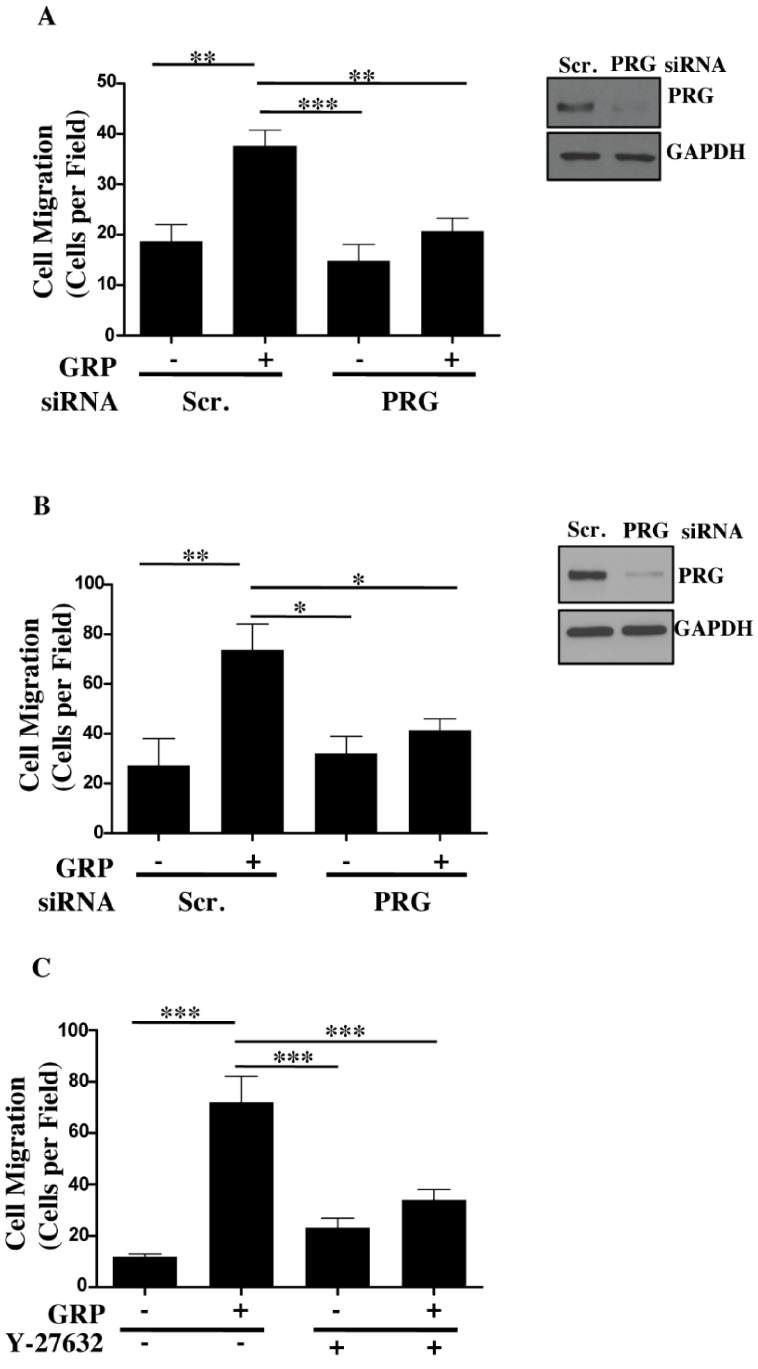
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Figure 13. PRG-RhoA-ROCK axis mediates GRP stimulated colon cancer cell migration. A-B. Caco-2 **(A)** or HT-29 **(B)** cells transfected with Scrambled or PRG siRNA for 48 hours. The transfected cells were serum starved over-night and plated on the top chamber of transwell insert at $5X10^5$ cells/well. The inserts were placed in 1% FBS containing media with or without 100 nM GRP (see methods). Representative images of PRG knockdown in Caco-2 and HT-29 cells. Statistical analysis of cell migration of n=3 repeated in duplicates. Shown are mean values $\pm$ SEM; (*, $p<0.05$, **, $p<0.01$, ***, $p<0.001$). **C.** Caco-2 cells were plated on the top of the transwell inserts at $5X10^5$ cells/well in media with or without GRP along with Y-27632 (20 µM) (see methods). Statistical analysis of cell migration of n=3 repeated in duplicates. Shown are mean values $\pm$ SEM; (**, $p<0.01$, ***, $p<0.001$).

G. GRP Stimulation Increases Cox-2 Expression in Colon Cancer Cells

Cox-2 plays a critical role in colon cancer development and progression. Studies have shown that 85% of colon cancers have increased Cox-2 expression (203). GRPR signaling has been implicated in regulation of Cox-2 expression in variety of tissues via different mechanisms (35,36). However, the role of $G\alpha_{13}$-mediated signaling pathways in regulation of Cox-2 expression in colon cancer cells has not been elucidated. First, we tested if GRP stimulation increases Cox-2 expression in Caco-2 and HT-29 cells. As shown in figure 14A-B, Cox-2 expression is increased upon GRP stimulation in both Caco-2 and HT-29 cells. Cox-2 expression is increased in these cancer cells at four and eight hours after GRP addition.

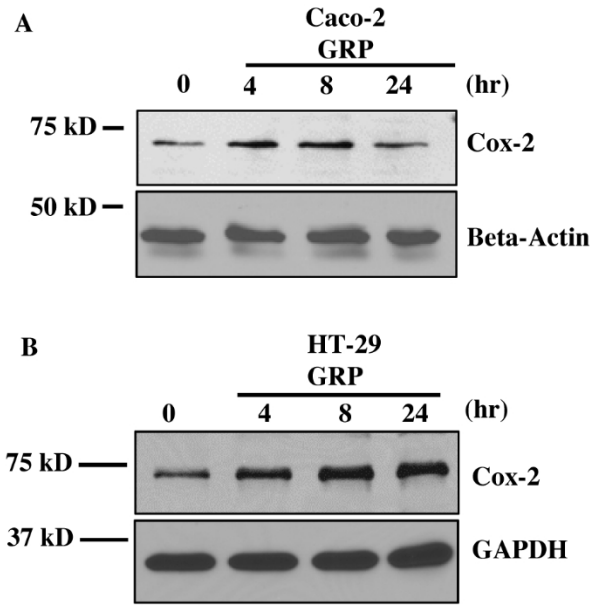


Figure 14: GRP stimulation increases Cox-2 expression in colon cancer cells: Time course of Cox-2 expression. Caco-2 **(A)** or HT-29 **(B)** cells were stimulated with GRP for the indicated time(s). Cox-2 expression was determined by Western blot utilizing Cox-2 specific antibody. Beta-actin and GAPDH used as loading control.

H. PRG Contributes to Cox-2 Expression in Colon Cancer Cells

Next we sought to determine if $G\alpha_{13}$ signaling downstream of GRPR, specifically the PRG-RhoA-ROCK axis, plays a role in regulation of Cox-2 expression in colon cancer cells. To test this hypothesis, we first downregulated PRG expression in Caco-2 and HT-29 cells to observe if PRG is required for GRP stimulated Cox-2 expression in these colon cancer cell lines. Downregulation of PRG expression using siRNA reduced Cox-2 expression after 8 hours of treatment with GRP (Fig 15A-D). It is well known that Cox-2 expression drives colon cancer progression through the production of PGE_2 (203,204). In fact, PGE_2 is the predominant prostaglandin found in colon cancer (205). So next we examined if the decrease in Cox-2 expression in PRG siRNA-transfected cells is associated with a decrease in PGE_2 production. We utilized enzyme-linked immunosorbent assay (ELISA) to quantitate PGE_2 concentration in the media of scrambled or PRG siRNA-transfected cells stimulated with GRP. As shown in figures 15E-F, PRG knockdown inhibited GRP-induced production of PGE_2 in comparison to scrambled siRNA treated cells stimulated with GRP. These data show that GRPR-$G\alpha_{13}$ signaling through PRG regulates Cox-2 expression and PGE_2 production.

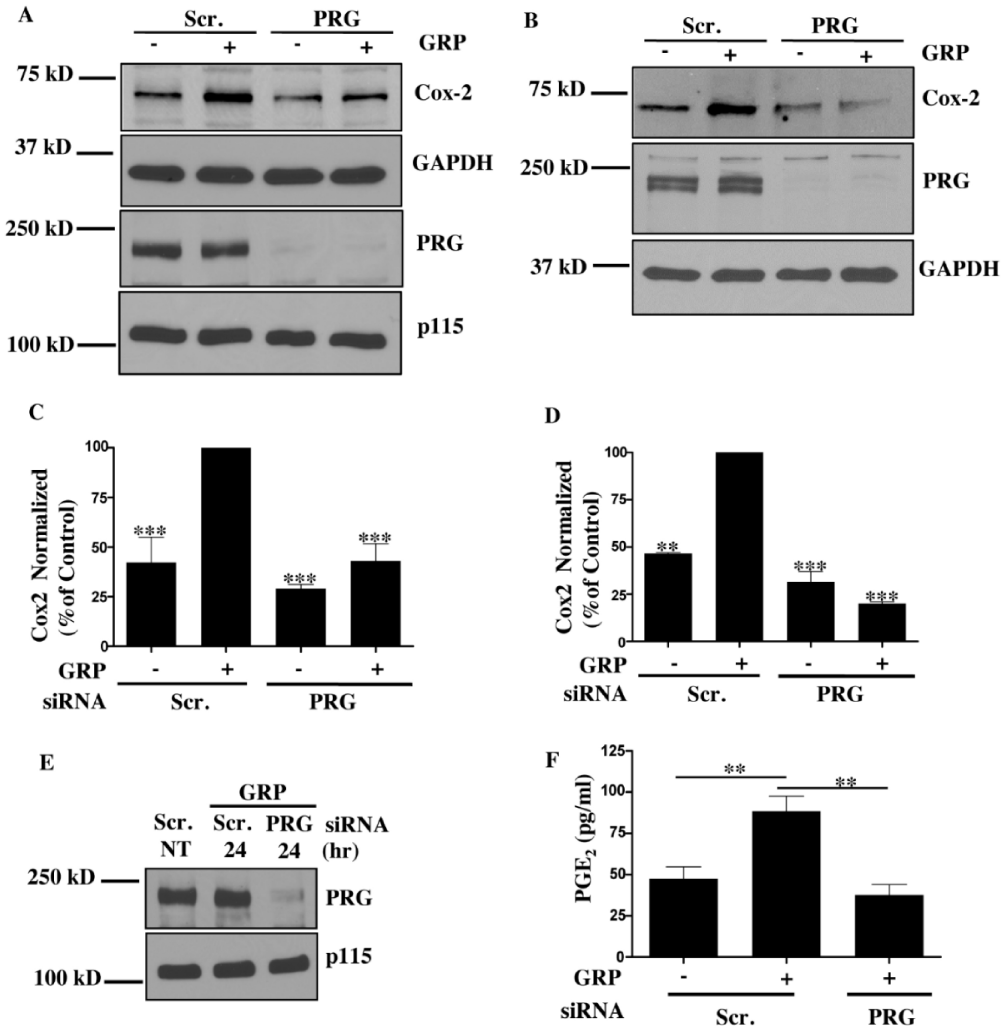


Figure 15: PRG contributes to Cox-2 expression downstream of GRPR. A-B. Cox-2 expression in Caco-2 **(A)** or HT-29 **(B)** cells transfected with Scrambled or PRG siRNA. The cells were incubated with GRP for 8 hours. Cox-2 expression and PRG knock down was verified by Western Blot. **C-D.** Statistical densitometric analysis of Cox-2 expression in Caco-2 **(C)** and HT-29 **(D)** cells from n=3. Shown are mean values $\pm$ SEM; (*** $p<0.001$). **E-F.** PGE_2 production in Caco-2 cells transfected with Scrambled or PRG siRNA. **E.** PRG knock down was confirmed with Western blot. **F.** Caco2 cells serum starved over-night and stimulated with GRP for 24 hours. Cell media for each condition was harvested and analyzed for PGE_2 concentration by ELISA (see methods). Statistical analysis of n=4. Shown are mean values $\pm$ SEM; (** $p<0.01$).

I. Rho-ROCK Mediated Regulation of Cox-2-PGE_2 Production Contributes to Overall GRP Stimulated Cancer Cell Migration

Having identified that PRG-RhoA signaling plays a role in GRP stimulated Cox-2 expression, we questioned if this regulation is mediated though ROCK. ROCK has previously been implicated in regulation of Cox-2 expression in different tissues (127,206). Here we utilized Y-27632 (20μM) to inhibit ROCK and assess its effect on Cox-2 expression in response to GRP. ROCK inhibition abrogates GRP-mediated stimulation of Cox-2 expression in Caco-2 and HT-29 cells (Fig 16A-D). We also observed that treatment with Y-27632 impedes GRP-stimulated PGE_2 production (Fig 16E). These data reveal that the PRG-RhoA-ROCK signaling axis downstream of GRPR activation contributes to Cox-2 expression and PGE_2 production in colon cancer cells.

Evidence from *in vitro* and *in vivo* studies have shown that Cox-2-PGE_2 signaling increases colon cancer cell migration and invasion (207-209). Therefore, we wanted to identify the contribution of Cox-2-PGE_2 signaling to overall GRP-stimulated colon cancer cell migration. Here we conducted a transwell cell migration assay with Caco-2 cells stimulated with GRP incubated with or without celecoxib, a Cox-2 specific inhibitor. It has been reported that celecoxib at 20 μM does not result in colon cancer cell apoptosis (210). Caco-2 cells incubated with celecoxib without GRP had no defect in basal cell migration in comparison to DMSO treated Caco-2 cells (Fig 16F). However, celecoxib treatment did result in a modest reduction (~35%) in GRP-stimulated migration of Caco-2 cells as compared to Caco-2 cells treated with both GRP and DMSO (Fig 16F). Thus, our data indicates that Cox-2 expression and activity contributes to overall GRP-stimulated colon cancer cell migration.

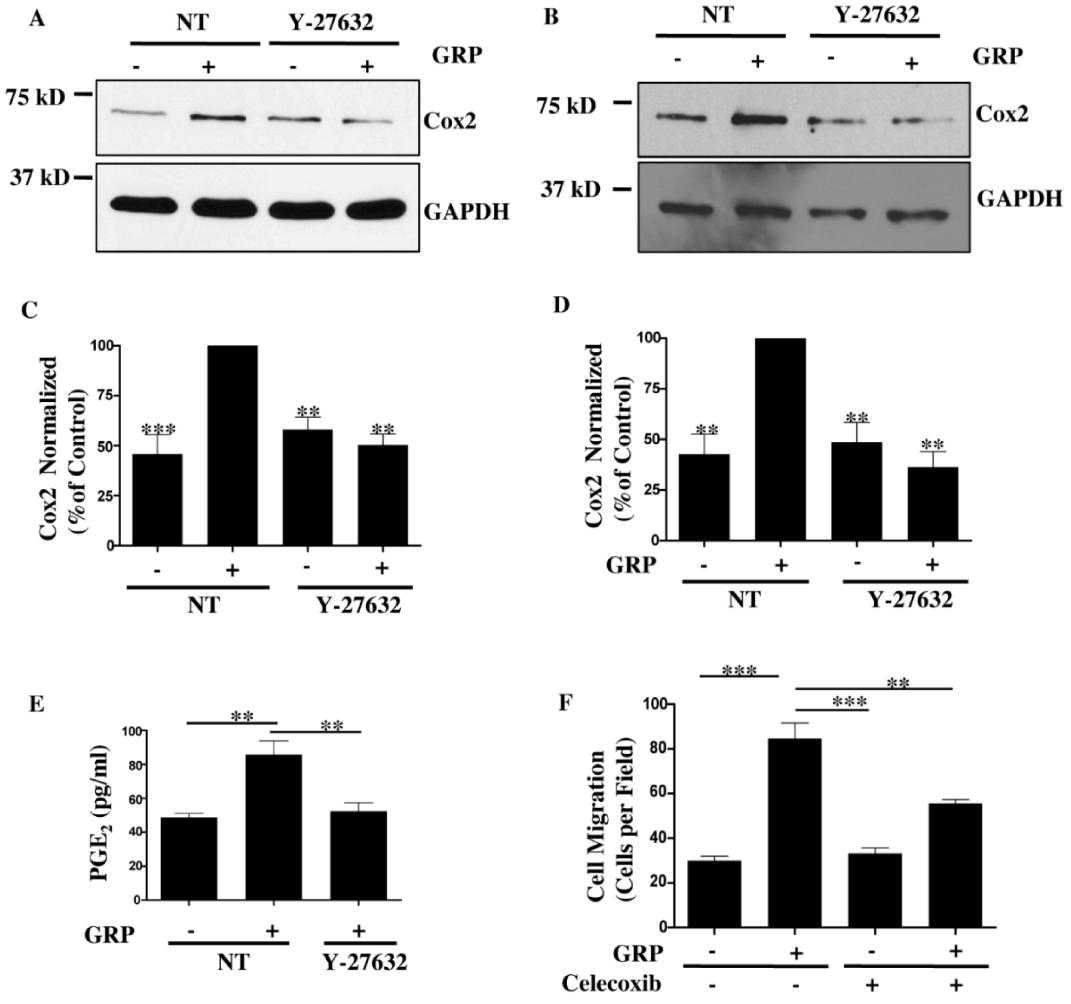


Figure 16: Rho-ROCK mediated regulation of Cox-2-PGE_2 production contributes to overall GRP stimulated cancer cell migration- A-D. Cox-2 expression in Caco-2 **(A)** and HT-29 **(B)** cells treated with or without Y-27632 (20 μM) along with GRP for 8 hours. Cox-2 expression was verified by Western Blot. **C-D.** Statistical densitometric analysis of Cox-2 expression in Caco-2 **(C)** and HT-29 **(D)** cells from n=3. Shown are mean values $\pm$SEM; (** $p<0.01$, *** $p<0.001$). **E.** Caco-2 cells serum starved over-night and stimulated with GRP with or without Y-27632 for 8 hours. Cell media for each condition was harvested and analyzed for PGE_2 concentration by ELISA (see methods). Statistical analysis of n=3. Shown are mean values $\pm$ SEM; (** $p<0.01$). **F.** Caco-2 cells serum starved overnight and plated on the upper chamber of transwell insert at $5X10^5$cells/well. Transwell inserts were contained in media supplemented with 1%FBS and with or without GRP along with celecoxib (20 μM) (see Methods). Statistical analysis of cell migration n=3 repeated in duplicates. Shown are mean values $\pm$ SEM; (**, $p<0.01$, ***, $p<0.001$).

J. PRG Expression is Upregulated in Colon Cancer Cells.

Our data show that PRG is critical in regulation of cell migration stimulated though GRPR. Enhanced propagation of GRPR- signaling in colon cancer cells might be achieved by elevated expression of PRG. Evaluation of PRG protein levels demonstrated higher PRG expression in Caco-2 and HT-29 colon cancer cells when compared to primary HCoEpiC and samples from normal human distal colonic mucosa (DCM) (Fig 17A). Furthermore, analysis of copy number variation for RH-RhoGEFs in Catalogue of Somatic Mutations in Cancer database (COSMIC) revealed that 17.1% of the 486 tested human colon cancers have gains in PRG gene copy number (COSMIC v68) (Fig 17B). These results indicate that PRG expression may be elevated in colon cancers, playing a critical role in regulation of colon cancer cell migration and invasion.

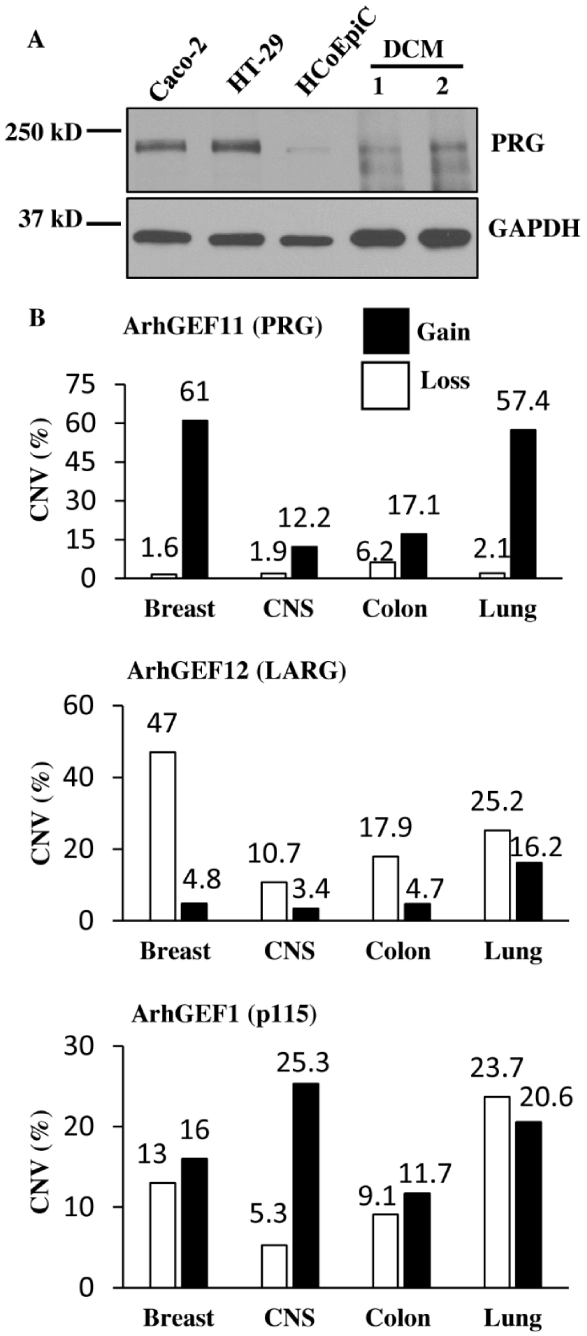


Figure 17: PRG expression is upregulated in colon cancer cells. A. Protein expression of PRG in Caco-2, HT-29, primary human colonic epithelial cells, and two different samples of human distal colonic mucosa. **B.** Copy number variation (CNV) of three RH-RhoGEF family members in four common types of solid tumors obtained from COSMIC v68. Depicting RH-RhoGEF gene gain or loss within these solid tumors.

V. Discussion

GPCRs coupled to $G\alpha_{12/13}$ have been implicated in cancer progression via increased cancer cell migration and invasion in SCLC, breast cancer, prostate cancer and colon cancer (38). In our current work, we have identified the molecular mechanism by which GRPR-activated $G\alpha_{13}$ signaling contributes to colon cancer cell migration. We have found that $G\alpha_{13}$ is the predominant mediator of RhoA activation downstream of GRPR, whereas $G\alpha_q$ makes small contributions to total RhoA activation. This observation, along with previous studies demonstrating that CXCR4 and LPA receptors mediate RhoA activation through $G\alpha_{13}$ (23,95), suggests that $G\alpha_{13}$ possibly is the predominant regulator of RhoA activity downstream of multiple GPCRs that couple to $G\alpha_{12/13}$.

Our studies identify PRG as the predominant RH-RhoGEF activated downstream of GRPR in colon cancer cells, whereas the other two RH-RhoGEFs, p115 and LARG, have little or no change in activity as indicated by our $RhoA^{G17A}$ pulldown data. Interestingly, downregulation of PRG expression leads to a similar decrease in RhoA activation as inhibition of $G\alpha_{13}$, indicating that $G\alpha_{13}$ regulates RhoA through PRG. The remaining $G\alpha_q$-mediated contribution to activation of RhoA may possibly be regulated through Trio, Kalirin, LARG, or p63RhoGEF. Previous report has demonstrated that LARG may be a downstream effector of $G\alpha_q$ (211), however our $RhoA^{G17A}$ pulldown data does not support this possibility as GRP stimulation brings about no further increase in LARG activation in colon cancer cells. It has been shown that p63RhoGEF, RhoA specific GEF, is activated through the direct interaction of AlF_4-activated $G\alpha_q$ subunit with the C-terminal extension of p63RhoGEF's PH domain (212,213). Indeed, we have observed that p63RhoGEF is activated upon GRP stimulation in Caco-2 cells and thus presumably contributes to $G\alpha_q$ mediated RhoA activation

(Patel and Kozasa, unpublished observations). However, this novel pathway requires further characterization.

GPCR-mediated RhoA-ROCK activation plays a critTical role in cell migration. Here, we report for the first time that GRP-stimulated colon cancer cell migration is regulated by the PRG-RhoA-ROCK signaling axis. Our findings are in line with previous studies, which have reported that PRG-RhoA-ROCK signaling regulates fibroblast cell migration and breast cancer cell migration. These studies have identified that PRG-RhoA-ROCK signaling regulates cell migration through induction of adhesion complexes and spatial regulation of actinomyosin contractile machinery (97,166). Prior work has also demonstrated that growth factor receptor tyrosine kinases also utilize Rho-ROCK signaling to promote tumor cell migration and invasion (214,215). Hence, it is clear that ROCK may be an ideal molecular target for prevention of tumor cell migration and metastasis.

GPCR-mediated regulation of Cox-2 expression contributes to colon cancer progression by regulating proliferation, migration and invasion (203). Here we show that GRP stimulation of Caco-2 and HT-29 cells leads to Cox-2 expression. Our data for the first time supports the role of PRG in regulation of Cox-2 expression and Cox-2-mediated PGE_2 production. Furthermore, we identified that ROCK, acting downstream of PRG-RhoA, contributes to Cox-2 expression in response to GRP stimulation. Our findings are in line with other studies that have also reported the role of ROCK in regulating Cox-2 expression downstream of another GPCR, Proteinase-activated receptor-2 (PAR-2)(206). Current evidence indicates that Cox-2-PGE_2 signaling stimulates colon cancer cell migration through activation of its cognate receptor EP4 or through transactivation of EGF-R (207,208). Overall our data suggests that the modest defect in cancer cell motility observed with celecoxib treatment, indicates that Cox-2-PGE_2 signaling is not the main regulator of GRP-

stimulated colon cancer cell migration, and most likely it is predominantly controlled by PRG-RhoA-ROCK pathway directly regulating actomyosin contractile machinery.

The role of RH-RhoGEFs in tumorigenesis has just recently gained recognition. Existing evidence suggests that the role of these RH-RhoGEFs is varied in tumor development and metastasis and their functions are tumor specific. It has been reported that p115 expression is upregulated in prostate cancer cells and invasive prostate tumors (216). However, the role of p115 in the context of its involvement in signaling downstream of GPCRs and its effect on cancer progression is not known. In contrast to elevated expression of p115 in prostate cancer, LARG expression in breast and colon cancers is reported to be decreased. In these cancers, LARG has been reported to act as a tumor suppressor (185). Loss of LARG expression in breast and colon cancer is also supported by data that shows that there is loss of gene copy number of LARG in these solid tumors. Here, we show that PRG is the major mediator of GRP-stimulated colon cancer cell migration. Data form the COSMIC database shows that PRG gene copy number is increased in a significant number of colon cancer samples. Furthermore, our results demonstrate that PRG is overexpressed in colon cancer cell lines. These results suggest that PRG may play a key role in regulation of tumorigenesis mediated by GRPR and other GPCRs. Indeed, a recent report by Struckhoff et al., concludes that PRG is essential for CXCR4-mediated breast cancer cell migration and invasion and found that PRG expression is increased at the leading edge of primary tumors and tumor cells that have undergone lymphatic invasion (97). Another study looking at PC-3 prostate cancer cells grown in 3-D organotypic culture reported increased PRG expression in the invasive cultures (186). Furthermore, PRG has been implicated as a pro-survival gene in human gliobastomas, where knockdown of PRG resulted in decreased cell viability (187,188). It should be noted that p115 nor LARG were identified as one of the 55 candidate cell survival genes in this

study (187). Further supporting the role of PRG in gliobastoma comes from a recent published abstract which reports that PRG plays a critical role in regulation of gliobastoma cell migration, invasion, and also mediates cancer cell proliferation and survival. The authors report that PRG, through largely activation of RhoC, regulates these various pathological processes. It should be noted that these data are not yet available for review as the authors have yet to publish these findings (217). Similarly, another published abstract also reports that PRG is over-expressed in ovarian cancer cells and human epithelial ovarian cancer samples. The authors report that PRG acts downstream of ET_A in ovarian cancer cells to activate RhoA/ROCK signaling axis. The abstract states that in ovarian cancer cells, ET_A activation leads to β-arrestin1/PRG interaction, which results in activation of RhoA/ROCK signaling axis. This novel pathway downstream of ET_A is implicated in promoting metastatic spread of ovarian cancer. However, data supporting these conclusions are also not yet published (218). Non-the-less it is interesting that in these common solid tumors there is reported to be increased gene-copy-number of PRG, whereas LARG gene-copy-number is lost in a manner that seems to be almost reciprocal with PRG. It is likely that perhaps in cancer PRG and LARG may have differing functions, as LARG has been suggested to be a candidate TSG, thus loss of its expression contributes to cancer progression (185). Whereas, current evidence supports the notion that gain in PRG gene-copy-number resulting in increased PRG expression and activity may contribute to cancer progression not only in colon cancer but also in other solid tumors by regulating pro-survival pathways, cancer cell migration and invasion.

Characterization of GRPR-mediated signaling pathway in colon cancer cells has revealed new potential therapeutic targets. Identification of the role of PRG in GRPR-mediated colon cancer cell migration and Cox-2 expression opens additional opportunities for developing novel therapeutic agents. Application of a recently developed inhibitor specific for RH-RhoGEFs (Y16)

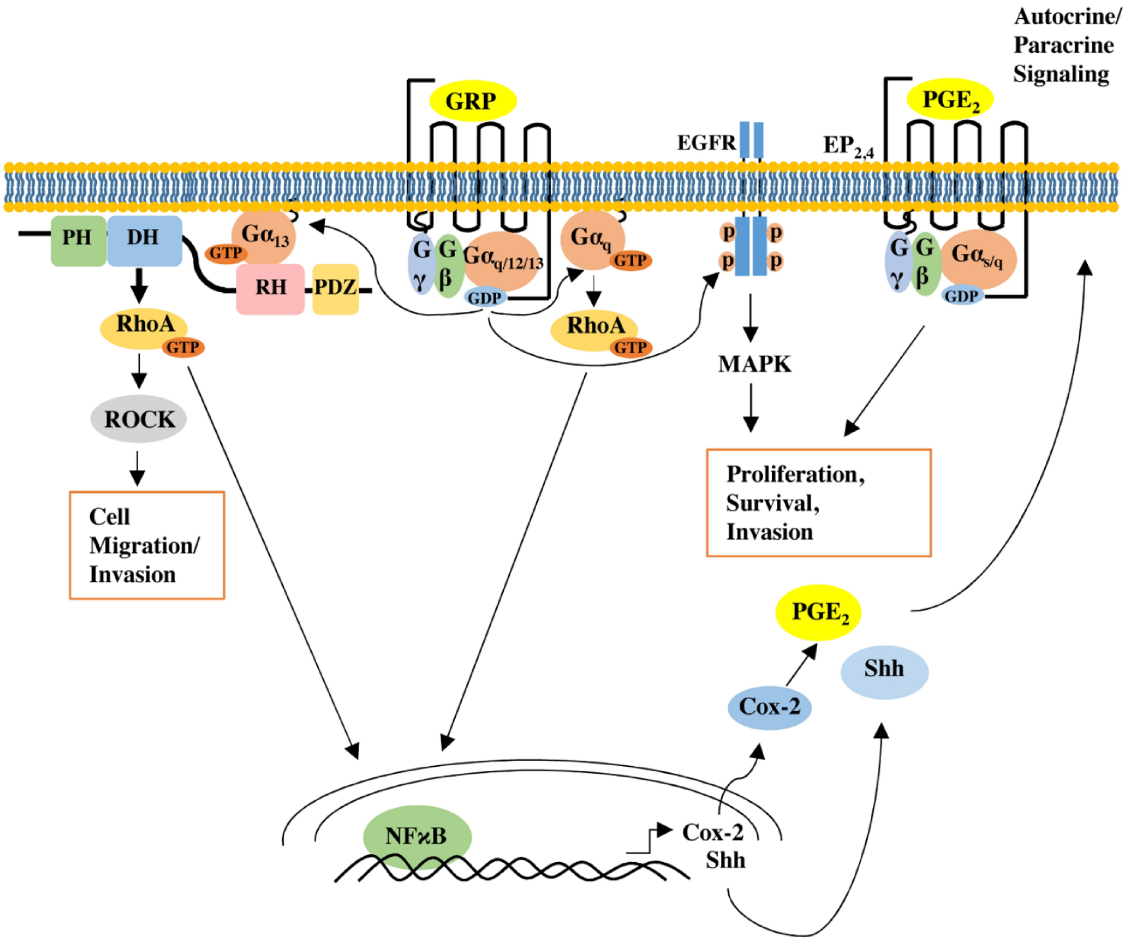
together with existing inhibitors for Cox-2 may prove to have therapeutic effects on colon cancer models (26). As the roles of the RH-RhoGEFs in tumorigenesis and metastasis become more well-defined, development of novel inhibitors specific for p115, LARG, or PRG would expand our choices for selection of therapeutic strategy.

VI. Future directions

One clear future direction that is illuminated based on the data and the conclusions made from this study would be addressing the role of this signaling pathway in regulation of tumor cell invasion and metastasis in an animal model. Our data suggests that perturbation of the $G\alpha_{13}$ arm of GRPR in colon cancer, specifically either by silencing PRG expression or through small molecule inhibition of PRG activity, would result in defective cancer cell invasion and metastatic spread. We propose to test this hypothesis by xenografting stable PRG KD HT-29 cells in the colon of SCID (severe combined immuno-deficiency) mice and observe if PRG KD has an impact on tumor cell invasion and metastatic spread. In these mice we would also preform secondary analysis of the primary tumor to see if there is also an impact on tumor growth and tumor vascularization. Similar study would be conducted in parallel with WT HT-29 cells xenografted into the colon of these SCID mice that are treated with Y16 compound that has been shown to be specific for RH-RhoGEFs (26). Although, this compound is not specific for PRG, it would still be of interest to see what effect, if any, does RH-RhoGEF inhibition have on colon cancer proliferation, invasion, and metastatic spread? It would be of interest to perform a comparative analysis of tumor growth, invasion, and metastatic spread, of mice with HT-29 PRG KD xenografts with mice that have WT HT-29 xenografts that were treated with Y16 compound as this may reveal similar outcome, which would then provide the evidence for further development of a compound that is specific for PRG.

GRPR signaling through RhoGTPases can activate signaling pathways that regulate cancer cell migration, invasion, and proliferation (Figure 18). Specifically, GRPR mediated RhoA signaling has not only been demonstrated to be important for cancer cell migration and invasion, but also to activate proliferation and pro-survival pathways such as Cox-2/PGE_2, and Shh regulated signaling pathways (102,127). Thus, targeting the upstream activators of RhoA may prove to also impede these pro-survival signaling pathways. Furthermore, it would also be

interesting to see if targeting PRG along with utilization of standard chemotherapy agents utilized for specific malignancies would result in synergistic inhibition of cancer growth and metastatic spread. It is of the author's contention that the best therapeutic target within this signaling pathway is PRG. As targeting GRPR has proved unsuccessful, primarily due to the inability to achieve target therapeutic concentrations *in vivo*, as a result of the intrinsic pharmacokinetic properties of the GRPR antagonists that have been developed. Even with formulation of small molecule antagonists for GRPR with better pharmacokinetic properties, we may observe un-intended and unforeseen adverse effects due to inhibition of GRPR, as it has wide expression profile, and its role in regulation of critical physiological functions. Also, it is clear that GRPR is only one of many GPCRs that are involved in colon cancer progression as summarized in (Table 2). Targeting GRPR alone most likely will not bring about the desired therapeutic effect as signaling downstream of other GPCRs that contribute to cancer progression is still active. However, by targeting a downstream molecule that is shared by many of these GPCRs, such as PRG, may result in therapeutically relevant inhibition of cancer progression.


Autocrine/
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ROCK
Proliferation,
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Cell
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Invasion
PGE2
Shh
Cox-2
NFκB
Cox-2
Shh

Figure 18: Schematic of GPRR signaling in solid malignancies. GRPR is known to couple to $G\alpha_q$ and $G\alpha_{/12/13.}$ Current evidence indicates that GRPR activation leads to both $G\alpha_{12/13}$ and $G\alpha_q$ mediated RhoA activation. We have provided evidence that PRG is the predominant RH-RhoGEF downstream of $G\alpha_{13}$ (as denoted by b arrow), and that PRG-RhoA-ROCK axis in-turn regulates colon cancer cell migration. A recent study also demonstrates that GRPR signaling through $G\alpha_{q/12/13}$-Rho signaling, transcriptionally regulates Shh production via activation of NFκB. Production of Shh and PGE_2 leads to an autocrine/paracrine growth factor signaling loop (It should be noted that the specific Rho isoforms, RhoA or RhoC, that are involved in this signaling pathway were not identified). Depicted here is PGE_2 activating its receptor, $EP_{1\text{-}4,}$ to initiate signaling cascades that contribute to cancer cell proliferation, survival, and invasion. It should also be noted that GRPR signaling has also been implicated in transactivation of EGFR (by unknown mechanisms), which further promotes cancer cell proliferation. Not depicted here is the Shh signaling, via activation of Ptch (patched) and Smo (Smoothened) receptors, which activates Gli transcription factors that ultimately promotes cancer cell proliferation and survival. Thus, it is clear that GRPR activation in variety of solid malignancies may be a central event that initiates other prosurvival signaling pathways to contribute to cancer development and progression. It should be noted that not all of these signaling pathways have been verified in different types of solid tumors, and more work needs to be carried out to verify if these signaling pathways are more general and operational in different cancers.

VII. Cited Literature

1. Jensen, R. T., Battey, J. F., Spindel, E. R., and Benya, R. V. (2008) International Union of Pharmacology. LXVIII. Mammalian Bombesin Receptors: Nomenclature, Distribution, Pharmacology, Signaling, and Functions in Normal and Disease States. *Pharmacological Reviews* **60**, 1-42

2. Radulovic, S., Miller, G., and Schally, A. V. (1991) Inhibition of Growth of HT-29 Human Colon Cancer Xenografts in Nude Mice by Treatment with Bombesin/Gastrin Releasing Peptide Antagonist (RC-3095). *Cancer Research* **51**, 6006-6009

3. Frucht, H., Gazdar, A. F., Park, J. A., Oie, H., and Jensen, R. T. (1992) Characterization of Functional Receptors for Gastrointestinal Hormones on Human Colon Cancer Cells. *Cancer Research* **52**, 1114-1122

4. Carroll, R. E., Matkowskyj, K. A., Chakrabarti, S., McDonald, T. J., and Benya, R. V. (1999) Aberrant expression of gastrin-releasing peptide and its receptor by well-differentiated colon cancers in humans. *American Journal of Physiology - Gastrointestinal and Liver Physiology* **276**, G655-G665

5. Glover, S., Nathaniel, R., Shakir, L., Perrault, C., Anderson, R. K., Tran-Son-Tay, R., and Benya, R. V. (2005) Transient upregulation of GRP and its receptor critically regulate colon cancer cell motility during remodeling. *American Journal of Physiology - Gastrointestinal and Liver Physiology* **288**, G1274-G1282

6. Cuttitta F, C. D., Mulshine J, Moody TW, Fedorko J, Fischler A, Minna JD. (1985) Bombesin-like peptides can function as autocrine growth factors in human small-cell lung cancer. *Nature* **316**, 823-826.

7. Rozengurt, E. (1998) Signal transduction pathways in the mitogenic response to G protein–coupled neuropeptide receptor agonists. *Journal of Cellular Physiology* **177**, 507-517

8. Wang, Q., Liu, M., Kozasa, T., Rothstein, J. D., Sternweis, P. C., and Neubig, R. R. (2004) Thrombin and Lysophosphatidic Acid Receptors Utilize Distinct rhoGEFs in Prostate Cancer Cells. *Journal of Biological Chemistry* **279**, 28831-28834

9. Mikelis, C. M., Palmby, T. R., Simaan, M., Li, W., Szabo, R., Lyons, R., Martin, D., Yagi, H., Fukuhara, S., Chikumi, H., Galisteo, R., Mukouyama, Y.-s., Bugge, T. H., and Gutkind, J. S. (2013) PDZ-RhoGEF and LARG Are Essential for Embryonic Development and Provide a Link between Thrombin and LPA Receptors and Rho Activation. *Journal of Biological Chemistry* **288**, 12232-12243

10. Hart, M. J., Sharma, S., elMasry, N., Qiu, R.-G., McCabe, P., Polakis, P., and Bollag, G. (1996) Identification of a Novel Guanine Nucleotide Exchange Factor for the Rho GTPase. *Journal of Biological Chemistry* **271**, 25452-25458

11. Hart, M. J., Jiang, X., Kozasa, T., Roscoe, W., Singer, W. D., Gilman, A. G., Sternweis, P. C., and Bollag, G. (1998) Direct Stimulation of the Guanine Nucleotide Exchange Activity of p115 RhoGEF by Gα13. *Science* **280**, 2112-2114

12. Kozasa, T., Jiang, X., Hart, M. J., Sternweis, P. M., Singer, W. D., Gilman, A. G., Bollag, G., and Sternweis, P. C. (1998) p115 RhoGEF, a GTPase Activating Protein for Gα12 and Gα13. *Science* **280**, 2109-2111

13. Fukuhara, S., Murga, C., Zohar, M., Igishi, T., and Gutkind, J. S. (1999) A Novel PDZ Domain Containing Guanine Nucleotide Exchange Factor Links Heterotrimeric G Proteins to Rho. *Journal of Biological Chemistry* **274**, 5868-5879

14. Fukuhara, S., Chikumi, H., and Gutkind, J. S. (2000) Leukemia-associated Rho guanine nucleotide exchange factor (LARG) links heterotrimeric G proteins of the G12 family to Rho. *FEBS Letters* **485**, 183-188

15. Tanabe, S., Kreutz, B., Suzuki, N., and Kozasa, T. (2004) Regulation of RGS-RhoGEFs by Gα12 and Gα13 Proteins. in *Methods in Enzymology* (David, P. S. ed.), Academic Press. pp 285-294

16. Chen, Z., Singer, W. D., Danesh, S. M., Sternweis, P. C., and Sprang, S. R. (2008) Recognition of the Activated States of Gα13 by the rgRGS Domain of PDZRhoGEF. *Structure* **16**, 1532-1543

17. Sahai, E., and Marshall, C. J. (2002) RHO-GTPases and cancer. *Nat Rev Cancer* **2**, 133-142

18. Fritz, G., Just, I., and Kaina, B. (1999) Rho GTPases are over-expressed in human tumors. *International Journal of Cancer* **81**, 682-687

19. Takami, Y., Higashi, M., Kumagai, S., Kuo, P., Kawana, H., Koda, K., Miyazaki, M., and Harigaya, K. (2008) The Activity of RhoA is Correlated with Lymph Node Metastasis in Human Colorectal Cancer. *Dig Dis Sci* **53**, 467-473

20. Wang H, L. P., Liang J, Yang K, Sui A, Liu JY. (2009) Expression of RhoA and RhoC in colorectal carcinoma and its relations with clinicopathological parameters. *Clinical Chemistry and Laboratory Medicine* **47**, 811-817

21. Vigil, D., Kim, T. Y., Plachco, A., Garton, A. J., Castaldo, L., Pachter, J. A., Dong, H., Chen, X., Tokar, B., Campbell, S. L., and Der, C. J. (2012) ROCK1 and ROCK2 Are Required for Non-Small Cell Lung Cancer Anchorage-Independent Growth and Invasion. *Cancer Research* **72**, 5338-5347

22. Zhang, C., Zhang, S., Zhang, Z., He, J., Xu, Y., and Liu, S. (2013) ROCK has a crucial role in regulating prostate tumor growth through interaction with c-Myc. *Oncogene*

23. Yagi, H., Tan, W., Dillenburg-Pilla, P., Armando, S., Amornphimoltham, P., Simaan, M., Weigert, R., Molinolo, A. A., Bouvier, M., and Gutkind, J. S. (2011) A Synthetic Biology Approach Reveals a CXCR4-G13-Rho Signaling Axis Driving Transendothelial Migration of Metastatic Breast Cancer Cells. *Science Signaling* **4**.

24. Kelly, P., Stemmle, L. N., Madden, J. F., Fields, T. A., Daaka, Y., and Casey, P. J. (2006) A Role for the G12 Family of Heterotrimeric G Proteins in Prostate Cancer Invasion. *Journal of Biological Chemistry* **281**, 26483-26490

25. Kelly, P., Moeller, B. J., Juneja, J., Booden, M. A., Der, C. J., Daaka, Y., Dewhirst, M. W., Fields, T. A., and Casey, P. J. (2006) The G12 family of heterotrimeric G proteins promotes breast cancer invasion and metastasis. *Proceedings of the National Academy of Sciences* **103**, 8173-8178

26. Shang, X., Marchioni, F., Evelyn, C. R., Sipes, N., Zhou, X., Seibel, W., Wortman, M., and Zheng, Y. (2013) Small-molecule inhibitors targeting G-protein–coupled Rho guanine nucleotide exchange factors. *Proceedings of the National Academy of Sciences* **110**, 3155-3160

27. Colorectal Cancer Facts & Figures 2014-2016 (2014). in *American Cancer Society.* Society., A. C. ed., American Cancer Society., Atlanta

28. Huxley, R. R., Ansary-Moghaddam, A., Clifton, P., Czernichow, S., Parr, C. L., and Woodward, M. (2009) The impact of dietary and lifestyle risk factors on risk of colorectal cancer: A quantitative overview of the epidemiological evidence. *International Journal of Cancer* **125**, 171-180

29. Vogelstein, B., Fearon, E. R., Hamilton, S. R., Kern, S. E., Preisinger, A. C., Leppert, M., Smits, A. M. M., and Bos, J. L. (1988) Genetic Alterations during Colorectal-Tumor Development. *New England Journal of Medicine* **319**, 525-532

30. Fearon, E. R. (2011) Molecular Genetics of Colorectal Cancer. *Annual Review of Pathology: Mechanisms of Disease* **6**, 479-507

31. Winawer, S. J., Zauber, A. G., Ho, M. N., O'Brien, M. J., Gottlieb, L. S., Sternberg, S. S., Waye, J. D., Schapiro, M., Bond, J. H., Panish, J. F., Ackroyd, F., Shike, M., Kurtz, R. C., Hornsby-Lewis, L., Gerdes, H., and Stewart, E. T. (1993) Prevention of Colorectal Cancer by Colonoscopic Polypectomy. *New England Journal of Medicine* **329**, 1977-1981

32. Pritchard, C. C., and Grady, W. M. (2011) Colorectal cancer molecular biology moves into clinical practice. *Gut* **60**, 116-129

33. Liu, C. H., Chang, S.-H., Narko, K., Trifan, O. C., Wu, M.-T., Smith, E., Haudenschild, C., Lane, T. F., and Hla, T. (2001) Overexpression of Cyclooxygenase-2 Is Sufficient to

Induce Tumorigenesis in Transgenic Mice. *Journal of Biological Chemistry* **276**, 18563-18569

34. Wang, D., and DuBois, R. N. (2010) Eicosanoids and cancer. *Nat Rev Cancer* **10**, 181-193

35. Corral, R. S., Iniguez, M. A., Duque, J., Lopez-Perez, R., and Fresno, M. (2006) Bombesin induces cyclooxygenase-2 expression through the activation of the nuclear factor of activated T cells and enhances cell migration in Caco-2 colon carcinoma cells. *Oncogene* **26**, 958-969

36. Slice, L. W., Walsh, J. H., and Rozengurt, E. (1999) Gα13 Stimulates Rho-dependent Activation of the Cyclooxygenase-2 Promoter. *Journal of Biological Chemistry* **274**, 27562-27566

37. Wen X, C. C., Ives K, Hellmich MR. (2011) Regulation of bombesin-stimulated cyclooxygenase-2 expression in prostate cancer cells. *BMC Molecular Biology* **12**

38. Lappano, R., and Maggiolini, M. (2011) G protein-coupled receptors: novel targets for drug discovery in cancer. *Nat Rev Drug Discov* **10**, 47-60

39. Dorsam, R. T., and Gutkind, J. S. (2007) G-protein-coupled receptors and cancer. *Nat Rev Cancer* **7**, 79-94

40. O'Hayre, M., Vazquez-Prado, J., Kufareva, I., Stawiski, E. W., Handel, T. M., Seshagiri, S., and Gutkind, J. S. (2013) The emerging mutational landscape of G proteins and G-protein-coupled receptors in cancer. *Nat Rev Cancer* **13**, 412-424

41. Oldham, W. M., and Hamm, H. E. (2008) Heterotrimeric G protein activation by G-protein-coupled receptors. *Nat Rev Mol Cell Biol* **9**, 60-71

42. Hopkins, A. L., and Groom, C. R. (2002) The druggable genome. *Nat Rev Drug Discov* **1**, 727-730

43. Downes, G. B., and Gautam, N. (1999) The G Protein Subunit Gene Families. *Genomics* **62**, 544-552

44. Pierce, K. L., Premont, R. T., and Lefkowitz, R. J. (2002) Seven-transmembrane receptors. *Nat Rev Mol Cell Biol* **3**, 639-650

45. Rasmussen, S. G. F., DeVree, B. T., Zou, Y., Kruse, A. C., Chung, K. Y., Kobilka, T. S., Thian, F. S., Chae, P. S., Pardon, E., Calinski, D., Mathiesen, J. M., Shah, S. T. A., Lyons, J. A., Caffrey, M., Gellman, S. H., Steyaert, J., Skiniotis, G., Weis, W. I., Sunahara, R. K., and Kobilka, B. K. (2011) Crystal structure of the [bgr]2 adrenergic receptor-Gs protein complex. *Nature* **477**, 549-555

46. Chung, K. Y., Rasmussen, S. G. F., Liu, T., Li, S., DeVree, B. T., Chae, P. S., Calinski, D., Kobilka, B. K., Woods, V. L., and Sunahara, R. K. (2011) Conformational changes in the G protein Gs induced by the [bgr]2 adrenergic receptor. *Nature* **477**, 611-615

47. Manglik, A., and Kobilka, B. (2014) The role of protein dynamics in GPCR function: insights from the β2AR and rhodopsin. *Current Opinion in Cell Biology* **27**, 136-143

48. Erspamer V, E. G., Inselvini M. (1970) Some pharmacological actions of alytesin and bombesin. *Journal of pharmacy and pharmacology* **22**, 875 - 876

49. Erspamer, V., Erspamer, G. F., Inselvini, M., and Negri, L. (1972) Occurrence of bombesin and alytesin in extracts of the skin of three European discoglossid frogs and pharmacological actions of bombesin on extravascular smooth muscle. *British Journal of Pharmacology* **45**, 333-348

50. McDonald, T. J., Jörnvall, H., Nilsson, G., Vagne, M., Ghatei, M., Bloom, S. R., and Mutt, V. (1979) Characterization of a gastrin releasing peptide from porcine non-antral gastric tissue. *Biochemical and Biophysical Research Communications* **90**, 227-233

51. Minamino, N., Kangawa, K., and Matsuo, H. (1983) Neuromedin B: A novel bombesin-like peptide identified in porcine spinal cord. *Biochemical and Biophysical Research Communications* **114**, 541-548

52. Benya, R. V., Kusui, T., Pradhan, T. K., Battey, J. F., and Jensen, R. T. (1995) Expression and characterization of cloned human bombesin receptors. *Molecular Pharmacology* **47**, 10-20

53. Maslen, G. L., and Boyd, Y. (1993) Comparative Mapping of the Grpr Locus on the X Chromosomes of Man and Mouse. *Genomics* **17**, 106-109

54. Xiao, D., Wang, J., Hampton, L. L., and Weber, H. C. (2001) The human gastrin-releasing peptide receptor gene structure, its tissue expression and promoter. *Gene* **264**, 95-103

55. Kris, R. M., Hazan, R., Villines, J., Moody, T. W., and Schlessinger, J. (1987) Identification of the bombesin receptor on murine and human cells by cross-linking experiments. *Journal of Biological Chemistry* **262**, 11215-11220

56. Kusui, T., Benya, R. V., Battey, J. F., and Jensen, R. T. (1994) Glycosylation of Bombesin Receptors: Characterization, Effect on Binding, and G-Protein Coupling. *Biochemistry* **33**, 12968-12980

57. Benya, R. V., Kusui, T., Katsuno, T., Tsuda, T., Mantey, S. A., Battey, J. F., and Jensen, R. T. (2000) Glycosylation of the Gastrin-Releasing Peptide Receptor and Its Effect on Expression, G Protein Coupling, and Receptor Modulatory Processes. *Molecular Pharmacology* **58**, 1490-1501

58. Walton, N. M., de Koning, A., Xie, X., Shin, R., Chen, Q., Miyake, S., Tajinda, K., Gross, A. K., Kogan, J. H., Heusner, C. L., Tamura, K., and Matsumoto, M. (2014) Gastrin-releasing peptide contributes to the regulation of adult hippocampal neurogenesis and neuronal development. *STEM CELLS*.

59. Sun, Y.-G., Zhao, Z.-Q., Meng, X.-L., Yin, J., Liu, X.-Y., and Chen, Z.-F. (2009) Cellular Basis of Itch Sensation. *Science* **325**, 1531-1534

60. Sun, Y.-G., and Chen, Z.-F. (2007) A gastrin-releasing peptide receptor mediates the itch sensation in the spinal cord. *Nature* **448**, 700-703

61. Shumyatsky, G. P., Tsvetkov, E., Malleret, G., Vronskaya, S., Hatton, M., Hampton, L., Battey, J. F., Dulac, C., Kandel, E. R., and Bolshakov, V. Y. (2002) Identification of a Signaling Network in Lateral Nucleus of Amygdala Important for Inhibiting Memory Specifically Related to Learned Fear. *Cell* **111**, 905-918

62. Hampton, L. L., Ladenheim, E. E., Akeson, M., Way, J. M., Weber, H. C., Sutliff, V. E., Jensen, R. T., Wine, L. J., Arnheiter, H., and Battey, J. F. (1998) Loss of bombesin-induced feeding suppression in gastrin-releasing peptide receptor-deficient mice. *Proceedings of the National Academy of Sciences* **95**, 3188-3192

63. Czepielewski, R. S., Porto, B. N., Rizzo, L. B., Roesler, R., Abujamra, A. L., Pinto, L. G., Schwartsmann, G., Cunha, F. d. Q., and Bonorino, C. (2012) Gastrin-releasing peptide receptor (GRPR) mediates chemotaxis in neutrophils. *Proceedings of the National Academy of Sciences* **109**, 547-552

64. Qiao, J., Kang, J., Ishola, T. A., Rychahou, P. G., Evers, B. M., and Chung, D. H. (2008) Gastrin-releasing peptide receptor silencing suppresses the tumorigenesis and metastatic potential of neuroblastoma. *Proceedings of the National Academy of Sciences* **105**, 12891-12896

65. Patel, O., Shulkes, A., and Baldwin, G. S. (2006) Gastrin-releasing peptide and cancer. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer* **1766**, 23-41

66. Carroll, R. E., Matkowskyj, K. A., Tretiakova, M. S., Battey, J. F., and Benya, R. V. (2000) Gastrin-releasing Peptide Is a Mitogen and a Morphogen in Murine Colon Cancer. *Cell Growth Differentiation* **11**, 385-393

67. MacKinnon, A. C., Waters, C., Jodrell, D., Haslett, C., and Sethi, T. (2001) Bombesin and Substance P Analogues Differentially Regulate G-protein Coupling to the Bombesin Receptor: DIRECT EVIDENCE FOR BIASED AGONISM. *Journal of Biological Chemistry* **276**, 28083-28091

68. Moody TW, Z. F., Venugopal R, Fagarasan M, Oie H, Hu V. (1996) GRP receptors are present in non small cell lung cancer cells. *Journal of cellular biochemistry. Supplement* **24**, 247 - 256

69. Lui, V. W. Y., Thomas, S. M., Zhang, Q., Wentzel, A. L., Siegfried, J. M., Li, J. Y., and Grandis, J. R. (2003) Mitogenic effects of gastrin-releasing peptide in head and neck squamous cancer cells are mediated by activation of the epidermal growth factor receptor. *Oncogene* **22**, 6183-6193

70. Thomas, S. M., Grandis, J. R., Wentzel, A. L., Gooding, W. E., Lui, V. W. Y., and Siegfried, J. M. (2005) Gastrin-Releasing Peptide Receptor Mediates Activation of the Epidermal Growth Factor Receptor in Lung Cancer Cells. *Neoplasia* **7**, 426-431

71. Adomeit, A., Graness, A., Gross, S., Seedorf, K., Wetzker, R., and Liebmann, C. (1999) Bradykinin B2 Receptor-Mediated Mitogen-Activated Protein Kinase Activation in COS-7 Cells Requires Dual Signaling via Both Protein Kinase C Pathway and Epidermal Growth Factor Receptor Transactivation. *Molecular and Cellular Biology* **19**, 5289-5297

72. Zheng, R., Iwase, A., Shen, R., Goodman, O. B., Jr., Sugimoto, N., Takuwa, Y., Lerner, D. J., and Nanus, D. M. (2006) Neuropeptide-stimulated cell migration in prostate cancer cells is mediated by RhoA kinase signaling and inhibited by neutral endopeptidase. *Oncogene* **25**, 5942-5952

73. Dhanasekaran, N., and Dermott, J. M. (1996) Signaling by the G12 class of G proteins. *Cellular Signalling* **8**, 235-245

74. Jones, T. L. Z., and Gutkind, J. S. (1998) Gα12 Requires Acylation for Its Transforming Activity. *Biochemistry* **37**, 3196-3202

75. Bhattacharyya, R., and Wedegaertner, P. B. (2000) Gα13 Requires Palmitoylation for Plasma Membrane Localization, Rho-dependent Signaling, and Promotion of p115-RhoGEF Membrane Binding. *Journal of Biological Chemistry* **275**, 14992-14999

76. Riobo, N. A., and Manning, D. R. (2005) Receptors coupled to heterotrimeric G proteins of the G12 family. *Trends in Pharmacological Sciences* **26**, 146-154

77. Hermans, E. (2003) Biochemical and pharmacological control of the multiplicity of coupling at G-protein-coupled receptors. *Pharmacology & Therapeutics* **99**, 25-44

78. Gong, H., Shen, B., Flevaris, P., Chow, C., Lam, S. C. T., Voyno-Yasenetskaya, T. A., Kozasa, T., and Du, X. (2010) G Protein Subunit Gα13 Binds to Integrin αIIbβ3 and Mediates Integrin "Outside-In" Signaling. *Science* **327**, 340-343

79. Kelly, P., Casey, P. J., and Meigs, T. E. (2007) Biologic Functions of the G12 Subfamily of Heterotrimeric G Proteins: Growth, Migration, and Metastasis†. *Biochemistry* **46**, 6677-6687

80. Suzuki, N., Hajicek, N., and Kozasa, T. (2009) Regulation and Physiological Functions of G12/13-Mediated Signaling Pathways. *Neurosignals* **17**, 55-70

81. Suzuki, N., Tsumoto, K., Hajicek, N., Daigo, K., Tokita, R., Minami, S., Kodama, T., Hamakubo, T., and Kozasa, T. (2009) Activation of Leukemia-associated RhoGEF by Gα13 with Significant Conformational Rearrangements in the Interface. *Journal of Biological Chemistry* **284**, 5000-5009

82. Barrett, K., Leptin, M., and Settleman, J. (1997) The Rho GTPase and a Putative RhoGEF Mediate a Signaling Pathway for the Cell Shape Changes in Drosophila Gastrulation. *Cell* **91**, 905-915

83. Parks, S., and Wieschaus, E. (1991) The drosophila gastrulation gene concertina encodes a Gα-like protein. *Cell* **64**, 447-458

84. Sweeton, D., Parks, S., Costa, M., and Wieschaus, E. (1991) Gastrulation in Drosophila: the formation of the ventral furrow and posterior midgut invaginations. *Development* **112**, 775-789

85. Yau, D. M., Yokoyama, N., Goshima, Y., Siddiqui, Z. K., Siddiqui, S. S., and Kozasa, T. (2003) Identification and molecular characterization of the Gα12–Rho guanine nucleotide exchange factor pathway in Caenorhabditis elegans. *Proceedings of the National Academy of Sciences* **100**, 14748-14753

86. Offermanns, S., Mancino, V., Revel, J.-P., and Simon, M. I. (1997) Vascular System Defects and Impaired Cell Chemokinesis as a Result of Gα13 Deficiency. *Science* **275**, 533-536

87. Ruppel, K. M., Willison, D., Kataoka, H., Wang, A., Zheng, Y.-W., Cornelissen, I., Yin, L., Xu, S. M., and Coughlin, S. R. (2005) Essential role for Gα13 in endothelial cells during embryonic development. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 8281-8286

88. Gu, J. L., Müller, S., Mancino, V., Offermanns, S., and Simon, M. I. (2002) Interaction of Gα12 with Gα13 and Gαq signaling pathways. *Proceedings of the National Academy of Sciences* **99**, 9352-9357

89. Chan, A. M., Fleming, T. P., McGovern, E. S., Chedid, M., Miki, T., and Aaronson, S. A. (1993) Expression cDNA cloning of a transforming gene encoding the wild-type G alpha 12 gene product. *Molecular and Cellular Biology* **13**, 762-768

90. Xu, N., Bradley, L., Ambdukar, I., and Gutkind, J. S. (1993) A mutant alpha subunit of G12 potentiates the eicosanoid pathway and is highly oncogenic in NIH 3T3 cells. *Proceedings of the National Academy of Sciences* **90**, 6741-6745

91. Xu, N. Z., Voynoyasenetskaya, T., and Gutkind, J. S. (1994) Potent Transforming Activity of the G13 α Subunit Defines a Novel Family of Oncogenes. *Biochemical and Biophysical Research Communications* **201**, 603-609

92. Radhika V., D. N. (2001) Transforming G proteins. *Oncogene* **20**, 1607-1614

93. Even-Ram S, U. B., Cohen P, Grisaru-Granovsky S, Maoz M, Ginzburg Y, Reich R, Vlodavsky I, Bar-Shavit R. (1998) Thrombin receptor overexpression in malignant and physiological invasion processes. *Nature medicine* **4**, 909 - 914

94. Daaka, Y. (2004) G Proteins in Cancer: The Prostate Cancer Paradigm. *Science Signaling* **2004**.

95. Bian, D., Mahanivong, C., Yu, J., Frisch, S. M., Pan, Z. K., Ye, R. D., and Huang, S. (2005) The G12//13-RhoA signaling pathway contributes to efficient lysophosphatidic acid-stimulated cell migration. *Oncogene* **25**, 2234-2244

96. Gardner, J. A., Ha, J. H., Jayaraman, M., and Dhanasekaran, D. N. (2013) The gep Proto-Oncogene Gα13 Mediates Lysophosphatidic Acid-Mediated Migration of Pancreatic Cancer Cells. *Pancreas* **42**

97. Struckhoff, A. P., Rana, M. K., Kher, S. S., Burow, M. E., Hagan, J. L., Del Valle, L., and Worthylake, R. A. (2013) PDZ-RhoGEF is essential for CXCR4-driven breast tumor cell motility through spatial regulation of RhoA. *Journal of Cell Science* **126**, 4514-4526

98. Sanchez, T., Thangada, S., Wu, M.-T., Kontos, C. D., Wu, D., Wu, H., and Hla, T. (2005) PTEN as an effector in the signaling of antimigratory G protein-coupled receptor. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 4312-4317

99. Lepley, D., Paik, J.-H., Hla, T., and Ferrer, F. (2005) The G Protein–Coupled Receptor S1P2 Regulates Rho/Rho Kinase Pathway to Inhibit Tumor Cell Migration. *Cancer Research* **65**, 3788-3795

100. Arikawa, K., Takuwa, N., Yamaguchi, H., Sugimoto, N., Kitayama, J., Nagawa, H., Takehara, K., and Takuwa, Y. (2003) Ligand-dependent Inhibition of B16 Melanoma Cell Migration and Invasion via Endogenous S1P2 G Protein-coupled Receptor: REQUIREMENT OF INHIBITION OF CELLULAR RAC ACTIVITY. *Journal of Biological Chemistry* **278**, 32841-32851

101. Grzelinski, M., Pinkenburg, O., Büch, T., Gold, M., Stohr, S., Kalwa, H., Gudermann, T., and Aigner, A. (2010) Critical Role of Gα12 and Gα13 for Human Small Cell Lung Cancer Cell Proliferation In vitro and Tumor Growth In vivo. *Clinical Cancer Research* **16**, 1402-1415

102. Castellone, M. D., Laukkanen, M. O., Teramoto, H., Bellelli, R., Ali, G., Fontanini, G., Santoro, M., and Gutkind, J. S. (2014) Cross talk between the bombesin neuropeptide receptor and Sonic hedgehog pathways in small cell lung carcinoma. *Oncogene*

103. Goldsmith, Z. G., Ha, J. H., Jayaraman, M., and Dhanasekaran, D. N. (2011) Lysophosphatidic Acid Stimulates the Proliferation of Ovarian Cancer Cells via the gep Proto-Oncogene Gα12. *Genes & Cancer* **2**, 563-575

104. Ha, J. H., Ward, J. D., Varadarajalu, L., Kim, S. G., and Dhanasekaran, D. N. (2014) The gep proto-oncogene Gα12 mediates LPA-stimulated activation of CREB in ovarian cancer cells. *Cellular Signalling* **26**, 122-132

105. Cheng, S.-J., Liu, Y.-C., Cheng, S.-L., Lee, J.-J., Chen, H.-M., Chang, H.-H., Kok, S.-H., Kuo, M. Y.-P., and Chiang, C.-P. (2013) Expression of Gα12 predicts progression and prognosis of oral squamous cell carcinomas in Taiwan. *Journal of Oral Pathology & Medicine* **42**, 565-569

106. Gokhale, A. S., Haddad, R. I., Cavacini, L. A., Wirth, L., Weeks, L., Hallar, M., Faucher, J., and Posner, M. R. (2005) Serum concentrations of interleukin-8, vascular endothelial growth factor, and epidermal growth factor receptor in patients with squamous cell cancer of the head and neck. *Oral Oncology* **41**, 70-76

107. St. John, M. R., Li, Y., Zhou, X., and et al. (2004) INterleukin 6 and interleukin 8 as potential biomarkers for oral cavity and oropharyngeal squamous cell carcinoma. *Archives of Otolaryngology–Head & Neck Surgery* **130**, 929-935

108. de Oliveira, M. V. M., de Carvalho Fraga, C. A., Gomez, R. S., and Paula, A. M. B. D. (2009) Immunohistochemical expression of interleukin-4, -6, -8, and -12 in inflammatory cells in surrounding invasive front of oral squamous cell carcinoma. *Head & Neck* **31**, 1439-1446

109. Wu, J.-Y., Yi, C., Chung, H.-R., Wang, D.-J., Chang, W.-C., Lee, S.-Y., Lin, C.-T., Yang, Y.-C., and Yang, W.-C. V. (2010) Potential biomarkers in saliva for oral squamous cell carcinoma. *Oral Oncology* **46**, 226-231

110. Love, C., Sun, Z., Jima, D., Li, G., Zhang, J., Miles, R., Richards, K. L., Dunphy, C. H., Choi, W. W. L., Srivastava, G., Lugar, P. L., Rizzieri, D. A., Lagoo, A. S., Bernal-Mizrachi, L., Mann, K. P., Flowers, C. R., Naresh, K. N., Evens, A. M., Chadburn, A., Gordon, L. I., Czader, M. B., Gill, J. I., Hsi, E. D., Greenough, A., Moffitt, A. B., McKinney, M., Banerjee, A., Grubor, V., Levy, S., Dunson, D. B., and Dave, S. S. (2012) The genetic landscape of mutations in Burkitt lymphoma. *Nat Genet* **44**, 1321-1325

111. Morin, R. D., Mungall, K., Pleasance, E., Mungall, A. J., Goya, R., Huff, R. D., Scott, D. W., Ding, J., Roth, A., Chiu, R., Corbett, R. D., Chan, F. C., Mendez-Lago, M., Trinh, D. L., Bolger-Munro, M., Taylor, G., Hadj Khodabakhshi, A., Ben-Neriah, S., Pon, J., Meissner, B., Woolcock, B., Farnoud, N., Rogic, S., Lim, E. L., Johnson, N. A., Shah, S., Jones, S., Steidl, C., Holt, R., Birol, I., Moore, R., Connors, J. M., Gascoyne, R. D., and Marra, M. A. (2013) Mutational and structural analysis of diffuse large B-cell lymphoma using whole-genome sequencing. *Blood* **122**, 1256-1265

112. Green, J. A., and Cyster, J. G. (2012) S1PR2 links germinal center confinement and growth regulation. *Immunological Reviews* **247**, 36-51

113. Takuwa N, D. W., Kaneko E, Okamoto Y, Yoshioka K, Takuwa Y. (2011) Tumor-suppressive sphingosine-1-phosphate receptor-2 counteracting tumor-promoting sphingosine-1-phosphate receptor-1 and sphingosine kinase 1 - Jekyll Hidden behind Hyde. *American journal of cancer research* **1**, 460 - 481

114. Van Raamsdonk, C. D., Griewank, K. G., Crosby, M. B., Garrido, M. C., Vemula, S., Wiesner, T., Obenauf, A. C., Wackernagel, W., Green, G., Bouvier, N., Sozen, M. M., Baimukanova, G., Roy, R., Heguy, A., Dolgalev, I., Khanin, R., Busam, K., Speicher, M. R., O'Brien, J., and Bastian, B. C. (2010) Mutations in GNA11 in Uveal Melanoma. *New England Journal of Medicine* **363**, 2191-2199

115. O'Hayre, M., Degese, M. S., and Gutkind, J. S. (2014) Novel insights into G protein and G protein-coupled receptor signaling in cancer. *Current Opinion in Cell Biology* **27**, 126-135

116. Burridge, K., and Wennerberg, K. (2004) Rho and Rac Take Center Stage. *Cell* **116**, 167-179

117. Bishop LA., H. A. (2000) Rho GTPases and their effector proteins. *Biochemical Journal* **348 Pt 2**, 241 - 255

118. Gosser, Y. Q., Nomanbhoy, T. K., Aghazadeh, B., Manor, D., Combs, C., Cerione, R. A., and Rosen, M. K. (1997) C-terminal binding domain of Rho GDP-dissociation inhibitor directs N-terminal inhibitory peptide to GTPases. *Nature* **387**, 814-819

119. Rossman, K. L., Der, C. J., and Sondek, J. (2005) GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. *Nat Rev Mol Cell Biol* **6**, 167-180

120. Olofsson, B. (1999) Rho Guanine Dissociation Inhibitors: Pivotal Molecules in Cellular Signalling. *Cellular Signalling* **11**, 545-554

121. Derewenda, U., Oleksy, A., Stevenson, A. S., Korczynska, J., Dauter, Z., Somlyo, A. P., Otlewski, J., Somlyo, A. V., and Derewenda, Z. S. (2004) The Crystal Structure of RhoA in Complex with the DH/PH Fragment of PDZRhoGEF, an Activator of the Ca2+ Sensitization Pathway in Smooth Muscle. *Structure* **12**, 1955-1965

122. Ihara, K., Muraguchi, S., Kato, M., Shimizu, T., Shirakawa, M., Kuroda, S., Kaibuchi, K., and Hakoshima, T. (1998) Crystal Structure of Human RhoA in a Dominantly Active Form Complexed with a GTP Analogue. *Journal of Biological Chemistry* **273**, 9656-9666

123. Zong, H., Raman, N., Mickelson-Young, L. A., Atkinson, S. J., and Quilliam, L. A. (1999) Loop 6 of RhoA Confers Specificity for Effector Binding, Stress Fiber Formation, and Cellular Transformation. *Journal of Biological Chemistry* **274**, 4551-4560

124. Zong, H., Kaibuchi, K., and Quilliam, L. A. (2001) The Insert Region of RhoA Is Essential for Rho Kinase Activation and Cellular Transformation. *Molecular and Cellular Biology* **21**, 5287-5298

125. Sahai, E. A. A. S. T. R. (1998) RhoA effector mutants reveal distinct effector pathways for cytoskeletal reorganization, SRF activation and transformation. *The EMBO Journal* **17**, 1350-1361

126. Vishnubhotla, R., Sun, S., Huq, J., Bulic, M., Ramesh, A., Guzman, G., Cho, M., and Glover, S. C. (2007) ROCK-II mediates colon cancer invasion via regulation of MMP-2 and MMP-13 at the site of invadopodia as revealed by multiphoton imaging. *Lab Invest* **87**, 1149-1158

127. Benitah, S. A., Valerón, P. F., and Lacal, J. C. (2003) ROCK and Nuclear Factor-κB–dependent Activation of Cyclooxygenase-2 by Rho GTPases: Effects on Tumor Growth and Therapeutic Consequences. *Molecular Biology of the Cell* **14**, 3041-3054

128. Kakiuchi, M., Nishizawa, T., Ueda, H., Gotoh, K., Tanaka, A., Hayashi, A., Yamamoto, S., Tatsuno, K., Katoh, H., Watanabe, Y., Ichimura, T., Ushiku, T., Funahashi, S., Tateishi, K., Wada, I., Shimizu, N., Nomura, S., Koike, K., Seto, Y., Fukayama, M., Aburatani, H., and Ishikawa, S. (2014) Recurrent gain-of-function mutations of RHOA in diffuse-type gastric carcinoma. *Nat Genet* **46**, 583-587

129. Vega, F. M., and Ridley, A. J. (2008) Rho GTPases in cancer cell biology. *FEBS Letters* **582**, 2093-2101

130. Leve, F., and Morgado-Díaz, J. A. (2012) Rho GTPase signaling in the development of colorectal cancer. *Journal of Cellular Biochemistry* **113**, 2549-2559

131. Pertz, O., Hodgson, L., Klemke, R. L., and Hahn, K. M. (2006) Spatiotemporal dynamics of RhoA activity in migrating cells. *Nature* **440**, 1069-1072

132. Machacek, M., Hodgson, L., Welch, C., Elliott, H., Pertz, O., Nalbant, P., Abell, A., Johnson, G. L., Hahn, K. M., and Danuser, G. (2009) Coordination of Rho GTPase activities during cell protrusion. *Nature* **461**, 99-103

133. Buhl, A. M., Johnson, N. L., Dhanasekaran, N., and Johnson, G. L. (1995) Gα12 and Gα13 Stimulate Rho-dependent Stress Fiber Formation and Focal Adhesion Assembly. *Journal of Biological Chemistry* **270**, 24631-24634

134. Gohla, A., Offermanns, S., Wilkie, T. M., and Schultz, G. (1999) Differential Involvement of Gα12 and Gα13 in Receptor-mediated Stress Fiber Formation. *Journal of Biological Chemistry* **274**, 17901-17907

135. Gohla, A., Harhammer, R., and Schultz, G. (1998) The G-protein G13 but Not G12 Mediates Signaling from Lysophosphatidic Acid Receptor via Epidermal Growth Factor Receptor to Rho. *Journal of Biological Chemistry* **273**, 4653-4659

136. Jaiswal, M., Gremer, L., Dvorsky, R., Haeusler, L. C., Cirstea, I. C., Uhlenbrock, K., and Ahmadian, M. R. (2011) Mechanistic Insights into Specificity, Activity, and Regulatory Elements of the Regulator of G-protein Signaling (RGS)-containing Rho-specific Guanine Nucleotide Exchange Factors (GEFs) p115, PDZ-RhoGEF (PRG), and Leukemia-associated RhoGEF (LARG). *Journal of Biological Chemistry* **286**, 18202-18212

137. Oleksy, A., Opaliński, L. u., Derewenda, U., Derewenda, Z. S., and Otlewski, J. (2006) The Molecular Basis of RhoA Specificity in the Guanine Nucleotide Exchange Factor PDZ-RhoGEF. *Journal of Biological Chemistry* **281**, 32891-32897

138. Kozasa, T., Hajicek, N., Chow, C. R., and Suzuki, N. (2011) Signalling mechanisms of RhoGTPase regulation by the heterotrimeric G proteins G12 and G13. *Journal of Biochemistry* **150**, 357-369

139. Worzfeld, T., Swiercz, J. M., Looso, M., Straub, B. K., Sivaraj, K. K., and Offermanns, S. (2012) ErbB-2 signals through Plexin-B1 to promote breast cancer metastasis. *The Journal of Clinical Investigation* **122**, 1296-1305

140. Suzuki, N., Nakamura, S., Mano, H., and Kozasa, T. (2003) Gα12 activates Rho GTPase through tyrosine-phosphorylated leukemia-associated RhoGEF. *Proceedings of the National Academy of Sciences* **100**, 733-738

141. Kourlas, P. J., Strout, M. P., Becknell, B., Veronese, M. L., Croce, C. M., Theil, K. S., Krahe, R., Ruutu, T., Knuutila, S., Bloomfield, C. D., and Caligiuri, M. A. (2000) Identification of a gene at 11q23 encoding a guanine nucleotide exchange factor: Evidence for its fusion with MLL in acute myeloid leukemia. *Proceedings of the National Academy of Sciences* **97**, 2145-2150

142. Swiercz, J. M., Kuner, R., and Offermanns, S. (2004) Plexin-B1/RhoGEF–mediated RhoA activation involves the receptor tyrosine kinase ErbB-2. *The Journal of Cell Biology* **165**, 869-880

143. Swiercz, J. M., Worzfeld, T., and Offermanns, S. (2009) Semaphorin 4D Signaling Requires the Recruitment of Phospholipase Cγ into the Plexin-B1 Receptor Complex. *Molecular and Cellular Biology* **29**, 6321-6334

144. Basile, J. R., Barac, A., Zhu, T., Guan, K.-L., and Gutkind, J. S. (2004) Class IV Semaphorins Promote Angiogenesis by Stimulating Rho-Initiated Pathways through Plexin-B. *Cancer Research* **64**, 5212-5224

145. Taya, S., Inagaki, N., Sengiku, H., Makino, H., Iwamatsu, A., Urakawa, I., Nagao, K., Kataoka, S., and Kaibuchi, K. (2001) Direct interaction of insulin-like growth factor-1 receptor with leukemia-associated RhoGEF. *The Journal of Cell Biology* **155**, 809-820

146. Okuhira, K., Fitzgerald, M. L., Tamehiro, N., Ohoka, N., Suzuki, K., Sawada, J., Naito, M., and Nishimaki-Mogami, T. (2010) Binding of PDZ-RhoGEF to ATP-binding Cassette Transporter A1 (ABCA1) Induces Cholesterol Efflux through RhoA Activation and Prevention of Transporter Degradation. *Journal of Biological Chemistry* **285**, 16369-16377

147. Yamada, T., Ohoka, Y., Kogo, M., and Inagaki, S. (2005) Physical and Functional Interactions of the Lysophosphatidic Acid Receptors with PDZ Domain-containing Rho Guanine Nucleotide Exchange Factors (RhoGEFs). *Journal of Biological Chemistry* **280**, 19358-19363

148. Hajicek, N., Kukimoto-Niino, M., Mishima-Tsumagari, C., Chow, C. R., Shirouzu, M., Terada, T., Patel, M., Yokoyama, S., and Kozasa, T. (2011) Identification of Critical Residues in Gα13 for Stimulation of p115RhoGEF Activity and the Structure of the Gα13-p115RhoGEF Regulator of G Protein Signaling Homology (RH) Domain Complex. *Journal of Biological Chemistry* **286**, 20625-20636

149. Chen, Z., Guo, L., Hadas, J., Gutowski, S., Sprang, S. R., and Sternweis, P. C. (2012) Activation of p115-RhoGEF Requires Direct Association of Gα13 and the Dbl Homology Domain. *Journal of Biological Chemistry* **287**, 25490-25500

150. Gasmi-Seabrook, G. M. C., Marshall, C. B., Cheung, M., Kim, B., Wang, F., Jang, Y. J., Mak, T. W., Stambolic, V., and Ikura, M. (2010) Real-time NMR Study of Guanine Nucleotide Exchange and Activation of RhoA by PDZ-RhoGEF. *Journal of Biological Chemistry* **285**, 5137-5145

151. Kristelly, R., Gao, G., and Tesmer, J. J. G. (2004) Structural Determinants of RhoA Binding and Nucleotide Exchange in Leukemia-associated Rho Guanine-Nucleotide Exchange Factor. *Journal of Biological Chemistry* **279**, 47352-47362

152. Chen, Z., Medina, F., Liu, M.-y., Thomas, C., Sprang, S. R., and Sternweis, P. C. (2010) Activated RhoA Binds to the Pleckstrin Homology (PH) Domain of PDZ-RhoGEF, a Potential Site for Autoregulation. *Journal of Biological Chemistry* **285**, 21070-21081

153. Medina, F., Carter, A. M., Dada, O., Gutowski, S., Hadas, J., Chen, Z., and Sternweis, P. C. (2013) Activated RhoA Is a Positive Feedback Regulator of the Lbc Family of Rho Guanine Nucleotide Exchange Factor Proteins. *Journal of Biological Chemistry* **288**, 11325-11333

154. Chikumi, H., Barac, A., Behbahani, B., Gao, Y., Teramoto, H., Zheng, Y., and Gutkind, J. S. (2004) Homo- and hetero-oligomerization of PDZ-RhoGEF, LARG and p115RhoGEF by their C-terminal region regulates their in vivo Rho GEF activity and transforming potential. *Oncogene* **23**, 233-240

155. Eisenhaure, T. M., Francis, S. A., Willison, L. D., Coughlin, S. R., and Lerner, D. J. (2003) The Rho Guanine Nucleotide Exchange Factor Lsc Homo-oligomerizes and Is Negatively Regulated through Domains in Its Carboxyl Terminus That Are Absent in Novel Splenic Isoforms. *Journal of Biological Chemistry* **278**, 30975-30984

156. Wells, C. D., Gutowski, S., Bollag, G., and Sternweis, P. C. (2001) Identification of Potential Mechanisms for Regulation of p115 RhoGEF through Analysis of Endogenous and Mutant Forms of the Exchange Factor. *Journal of Biological Chemistry* **276**, 28897-28905

157. Artamonov, M. V., Momotani, K., Stevenson, A., Trentham, D. R., Derewenda, U., Derewenda, Z. S., Read, P. W., Gutkind, J. S., and Somlyo, A. V. (2013) Agonist-induced Ca2+ Sensitization in Smooth Muscle: REDUNDANCY OF RHO GUANINE NUCLEOTIDE EXCHANGE FACTORS (RhoGEFs) AND RESPONSE KINETICS, A CAGED COMPOUND STUDY. *Journal of Biological Chemistry* **288**, 34030-34040

158. Häcker, U., and Perrimon, N. (1998) DRhoGEF2 encodes a member of the Dbl family of oncogenes and controls cell shape changes during gastrulation in Drosophila. *Genes & Development* **12**, 274-284

159. Rogers, S. L., Wiedemann, U., Häcker, U., Turck, C., and Vale, R. D. (2004) Drosophila RhoGEF2 Associates with Microtubule Plus Ends in an EB1-Dependent Manner. *Current Biology* **14**, 1827-1833

160. Panizzi, J. R., Jessen, J. R., Drummond, I. A., and Solnica-Krezel, L. (2007) New functions for a vertebrate Rho guanine nucleotide exchange factor in ciliated epithelia. *Development* **134**, 921-931

161. Marlow, F., Topczewski, J., Sepich, D., and Solnica-Krezel, L. (2002) Zebrafish Rho Kinase 2 Acts Downstream of Wnt11 to Mediate Cell Polarity and Effective Convergence and Extension Movements. *Current Biology* **12**, 876-884

162. Matsui, T., Raya, Á., Kawakami, Y., Callol-Massot, C., Capdevila, J., Rodríguez-Esteban, C., and Izpisúa Belmonte, J. C. (2005) Noncanonical Wnt signaling regulates midline convergence of organ primordia during zebrafish development. *Genes & Development* **19**, 164-175

163. Ye, D., and Lin, F. (2013) S1pr2/Gα13 signaling controls myocardial migration by regulating endoderm convergence. *Development* **140**, 789-799

164. Chikumi, H., Fukuhara, S., and Gutkind, J. S. (2002) Regulation of G Protein-linked Guanine Nucleotide Exchange Factors for Rho, PDZ-RhoGEF, and LARG by Tyrosine Phosphorylation: EVIDENCE OF A ROLE FOR FOCAL ADHESION KINASE. *Journal of Biological Chemistry* **277**, 12463-12473

165. Chikumi, H., Barac, A., Behbahani, B., Gao, Y., Teramoto, H., Zheng, Y., and Gutkind, J. S. (0000) Homo- and hetero-oligomerization of PDZ-RhoGEF, LARG and p115RhoGEF by their C-terminal region regulates their in vivo Rho GEF activity and transforming potential. *Oncogene* **23**, 233-240

166. Iwanicki, M. P., Vomastek, T., Tilghman, R. W., Martin, K. H., Banerjee, J., Wedegaertner, P. B., and Parsons, J. T. (2008) FAK, PDZ-RhoGEF and ROCKII cooperate to regulate adhesion movement and trailing-edge retraction in fibroblasts. *Journal of Cell Science* **121**, 895-905

167. Ying, Z., Giachini, F. R. C., Tostes, R. C., and Webb, R. C. (2009) PYK2/PDZ-RhoGEF Links Ca2+ Signaling to RhoA. *Arteriosclerosis, Thrombosis, and Vascular Biology* **29**, 1657-1663

168. Ohtsu, H., Mifune, M., Frank, G. D., Saito, S., Inagami, T., Kim-Mitsuyama, S., Takuwa, Y., Sasaki, T., Rothstein, J. D., Suzuki, H., Nakashima, H., Woolfolk, E. A., Motley, E. D., and Eguchi, S. (2005) Signal-Crosstalk Between Rho/ROCK and c-Jun NH2-Terminal Kinase Mediates Migration of Vascular Smooth Muscle Cells Stimulated by Angiotensin II. *Arteriosclerosis, Thrombosis, and Vascular Biology* **25**, 1831-1836

169. Wirth, A., Benyo, Z., Lukasova, M., Leutgeb, B., Wettschureck, N., Gorbey, S., Orsy, P., Horvath, B., Maser-Gluth, C., Greiner, E., Lemmer, B., Schutz, G., Gutkind, J. S., and Offermanns, S. (2008) G12-G13-LARG-mediated signaling in vascular smooth muscle is required for salt-induced hypertension. *Nat Med* **14**, 64-68

170. Hilgers, R. H. P., Todd, J., Jr., and Webb, R. C. (2007) Increased PDZ-RhoGEF/RhoA/Rho kinase signaling in small mesenteric arteries of angiotensin II-induced hypertensive rats. *Journal of Hypertension* **25**

171. Guilluy, C., Bregeon, J., Toumaniantz, G., Rolli-Derkinderen, M., Retailleau, K., Loufrani, L., Henrion, D., Scalbert, E., Bril, A., Torres, R. M., Offermanns, S., Pacaud, P., and Loirand, G. (2010) The Rho exchange factor Arhgef1 mediates the effects of angiotensin II on vascular tone and blood pressure. *Nat Med* **16**, 183-190

172. Das, S., and Elbein, S. (2007) The search for type 2 diabetes susceptibility loci: The chromosome 1q story. *Curr Diab Rep* **7**, 154-164

173. Fu, M., Sabra, M. M., Damcott, C., Pollin, T. I., Ma, L., Ott, S., Shelton, J. C., Shi, X., Reinhart, L., O'Connell, J., Mitchell, B. D., Baier, L. J., and Shuldiner, A. R. (2007) Evidence That Rho Guanine Nucleotide Exchange Factor 11 (ARHGEF11) on 1q21 is a Type 2 Diabetes Susceptibility Gene in the Old Order Amish. *Diabetes* **56**, 1363-1368

174. Jin, Q. S., Kim, S. H., Piao, S.-J., Lim, H. A., Lee, S. Y., Hong, S. B., Kim, Y. S., Lee, H.-J., and Nam, M. (2010) R1467H Variants of Rho Guanine Nucleotide Exchange Factor 11 (ARHGEF11) are Associated with Type 2 Diabetes Mellitus in Koreans. *Korean Diabetes J* **34**, 368-373

175. Liu, J., Chen, X., Guo, Q., Ma, X., Zhang, J., Huang, X., Zhang, X., and Zhang, S. (2011) Association of ARHGEF11 R1467H polymorphism with risk for type 2 diabetes mellitus and insulin resistance in Chinese population. *Mol Biol Rep* **38**, 2499-2505

176. Bottcher, Y., Schleinitz, D., Tonjes, A., Bluher, M., Stumvoll, M., and Kovacs, P. (2008) R1467H variant in the rho guanine nucleotide exchange factor 11 (ARHGEF11) is associated with impaired glucose tolerance and type 2 diabetes in German Caucasians. *J Hum Genet* **53**, 365-367

177. Jang, Y.-J. (2011) *Characterization of the Physiological Role of PDZ-RhoGEF in Drosophila and Mice.* Doctor of Philosophy Doctoral University of Toronto.

178. Zohar M, T. H., Katz BZ, Yamada KM, Gutkind JS. (1998) Effector domain mutants of Rho dissociate cytoskeletal changes from nuclear signaling and cellular transformation. *Oncogene* **17**, 991 - 998

179. Karlsson, R., Pedersen, E. D., Wang, Z., and Brakebusch, C. (2009) Rho GTPase function in tumorigenesis. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer* **1796**, 91-98

180. Huang, C., Hydo, L. M., Liu, S., and Miller, R. T. (2009) Activation of choline kinase by extracellular Ca2+ is Ca2+-sensing receptor, Gα12 and Rho-dependent in breast cancer cells. *Cellular Signalling* **21**, 1894-1900

181. Bourguignon, L. Y. W., Gilad, E., Brightman, A., Diedrich, F., and Singleton, P. (2006) Hyaluronan-CD44 Interaction with Leukemia-associated RhoGEF and Epidermal Growth Factor Receptor Promotes Rho/Ras Co-activation, Phospholipase Cϵ-Ca2+ Signaling, and Cytoskeleton Modification in Head and Neck Squamous Cell Carcinoma Cells. *Journal of Biological Chemistry* **281**, 14026-14040

182. Lee, A. S. G., Seo, Y. C., Chang, A., Tohari, S., Eu, K. W., Seow-Choen, F., and McGee, J. O. D. (2000) Detailed deletion mapping at chromosome 11q23 in colorectal carcinoma. *Br J Cancer* **83**, 750-755

183. Carter, S. L., Negrini, M., Baffa, R., Gillum, D. R., Rosenberg, A. L., Schwartz, G. F., and Croce, C. M. (1994) Loss of Heterozygosity at 11q22–q23 in Breast Cancer. *Cancer Research* **54**, 6270-6274

184. Rasio, D., Negrini, M., Manenti, G., Dragani, T. A., and Croce, C. M. (1995) Loss of Heterozygosity at Chromosome 11q in Lung Adenocarcinoma: Identification of Three Independent Regions. *Cancer Research* **55**, 3988-3991

185. Ong, D. C. T., Ho, Y. M., Rudduck, C., Chin, K., Kuo, W. L., Lie, D. K. H., Chua, C. L. M., Tan, P. H., Eu, K. W., Seow-Choen, F., Wong, C. Y., Hong, G. S., Gray, J. W., and Lee, A. S. G. (2009) LARG at chromosome 11q23 has functional characteristics of a tumor suppressor in human breast and colorectal cancer. *Oncogene* **28**, 4189-4200

186. Harma, V., Knuuttila, M., Virtanen, J., Mirtti, T., Kohonen, P., Kovanen, P., Happonen, A., Kaewphan, S., Ahonen, I., Kallioniemi, O., Grafstrom, R., Lotjonen, J., and Nees, M. (2012) Lysophosphatidic acid and sphingosine-1-phosphate promote morphogenesis and block invasion of prostate cancer cells in three-dimensional organotypic models. *Oncogene* **31**, 2075-2089

187. Thaker, N. G., Zhang, F., McDonald, P. R., Shun, T. Y., Lewen, M. D., Pollack, I. F., and Lazo, J. S. (2009) Identification of Survival Genes in Human Glioblastoma Cells by Small Interfering RNA Screening. *Molecular Pharmacology* **76**, 1246-1255

188. Masica, D. L., and Karchin, R. (2011) Correlation of Somatic Mutation and Expression Identifies Genes Important in Human Glioblastoma Progression and Survival. *Cancer Research* **71**, 4550-4561

189. Kim JH, K. H., Lee KT, Lee JK, Choi SH, Paik SW, Rhee JC, Lowe AW. (2008) Gene expression profiles in gallbladder cancer: the close genetic similarity seen for early and advanced gallbladder cancers may explain the poor prognosis. *Tumor biology* **29**, 41 - 49

190. Singer, W. D., Miller, R. T., and Sternweis, P. C. (1994) Purification and characterization of the alpha subunit of G13. *Journal of Biological Chemistry* **269**, 19796-19802

191. García-Mata, R., Wennerberg, K., Arthur, W. T., Noren, N. K., Ellerbroek, S. M., and Burridge, K. (2006) Analysis of Activated GAPs and GEFs in Cell Lysates. in *Methods in Enzymology* (William E. Balch, C. J. D., and Alan, H. eds.), Academic Press. pp 425-437

192. Wiznerowicz, M., and Trono, D. (2003) Conditional Suppression of Cellular Genes: Lentivirus Vector-Mediated Drug-Inducible RNA Interference. *Journal of Virology* **77**, 8957-8951

193. Tesmer, V. M., Kawano, T., Shankaranarayanan, A., Kozasa, T., and Tesmer, J. J. G. (2005) Snapshot of Activated G Proteins at the Membrane: The Gαq-GRK2-Gßγ Complex. *Science* **310**, 1686-1690

194. Carroll, R. E., Ostrovskiy, D., Lee, S., Danilkovich, A., and Benya, R. V. (2000) Characterization of Gastrin-Releasing Peptide and Its Receptor Aberrantly Expressed by Human Colon Cancer Cell Lines. *Molecular Pharmacology* **58**, 601-607

195. Ferris, H. A., Carroll, R. E., Rasenick, M. M., and Benya, R. V. (1997) Constitutive activation of the gastrin-releasing peptide receptor expressed by the nonmalignant human colon epithelial cell line NCM460. *The Journal of Clinical Investigation* **100**, 2530-2537

196. Ren, X.-D., and Schwartz, M. A. (2000) Determination of GTP loading on Rho. in *Methods in Enzymology* (W.E. Balch, C. J. D., and Alan, H. eds.), Academic Press. pp 264-272

197. Aittaleb, M., Boguth, C. A., and Tesmer, J. J. G. (2010) Structure and Function of Heterotrimeric G Protein-Regulated Rho Guanine Nucleotide Exchange Factors. *Molecular Pharmacology* **77**, 111-125

198. Carman, C. V., Parent, J.-L., Day, P. W., Pronin, A. N., Sternweis, P. M., Wedegaertner, P. B., Gilman, A. G., Benovic, J. L., and Kozasa, T. (1999) Selective Regulation of Gαq/11 by an RGS Domain in the G Protein-coupled Receptor Kinase, GRK2. *Journal of Biological Chemistry* **274**, 34483-34492

199. Scheschonka, A., Dessauer, C. W., Sinnarajah, S., Chidiac, P., Shi, C.-S., and Kehrl, J. H. (2000) RGS3 Is a GTPase-Activating Protein for Giα and Gqα and a Potent Inhibitor of Signaling by GTPase-Deficient Forms of Gqα and G11α. *Molecular Pharmacology* **58**, 719-728

200. Ridley, A. J., and Hall, A. (1992) The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70**, 389-399

201. Hopkins, A. M., Pineda, A. D. A., Winfree, L. M., Brown, G. T., Laukoetter, M. G., and Nusrat, A. (2007) Organized migration of epithelial cells requires control of adhesion and protrusion through Rho kinase effectors. *American Journal of Physiology - Gastrointestinal and Liver Physiology* **292**, G806-G817

202. Makrodouli, E., Oikonomou, E., Koc, M., Andera, L., Sasazuki, T., Shirasawa, S., and Pintzas, A. (2011) BRAF and RAS oncogenes regulate Rho GTPase pathways to mediate migration and invasion properties in human colon cancer cells: a comparative study. *Molecular Cancer* **10**, 118

203. Wang, D., and Dubois, R. N. (2010) The role of COX-2 in intestinal inflammation and colorectal cancer. *Oncogene* **29**, 781-788

204. Mutoh, M., Watanabe, K., Kitamura, T., Shoji, Y., Takahashi, M., Kawamori, T., Tani, K., Kobayashi, M., Maruyama, T., Kobayashi, K., Ohuchida, S., Sugimoto, Y., Narumiya, S., Sugimura, T., and Wakabayashi, K. (2002) Involvement of Prostaglandin E Receptor Subtype EP4 in Colon Carcinogenesis. *Cancer Research* **62**, 28-32

205. Rigas B, G. I., Levine L. (1993) Altered eicosanoid levels in human colon cancer. *J Lab Clin Med* **122**, 518-523.

206. Hirota, C. L., Moreau, F., Iablokov, V., Dicay, M., Renaux, B., Hollenberg, M. D., and MacNaughton, W. K. (2012) Epidermal growth factor receptor transactivation is required for proteinase-activated receptor-2-induced COX-2 expression in intestinal epithelial

cells. *American Journal of Physiology - Gastrointestinal and Liver Physiology* **303**, G111-G119

207. Buchanan, F. G., Wang, D., Bargiacchi, F., and DuBois, R. N. (2003) Prostaglandin E2 Regulates Cell Migration via the Intracellular Activation of the Epidermal Growth Factor Receptor. *Journal of Biological Chemistry* **278**, 35451-35457

208. Hawcroft, G., Volpato, M., Marston, G., Ingram, N., Perry, S. L., Cockbain, A. J., Race, A. D., Munarini, A., Belluzzi, A., Loadman, P. M., Coletta, P. L., and Hull, M. A. (2012) The omega-3 polyunsaturated fatty acid eicosapentaenoic acid inhibits mouse MC-26 colorectal cancer cell liver metastasis via inhibition of PGE2-dependent cell motility. *British Journal of Pharmacology* **166**, 1724-1737

209. Ninomiya, I., Nagai, N., Oyama, K., Hayashi, H., Tajima, H., Kitagawa, H., Fushida, S., Fujimura, T., and Ohta, T. (2012) Antitumor and anti-metastatic effects of cyclooxygenase-2 inhibition by celecoxib on human colorectal carcinoma xenografts in nude mouse rectum. *Oncology reports* **28**, 777-784

210. Xiao, H., Zhang, Q., Lin, Y., Reddy, B. S., and Yang, C. S. (2008) Combination of atorvastatin and celecoxib synergistically induces cell cycle arrest and apoptosis in colon cancer cells. *Int J Cancer* **122**, 2115-2124

211. Booden, M. A., Siderovski, D. P., and Der, C. J. (2002) Leukemia-Associated Rho Guanine Nucleotide Exchange Factor Promotes Gαq-Coupled Activation of RhoA. *Molecular and Cellular Biology* **22**, 4053-4061

212. Lutz, S., Freichel-Blomquist, A., Yang, Y., Rümenapp, U., Jakobs, K. H., Schmidt, M., and Wieland, T. (2005) The Guanine Nucleotide Exchange Factor p63RhoGEF, a Specific Link between Gq/11-coupled Receptor Signaling and RhoA. *Journal of Biological Chemistry* **280**, 11134-11139

213. Rojas, R. J., Yohe, M. E., Gershburg, S., Kawano, T., Kozasa, T., and Sondek, J. (2007) Gαq Directly Activates p63RhoGEF and Trio via a Conserved Extension of the Dbl Homology-associated Pleckstrin Homology Domain. *Journal of Biological Chemistry* **282**, 29201-29210

214. Harrison, S., Knifley, T., Chen, M., and O'Connor, K. (2013) LPA, HGF, and EGF utilize distinct combinations of signaling pathways to promote migration and invasion of MDA-MB-231 breast carcinoma cells. *BMC Cancer* **13**, 501

215. Somlyo, A. V., Bradshaw, D., Ramos, S., Murphy, C., Myers, C. E., and Somlyo, A. P. (2000) Rho-Kinase Inhibitor Retards Migration and in Vivo Dissemination of Human Prostate Cancer Cells. *Biochemical and Biophysical Research Communications* **269**, 652-659

216. Huang, C., Liu, S., and Miller, R. T. (2011) Role of p115RhoGEF in the regulation of extracellular Ca2+-induced choline kinase activation and prostate cancer cell proliferation. *International Journal of Cancer* **128**, 2833-2842

217. Kwiatkowska A, D. Z., Paulino MV, Tran LN, Ruggieri R, Symons HM. (2012) PDZ-RhoGEF promotes glioblastoma cell invasion, proliferation and survival. in *American Association for Cancer Research*, AACR, Chicago, IL.

218. Tocci P, C. R., Semprucci E, Di Castro V, Bagnato A, Rosanò L, Elena R. . (2014) PDZ-RhoGEF/β-arrestin-1 interaction mediates endothelin A receptor-induced RhoA activation and cell motility in ovarian tumor cells. in *American Association for Cancer Research*, AACR, San Diego, CA.

VIII. APPENDICES

A.

August 5, 2014

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Dear Maulik Patel:

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IX. VITA

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Publications	**Patel M,** Kawano T, Suzuki N, Hamakubo T, Karginov AV and Kozasa T. (2014a) Gα13/PDZ-RhoGEF/RhoA Signaling Is Essential for Gastrin-Releasing Peptide Receptor–Mediated Colon Cancer Cell Migration. Molecular Pharmacology 86(3): 252-262.
	Patel, M., and Karginov, A. V. (2014) Phosphorylation-mediated regulation of GEFs for RhoA. *Cell Adhesion & Migration* **8**, 11-18.
	Gan, X., Wang, C., **Patel, M.,** Kreutz, B., Zhou, M., Kozasa, T., and Wu, D. (2013) Different Raf Protein Kinases Mediate Different Signaling Pathways to Stimulate E3 Ligase RFFL Gene Expression in Cell Migration Regulation. *Journal of Biological Chemistry* **288**, 33978-33984.
	Hajicek, N., Kukimoto-Niino, M., Mishima-Tsumagari, C., Chow, C. R., Shirouzu, M., Terada, T., **Patel, M**., Yokoyama, S., and Kozasa, T. (2011) Identification of Critical Residues in Gα13 for Stimulation of p115RhoGEF Activity and the Structure of the Gα13-p115RhoGEF Regulator of G Protein Signaling Homology (RH) Domain Complex. *Journal of Biological Chemistry* **286**, 20625-20636

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Presentations	**Patel M,** Kawano T, Karginov A, and Kozasa T. Gα13/PDZ-RhoGEF/RhoA Signaling is Essential for Gastrin Releasing Peptide-Receptor Mediated Colon Cancer Cell Migration, Chicago Symposium on Cell Signaling - Chicago, IL. 2014
	Patel M, Kawano T, Karginov A, and Kozasa T. Gα13/PDZ-RhoGEF/RhoA Signaling is Essential for Gastrin Releasing Peptide-Receptor Mediated Colon Cancer Cell Migration, UIC Cancer Research Forum - Chicago, IL. 2014
	Patel M, Kawano T, Karginov A, and Kozasa T. GRP-R Mediated Activation of Gα13-PRG-RhoA Signaling Pathway Drives Colon Cancer Cell Migration, 14th Annual Great Lakes GPCR Retreat - Cleveland, Ohio. 2013
	Patel M, Kawano T, and Kozasa T. Regulation of RhoA Activation Downstream of Gastrin Releasing Peptide-Receptor in Colon Cancer, Gordon Research Conference: Phosphorylation & G-Protein Mediated Signaling Networks - University of New England, Biddeford, Maine. 2012
	Patel M, and Kozasa T. Characterization of PDZ-RhoGEF Monoclonal Antibody, University of Tokyo – Laboratory of Systems Biology and Medicine, Tokyo, Japan. 2010