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Stability and Activity of Naturally Occurring RGS2 Variants

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Physiology and Pharmacology

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ABSTRACT

Regulator of G protein signaling protein 2 (RGS2) attenuates G Protein-Coupled Receptor (GPCR) signaling by promoting the hydrolysis of GTP in the activated Gα subunit to GDP, thereby governing many physiological and pathophysiological signals. However, how RGS2 itself is regulated remains to be elucidated. In this study, our principal goal was to discover the molecular mechanisms controlling RGS2 degradation and if altered degradation affects Gα signaling. RGS2 has four initiation sites (at residues 1, 5, 16, and 33), resulting in the existence of four distinct N-terminal initiation site variants. Additionally, there are naturally occurring mutations in this region at residues 5 (RGS2 M5V), 14 (RGS2 R14I), 18 (RGS2 K18N) and 23 (RGS2 G23D), which may be associated with a phenotypic profile seen in individuals with the mutant forms. Here we report that the use of each initiator methionine residue, as well as mutations within the N-terminus of RGS2, can have profound effects on RGS2 half-life. Additionally, we show a correlation between RGS2 half-life and the ability to attenuate $G\alpha_{q/11}$ signaling. Finally, we provide evidence that RGS2 is degraded via the ubiquitin-proteasome pathway. Considering the importance of RGS2 in pathophysiological conditions, altered degradation associated with initiation variants or mutant isoforms could be contributing to such conditions.

Key Words

G protein-coupled receptors (GPCR), Regulator of G protein signaling proteins 2 (RGS2), GTPase accelerating proteins (GAPs), protein degradation, single nucleotide polymorphisms (SNPs), cell signaling.

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List of Abbreviations

5-HT$_{2A}$R	Serotonin 2A Receptor
AC	Adenylyl Cyclase
AHO	Albright's Hereditary Osteodystrophy
AlF_4^-	Aluminum Fluoride
ANOVA	Analysis of Variance
ATE1	Arginyl-tRNA Protein Transferase 1
ATF	Activating Transcription Factor
ATP	Adenosine Triphosphate
Ca^{2+}	Calcium
CaM	Calmodulin
cAMP	Cyclic Adenosine Monophosphate
CaN	Calcineurin
CHX	Cycloheximide
CREB	cAMP Response Element-binding Protein
CREM	cAMP-responsive Element Modulator
DAG	Diacylglycerol
DEP	Dishevelled, Worm EGL-10, and Mammalian Pleckstrin
DMEM	Dulbecco's Modified Eagle Medium

DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPM	Disintegrations Per Minute
E1	Ubiquitin-activating Enzyme
E2	Ubiquitin-conjugating Enzyme
E3	Ubiquitin-ligase Enzyme
EC_{50}	Half Maximal Effective Concentration
EDTA	Ethylenediaminetetraacetic Acid
eIF2	Eukaryotic Initiation Factor 2
eIF2B	Eukaryotic Initiation Factor 2B
E_{max}	Maximal Effective Concentration
EPAC	Exchange Protein Directly Activated by cAMP
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
FL	Full-length
FLAG	Fusion tag (DYKDDDDK)
GAIP	Gα-interacting Protein
GAP	GTPase Accelerating Protein
GDI	GDP Dissociation Inhibitor

GDP	Guanosine Diphosphate
GEF	Guanine Nucleotide Exchange Factor
GGL	G Protein Gamma Subunit-like
GIRK	G Protein-coupled Inwardly-rectifying Potassium Channels
GPCR	G Protein-coupled Receptor
GPR	G Protein Regulatory Domain
GPSM	G Protein Signaling Modulator
GRK	G Protein-coupled Receptor Kinase
GTP	Guanosine Triphosphate
HA	Human Influenza Hemagglutinin
HBSS	Hank's Balanced Salt Solution
HEK293	Human Embryonic Kidney 293 cells
IgG	Immunoglobulin G
IP_3	Inositol 1,4,5-trisphosphate
K^+	Potassium Ion
Mg^{2+}	Magnesium Ion
mRNA	Messenger RNA
NFAT	Nuclear Factor of Activated T-cells
NMR	Nuclear Magnetic Resonance

NP-40	Nonyl Phenoxypolyethoxyethanol
NTAN	Amino-terminal Aminohydrolase
NTAQ	Amino-terminal Glutamine Aminohydrolase
N-terminal	Amino-terminal
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PDZ	Post Synaptic Density Protein, Drosophila Disc Large Tumor Suppressor, and Zonula Occludens-1 Protein
PEST	Proline, Glutamic Acid, Serine, Threonine sequence
PI3-Kinase	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-Bisphosphate
PKA	Protein Kinase A or cAMP-dependent Protein Kinase
PKC	Protein Kinase C
PLC-β	Phospholipase C-beta
PTB	Protein Tyrosine Binding
PVDF	Polyvinylidene Fluoride
r^2	Correlation Coefficient
R7H	R7 Binding Protein
Rac	Receptor-adenylate Cyclase Protein

Rap1	Ras-proximate-1 or Ras-related Protein 1
RGS	Regulator of G Protein Signaling Protein
RhoA	Ras Homolog Family Member A
RhoGEF	Rho Guanine Nucleotide Exchange Factor
Ric-8	Resistance to Inhibitors of Cholinesterase 8
RID	Ras Interaction Domain
RING	Really Interesting New Gene
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
Smad	SMA and MAD-Related Protein
SNP	Single Nucleotide Polymorphism
SR	Sarcoplasmic Reticulum
TBST	Tris-buffered Saline and Tween-20
TGF-β	Transforming Growth Factor beta
UBA	Ubiquitin-associated Domain
UBR	Ubiquitin-protein Ligase E3 Component N-recognin
USE1	Unconventional SNARE in the ER 1 Homolog
WT	Wild-type

Chapter 1: Introduction

1 INTRODUCTION

1.1 G PROTEIN SIGNALING

1.1.1 G PROTEIN SIGNALING OVERVIEW

G Protein-Coupled Receptors (GPCRs) are seven transmembrane domain-spanning proteins that constitute one of the largest receptor classes within the human body[1]. The human genome is reported to contain greater than 720 GPCRs[2]. GPCRs have numerous physiological and pathological roles due to the transduction of extracellular signals into intracellular effector pathways through receptor activation[1]. Receptor activation may occur from a broad range of ligands including proteins, peptides, and organic compounds[1]. Upon activation, GPCRs turn on heterotrimeric G proteins by promoting the binding of the activating nucleotide GTP in exchange for GDP on the G protein's Gα subunit[1]. The Gα subunit, along with the bound GTP, is thought to dissociate from the Gβγ dimer, with both complexes subsequently able to modulate the activities of a variety of effectors[3]. Gα signaling is dependent on which Gα family (i.e., $G\alpha_s$, $G\alpha_{q/11}$, $G\alpha_{i/o}$, $G\alpha_{12/13}$) is activated (*Table 1.1)*. $G\alpha_s$ activates, while $G\alpha_{i/o}$ inhibits, receptor-dependent adenylyl cyclase function, leading respectively to an increase or decrease in the second messenger cyclic adenosine monophosphate (cAMP)[3]. The $G\alpha_{q/11}$ G protein family couple receptors to phospholipase Cβ (PLC-β) to increase the levels of the second messengers inositol (1,4,5)-trisphosphate (IP_3) and diacylglycerol (DAG), subsequently leading to an increase in cytosolic calcium (Ca^{2+}) and the activation of protein kinase C (PKC)[3]. The $G\alpha_{12/13}$ G protein family members activate RhoGTPase nucleotide exchange factors (RhoGEFs) which in turn activate a small monomeric GTPase, RhoA, and other downstream effectors[2]. These GPCR-mediated signaling pathways are involved in many human diseases, and are thus the focus of approximately 30-40% of today's commercially available drugs[4].

Table 1.1. Classes of Gα subunits, their expression pattern, and their effectors

Members of the Gα family, their expression patterns, and their signaling functions			
$G\alpha_s$	$G\alpha_s$	Ubiquitous	Stimulation of adenylyl cyclase
	$G\alpha_{olf}$	Olfactory neurons	
$G\alpha_{i/o}$	$G\alpha_{i1/2/3}$	Ubiquitous	Inhibition of adenylyl cyclase Closes Ca^{2+} channels
	$G\alpha_{oA/B}$	Brain	
	$G\alpha_{ot1/2}$	Retina	Stimulation of cGMP-phosphodiesterase
	$G\alpha_z$	Brain/platelets	Inhibition of adenylyl cyclase K^+ channel closure
$G\alpha_{q/11}$	$G\alpha_{q/11}$	Ubiquitous	Stimulation of PLC-β Activate RhoGEFs
	$G\alpha_{15/16}$	Hematopoietic cells	
	$G\alpha_{14}$	Lung, kidney, liver	
$G\alpha_{12/13}$	$G\alpha_{12/13}$	Ubiquitous	Activate RhoGEFs

G protein activation is terminated by the intrinsic GTPase activity of the Gα subunit, which hydrolyzes the GTP back to GDP, resulting in the reformation of the inactive Gαβγ heterotrimer (*Figure 1.1)*. After activation, GPCRs may undergo receptor internalization, and many are able to signal from endosomal compartments, primarily via G protein-independent mechanisms[5]. This process appears to be regulated by phosphorylation by GPCR Kinases (GRKs) and arrestin binding[5,6]. There are diverse mechanisms within a cell that regulate the magnitude and duration of G protein signaling. Nucleotide exchange can be modulated by Guanine nucleotide Exchange Factors (GEFs) and Guanine nucleotide Dissociation Inhibitors (GDIs), whereas the GTPase activity of the Gα subunit may be enhanced by GTPase Accelerating Proteins (GAPs)[7] (*Figure 1.2)*. One family of GAPs are the Regulator of G protein Signaling (RGS) proteins, which promote the hydrolysis of GTP on the activated Gα subunit to GDP, thereby curtailing GPCR signaling[8]. The RGS protein family, including RGS-like proteins, contain more than 30 members, of which most act as GAPs by binding to and deactivating Gα proteins[9]. The majority of RGS proteins act upon $G\alpha_{i/o}$, with a subset acting upon $G\alpha_{q/11}$[10]. One protein, RGS2, however, is unique in that it preferentially acts upon $G\alpha_{q/11}$ over $G\alpha_{i/o}$, while maintaining the ability to impede adenylyl cyclase activity to obstruct signaling via $G\alpha_s$. The mechanism resulting in the inhibition of $G\alpha_s$ signaling by RGS2 remains controversial. It has been suggested to be caused by the binding of $G\alpha_s$ and RGS2, but also the binding of certain isoforms of adenylyl cyclase with RGS2[11]. The fundamental role of RGS2 has been extensively researched as it is important to understand how such an abundant receptor class such as GPCRs are regulated. However, it is unknown how RGS2 itself is regulated. In the following study, we used an RGS2 overexpression model as a tool to study how RGS2 is regulated, specifically the rate of degradation of RGS2, and how this could contribute to Gα signaling.

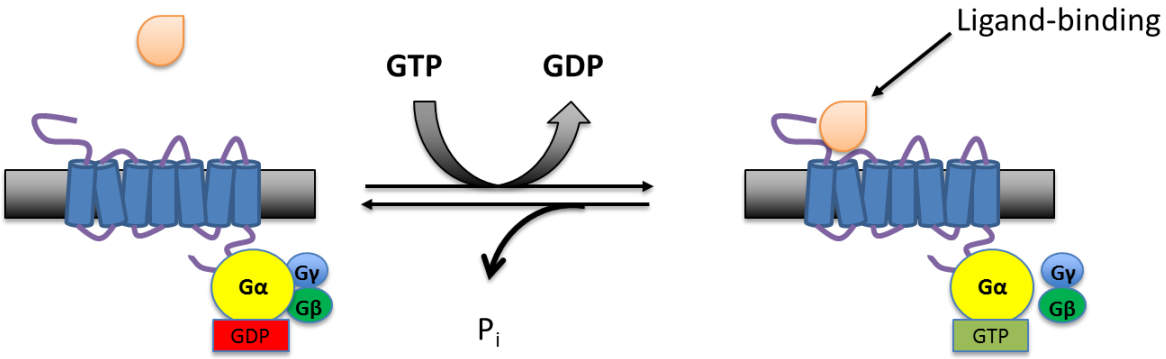

Fig 1.1. Receptor-mediated activation of G proteins. The binding of an extracellular ligand to the GPCR causes a conformational change in the receptor, which leads to the activation of the Gα subunit. This activation promotes the exchange of GDP for the activating nucleotide GTP and is thought to cause the dissociation of the Gβγ dimer from the complex. Both the GTP-bound Gα and free Gβγ are capable of initiating downstream signals by interacting with effectors. This process is terminated by the intrinsic GTPase activity of the Gα subunit which can hydrolyze the GTP back to GDP, forming the inactive Gαβγ heterotrimer complex.

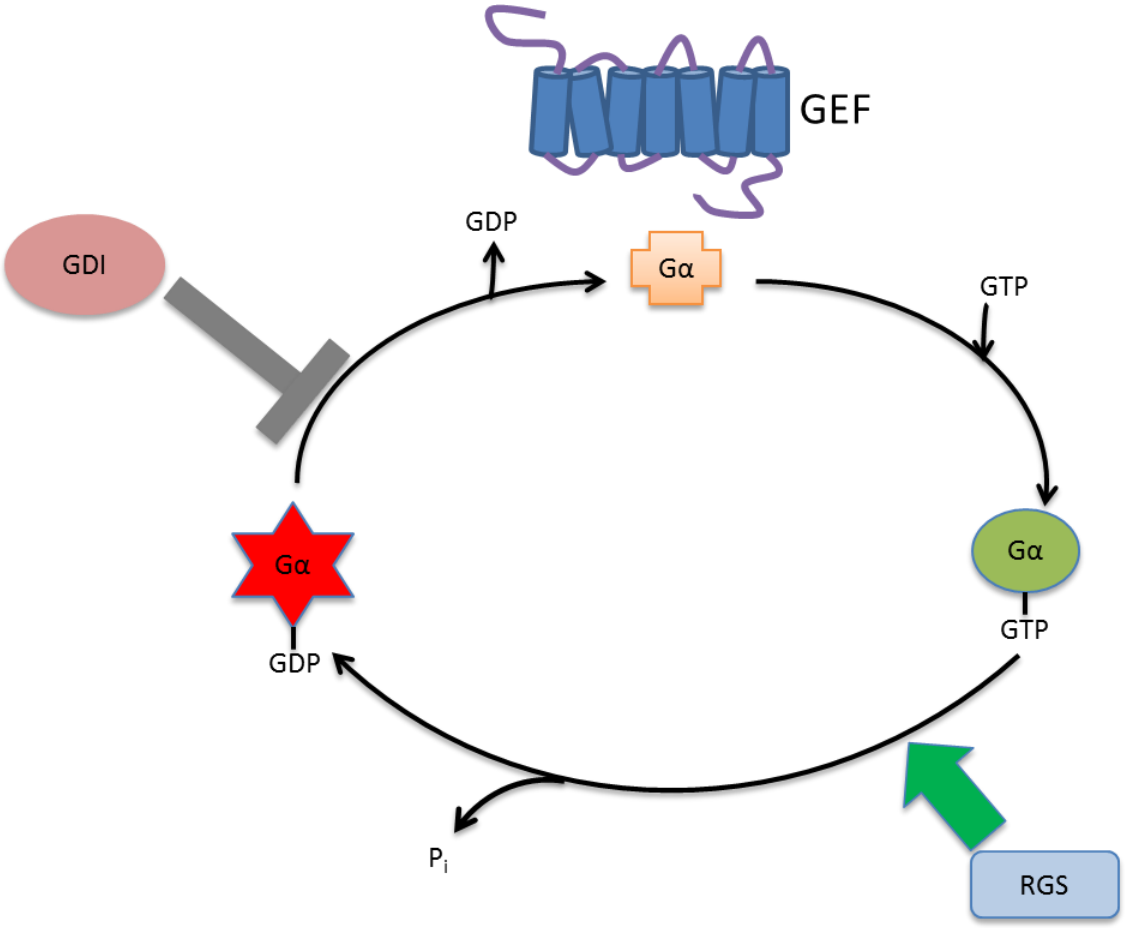

Fig 1.2 Regulation of G protein signaling. The rate of nucleotide exchange can be altered by guanine nucleotide dissociation inhibitors (GDIs) such as RGS12 and guanine nucleotide exchange factors (GEFs) such as GPCRs. GTP hydrolysis can be regulated by GTPase accelerating proteins (GAPs) such as RGS proteins.

1.1.2 $G\alpha_{q/11}$ SIGNALING

Upon ligand binding, GPCRs have the ability to activate G proteins by promoting the exchange of a GDP nucleotide for the activating GTP nucleotide on the Gα subunit of the G protein[3]. Intracellular signaling is dependent upon which G protein family is activated. $G\alpha_{q/11}$ signaling begins when the enzyme PLC-β is activated by the G protein[3]. PLC-β catalyzes the hydrolysis of the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) into two secondary messengers: IP_3 and DAG[12]. IP_3 diffuses throughout the cytosol and binds to IP_3 receptors located on the endoplasmic reticulum (ER) or the sarcoplasmic reticulum (SR) in order to release calcium (Ca^{2+})[13]. Due to the higher Ca^{2+} concentration within the ER compared to the cytosol, activation of IP_3 receptors causes a rapid rise in cytosolic Ca^{2+} levels[12].

One role of Ca^{2+} and DAG is to promote the activation of some isoforms of the enzyme protein kinase C (PKC)[12]. PKC has a major role in regulating many cellular functions including transcription of certain genes, regulating many membrane receptors and ion channels, regulating many cellular phosphorylation cascades[12], cytoskeleton remodelling, and cellular survival signals[14]. Ca^{2+} has other intracellular functions, including binding to and activating the intermediate messenger calmodulin (CaM)[15,16]. CaM mediates many cellular processes such as inflammation, metabolism, apoptosis, smooth muscle contraction, memory, and immune response[15]. CaM is able to activate a protein phosphatase, calcineurin (CaN), by binding to a regulatory domain within CaN, causing a conformational change and resulting in activation of the phosphatase[17]. CaN can trigger the activation of the transcription factor Nuclear Factor of Activated by T-cells (NFAT)[18]. NFAT can regulate gene transcription including the upregulation of hypertrophic response genes, which may lead to cardiac hypertrophy[18]. $G\alpha_{q/11}$ can also signal in a PLC-β-independent manner. The Rho GTPase family

belongs to the Ras superfamily and have a role in many cellular processes such as secretion, smooth muscle contraction, migration, neurite retraction, and gene transcription[19]. Rho proteins cycle between inactive GDP-bound and an active GTP-bound state, and this activation is catalyzed by GEFs (i.e., RhoGEFs)[19]. It is well known that $G\alpha_{12/13}$ G proteins activate RhoGEFs. It has only been recently shown that p63RhoGEF links specifically $G\alpha_{q/11}$-coupled receptors to RhoA by a direct interaction with GTP-liganded $G\alpha_{q/11}$ proteins[19]. This discovery provides another avenue for $G\alpha_{q/11}$ to signal through, adding to the complexity of G protein signaling. Clearly, the $G\alpha_{q/11}$ signaling pathway followed can depend on many factors such as which $G\alpha_{q/11}$-coupled GPCR is activated.

1.1.3 $G\alpha_s$ SIGNALING

$G\alpha_s$ signaling begins the same way as $G\alpha_{q/11}$ signaling – ligand binding causes a conformational change in the GPCR, allowing for the activation of the G protein. $G\alpha_s$ signaling is mediated by the activation of adenylyl cyclase from the $G\alpha_s$-GTP complex[20]. Adenylyl cyclase catalyzes the cyclization of 5'-Adenosine Triphosphate (ATP) to the second messenger cyclic adenosine monophosphate (cAMP)[21]. cAMP is an important second messenger that can activate several different signaling proteins including the enzyme Protein Kinase A (PKA, or cAMP-dependent protein kinase)[22]. PKA is a tetramer composed of two catalytic domains and two regulatory domains. When present, the regulatory subunits bind cAMP and release the catalytic domains which can phosphorylate target proteins[22]. PKA has several cellular functions including regulation of glycogen, sugar, and lipid metabolism[23], sequestering Rac to control cytoskeleton remodeling[24], activating the reward system[25], vasodilation[26] and renin secretion[27]. PKA is well-known for regulating transcription by phosphorylating various transcription factors including

cAMP-response element-binding protein (CREB), cAMP-responsive element modulator (CREM), and activating transcription factor 1 (ATF1)[28].

An important downstream effector in the cAMP-dependent pathway is Exchange Protein directly Activated by cAMP (EPAC). EPAC is a Guanine nucleotide Exchange Factor (GEF) that promotes the activation of small GTPases, such as Rap1, whose major function is to increase cell adhesion via integrin receptors[28,29]. Another cAMP-dependent function is to bind to and modulate a family of cyclic-nucleotide-gated ion channels that conduct calcium[28]. The $G\alpha_s$ pathway can be quite variable and has many pathways to choose from. It is thought that complex molecular mechanisms must be occurring to allow cross-talk between the pathways. This cross talk will allow the pathways to agonize or antagonize each other to ultimately get the desired response of the specific receptor-bound ligand[29].

1.1.4 STRUCTURAL BASIS OF Gα ACTIVATION

All Gα proteins are composed of two domains: a GTPase domain and a helical domain[30]. The GTPase domain contains three flexible loops that undergo substantial structural changes during nucleotide exchange and the hydrolysis cycle[31]. The GTPase domain hydrolyses GTP and also contains binding sites for Gβγ, receptors, and effectors[30]. The helical domain of the Gα subunit is composed of a six α-helix bundle that forms a cap over the nucleotide-binding site in order to bury the bound nucleotide within the protein[30,31]. The helical domain is unique to each of the heterotrimeric G proteins suggesting it may regulate coupling of specific G proteins and other regulators[32].

Although GPCR-stimulated GDP release is not fully understood, several mechanisms have been proposed[30]. One model suggests that upon GPCR activation, the receptor uses the N-

terminal helix of Gα as a lever arm to pull Gβγ away from Gα, resulting in GDP release[30]. A second model suggests that the receptor uses the N-terminal helix of Gα to force Gβγ into Gα, allowing Gγ to engage the helical domain of Gα, resulting in GDP release[30]. Independent of either model, the higher cellular GTP concentration relative to GDP means that GTP will more likely bind to the transient nucleotide-free state of Gα[30], causing Gα protein activation. In addition, the binding of GTP to Gα can be facilitated and stabilized by magnesium (Mg^{2+}), which has been suggested to act as a keystone locking the Gα in a conformation that favours dissociation from Gβγ and effector binding[33]. The intrinsic GTP hydrolysis varies among different Gα proteins[34], however, the relatively slow GTPase activity of Gα subunits (other than $G\alpha_s$) can be enhanced by GAPs.

1.2 REGULATOR OF G PROTEIN SIGNALING (RGS) PROTEINS

The duration of G protein signaling is determined by the length of time that the Gα subunit is in the GTP-bound or activated state[35]. GTP hydrolysis was originally thought to be an unregulated function of Gα subunit that provides intrinsic control over the activation period of a G protein[35]. However, there remained an inconsistency between the rapid G protein signal inactivation rates *in vivo* and relatively slow GTP hydrolysis rate *in vitro*[36,37]. This suggested there is a mechanism *in vivo* regulating the intrinsic GTPase activity of Gα that could accelerate the process. One family of proteins that are known to serve such a role are Regulators of G protein Signaling (RGS) proteins.

SST2, Egl-10, G0S8 (later renamed RGS2), and GAIP (RGS19) were among the first RGS proteins identified in the mid 1990s[38,39,40,41]. Since the 1990s, more than 30 RGS proteins, including RGS-like proteins, have been discovered and added to the RGS family[9,42]. All RGS

proteins share an RGS domain of approximately 120 amino acids which mediates the GAP activity of the RGS protein. There are four subfamilies based on their sequence similarity in the RGS domain: A/RZ, B/R4, C/R7, and D/R12 (*Figure 1.3*). Although many RGS proteins are relatively simple proteins, containing little more than an RGS domain, some RGS proteins contain additional functional domains other than the RGS domain. Thus, RGS protein may have non-canonical functions distinct from deactivating Gα subunits[43].

1.2.1 RGS PROTEIN-G PROTEIN BINDING

RGS proteins have the ability to bind to the active state of Gα proteins and increase the rate of GTP hydrolysis upwards of 2000 fold[45]. The molecular and structural mechanism of RGS proteins GAP activity has been extensively studied. The classical RGS domain consists of 9 α-helices bundled into two lobes. One lobe is formed by helices αI, αII, αIII, αVIII, and αIX whereas the other lobe consists of the αIV, αV, αVI, and αVII helices[46,47]. The RGS domain has been shown to be crucial in the G protein-RGS protein interface by both NMR and crystallography, and this interaction is important to mediate a RGS protein's GAP activity[46,48]. It is likely that the interaction between the αVII and αVIII helices of RGS domain and the Gα helical domain is what allows for selectivity between different RGS protein and G proteins[47,49].

1.2.2 MECHANISMS OF RGS PROTEIN GAP ACTIVITY

The mechanism of RGS protein GAP activity was first studied using RGS4-Gαi1 as a model[46,50]. It was concluded that RGS4 stimulates GTP hydrolysis primarily by binding to and

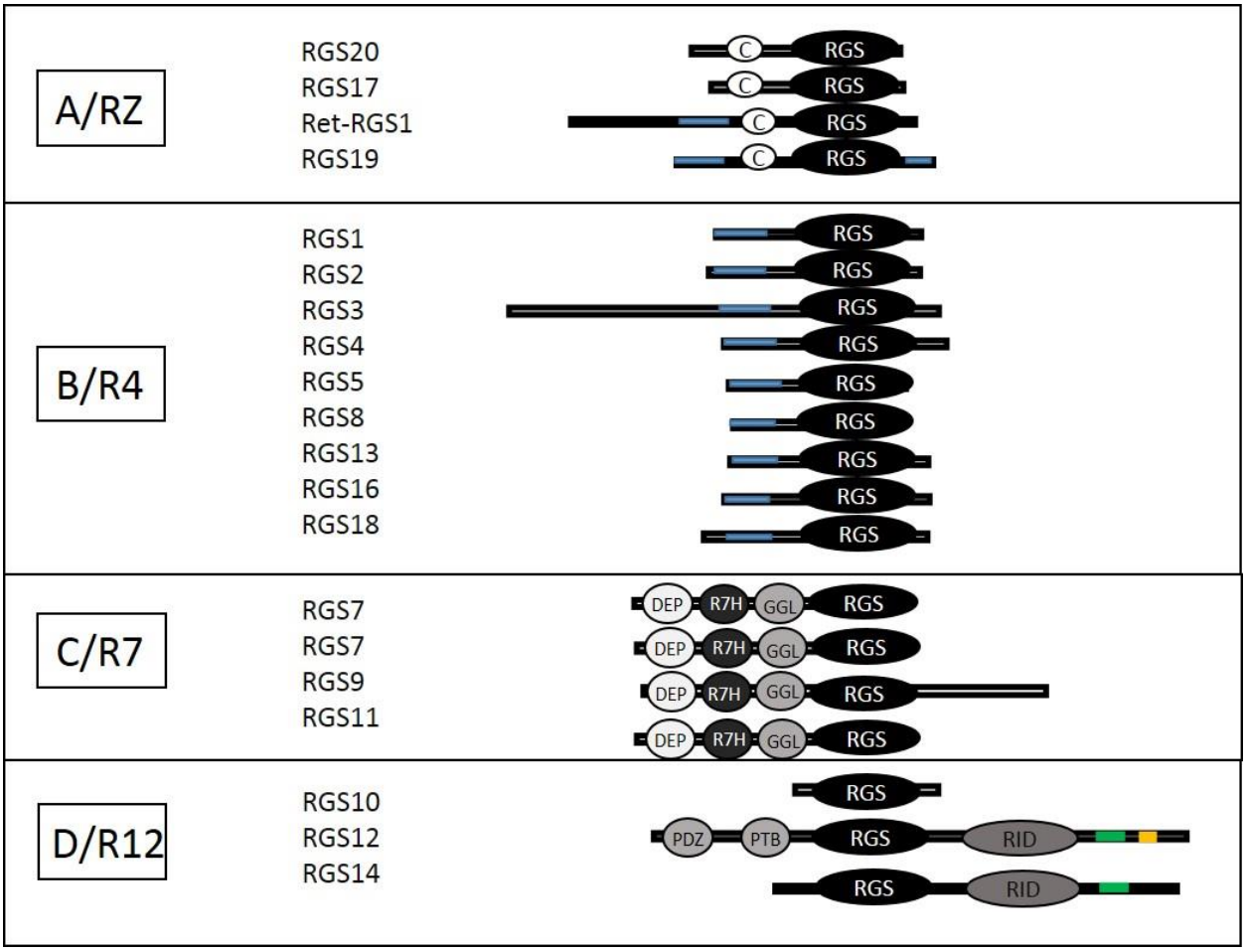

Fig. 1.3. Structures and classification of mammalian RGS proteins. RGS proteins are classified into subfamilies based on the alignment of RGS domain amino acid sequences. Proteins are oriented with their N termini on the left and their C termini on the right. Abbreviations: RGS: Regulator of G protein signaling; DEP: Dishevelled, worm EGL-10, and mammalian Pleckstrin; R7H: R7 binding proteins; GGL: G game like; PDZ: PSD95, Dgl and ZO-1/2; PTB: protein tyrosine binding; RID: Ras interaction domain. Colours: Blue: Amphipathic helix; Green: GoLoco motif; Yellow: Coiled coil domain. Adapted from Hollinger and Hepler, 2002, with permission[44].

creating an environment that favours the transition state conformation of the Gα subunit which is most likely to hydrolyse GTP[50]. The crystal structure of RGS4 bound to the transition state of Gαi1 (i.e., with GDP and AlF_4^- in the binding pocket, mimicking the conformation of the GTP-activated form that precedes GTP hydrolysis) provided supplementary information about the interaction between RGS protein and G protein. It was shown that RGS4 does not directly interact with either GDP or AlF_4^-[51]. Instead, RGS4 catalyzes GTP hydrolysis by reducing the energy of the transition state of the Gα subunit and destabilizing the Gα-GTP complex[46]. Additional studies using other RGS-Gα complexes such as RGS16/$G\alpha_t$ and RGS9/$G\alpha_t$[52,53], p115-RhoGEF/$G\alpha_{13/i1}$[54], RGS1/ $G\alpha_{i/o}$, and RGS19/$G\alpha_{i/o}$[55], each confirmed that the RGS domains bind to and stabilize the flexible regions of Gα during the transition state of GTP hydrolysis.

1.2.3 SELECTIVE REGULATION BY RGS PROTEINS

The selectivity of RGS proteins is dependent on the amino acid residue sequence elements within the RGS domain, as well as the helical domain of the Gα protein[39]. The vast majority of RGS proteins are selective for the $G\alpha_{i/o}$ and $G\alpha_q$ subfamilies of G proteins, but their affinity toward different G proteins varies somewhat within these two subfamilies. For example, RGS4 is known to interact strongly with both $G\alpha_{i/o}$ and $G\alpha_q$[56] but RGS19 interacts strongly with $G\alpha_{i1}$, $G\alpha_{i3}$, and $G\alpha_o$, while maintaining the ability to weakly bind $G\alpha_{i2}$ but not appearing to bind with $G\alpha_s$ or $G\alpha_q$ at all[57]. The available crystal data suggests that RGS19 has a serine at the position corresponding to the asparagine position in RGS4, which may contribute to the difference in G protein selectivity[46,58]. Recently, studies have identified many other RGS proteins can also regulate $G\alpha_{q/11}$ signaling functions, although not block $G\alpha_{q/11}$ signaling by accelerating Gα-GTPase activity[59]. For example, GRK2 contains an RGS domain that binds tightly to $G\alpha_q$ to

block its interactions with PLC-β without affecting $G\alpha_{q/11}$-GTPase activity[59]. Thus, the effector antagonism of RGS proteins can be more complex than simply the binding to G proteins.

The N-terminal regions of RGS proteins serve as important determinants for their biochemical selectivity. Each of the subfamilies of RGS proteins contain N-terminal regions with diverse structural features. The R4/B subfamily each have an amphipathic α-helix of about 30 amino-acid residues with multiple palmitoylation sites[60]. The RZ/A subfamily have a cysteine-rich domain referred to as a cysteine string motif[61]. In other RGS subfamilies, molecular domains such as the DEP domain (R7/C subfamily) or PDZ domain (R12/D) are near the N-terminus of the protein. The N-terminus of RGS proteins regulates selectivity by either mediating RGS protein sub-cellular localization or making direct contact with specific GPCRs or effector proteins[62]. For example, deletion of the N-terminus of RGS2 greatly reduces its plasma membrane and nuclear localization[63].

Though all RGS proteins share a similar RGS domain, there are very different tissue expression patterns among different RGS proteins. For example, RGS2 is ubiquitously expressed throughout all cells, suggesting a more general function[39]. On the other hand, RGS9-1 is solely expressed in the retina, while its splice variant RGS9-2 is expressed in certain regions of the brain[64,65]. Other examples include RGS5 being highly expressed in the vascular tissue with lower expression in skeletal muscle and kidney tissue[66] and RGS21 expression in taste bud cells[67]. The relatively specific tissue distribution of RGS proteins suggests that there may be specialized roles of each RGS protein.

1.2.4 NON-CANONICAL FUNCTIONS OF RGS PROTEINS

RGS proteins contain other molecular domains outside of the conserved RGS domain that have binding partners other than Gα subunits of G proteins and thus function to regulate either their subcellular localization, GAP activity, or receptor coupling. These additional domains may enable RGS proteins to serve non-canonical functions and limit signaling via GAP-independent mechanisms such as effector antagonism and GDI[43]. RGS proteins can also bind many different effector proteins such as adenylyl cyclases, PLC-β, and G protein-coupled inwardly-rectifying potassium (GIRK) channels[42]. In some cases, RGS proteins can bind to effectors and interfere with the productive interaction between these two proteins, thus, RGS proteins function as effector antagonists[68]. On the other hand, RGS proteins may also serve as anchors and create RGS-G protein-effector complexes, resulting in rapid transduction and temporal focusing of the G protein signal[9].

Regulation of non-G protein signaling by RGS proteins has also been investigated. Both RGS13 and RGS16 have been found to interact with the p85α subunit of phosphatidylinositol 3-kinase (PI3 kinase) in a G protein-independent manner, and thus inhibit signaling events downstream of PI3 kinase[69,70]. RGS3 has been shown to interact with the Smad family of proteins to interfere to TGF-β-induced dimerization of Smad3 and Smad4, thereby inhibiting Smad-mediated gene transcription[71]. Other RGS or RGS-like proteins have been suggested to play a role in regulating nuclear signaling, for example, by modulating gene transcription[43]. Work done by our lab has identified a novel inhibitory role of RGS2 on global protein synthesis, wherein RGS2 interacts with the ε subunit of eIF2B to inhibit its GEF activity on eIF2[72].

1.2.5 PATHOPHYSIOLOGICAL FUNCTIONS OF RGS PROTEINS

Characterizing the specific roles of RGS proteins in the context of whole-organism homeostasis and pathophysiology has been a major concerted effort in the field and knockout mouse strains have been essential to these discoveries[73]. Knockout animals have shed light on the importance of RGS proteins in many physiological conditions. RGS1-deficient mice showed abnormal responses to chemokines and improper maturation of germinal centers[74], whereas RGS13-deficient mice showed a different immune system phenotype which resulted in increased mast-cell degranulation and anaphylaxis[69]. RGS2-deficient mice have a completely different immune issue in that their B cell quantities and differentiation are normal, however, they are unable to mount a robust T cell immune response[73]. RGS2 is ubiquitously expressed throughout all cells types which is presumably why RGS2-deficient mice exhibit many abnormal phenotypes. Besides immune issues, RGS2-deficient mice also show increased anxiety and decreased male agression[75], decreased fat stores[76], and constitutive hypertension due to a decreased inhibitory influence on $G\alpha_{q/11}$-mediated vasoconstriction[77–79].

Other RGS-deficient mice also show dysregulation in the cardiovascular system. Mice deficient in RGS5, which is mainly found in vascular smooth muscle and pericytes, exhibit constitutive hypotension[73]. Mice deficient in RGS4, a RGS protein with high expression in the sinoatrial node of the heart, experience exaggerated decreases in heart rate when the parasympathetic nervous system is activated[73]. Finally, RGS6-deficient mice have a mild resting bradycardia and altered heart rate responses to pharmacological agents[73].

Alterations in RGS pathways have been implicated in several disease states, especially cancer[80]. Prostate cancer, head and neck squamous cell carcinoma, breast cancer, and lung

cancer all demonstrate variable risk with alterations in the RGS pathway[80]. There is increasing evidence that point mutations or Single Nucleotide Polymorphisms (SNPs) within RGS genes may predispose individuals to disease states associated with RGS protein pathways. For example, SNPs located on the RGS4 gene have been associated with bladder cancer[80] and also with schizophrenia[81]. RGS SNPs have also been linked to several other human diseases including celiac disease[82], anxiety and panic disorders[83], platelet hypofunction[84], and hypertension[85,86]. Extensive research has not been performed on these SNPs, as most have just been identified as potential contributors to physiological disease states. Both genome-wide association studies and molecular mechanistic studies need to be performed in order to confirm the role RGS SNPs play in disease development.

1.2.6 RGS PROTEINS AS POTENTIAL DRUG TARGETS

GPCRs and their linked signaling pathways are the direct targets for a vast majority of currently used pharmaceuticals[87,88]. RGS proteins have a unique ability to modulate G protein signaling combined with highly regionalized localization, for example, within the nervous system[87]. Developing small molecules that can inhibit RGS protein/Gα binding have been proposed as molecular targets to potentiate the actions of endogenous neurotransmitters in a multitude of disease states such as Alzheimer's disease, depression, and epilepsy[89]. Also, targeting RGS proteins has been proposed to boost efficacy of current GPCR-directed drugs while decreasing the therapeutic dose in order minimize adverse side effects[89]. Targeting RGS proteins have also been proposed to decrease tolerance and possibly reduce desensitization to agonist drugs. For example, opioid tolerance depends on GRK-mediated phosphorylation of agonist-bound receptor and binding of arrestin. RGS inhibitors should also reduce desensitization and tolerance by reducing the fractional receptor occupancy that is required for an analgesic

effect[89]. Currently, these are proposed outcomes of RGS protein drug targeting. If the physiological and pathophysiological roles of RGS proteins are well-established, then targeting RGS proteins could become a viable pharmacological approach in animal studies and human diseases.

1.2.7 OVERVIEW OF RGS2

The R4/B subfamily of RGS proteins contain ten members including, RGS1-5, RGS8, RGS13, RGS16, RGS18, and RGS21. The R4/B subfamily contain the smallest RGS proteins, containing relatively short peptide sequences (2-80 amino acids) flanking the N- and C-terminal ends of the RGS domain, with just one exception (RGS3)[90]. RGS3 exists as several isoforms that are all splice variants from the *RGS3* gene. The shortest splice variant contains little more than the RGS domain, synonymous with the other R4/B subfamily, whereas the longer isoforms may contain PDZ, PEST, and/or acidic domains[90]. PDZ domains are protein-protein interaction domains that are specialized for binding to short peptide motifs at the extreme carboxy termini of other proteins, although they can have other modes of action[91]. A PEST domain is rich in proline (P), glutamic acid (E), serine (S), and threonine (T), which is associated with proteins that have a short intracellular half-life[92].

RGS2, the focus of this thesis, is unique among the RGS protein family. RGS2 is a 211 amino acid protein that contains an ~120 amino acid conserved RGS domain between residues 80-205 that mediates it GAP activity[93]. RGS2 also contains four initiator methionine residues at positions 1, 5, 16, and 33, any of which can initiate protein production in mammals[94] (*Figure 1.4*). RGS2 is found ubiquitously throughout the body[72,95–100], which allows it to play an important role in many physiological processes. Indeed, RGS2 appears to play an important

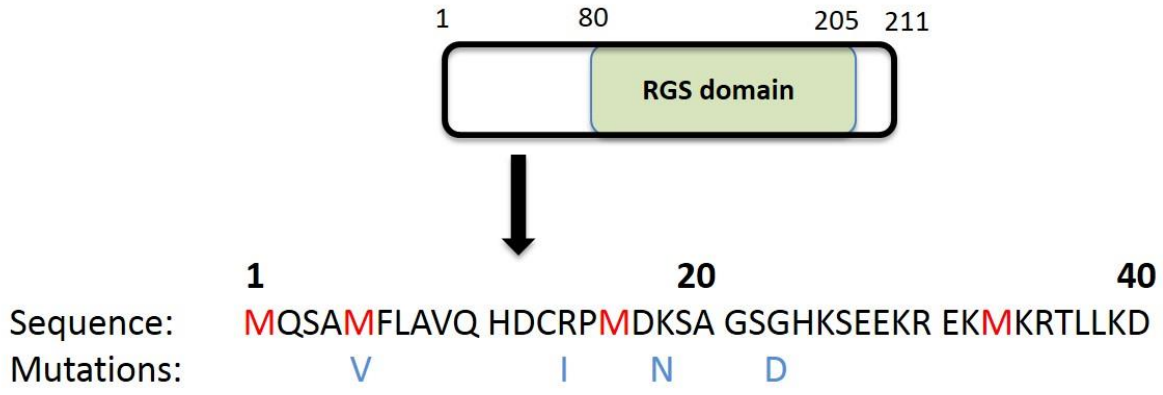

Fig 1.4. Representative RGS2 protein with the first 40 amino acids. The RGS domain is located between amino acids 80 and 205. Alternate start sites (initiation variants) are highlighted in red. Point substitutions are highlighted in blue.

role in pathophysiological conditions such as hypertension[101–103], anxiety[104], aggression[75], and oxidative stress[105]. RGS2 is upregulated by $G\alpha_s$- and $G\alpha_{q/11}$-mediated signals, which appear to cause cross-desensitization between the pathways[106]. RGS2 is also upregulated by factors such as heat-shock[105], electrical stimulation[107], ischemia[108], and other cellular stress inducing agents. Interestingly, other stress related proteins (such as ATF4) are upregulated by cellular stress and also contain multiple initiation sites, similar to that of RGS2. Hence, RGS2 may have multiple sites of initiation in order to synthesize important isoforms of the protein during stressful cellular conditions.

While RGS4, another R4/B subfamily member, possesses good GAP activity for $G\alpha_{i/o}$ and $G\alpha_{q/11}$, RGS2 lacks *in vitro* GAP activity for $G\alpha_{i/o}$, but has been reported to be quite potent in blocking $G\alpha_{q/11}$-directed activation of PLC-β[93]. The structural basis for RGS2's poor GAP activity for $G\alpha_{i/o}$ compared to RGS4 is thought to be due to a three amino acid substitutions in the Gα binding surface of RGS2[93,109]. It is suggested that these structural differences in RGS2 may impair the binding of RGS2 to $G\alpha_{i/o}$ but in theory could favour the binding to $G\alpha_{q/11}$[93]. An important factor in the binding of RGS2 to Gα proteins is plasma membrane targeting. Truncation of the first 78 amino acids (N-terminus) of RGS2 results in the loss of effector antagonism of RGS2 but not its GAP activity, suggesting that the N-terminus may have a plasma membrane targeting function[93,110].

RGS2 also has the ability to inhibit $G\alpha_s$ pathways. The structural basis for its inhibitory effects remains unknown and controversial as to whether RGS2 binds $G\alpha_s$, isoforms of adenylyl cyclase, or both. When purified recombinant RGS2 was added to purified recombinant adenylyl cyclase V cytoplasmic domains, it was able to decrease cAMP production stimulated by either $G\alpha_s$ or by forskolin[111]. However, others have demonstrated that RGS2 protein can be

immunoprecipitated with purified $G\alpha_s$[112]. Additionally, RGS2 is recruited to the plasma membrane when co-expressed with $G\alpha_s$ and when RGS2 is co-expressed with isoforms of adenylyl cyclase[11]. Regardless of how RGS2 inhibits $G\alpha_s$, this function of RGS2 is important in many physiological processes including the olfactory system. Addition of recombinant RGS2 to olfactory epithelia membranes blocked odorant-induced cAMP production suggesting that RGS2 negatively regulates $G\alpha_s$-mediated pathways, as $G\alpha_{olf}$ belongs to the $G\alpha_s$-family[93].

RGS2 is interesting in that it can have numerous SNPs, several of which can affect its regulation and function[84,86] (*Figure 1.4*). One point mutation, RGS2 M5V, is found within a Japanese population of hypertensive patients, but it was not identified in healthy control patients[86]. Another point mutation, RGS2 G23D, has been associated with a phenotypic patient profile that includes borderline IQ, hirsutism, upregulation of bone alkaline phosphatase due to an increase in bone mass, and decreased platelet $G\alpha_s$ function leading to enlarged, round platelets with abnormal α-granules[84]. The authors concluded that the genetic defect in RGS2 causes a preference for translation via the first two initiator methionine residues at amino acid positions 1 and 5, rather than 16 and 33, to initiate protein production.

Within the larger two forms of RGS2 is a putative adenylyl cyclase binding domain (amino acids 9-11), which is absent in the two shorter forms of RGS2[84]. Therefore, if the altered genetic sequence of RGS2 G23D is causing a shift towards production of the longer forms of RGS2, $G\alpha_s$ signaling may be inhibited more strongly, resulting in the pathological conditions seen in patients[84]. The mechanism as to how these pathophysiological conditions occur remains controversial as it is still unclear if RGS2 in fact directly binds to all forms of adenylyl cyclase[11].

Mice lacking RGS2 have previously been shown to spontaneously develop hypertension and cardiac hypertrophy and this correlates with decreased attenuation of $G\alpha_{q/11}$ signaling[113]. Authors concluded that it could also be due to altered kidney function[114] and increased circulating catecholamines[115]. This has led us to hypothesize RGS2 M5V may not be attenuate $G\alpha_{q/11}$ signaling as well as wild-type RGS2, thus potentially leading to hypertension[86]. By looking at the phenotypic profile of a RGS2 G23D patient, it was shown that there is a decreased platelet $G\alpha_s$ function, suggesting that this mutant will attenuate $G\alpha_s$-linked GPCR signaling to a greater degree than wild-type RGS2 protein. There are two RGS2 SNPs without any known pathophysiological effects but are found in between the RGS2 M5V and RGS2 G23D mutations; RGS2 R14I and RGS2 K18N. Although there is little known about these mutations, RGS2 R14I is close to, but is not part of the putative adenylyl cyclase binding region at amino acids 9-11. RGS2 K18N also contains a SNP between the RGS2 M5V and RGS2 G23D SNPs, and appears to not play a role in hypertension[85]. Currently, it is unknown if and how the mutations affect Gα signaling, but we hypothesize that these RGS2 SNPs may affect the stability of the protein, thus influencing Gα signal attenuation.

1.3 KINETIC REGULATION OF G PROTEIN ACTIVITY

To understand how G protein-mediated signaling occurs, the kinetics of G protein activation and deactivation must first be understood. The first step in the G protein activation/deactivation cycle is GDP dissociation. The relatively high concentration of GTP in a cell (while free GDP is relatively low) leads to a rapid association of GTP after GDP dissociation[30]. Thus, the rate of nucleotide exchange depends highly on the rate of GDP dissociation. GTP dissociation is relatively slow and as a result, GTP is normally hydrolyzed by the G protein even before it dissociates[30]. Overall, nucleotide exchange and GTP hydrolysis are two key reactions that

determine the duration of G protein signaling[30]. As alluded to in section 1.1.1, the kinetics of G protein signaling are tightly regulated by GEFs, GDIs, and GAPs, and the delicate balance among these regulatory mechanisms can affect the rate and magnitude of G protein signaling.

1.3.1 GEF-MEDIATED ACTIVATION OF G PROTEINS

From a kinetic point of view, the fractional activation of the G protein reflects the balance between GEF-promoted activation and GAP promoted deactivation[116]. GEFs, such as GPCRs, dramatically increase the rate of GDP dissociation, resulting in an increase in GTP association and ultimately, G protein activation.

An interesting non-receptor G protein activator is Ric-8 (resistance to inhibitors of cholinesterase 8A), which shares similar but not identical mechanism with that of the GPCR. Ric-8 favours binding with high affinity to the open conformation of the G protein thus, the effect of Ric-8 on fractional activation of G protein may be nucleotide concentration dependent[116]. Ric-8 also promotes the dissociation of both GDP and GTP which, at lower concentrations of GTP (< 500 nM), helps to reduce GTP turnover[116]. At higher GTP concentrations, as are found intracellularly (~150 μM), GTP association is greater than dissociation and the Gα-GTP form predominates[116]. Ric-8 has also been reported to increase cellular G protein levels by stabilizing G proteins in a nucleotide-free conformation thus preventing denaturation[117]. This finding has profound implications with respect to $G\alpha_{q/11}$ activation kinetics as $G\alpha_{q/11}$ tends to have a fast denaturation rate when in the nucleotide-free state[118]. As the binding of GTP competes with the denaturation of ligand-free $G\alpha_{q/11}$, increasing the concentration of GTP decreases denaturation and thus enables the formation of $G\alpha_{q/11}$-GTP.

The fact that Ric-8 is able to decrease the denaturation rate of the G protein will further benefit the formation of the active GTP-bound G protein.

1.3.2 GDI-MEDIATED INHIBITION OF G PROTEIN ACTIVATION

Nucleotide exchange is intrinsically limited by the relatively slow rate of nucleotide dissociation. GDI activity, which decreases the GDP dissociation, may also lead to an overall reduction in G protein activation. Among the GDIs identified for heterotrimeric G proteins, the best studied proteins are those containing the GoLoco motif (also called GPR domain and GPSM domain) – a highly conserved 19 amino acid motif[119] that has a much higher binding affinity for GDP-bound Gα relative to either nucleotide-free or GTP-bound Gα[120]. It has been shown that the rate of GTPγS binding, which reflects nucleotide exchange, is decreased up to 80% in the presence of GoLoco proteins or peptide derived from the GoLoco motifs of RGS12 and RGS14[121,122]. The function of GoLoco motifs becomes more complicated by its propensity to compete with Gβγ in binding to Gα. The Gβγ dimer is also able to slow down the rate of intrinsic GDP dissociation from the Gα subunit up to 50 fold, depending on the specific G protein in question[123]. Notwithstanding this, Gβγ is necessary for receptor-stimulated Gα activation since it can stabilize Gα-receptor coupling.

1.3.3 GAP-MEDIATED GTP HYDROLYSIS

The GTP hydrolysis rate of proteins can be increased up to 2000 fold by GAPs such as RGS proteins[124]. As a result, RGS proteins negatively regulate the G protein cycle by both dampening signaling output and by rapidly terminating G protein activation upon removal of a stimulus[124]. Interestingly, kinetic characterization indicated that the rate of the overall steady-state GTPase reaction measured in the presence of PLC-β is 10 times faster than the rate of

GTP binding to G protein in the absence of PLC-β[125]. Moreover, even though the GTP hydrolysis is dramatically increased by GAPs, the fact that the fractional activation of G protein still remains high suggests that either the activation rate is also increased or the GAP activity is inhibited while the receptor is activated.

One theory that supports the idea that RGS proteins are able to potentiate receptor-mediated G protein activation is a proposed kinetic scaffolding mechanism[125,126]. In this model, GAPs are able to reduce depletion of local Gα-GDP levels to permit rapid recoupling to receptor and sustained G protein activation. In combination with the kinetic scaffolding mechanism, there is another model based on the idea of physical scaffolds. This model suggests that RGS proteins may directly or indirectly interact with the receptor and facilitate receptor-G protein coupling and promote signal onset thus, RGS proteins may act as a scaffold to assemble different signaling components[127]. The theories described provide insights into how G protein signaling is regulated by different factors. It is prudent to understand how the mechanisms are regulated themselves, whether it be due to transcriptional differences in the mechanisms or alterations in protein levels.

1.4 PROTEIN DEGRADATION

The half-life of a protein depends on how rapidly it is produced and degraded. The majority of proteins are degraded in a selective protein ubiquitin-proteasomal fashion which depends on three classes of enzymes; E1, E2, and E3[128]. E1 enzymes attach an ATP to ubiquitin, E2 enzymes are ubiquitin-conjugating enzymes, and E3 are ubiquitin-ligase enzymes bind E2 enzymes and the target protein to mediate ubiquitination; this cycle repeats itself until the ubiquitinated proteins are recognized and the protein is ultimately degraded by the

proteasome[128]. Selective protein turnover results in protein levels that can rapidly change in response to external stimuli that alter degradation rates. Furthermore, abnormal products, such as misfolded proteins, can be rapidly degraded[128]. Long lived proteins typically are degraded by the lysosomal pathway, which is a non-selective pathway[129]. It is often assumed that lysosomal degradation is only for degrading endocytosed particles, but endogenous proteins also have the ability to bind to the lysosomal membrane, which controls the rate of entry into the lysosome and subsequent degradation of the protein[129]. This process is relatively slow compared to the rapid ubiquitin-proteasome pathway, which is why it is thought that only long lived proteins are degraded via the lysosomal pathway[129]. RGS proteins, specifically RGS4 and RGS5, have previously been shown to be degraded by the ubiquitin-proteasome pathway, and their rapid degradation was attributed to the presence of specific amino acids within their N-termini[130,131].

1.4.1 N-END RULE PATHWAY

In 1986, Alexander Varshavsky and co-workers developed the N-end rule which proposes that the N-terminal amino acid of a protein determines its half-life or likelihood of being degraded[132] (*Figure 1.5*). Specific amino acids within the N-termini, called N-degrons, present degradation signals that may promote the rapid breakdown of proteins[131,133]. The idea behind the N-end rule is that degrons on short-lived proteins in eukaryotes are recognized by ubiquitin ligases, which mediate the conjugation of ubiquitin to an internal lysine residue of the substrate, resulting in the ATP-dependent degradation by the 26S proteasome[128,130,133,134].

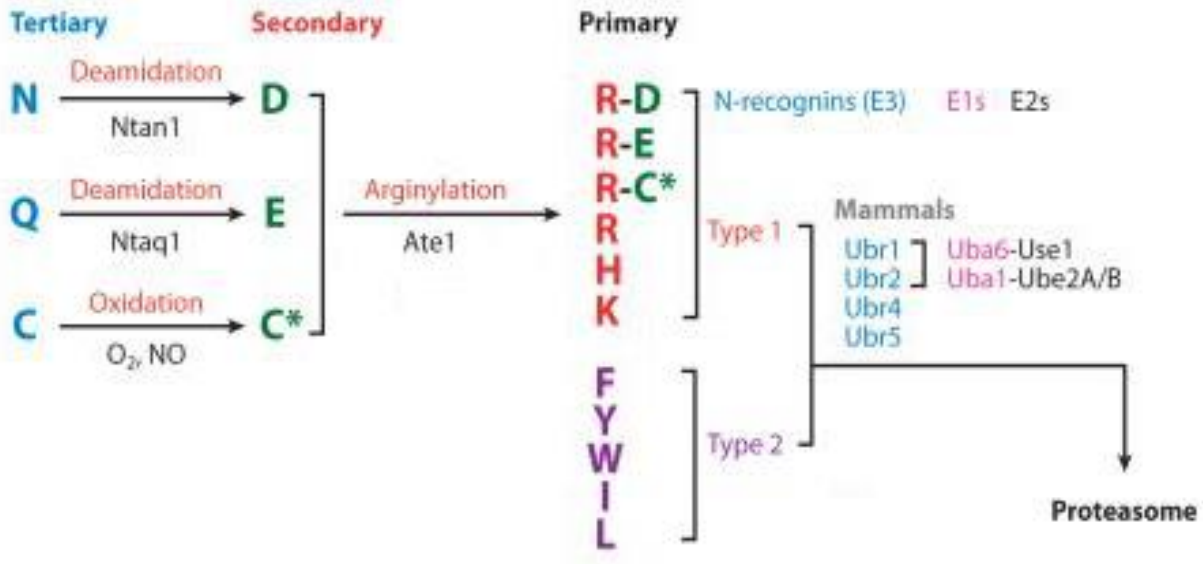

Fig. 1.5. The classical N end rule pathway. Tertiary destabilizing residues asparagine and glutamine are, respectively, deamidated into secondary destabilizing residues aspartic acid and glutamic acid by NTAN Nt^N-amidase and NTAQ Nt^Q-amidase, which are in turn arginylated by *ATE1*-encoded arginyl (R)-transferase isoforms generating the degron arginine. N terminal cysteine is converted to a substrate of arginylation through its oxidation. N terminal arginine, as well as type 1 and type 2 residues, are recognized and bound by the N-recognin family members, which mediate ubiquitylation and proteasomal degradation, via detection by the UBR box. Adapted from Tasaki *et al*., 2012, with permission[134].

1.4.2 RECOGNITION OF N-DEGRONS

The recognition of N-degrons is mediated by N-recognins which induce protein ubiquitylation and proteolysis via the proteasome. The mechanism of substrate selectivity was revealed by the discovery that the UBR box, conserved in many N-recognins, is the substrate recognition domain[135,136]. The mammalian genome encodes seven known UBR box proteins, UBR1-UBR7, which all contain signature substrate recognition components of the ubiquitin-proteasome system, with exception of UBR4[134]. UBR1-UBR3 are referred to as canonical due to their sequence homology, size (~200 kDa), and conserved domains including the UBR box (type 1 binding site), N domain (type 2 binding site), RING finger (ubiquitinylation domain), and autoinhibitory domain (which sterically blocks the UBR box and N domain)[134]. UBR4-UBR7 are referred to as non-canonical UBR box proteins as they are non-sequelogous to one another[134].

The recognition of N-end rule substrates initiates with hydrogen bonding with the free α-amino group of the N-terminal residue[134]. Once engaged, N-recognin establishes a substrate-selective interaction through hydrogen bonds with the positively charged side chains. Overall, N-end rule interactions are largely confined to the first two residues, enabling N-recognins to select substrates on the basis of destabilizing N-terminal residues[137,138]. UBR1 and UBR2 also have a second substrate-binding domain, the N domain, which binds to type 2 degrons (*Figure 1.5*)[139].

1.4.3 UBIQUITIN ACTIVATION AND CONJUGATION

Ubiquitin can be activated by two ubiquitin-activating enzymes (E1), UBA1 and UBA6[140]. UBA1 is the major E1 ubiquitin-activating enzyme responsible for the bulk of ubiquitin

conjugation to E2 enzymes, whereas UBA6 is an alternate E1 enzyme with a designated E2 enzyme, USE1[140]. Though UBA1 is used more often, it has been shown that mice lacking UBA6 die *in utero*, suggesting that UBA6 plays an essential role in mammalian development[141]. Recently, it has been shown that UBA6 mediates the ubiquitin activation and conjugation for the canonical N-recognins, UBR1-UBR3, and one substrate of UBA6-activated N-recognins includes RGS4[142]. Once ubiquitin has been activated by E1 enzymes, the ubiquitin molecule is transferred to a member of the ubiquitin-conjugating enzymes (E2). This transfer forms a thioester-charged E2 intermediate that can associate with an E3 ubiquitin-ligase enzyme to promote ubiquitin transfer from the E2 to the substrate of interest and ultimately lead to degradation[142].

1.4.4 RGS PROTEINS AND THE N END RULE

Although all proteins are not intrinsically unstable, they may become so via N-arginylation or the substitution of an amino acid for a destabilizing arginine residue[130]. RGS4 and RGS5 are two proteins known to undergo N-arginylation and ultimately follow the ubiquitin-proteasome degradation pathway[131]. Both RGS4 and RGS5 have a conserved N-terminal

cysteine at amino acid position 2[130], and cysteine is known to undergo N-arginylation[131]. After mutating the N-terminal cysteine of RGS4 and RGS5 to a serine, these proteins were no longer found to be rapidly degraded, suggesting that rapid degradation of RGS4 and RGS5 was reliant on the conserved cysteine[130]. An N terminal cysteine is rapidly oxidized after the removal of the initiator methionine (by a methionine aminopeptidase), which results in a pre-N-degron protein, allowing arginylation to occur by *ATE1*-encoded Arg-transferase[131]. An N-terminal arginine allows E3 enzymes (ubiquitin-protein ligase E3 component n-recognin 1 and 2, or

UBR1 and UBR2) to recognize the amino acid, and thus ubiquitin can be added to the protein. Further additions of ubiquitin ultimately result in degradation of the protein[143]. The physiological importance of protein arginylation has been established by the discovery that *ATE1*-deficient mouse embryos die due to cardiac and vascular development abnormalities[134].

The rapid ubiquitin-proteasome degradation caused by the conserved cysteine residues of RGS4 and RGS5 has also been reported to occur when there is a conserved glutamine or asparagine residue at amino acid position 2[131]. The mechanism to generate N-degrons from a glutamine or asparagine differs from that involving a conserved cysteine. Instead of oxidation, glutamine and asparagine residues undergo deamidation[134]. Deamidation is a chemical reaction in which an amide functional group is removed or replaced. N-terminal glutamine and asparagine are respectively deamidated by NTAQ1-encoded Nt^{Q}-amidase and NTAN1-encoded Nt^{N}-amidase, which are not analogous to each other[134]. The physiological function of deamidation was initially identified in NTAN1-deficient mice who were found to exhibit impaired memory, learning, and social behaviour which appeared to be primarily due to dysregulation of proteasomal degradation of the microtubule-associated protein 2 in the hippocampal neurons[134]. Glutamine and asparagine are respectively deamidated into the secondary destabilizing residues glutamic acid and aspartic acid, which are in turn arginylated by *ATE1*-encoded arginyl (R)-transferase isoforms generating the degron arginine and continuing on with degradation of the protein[134]. Although this process has not been reported in RGS2, which has a conserved glutamine at amino acid position 2, it is plausible that RGS2 protein may also be rapidly ubiquitinated and degraded in a similar fashion to RGS4 and RGS5. There is evidence that RGS2 is rapidly ubiquitinated and degraded. When HEK293 cells were transiently transfected with RGS2 and HA-ubiquitin, they were shown to co-

immunoprecipitate together and to a greater extent in the presence of the proteasome inhibitor MG132[144]. Also, since mutations in the N-terminal domains of RGS4 and RGS5 lead to altered degradation rates[130], it is possible that RGS2 SNPs may also have altered degradation rates compared to the wild-type RGS protein.

1.5 RESEARCH GOALS AND SIGNIFICANCE

RGS proteins, specifically RGS4 and RGS5, appear to be rapidly turned over unless there are specific mutations within the N-terminus[130]. Slight variations in the rapid turnover of RGS proteins can affect the way GPCR signals are attenuated. For instance, if the presence of RGS2 within a cell was increased or decreased from basal levels, this would be expected to cause GPCR signaling to be decreased or increased, respectively. In this study, our principal goal was to examine the molecular mechanisms controlling the degradation of RGS2. These studies were done in cellular *in vitro* models to establish a basic understanding of what is potentially occurring at the molecular level. Our **major goals** were as follows:

i. To determine the stability of wild-type and mutant forms of RGS2 (Study A)
ii. To determine the effects of wild-type and mutant forms of RGS2 on G protein-mediated signaling (Study B)

1.5.1 STUDY A: STABILITY OF WILD-TYPE AND MUTANT FORMS OF RGS2 *IN VITRO*

Regardless of the anticipated function of a protein, if it is targeted for degradation before it can perform its task, it can be rendered useless. Evidence in our lab has shown that wild-type RGS2 is a rapidly degraded protein. Here we report that the use of alternative initiator methionine

residues alters the half-life of RGS2. Furthermore, mutations within the N-terminus of RGS2 also have severe effects on the half-life. We also provide evidence that RGS2 is degraded via the ubiquitin-proteasome pathway. However, the degree to which expression levels change depend on which initiator methionine is used or if RGS2 contains a SNP.

The **objectives** in this study were to:

1) Determine the effects of different methionine initiation sites and N-terminal mutants on RGS2 stability
2) Determine the effects of inhibitors of degradation on RGS2 expression levels

1.5.2 STUDY B: EFFECTS OF WILD-TYPE AND MUTANT FORMS OF RGS2 ON $G\alpha_{q/11}$ SIGNALING

RGS2 is known to attenuate $G\alpha_{q/11}$ signaling but the degree to which each initiation variant can inhibit signaling is unknown. Here we report that each initiation variant, as well as each RGS2 SNP, has an altered ability to attenuate $G\alpha_{q/11}$ signaling compared to the full-length wild-type RGS2.

The **objective** in this study was to:

1) Determine the effects of RGS2 methionine initiation sites and N-terminal mutants on the ability to attenuate $G\alpha_{q/11}$ signaling

Based on the results presented here, we propose that RGS2 is a rapidly degraded protein that is usually degraded via the ubiquitin-proteasomal pathway. Which initiator methionine initiates translation can have profound effects on the half-life of the protein and also the ability to attenuate $G\alpha_{q/11}$ signaling. Also, mutations within the N-terminus of RGS2 can alter the protein's half-life and ability to attenuate $G\alpha_{q/11}$ signaling. Understanding the molecular

mechanisms behind the regulation of RGS2 may provide insight into why individuals with RGS2 mutations show the phenotypic profile they do.

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Chapter 2: Materials and Methods

2 MATERIALS AND METHODS

2.1 CELL LINE

In vitro based studies using the well-established Human Embryonic Kidney 293 (HEK293) cell line were used to assess how RGS2 is regulated. HEK293 cells are commonly used for *in vitro* experiments due to their ease of transient transfection[1]. HEK293 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco Life Technologies) supplemented with 10% Fetal Bovine Serum (FBS, Gibco Life Technologies) at 37ºC with 5% CO_2. Cells were seeded every 48 or 72 h at a density of $2.2x10^6$ or $0.8x10^6$ cells, respectively, in 10 ml medium in a 10 cm dish. Seeding density into smaller dishes were calculated depending on the surface area (cm^2) of the plate.

2.2 DNA CONSTRUCTS

A mammalian expression vector encoding full-length, C-terminally FLAG-tagged, wild-type RGS2 in pcDNA3.1$^+$ (*Figure 2.1*) was custom generated by the University of Missouri-Rolla cDNA Resource Center (www.cdna.org). Mutant constructs derived from the initial plasmid were generated in our laboratory. RGS2 M5V, RGS2 R14I, RGS2 K18N, and RGS2 G23D were generated by performing QuikChange site-directed mutagenesis according to the manufacturer's protocol (Agilent, 200522). tM5 RGS2, tM16 RGS2, tM33 RGS2, RGS2 M5V, RGS2 R14I, RGS2 K18N, and RGS2 G23D (*Table 2.1)* were amplified by polymerase chain reaction (PCR) as Kpn1-Eco81I fragments in which the primers introduced the mutations. Primers (*Table 2.2)* introduced a pseudo-Kozak sequence in the full length wild-type RGS2, tM5 RGS2, tM16 RGS2, and tM33 RGS2. Plasmid DNA was purified using Qiagen Plasmid Maxi Kit (Qiagen, 12163). FLAG-tagged 5-HT_AR was a gift from Dr. Stephen Ferguson,

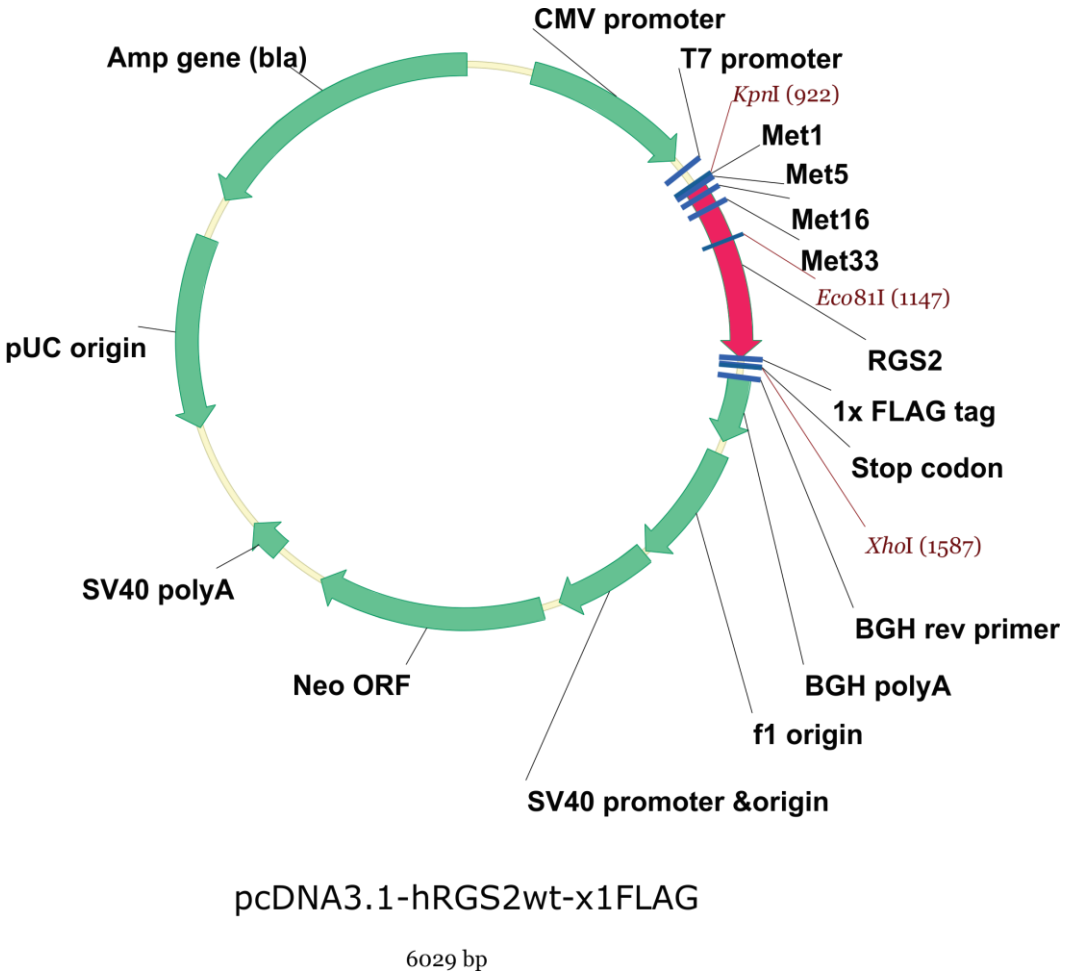

Figure 2.1. Full-length wild-type RGS2 construct.

Table 2.1. Amino acid sequence of RGS2 constructs

First 40 amino acids of RGS2 constructs with initiator methionines and mutations	
FL-WT RGS2	MQSAMFLAVQ HDCRPMDKSA GSGHKSEEKR EKMKRTLLKD
tM5 RGS2	MFLAVQHDCR PMDKSAGSGH KSEEKREKMK RTLLKDWKTR
tM16 RGS2	MDKSAGSGHK SEEKREKMKR TLLKDWKTRL SYLFQNSSTP
tM33 RGS2	MKRTLLKDWK TRLSYFLQNS STPGKPKTGK KSKQQAFIKP
RGS2 M5V	MQSAVFLAVQ HDCRPMDKSA GSGHKSEEKR EKMKRTLLKD
RGS2 R14I	MQSAMFLAVQ HDCIPMDKSA GSGHKSEEKR EKMKRTLLKD
RGS2 K18N	MQSAMFLAVQ HDCRPMDNSA GSGHKSEEKR EKMKRTLLKD
RGS2 G23D	MQSAMFLAVQ HDCRPMDKSA GSDHKSEEKR EKMKRTLLKD

The first 40 amino acid residues for RGS2 constructs. Alternate start sites (initiation variants) are highlighted in red. Point substitutions are highlighted in blue.

Table 2.2. Primers for making wild-type RGS2 and its mutants

Construct	5’ primer	3’ primer
FL-WT RGS2	ACTAGTGGATCCGCCACCATGGCGCAAAGTGCTATGTTCTTG	ACCGGTCGGTTCAAGTCTTCTTCTGA
tM5 RGS2	ACTAGTGGATCCGCCACCATGGCGTTCTGGCTGTTCAACAC	ACCGGTCGGTTCAAGTCTTCTTCTGA
tM16 RGS2	ACTAGTGGATCCGCCACCATGGCGGACAAGAGCGCAGGCAGT	ACCGGTCGGTTCAAGTCTTCTTCTGA
tM33 RGS2	ACTAGTGGATCCGCCACCATGGCGAAACGGACCCTTTTAAAAGATTGG	ACCGGTCGGTTCAAGTCTTCTTCTGA
RGS2 M5V	ACCATGCAAAGTGCTGTGTTCTTGGCTGTTC	GAACAGCCAAGAACACAGCACTTTGCATGGT
RGS2 R14I	TTCAACACGACTGCATACCCATGGACAAGAG	CTCTTGTCCATGGGTATGCAGTCGTGTTGAA
RGS2 K18N	CAGACCCATGGACAACAGCGCAGGCAGTGGC	GCCACTGCCTGCGCTGTTGTCCATGGGTCTG
RGS2 G23D	AGAGCGCAGGCAGTGACCACAAGAGCGAGGA	TCCTCGCTCTTGTGGTCACTGCCTGCGCTCT

University of Western Ontario[2]. All sequences were confirmed using Robarts Research Institute DNA Sequencing Facility.

2.3 TRANSFECTION

For immunoblotting, HEK293 cells were cultured in a 12-well plate under standard conditions of 37ºC and 5% CO_2, in DMEM with 10% FBS and transient transfections were performed using Lipofectamine 2000 (Life Technologies, 11668-019) according to the manufacturer's protocol. To summarize the protocol, cells were seeded and grown to 70% confluence before transfection. Once 70% confluent, 4 µl of Lipofectamine 2000 reagent was diluted into 100 µl of Opti-MEM® I Reduced Serum Medium (Life Technologies, 31985-070) in an Eppendorf tube. 1 µg plasmid DNA was diluted in a separate tube containing 100 µl of Opti-MEM® I Reduced Serum Medium and both tubes were incubated at room temperature for 5 min. The tubes were thoroughly mixed together and were incubated at room temperature for 20 min. The combined mixture was added to the cells and allowed to incorporate into the cells for 24 h.

For inositol-phosphate turnover experiments, HEK293 cells were transiently transfected in suspension and seeded in a 24-well plate. Briefly, 10 µl of Lipofectamine 2000 reagent was diluted into 250 µl of Opti-MEM® I Reduced Serum Medium in an Eppendorf tube. 2 µg 5-HT_{2A}R plasmid DNA and either 2 µg RGS2 plasmid DNA or 2 µg pcDNA3.1$^+$ DNA was diluted in a separate tube containing 250 µl of Opti-MEM® I Reduced Serum Medium and both tubes were incubated at room temperature for 5 min. The tubes were mixed thoroughly together and were incubated at room temperature for 20 min. HEK293 cells from a 70% confluent 10 cm plate were sedimented, mixed with 20 ml of fresh DMEM with 10% FBS and the combined mixture of Opti-MEM®, Lipofectamine 2000, and plasmid DNA were added to

the cells. Cells were seeded at 0.1 x 10^6 cells per well of a 24-well plate and were allowed to incorporate into DNA for 24 h.

2.4 DRUG TREATMENT

24 h post-transfection, cells were treated with well-established pharmacological agents to either inhibit proteasomal degradation or to attenuate global protein synthesis. HEK293 cells were subjected to MG-132 (Sigma-Aldrich, M7449-200UL) (20 μM) treatment (2 h) in order to inhibit the ubiquitin-proteasome pathway[3]. This was performed in parallel with 0.05% DMSO (Fisher Scientific, D128-500**)** vehicle controls. HEK293 cells were subject to cycloheximide (Sigma-Aldrich, C4859-1ML**)** (CHX, 20 μM) treatment over a period of 320 min in order to attenuate protein synthesis at varying times[4]. This was performed in parallel with 0.05% DMSO vehicle controls.

2.5 PROTEIN ISOLATION

Cell lysates were prepared by washing twice with ice-cold 1X phosphate-buffered saline (PBS, Fisher Scientific, BP399-4) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, pH 7.4) and scraped into 150 μl of ice-cold lysis buffer (250 mM NaCl, 50 mM Tris pH 8, 5 mM EDTA, 0.5% NP-40 (IGEPAL), phenylmethylsulfonyl fluoride protease inhibitor tablet (Roche, 04693116001), 20 mM $Na_4P_2O_7$, 10 mM NaF, and 20 mM Na_3VO_4). Cell lysates were homogenized by vigorous pipetting and underwent three consecutive freeze-thaw cycles via flash freezing with liquid nitrogen. Pellets were sedimented by centrifugation at 11 000 x g for 15 min at 4ºC. Supernatants were collected and protein concentrations were determined using Bradford Protein Assay (Bio-Rad, 500-0006).

2.6 IMMUNOBLOTTING

Protein samples were prepared in 5X Laemmli loading (sample) buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.02% bromophenol blue) and balanced with 1X sample buffer for equal protein concentration. Samples were heated at 99ºC for 5 min prior to gel loading and gel electrophoresis in order to denature the proteins. Equal amounts of protein (20 μg) were separated by 12% SDS-PAGE and wet transferred onto PVDF membrane (Millipore, IPVH00010). Membranes were incubated for 1 h in blocking buffer (Tris-buffered saline, 0.1% Tween 20, 5% skim milk) and rocked at room temperature before overnight incubation at 4ºC, rocking with either: anti-FLAG (1:1000, Sigma F3165) or anti-β tubulin for protein loading control (1:1000, Pierce PA5-16863). Following overnight incubation, membranes were washed 4 times for 5 min with TBST (Tris-buffered saline, 0.1% Tween 20) and incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies: anti-mouse IgG (1:5000, Pierce 31168) or anti-rabbit IgG (1:5000, Pierce 31463). Immunoblots were then washed 4 times for 5 min with TBST. Immunoblots were visualized with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, 34080) and digitally imaged using Bio-Rad VersaDoc camera and Quantity One program (Bio-Rad, model GS-700).

2.7 INOSITOL-PHOSPHATE TURNOVER

HEK293 cells were seeded in 24-well plates and transiently transfected with 5-HT_{2A}R and RGS2 plasmids as described in section 2.3 and the figure legends. Experiments were performed according to previous protocols[2,5]. 24 h post-transfection, cells were incubated overnight in 500 μl serum-free DMEM with 1 μCi/ml myo-[^{3}H]-inositol (PerkinElmer, NET1168001MC). For all experiments, cells were incubated for 1 h in Hank's Balance Salt Solution (HBSS –Life

Technologies, 14025-092) (1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 5.3 mM KCl, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 138 mM NaCl, 0.34 mM Na_2HPO_4, and 5.6 mM D-glucose) and were then incubated at 37ºC with 5% CO_2 with 500 µl of 10 mM LiCl alone for 10 min followed by increasing doses of serotonin hydrochloride (Sigma-Aldrich, H9523-25MG) in 500 µl 10 mM LiCl for 30 min. Cells were placed on ice and the reaction was stopped with 500 µl of 0.8 M perchloric acid and was neutralized with 400 µl of 0.72 M KOH, 0.6 M $KHCO_3$ overnight in 4ºC. Total cellular [^{3}H]-inositol incorporation was determined in 50 µl of cell lysate with 5 ml EcoLite(+)^{TM} Liquid Scintillation Cocktail (MP Biomedicals, 0188247504). Total inositol phosphate was purified with 4 ml 0.1 M formic acid / 1 M ammonium formate by anion exchange chromatography using 2 ml Dowex 1-X8 (formate form) 200-400 mesh anion exchange resin[6] (BioRad, 140-1454) after two consecutive 5 mL water and 60 mM ammonium formate washes. [^{3}H]-inositol phosphate formation was determined by liquid scintillation in 15 ml EcoLite(+)^{TM} Liquid Scintillation Cocktail using a Beckman LS 5500 scintillation system and calculations are shown below.

2.8 DENSITOMETRY AND STATISTICAL ANALYSIS

Immunoblots for MG132 experiments were analyzed using densitometry software (Quantity One, Bio-Rad) and expression levels were normalized to β-tubulin expression levels. Group data are presented as means ±SEM. Data were compared by two-way analysis of variance (ANOVA) and were further evaluated by Bonferroni post-test. Statistical analyses were performed using GraphPad Prism® 5.0 and p-values of <0.05 were considered statistically significant.

Immunoblots for CHX experiments were analyzed using Quantity One and expression levels were normalized to β-tubulin expression levels. RGS2 protein (relative to β-tubulin) at each

time point was normalized to the corresponding 0 time point. Group data are presented as means ± SEM and are fit according to the one phase decay equation.

$Y=(Y_o - \text{plateau})^{-kx} + \text{plateau}$

Statistical analyses were performed using GraphPad Prism® 5.0.

Percent conversion of [^{3}H]-inositol to [^{3}H]-inositol phosphates were solved using:

$$(\text{DPM from column})(1.2/0.8) / (\text{DPM in 50 } \mu\text{l})(1.2/0.05)$$

Where 1.2 = total neutralized cell extract (ml), 0.8 = 800 µl placed into the column, and 0.05 = 50 µl control cell extract. The data are representative of the mean ± SEM of three independent experiments normalized to the largest value of 5-HT_{2A}R + pcDNA3.1$^+$ within the data set. Each graph is representative of experiments done simultaneously. Thus, 5-HT_{2A}R + pcDNA3.1$^+$ and 5-HT_{2A}R + FL-WT RGS2 experiments were performed with the corresponding conditions in each of the graphs. Dose-response curves were fit by using nonlinear regression specifically the sigmodal dose-response equation was used:

$$Y = \text{Bottom} + (\text{Top-Bottom}) / (1 + 10^{\text{logEC50-x}})$$

This equation is also referred to as a three-parameter logistic equation. The bottom value of each graph was constrained to a common value for all data sets. EC_{50} values were compared by one-way analysis of variance (ANOVA) and were further evaluated by Bonferroni post-test. All analyses were performed using GraphPad Prism® 5.0.

2.9 REFERENCES

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Chapter 3: Results

3 RESULTS

3.1 STABILITY OF WILD-TYPE AND MUTANT FORMS OF RGS2 *IN VITRO*

Due to the importance of RGS2 within a cell, we were particularly interested in determining the half-life of full-length wild-type RGS2 as well as the half-lives of different point mutations and alternative initiation variants. Differences in the half-lives of various RGS2 constructs compared to the full-length wild-type form may underlie corresponding differences in GPCR signaling levels; a longer RGS2 half-life could result in greater GPCR signal attenuation.

3.1.1 RGS2 SNPS AFFECT PROTEIN TURNOVER RATE

Cycloheximide (CHX) is a well-established blocker of translational elongation and thus it acts as an inhibitor of protein synthesis[1]. This property allows for the determination of the half-life of a protein of interest[1]. The half-life of a protein is taken as the time after CHX addition for it to be reduced by 50% from baseline levels. Transfected HEK293 cells were treated with CHX at different time points, lysed simultaneously, and subject to immunoblotting. RGS2 levels were normalized to β-tubulin due to its abundance within a cell and long half-life of ~50 h. Full-length wild-type RGS2 was found to have a half-life of 17.7 ± 6.5 min (*Figure 3.1*). RGS2 M5V, RGS2 G23D, RGS2 R14I, and RGS2 K18N mutations were found to have half-lives of 10.3 ± 3.7, 34.7 ± 9.7, 6.3 ± 1.4, and 13.0 ± 4.7 min, respectively (*Figure 3.1-Figure 3.5*). The gray line represents the full-length wild-type RGS2 degradation curve for comparison (*Figure 3.2-3.5)*. The half-life of full-length wild-type RGS2 is consistent with the findings of a previous group who reported it to be 17.5 ± 5.8 min[2]. Simply by increasing or decreasing the turnover time of RGS2 could have significant effects on GPCR signaling. RGS2 M5V appeared to have a shorter half-life than full-length wild-type RGS2, which may be why there

is a possible association between this SNP and hypertension; Gα signaling is not being attenuated to the same degree. In contrast, RGS2 G23D appeared to have a half-life nearly double that of the full-length wild-type protein. This suggests that Gα signaling could be attenuated more efficiently in someone with this mutation compared to the wild-type RGS2. This may explain why individuals with this mutation develop pathophysiological conditions synonymous with decreased $G\alpha_s$ signaling. RGS2 R14I had a much shorter half-life than full-length wild-type RGS2 whereas RGS2 K18N had a similar half-life. Though neither of these SNPs is associated with any known phenotype, it would be interesting to screen individuals with the RGS2 R14I mutation to see if they have a hypertensive phenotype. The mechanism for why we see altered levels due to one point mutation remains unknown. However, it may be due to the specific amino acids mutated or located near the mutation affecting degradation via the N-end rule.

3.1.2 RGS2 INITIATION VARIANTS EXHIBIT DIFFERNT PROTEIN TURNOVER RATES

RGS2 contains four different initiator methionine residues, all of which can serve as the points for the initiation of translation[3]. It is important to determine if each initiation variant causes a change in half-life compared to the full length wild-type RGS2, as their relative levels could conceivably vary depending on cellular conditions[4]. Unfortunately, we were unable to determine the half-life of tM5 RGS2 due to minimal detectability of this protein construct at basal levels; this was further exacerbated by CHX treatment (*Figure 3.8*). tM16 RGS2 and tM33 RGS2 had half-lives of 34.5 ± 1.8 and 17.3 ± 4.3 min, respectively (*Figure 3.6*-*Figure 3.7*). These results suggest that tM5 RGS2 is highly unstable intracellularly, and is likely degraded at a rate that results in barely measureable protein levels. tM16 RGS2 exhibited

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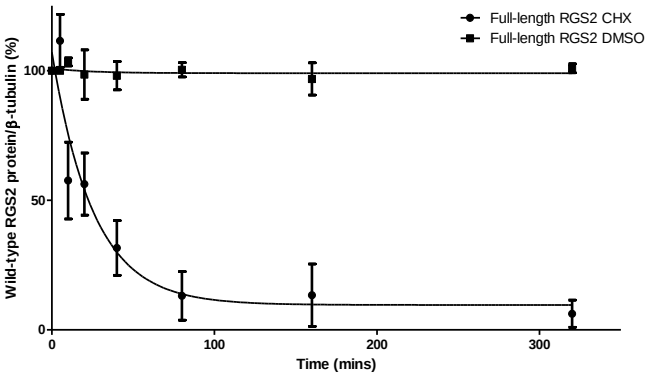

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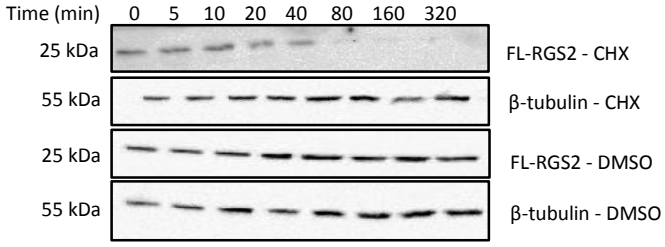
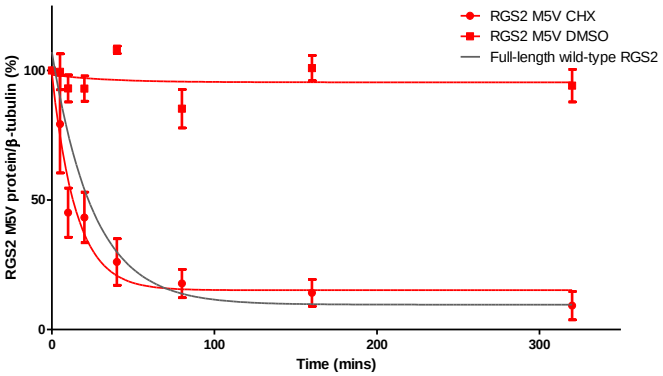


Figure 3.1. *Full-length wild-type RGS2 degradation curve* **(A)** effect of 20 μM cycloheximide (CHX) or vehicle (DMSO) treatment over a period of 320 minutes (n=5). RGS2 protein (relative to β-tubulin) at each time point was normalized to the corresponding 0 minute time point. The resultant half-life of full-length wild-type RGS2 is 17.7 ± 6.5 minutes. **(B)** Representative immunoblots of full-length wild-type RGS2 after treatment with CHX or vehicle. The first panel represents full-length wild-type RGS2 treated with CHX and the second panel represents the analogous β-tubulin treated with CHX. The lower two immunoblots represent full-length wild-type RGS2 in cells treated with DMSO at 0.05% and cells treated with DMSO and probed for β-tubulin.

A



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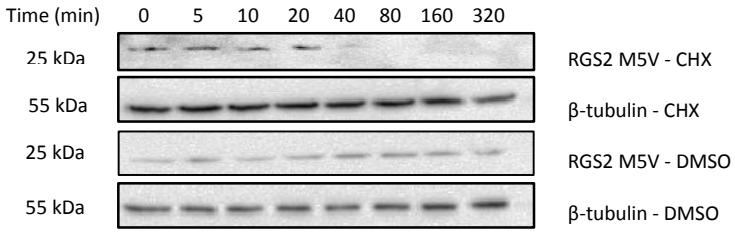

Figure 3.2. *RGS2 M5V degradation curve* **(A)** effect of 20 μM cycloheximide (CHX) or vehicle (DMSO) treatment over a period of 320 minutes (n=5). RGS2 protein (relative to β-tubulin) at each time point was normalized to the corresponding 0 minute time point. The resultant half-life of RGS2 M5V 10.3 ± 3.7 minutes. The gray line represents full-length wild-type RGS2 degradation in Figure 3.1. **(B)** Representative immunoblots of RGS2 M5V after treatment with CHX or vehicle. The first panel represents RGS2 M5V treated with CHX and the second panel represents the analogous β-tubulin treated with CHX. The lower two immunoblots represent RGS2 M5V in cells treated with DMSO at 0.05% and cells treated with DMSO and probed for β-tubulin.

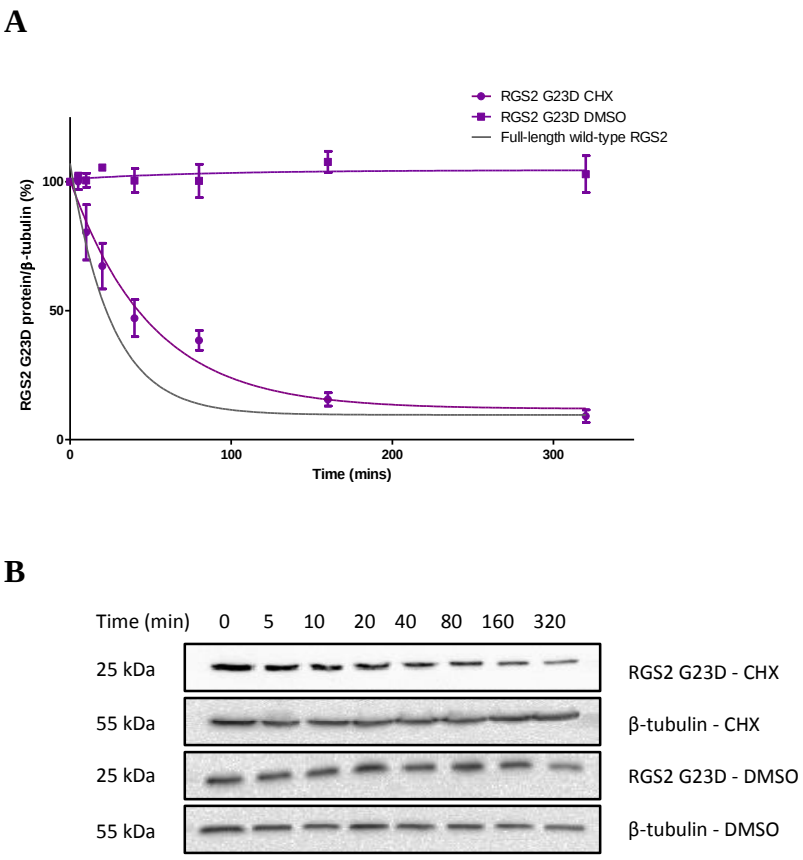

Figure 3.3. *RGS2 G32D degradation curve* **(A)** effect of 20 μM cycloheximide (CHX) or vehicle (DMSO) treatment over a period of 320 minutes (n=4). RGS2 protein (relative to β-tubulin) at each time point was normalized to the corresponding 0 minute time point. The resultant half-life of RGS2 G23D 34.7 ± 9.7 minutes. The gray line represents full-length wild-type RGS2 degradation in Figure 3.1. **(B)** Representative immunoblots of RGS2 G23D after treatment with CHX or vehicle. The first panel represents RGS2 G23D treated with CHX and the second panel represents the analogous β-tubulin treated with CHX. The lower two immunoblots represent RGS2 G23D in cells treated with DMSO at 0.05% and cells treated with DMSO and probed for β-tubulin.

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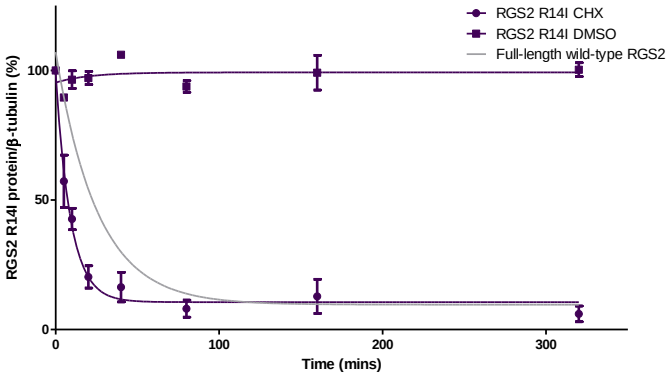

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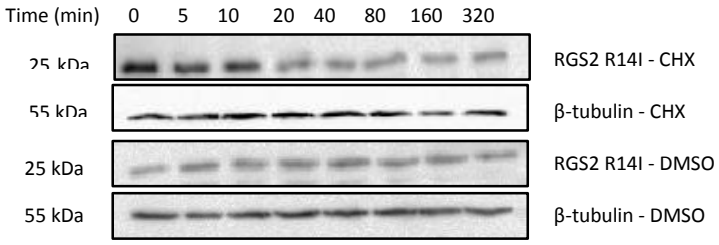

Figure 3.4. *RGS2 R14I degradation curve* **(A)** effect of 20 μM cycloheximide (CHX) or vehicle (DMSO) treatment over a period of 320 minutes (n=4). RGS2 protein (relative to β-tubulin) at each time point was normalized to the corresponding 0 minute time point. The resultant half-life of RGS2 R14I 6.3 ± 1.4 minutes. The gray line represents full-length wild-type RGS2 degradation in Figure 3.1. **(B)** Representative immunoblots of RGS2 R14I after treatment with CHX or vehicle. The first panel represents RGS2 R14I treated with CHX and the second panel represents the analogous β-tubulin treated with CHX. The lower two immunoblots represent RGS2 R14I in cells treated with DMSO at 0.05% and cells treated with DMSO and probed for β-tubulin.

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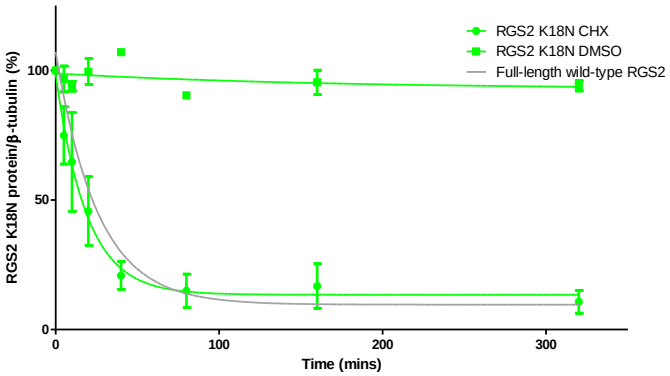

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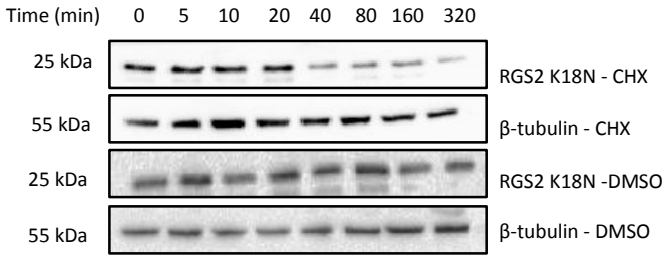
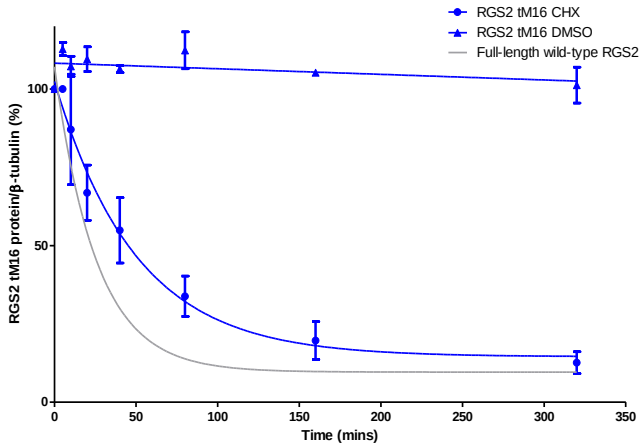


Figure 3.5. *RGS2 K18N degradation curve* **(A)** effect of 20 μM cycloheximide (CHX) or vehicle (DMSO) treatment over a period of 320 minutes (n=4). RGS2 protein (relative to β-tubulin) at each time point was normalized to the corresponding 0 minute time point. The resultant half-life of RGS2 K18N 13.0 ± 4.7 minutes. The gray line represents full-length wild-type RGS2 degradation in Figure 3.1. **(B)** Representative immunoblots of RGS2 K18N after treatment with CHX or vehicle. The first panel represents RGS2 K18N treated with CHX and the second panel represents the analogous β-tubulin treated with CHX. The following immunoblot represents RGS2 K18N in cells treated with DMSO at 0.05% and cells treated with DMSO and probed for β-tubulin.

A



B

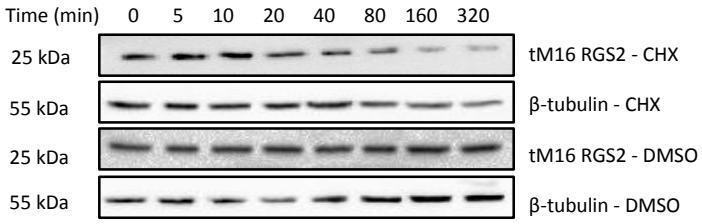
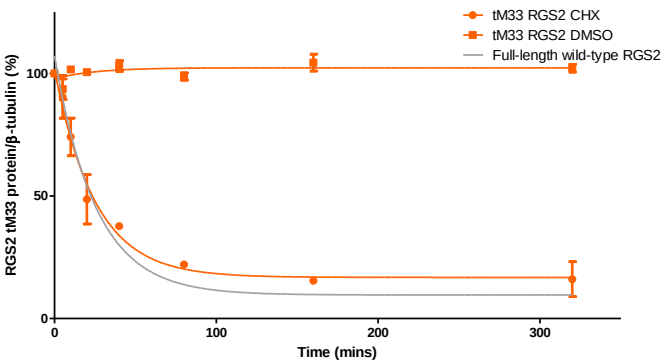


Figure 3.6. *tM16 RGS2 degradation curve* **(A)** effect of 20 μM cycloheximide (CHX) or vehicle (DMSO) treatment over a period of 320 minutes (n=5). RGS2 protein (relative to β-tubulin) at each time point was normalized to the corresponding 0 minute time point. The resultant half-life of tM16 RGS2 34.5 ± 1.8 minutes. The gray line represents full-length wild-type RGS2 degradation in Figure 3.1. **(B)** Representative immunoblots of tM16 RGS2 after treatment with CHX or vehicle. The first panel represents tM16 RGS2 treated with CHX and the second panel represents the analogous β-tubulin treated with CHX. The lower two immunoblots represent tM16 RGS2 in cells treated with DMSO at 0.05% and cells treated with DMSO and probed for β-tubulin.

A



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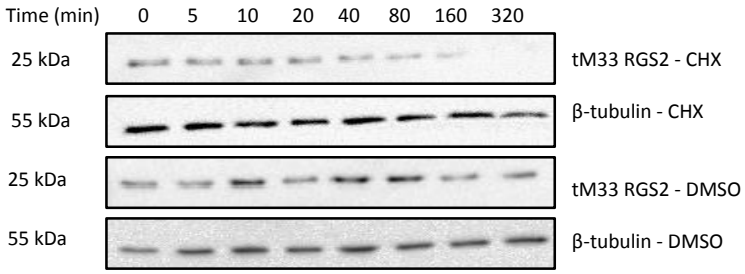

Figure 3.7. *tM33 RGS2 degradation curve* **(A)** effect of 20 μM cycloheximide (CHX) or vehicle (DMSO) treatment over a period of 320 minutes (n=3). RGS2 protein (relative to β-tubulin) at each time point was normalized to the corresponding 0 minute time point. The resultant half-life of tM33 RGS2 17.3 ± 4.3 minutes. The gray line represents full-length wild-type RGS2 degradation in Figure 3.1. **(B)** Representative immunoblots of tM33 RGS2 after treatment with CHX or vehicle. The first panel represents tM33 RGS2 treated with CHX and the second panel represents the analogous β-tubulin treated with CHX. The lower two immunoblots represent tM33 RGS2 in cells treated with DMSO at 0.05% and cells treated with DMSO and probed for β-tubulin.

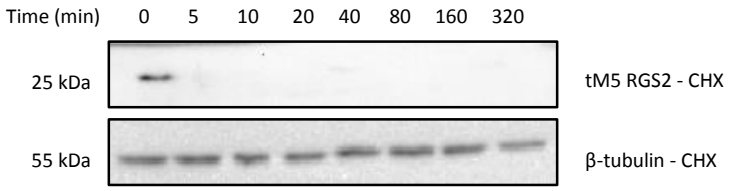

Figure 3.8. *tM5 RGS2 Degradation*. tM5 RGS2 was treated with CHX over 320 min. The figure represents the only instance out of 8 experiments in which tM5 RGS2 was detectable. Treatment with CHX further exacerbated the limited detection. Thus, the half-life for tM5 RGS2 could not be determined.

approximately double the half-life of full-length RGS2, whereas tM33 RGS2 showed a half-life comparable to full-length RGS2. The mechanism underlying these different half-lives remains unknown, but may reflect altered recognition by the ubiquitin-ligase enzymes which attach ubiquitin proteins for recognition by proteasomes. It might be advantageous to take a closer look at the amino acids near the N-terminus of each construct; it has been suggested that some specific amino acids are more likely to support degradation than others[5–9]. It should also be noted that tM16 RGS2 in some experiments increased in expression at the 5 and 10 min time points compared to the non-treated lysates. Cellular stress is known to increase RGS2 mRNA and protein expression[10]. However, this only seemed to occur in the tM16 RGS2 construct. tM33 RGS2 had a half-life comparable to that of full-length wild-type RGS2 suggesting that there is not a need for this initiation variant to be upregulated.

3.2 DEGRADATION PATHWAY FOLLOWED BY RGS2

Due to the altered half-lives associated with RGS2 mutations and initiation variants, we were particularly interested in determining how RGS2 is degraded. The most common intracellular protein degradation pathway is the ubiquitin-proteasome pathway, where ubiquitin is added to a protein that is then recognized by the proteasome and ultimately degraded. This is a rapid, common process, and by inhibiting the pathway, we can determine if levels of RGS2 are changed via this route.

3.2.1 INHIBITION OF PROTEASOMAL DEGRADATION ALTERS CELLULAR LEVELS OF WILD-TYPE RGS2 AND THE MAJORITY OF RGS2 SNPS

MG132 is a well-established peptide aldehyde that can inhibit many types of proteases[11–13]. Due to this property, MG132 is considered a proteasome inhibitor and has been used extensively to determine whether a particular protein is degraded via proteasomes[11–14]. If the

expression level of a protein increases upon MG132 treatment, it suggests that under normal conditions, the protein is degraded by the ubiquitin-proteasome pathway. Transfected HEK293 cells were subjected to MG132 or DMSO (vehicle) treatment for 2 h, lysed, and immunoblotted. Full-length wild-type RGS2 as well as RGS2 M5V expression increased when cells were treated with MG132 (Fi*gure 3.9*). However, RGS2 G23D levels did not change upon MG132 treatment (*Figure 3.9*). This result was unexpected as we hypothesized that each RGS2 construct would increase expression level in the presence of MG132. It is interesting to note that RGS2 G23D had a longer half-life than full length wild-type RGS2 (*Figure 3.3*). This could be attributed to RGS2 G23D being more stable intracellularly. It would thus appear not be degraded by the ubiquitin-proteasome pathway. Ultimately, our results demonstrate that full-length wild-type RGS2 levels increase in the presence of a proteasome inhibitor. This suggests that under basal conditions, RGS2 is rapidly degraded by proteasomes.

Analogous experiments were performed with RGS2 R14I and RGS K18N. Transfected HEK293 cells were subject to MG132 or DMSO treatment. Full-length wild-type RGS2 was again significantly increased when treated with MG132. RGS2 R14I and RGS2 K18N were also increased with MG132 treatment (*Figure 3.10*). RGS2 R14I and RGS2 K18N were found to have half-lives respectively shorter than and comparable to full-length wild-type RGS2, and taken together the data suggest that these RGS2 SNPs could also be degraded via the ubiquitin-proteasomal pathway. These results suggest that under basal conditions, RGS2 is rapidly degraded via proteasomes and inhibiting these proteasomes significantly increases intracellular levels of RGS2.

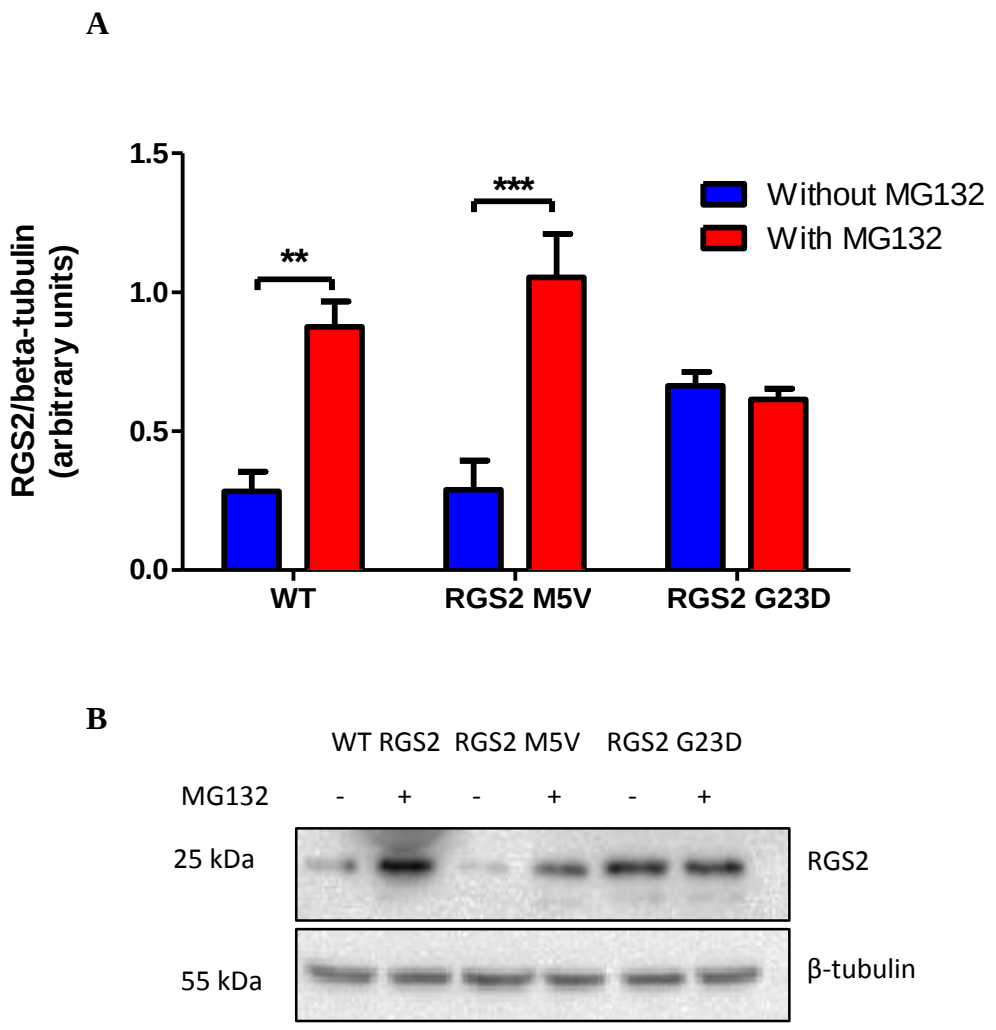

Figure 3.9. *RGS2 expression in the absence or presence of MG132.* **(A)** Effect of 2 h, 20 μM MG132 (proteasome inhibitor) treatment and single point mutations on cellular levels of FLAG-tagged RGS2 constructs, normalized to β tubulin (n=4). Group data are presented as means ± SEM. Data were compared by two-way analysis of variance (ANOVA) and were further evaluated by Bonferroni post-test. Observed levels of RGS2 WT and RGS2 M5V **p<0.01 and ***p<0.001, respectively. **(B)** Representative immunoblot of mutant RGS2 constructs in the absence and presence of MG132

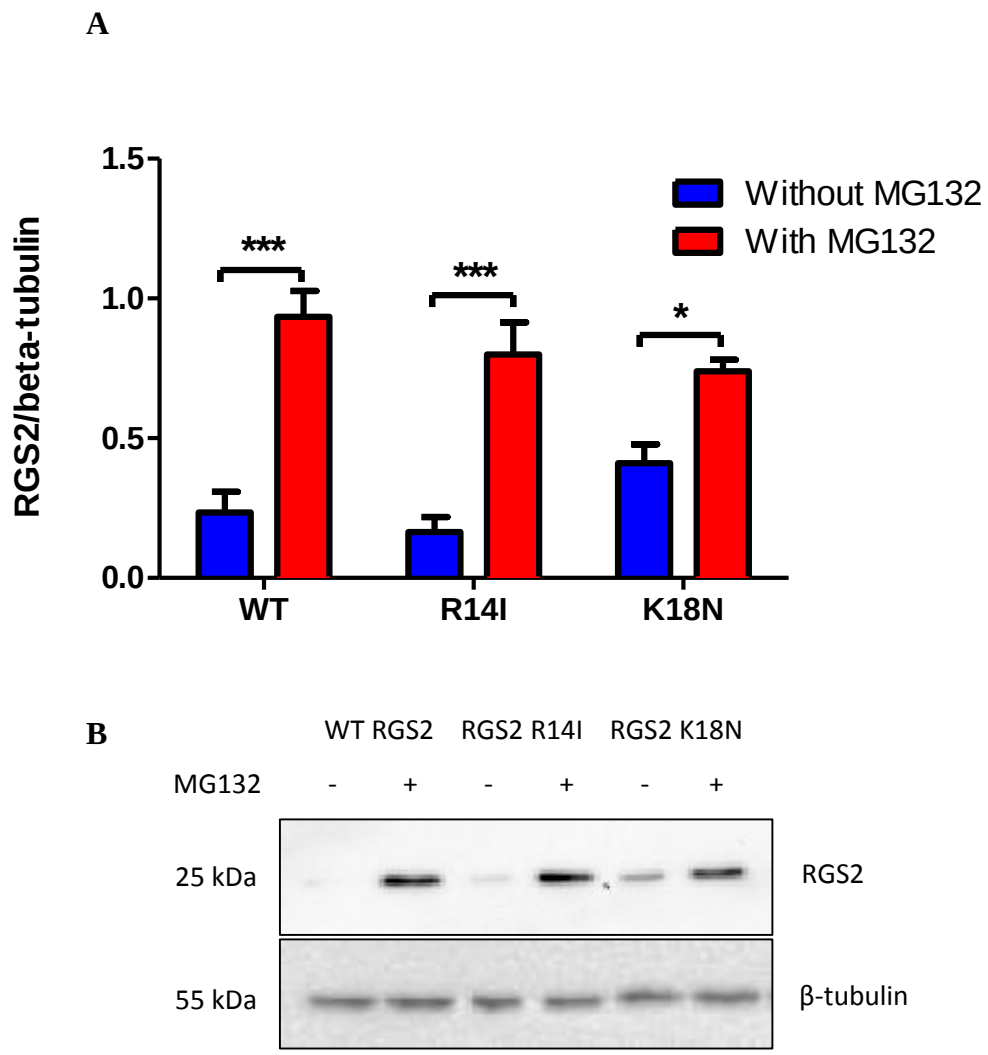

Figure 3.10. *RGS2 expression in the absence or presence of MG132.* **(A)** Effect of 2 h, 20 μM MG132 (proteasome inhibitor) treatment and single point mutations on cellular levels of FLAG-tagged RGS2 constructs, normalized to β tubulin (n=5). Group data are presented as means ± SEM. Data were compared by two-way analysis of variance (ANOVA) and were further evaluated by Bonferroni post-test. Observed levels of RGS2 WT, RGS2 R14I, RGS2 K18N ***$p<0.001$, and *$p<0.05$. **(B)** Representative immunoblot of mutant RGS2 constructs in the absence and presence of MG132

3.2.2 INIHIBITION OF PROTEASOMAL DEGRADATION INCREASES CELLULAR LEVELS OF ALL RGS2 INITIATION VARIANTS

Similar to our experiments on the mutant forms of RGS2, we examined whether the different initiation site variants had altered expression levels in the presence of MG132. A pseudo-Kozak sequence was placed before each methionine to ensure that the methionine of interest was initiating translation. Independent of which initiation variant was produced, the relative levels of protein increased with MG132 treatment (*Figure 3.11*). This result is consistent with the half-life experiments, where tM16 RGS2 had a much longer half-life than the other constructs (Fig*ure 3.6*).

3.3 INOSITOL-PHOSPHATE TURNOVER

Further studies were carried out to determine whether the altered half-lives of RGS2 mutations and initiation variants might correspond to any effect on the ability of RGS2 to attenuate $G\alpha_{q/11}$ signaling. Wild-type RGS2 has been reported to attenuate $G\alpha_{q/11}$ signaling[15]. It is possible that RGS2 mutations or initiation variants may differ from the full-length wild type protein in their abilities to attenuate $G\alpha_{q/11}$ signaling, however relatively few alternate forms of RGS2 have been tested.

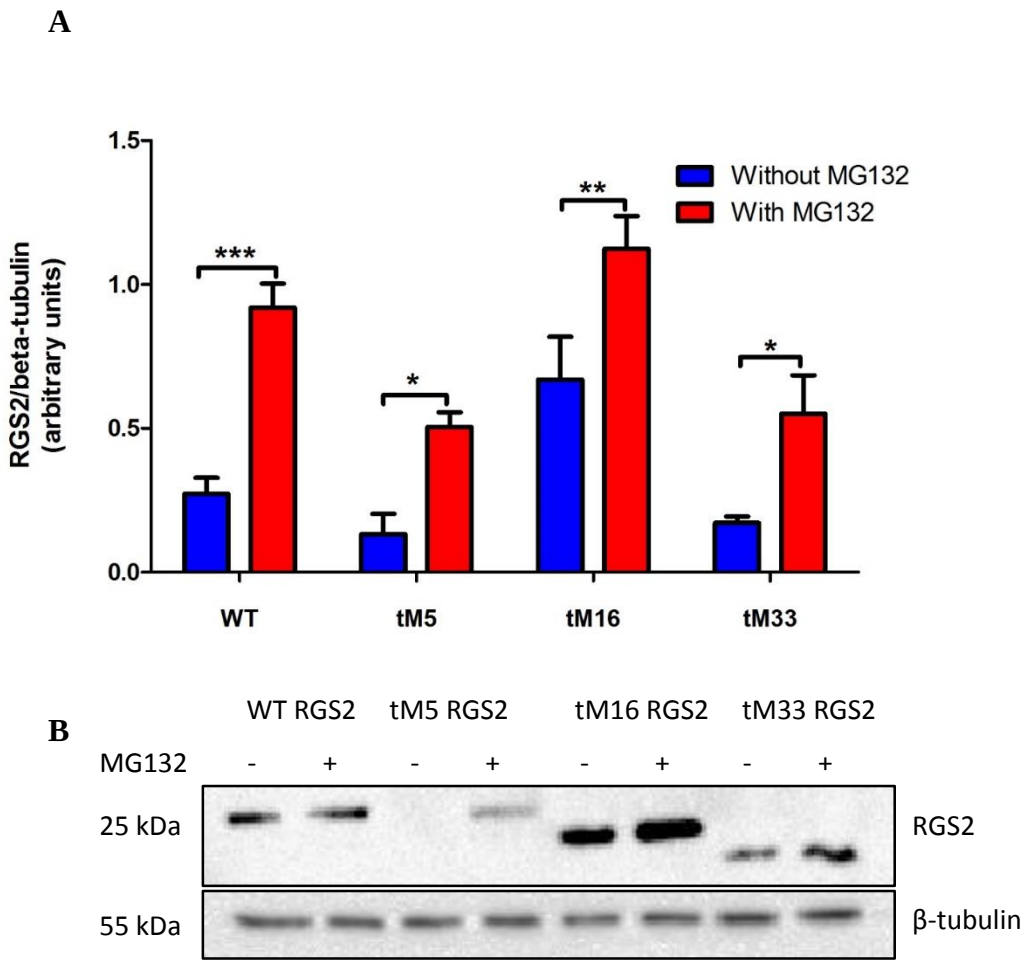

Figure 3.11. *RGS2 expression in the absence or presence of MG132.* **(A)** Effect of 2 h, 20 μM MG132 treatment on full-length RGS2 and truncations prior to each initiator methionine residue on the cellular levels of FLAG-tagged RGS2 constructs, normalized to β tubulin (n=5). Group data are presented as means ± SEM. Data were compared by two-way analysis of variance (ANOVA) and were further evaluated by Bonferroni post-test. Observed levels of full length wild-type RGS2, and variants with a pseudo-Kozak sequence prior to M5, M16 and M33 all increased in the presence of MG132, ***$p<0.001$, **$p<0.01$, and *$p<0.05$. **(B)** Representative immunoblot of full-length wild-type RGS2 expression and truncation mutants in the absence and presence of MG132.

3.3.1 RGS2 SNPS AFFECT $G\alpha_{q/11}$ SIGNAL ATTENUATION

IP_3 is generated by the activation of phospholipase Cβ by $G\alpha_{q/11}$. However, it is a challenge to measure IP_3 levels due to its rapid turnover[16]. Fortunately, IP_3 is broken down into inositol-phosphates which are stable in the presence of lithium and can be measured using a well-established technique[16,17]. Inhibitory effects of RGS proteins on $G\alpha_{q/11}$-PLC-β signaling are manifested as decreases in GPCR agonist potency and/or maximal effect. HEK293 cells transiently transfected with plasmid encoding the 5-HT_{2A}R were loaded with myo-[^{3}H]-inositol overnight and then stimulated with increasing concentrations of 5-HT. Full-length wild-type RGS2 (EC_{50} = 0.28 ± 0.18 μM) as well as RGS2 M5V (EC_{50} = 0.084 ± 0.032 μM) and RGS2 G23D (EC_{50} = 0.75 ± 0.68 μM) each yielded a rightward shift in the 5-HT dose-response curve compared to the 5-HT_{2A}R (EC_{50} = 0.066 ± 0.036 μM) by itself (*Figure 3.12*). Though RGS2 M5V yielded a rightward shift, it is much smaller than the effect of full-length wild-type RGS2 suggesting that this construct has decreased stability. The greater rightward shift of RGS2 G23D suggests that this construct has an increased ability to attenuate 5-HT_{2A}R compared to full-length wild-type RGS2, consistent with its slower degradation. The reduced E_{max} observed in cells transfected with RGS2 G23D, along with the increased EC_{50}, imply decreased receptor reserve with RGS2 G23D present. Ultimately, our results show that 1) RGS2 has the ability to attenuate the $G\alpha_{q/11}$ coupled 5-HT_{2A}R alone and 2) mutations within the N-terminus of RGS2 affect the degree to which it can attenuate $G\alpha_{q/11}$ signaling.

An analogous set of experiments was performed using two other RGS2 mutants, RGS2 R14I and RGS2 K18N. Both RGS2 R14I (EC_{50} = 0.28 ± 0.15 μM) and RGS2 K18N (EC_{50} = 0.50 ± 0.23 μM) yielded a rightward shift in the dose-response curve compared to 5-HT_{2A}R alone (EC_{50} = 0.065 ± 0.025 μM) (*Figure 3.13*). This suggests that both of these RGS2 constructs

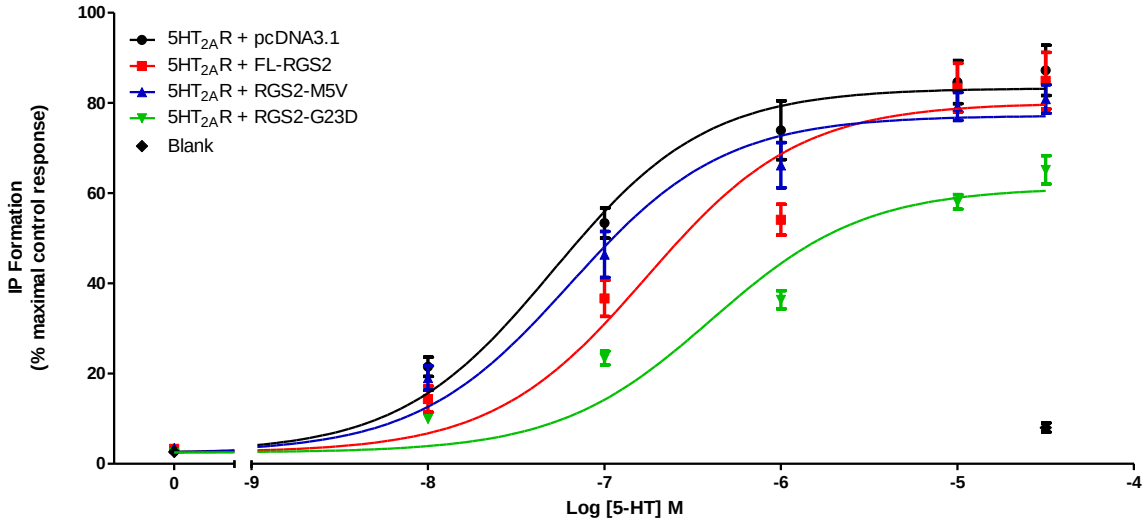

Figure 3.12. *Stimulation of phospholipase Cβ activity by 5-HT$_{2A}$R signaling*. Dose response curve for 5-HT mediated IP formation in response to treatment with increasing concentrations of 5-HT for 30 minutes in HEK293 cells transfected with a total of 2 µg of plasmid DNA expressing 5-HT$_{2A}$R, plus either an RGS2 construct, or pcDNA3.1 as a transfection control. The data shown are the means ± S.E.M. of three independent experiments. For each experiment, during the fitting procedure the lower asymptote was constrained to be equal for all four data sets. The resultant fitted parameters were then averaged for each of the four experimental conditions and used to generate the lines shown. The EC$_{50}$ for 5-HT$_{2A}$R-mediated IP formation in the absence of transfected RGS2 was found to be 0.066 ± 0.036 µM, whereas the corresponding values with full-length wild-type RGS2, RGS2 M5V, and RGS2 G23D were found to be 0.28 ± 0.18 µM, 0.084 ± 0.032 µM, and 0.75 ± 0.68 µM, respectively. EC$_{50}$ values were compared by one-way analysis of variance (ANOVA) and were further evaluated by Bonferroni post-test. Observed EC$_{50}$ values for 5HT$_{2A}$R + FL-RGS2 and 5HT$_{2A}$R + RGS2 G23D compared to 5HT$_{2A}$R + pcDNA3.1 increased, $p<0.01$ and $p<0.001$, respectively. 5HT$_{2A}$R + RGS2 M5V did not significantly change ($p>0.05$).

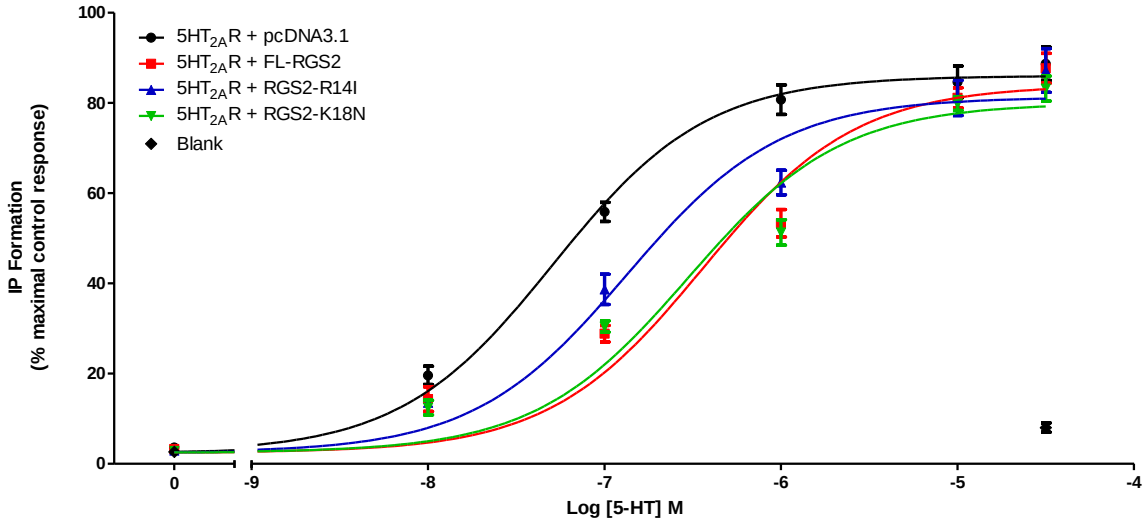

Figure 3.13. *Stimulation of phospholipase Cβ activity by 5-HT$_{2A}$R signaling*. Dose response curve for 5-HT mediated IP formation in response to treatment with increasing concentrations of 5-HT for 30 minutes in HEK293 cells transfected with a total of 2 µg of plasmid DNA expressing 5-HT$_{2A}$R, plus either an RGS2 construct, or pcDNA3.1 as a transfection control. The data shown are the means ± S.E.M. of three independent experiments. For each experimental, during the fitting procedure the lower asymptote was constrained to be equal for all four data sets. The resultant fitted parameters were then averaged for each of the four experimental conditions and used to generate the lines shown. The EC$_{50}$ for 5-HT$_{2A}$R-mediated IP formation in the absence of transfected RGS2 was found to be 0.065 ± 0.25 µM, whereas the corresponding values with full-length wild-type RGS2, RGS2 R14I and RGS2 K18N were found to be 0.55 ± 0.25 µM, 0.19 ± 0.15 µM and 0.50 ± 0.23 µM, respectively. EC$_{50}$ values were compared by one-way analysis of variance (ANOVA) and were further evaluated by Bonferroni post-test. Observed EC$_{50}$ values for 5HT$_{2A}$R + FL-RGS2, 5HT$_{2A}$R + RGS2 R14I, and 5HT$_{2A}$R + RGS2 K18N compared to 5HT$_{2A}$R + pcDNA3.1 increased, $p<0.001$, $p<0.01$, and $p<0.001$, respectively.

are able to attenuate $G\alpha_{q/11}$ signaling but to different degrees. RGS2 K18N, which had a half-life comparable to that of full-length wild-type RGS2, also had a similar log EC_{50} value to the wild-type construct (EC_{50} = 0.55 ± 0.25 μM). These results, along with the RGS2 M5V and RGS2 G23D experiments, suggest that the ability of RGS2 to attenuate $G\alpha_{q/11}$ can depend on the half-life of the protein as the constructs with the longer half-lives (*e.g.*, RGS2 G23D) tended to produce greater rightward shifts in the 5-HT dose-response curve.

3.3.2 RGS2 INITIATION VARIANTS AFFECT $G\alpha_{q/11}$ SIGNAL ATTENUATION

Experiments analogous to those performed using the RGS2 mutations (Section 3.2.1) were also performed with the four RGS2 initiation variants. Regardless of which RGS2 construct was transfected with 5-HT_{2A}R, there was a rightward shift in the EC_{50} (*Figure 3.14*), including the minimal shift with that tM5 RGS2 construct that had an immeasurably short half-life. The EC_{50} values of 5-HT in cells transfected with the 5-HT_{2A}R plus full-length RGS2, tM5 RGS2, tM16 RGS2, and tM33 RGS2 were found to be 0.27 ± 0.17 μM, 0.11 ± 0.04 μM, 0.67 ± 0.60 μM, and 0.46 ± 0.30μM respectively, compared to 0.072 ± 0.035 μM for 5-HT_{2A}R alone. The rightward shifts in these dose-response curves suggest that independent of which initiation site is used, RGS2 has the ability to attenuate $G\alpha_{q/11}$ coupled receptor signaling, but to varying extents. The degree to which RGS2 attenuates receptor signaling is roughly proportional to the half-life of a given initiation variant, as shown in Section 3.1.2. For example, tM5 RGS2 had an immeasurably short half-life (<5 min) and was the least able to attenuate inositol-phosphate formation, whereas tM16 RGS2 had the longest half-life (34.5 min) and also had the greatest ability to attenuate inositol phosphate formation and even lowered the E_{max}.

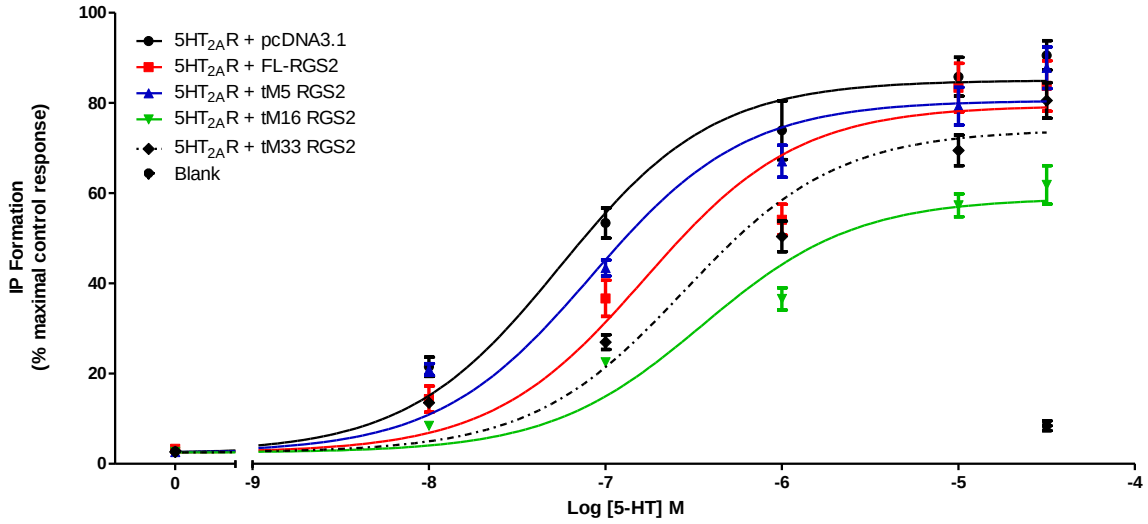

Figure 3.14. *Stimulation of phospholipase Cβ activity by 5-HT$_{2A}$R signaling*. Dose response curve for 5-HT mediated IP formation in response to treatment with increasing concentrations of 5-HT for 30 minutes in HEK293 cells transfected with a total of 2 µg of plasmid DNA expressing 5-HT$_{2A}$R, plus either an RGS2 construct, or pcDNA3.1 as a transfection control. The data shown are the means ± S.E.M. of three independent experiments. For each experimental, during the fitting procedure the lower asymptote was constrained to be equal for all four data sets. The resultant fitted parameters were then averaged for each of the four experimental conditions and used to generate the lines shown. The EC$_{50}$ for 5-HT$_{2A}$R-mediated IP formation in the absence of transfected RGS2 was found to be0.072 ± 0.035 µM, whereas the corresponding values with full-length RGS2, tM5 RGS2, tM16 RGS2, and tM33 RGS2 0.27 ± 0.17 µM, 0.11 ± 0.04 µM, 0.67 ± 0.60 µM, 0.46 ± 0.30 µM respectively. EC$_{50}$ values were compared by one-way analysis of variance (ANOVA) and were further evaluated by Bonferroni post-test. Observed EC$_{50}$ values for 5HT$_{2A}$R + FL-RGS2, 5HT$_{2A}$R + tM16 RGS2, and 5HT$_{2A}$R + tM33 RGS2 compared to 5HT$_{2A}$R + pcDNA3.1 increased, $p<0.01$, $p<0.001$, and $p<0.01$, respectively. 5HT$_{2A}$R + tM5 RGS2 did not significantly change ($p>0.05$).

3.3.3 CORRELATION BETWEEN RGS2 AND $G\alpha_{q/11}$ SIGNAL ATTENUATION

We wanted to determine if there was a correlation between the half-life of RGS2 constructs and their abilities to attenuate $G\alpha_{q/11}$ signaling. The average half-life and EC_{50} values from Sections 3.1 and 3.2, respectively, were graphed (*Figure 3.15*). tM5 RGS2 was arbitrarily given a half-life of 5 min due to our inability to consistently detect this construct via immunoblotting (*Figure 3.8*). There appears to be a correlation between the half-life and EC_{50} in that as the half-life increases, so does the EC_{50}. This suggests that when RGS2 is more stable in a cell, it also functions to attenuate signal to a greater degree. It should be noted that this is not a perfect correlation ($r^2 = 0.8004$). The low r^2 value could be due to inter-experimental variability and/or differences in translation efficiency between RGS2 variants.

To summarize, our data reveal that small changes in the amino acid sequence of RGS2 can have substantive effects on its rate of degradation, and thus on its ability to regulate signaling. Simply mutating one amino acid in the N-terminal region of RGS2 can alter its half-life, which in turn can affect the ability of the protein to attenuate $G\alpha_{q/11}$ signaling. For instance, RGS2 M5V was shown to have a half-life shorter than full-length wild-type RGS2 (*Figure 3.2*) which may result in the decreased ability to attenuate $G\alpha_{q/11}$ signaling (*Figure 3.12*). The opposite outcome was seen for the most stable variant tested, RGS2 G23D (*Figure 3.3*). Additionally, the majority of RGS2 construct's intracellular levels increased with proteasomal inhibition suggesting, that under basal conditions, RGS2 is degraded via the ubiquitin proteasomal pathway (*Figure3.9-Figure 3.11*).

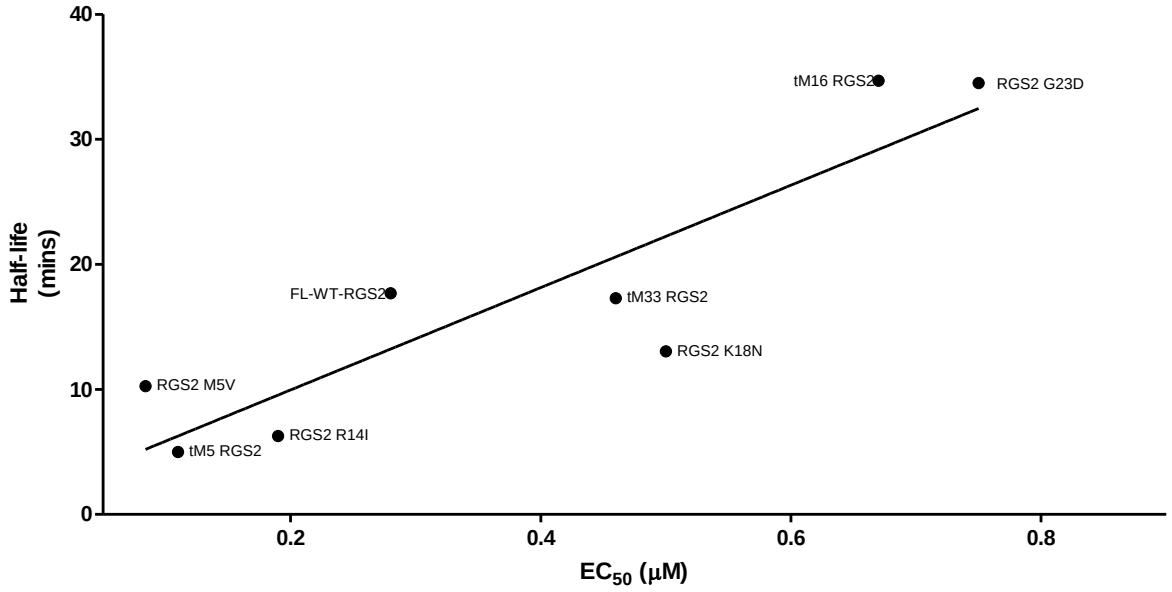

Figure 3.15. *Correlation between half-life and EC_{50}.* Positive correlation between the average half-life of an RGS2 construct and the EC_{50} of 5-HT in cells expressing 5-HT_{2A}R plus the given RGS2 variant. tM5 RGS2 was arbitrarily assigned a half-life of 5 min. Data shown are representative of the averages found in 3-5 independent experiments. Experiments were not done simultaneously.

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Chapter 4: Discussion

4 DISCUSSION

4.1 SUMMARY OF NOVEL FINDINGS AND CONCLUSIONS

Although recent studies have indicated that RGS2 contains multiple initiation sites[1] and can contain numerous SNPs[2,3], it is unknown whether these different isoforms have any effect on RGS2 regulation. Our data suggest that even minor changes in the N-terminus of RGS2 can have a profound effect on RGS2 regulation. We show for the first time altered degradation rates between RGS2 initiation variants and also RGS2 mutations. Additionally, the altered half-lives appear to be causing differences in RGS2 mediated $G\alpha_{q/11}$ signal attenuation. Furthermore, we present data suggesting that RGS2 is rapidly degraded via the ubiquitin-proteasome pathway. The altered degradation rates may be due to how quickly each RGS2 protein is recognized and degraded via the ubiquitin-proteasome pathway. Since RGS2 is a ubiquitously expressed protein and regulates the signaling of many GPCRs, it is most prudent to understand how any changes within the N-terminus of RGS2 could have significant effects on Gα signaling.

Study A: To determine the stability of wild-type and mutant forms of RGS2.

We demonstrated that the use of each initiator methionine residue alters the half-life of RGS2. Furthermore, mutations within the N-terminal region of RGS2 also have a strong impact on protein half-life. This becomes important when one considers the major biological function RGS2 has within a cell, which is to attenuate Gα signaling. We also presented evidence suggesting that under basal conditions, wild-type RGS2 is rapidly degraded via the ubiquitin-proteasome pathway. This holds true regardless of which initiator methionine begins

translation. It also appears the mutant forms of RGS2 are degraded via the same pathway with exception to RGS2 G23D. It was interesting that the RGS2 mutant with the longest half-life, RGS2 G23D, was unaffected by MG132 treatment. MG132 is a nonspecific inhibitor of the ubiquitin-proteasome pathway meaning when the drug is present within a cell, the vast majority proteins that are degraded in such a fashion should increase in cellular abundance. Individuals carrying this point substitution have a phenotypic profile including borderline IQ, hirsutism, increased bone alkaline phosphatase and decreased platelet $G\alpha_s$ function; all phenotypes indicative of decreased $G\alpha_s$ signaling[3]. It has been shown that RGS2 G23D causes a preferential shift in translation to the two longest isoforms of RGS2 which contains a putative adenylyl cyclase binding domain[3]. Since RGS2 G23D levels were unaffected by proteasome inhibition and had an increased half-life relative to full-length wild-type RGS2 levels, it would be reasonable to assume these attributes of the point substitution are what is leading to such a robust phenotype. Gα signaling is undoubtedly an important signaling pathway and if an RGS2 mutant attenuates the signal to a greater degree than what is expected, one would anticipate some sort of physiological complication.

Study B: To determine the effects of wild-type and mutant forms of RGS2 on G protein-mediated signaling

We showed that an increase or decrease in the half-life of RGS2 compared to the full-length wild-type RGS2 was respectively proportional to an increase or decrease in $G\alpha_{q/11}$ signal attenuation (*Figure 3.15)*. Interestingly, RGS2 M5V and RGS2 G23D, which have been associated with physiological consequences, had a respective decrease or increase in the ability to attenuate $G\alpha_{q/11}$-mediated signaling (*Figure 3.12).* Furthermore, RGS2 initiation variants had altered abilities to mitigate $G\alpha_{q/11}$ signaling (*Figure 3.14*) proportional to the half-lives

determined in Study A. Therefore, individuals carrying a mutant form of RGS2 may experience phenotypes associated with Gα signaling, not because RGS2 is ineffective but because the altered half-life of RGS2 has caused a shift in the expected degree of Gα signaling.

4.2 CONTRIBUTIONS OF RESEARCH TO CURRENT STATE OF KNOWLEDGE

GPCRs are an integral part of signaling systems, allowing extracellular signals from a broad range of ligands to be turned into intracellular responses[4]. Excessive GPCR signaling can lead to many pathophysiological conditions including hypertension[5], stress, anxiety, depression[6], and many endocrine disorders[7]. It is therefore necessary to have well regulated mechanisms for GPCR deactivation. There are intrinsic mechanisms for GPCR deactivation[8] but this process can be accelerated by GAPs such as RGS proteins, and indeed RGS proteins in many instances are necessary for normal signaling to take place[9]. There are many RGS proteins each with variable tissue distribution profiles, Gα protein specificity, and all must have mechanisms for regulation. For example, RGS4 and RGS5 have been extensively researched and they both have been identified as proteins that are rapidly degraded via the ubiquitin proteasome pathway[10,11]. We have shown that wild-type RGS2 is likely degraded in the same fashion, although its degradation is somewhat less rapid than occurs with RGS4 and RGS5[11]. We have also shown that the N-terminus of RGS2 is important in determining how it is regulated. Previous research showed that truncation of the N-terminus of RGS2 results in loss of function, suggesting that the N-terminus may have a role in plasma membrane targeting[12]. We indicated that N-terminal modifications, whether due to the use of initiation variants or introducing mutations, can have severe effects on RGS2 regulation and therefore, G protein signal attenuation. The N-end rule is only interested in the first two amino acids yet we found

substitutions farther into the N-terminus had profound effects on degradation. This concept will be discussed in Section 4.3.

Individuals carrying certain RGS2 SNPs show particular phenotypes[2,3] but it remains unknown as to why these pathological consequences occur. RGS2 M5V has been tentatively associated with hypertension[2] but the evidence is not yet compelling due in part to the low population frequency of the SNP. However, our data suggest that RGS2 M5V has a relatively short half-life compared to the full-length wild-type RGS2 and also has a decreased ability to attenuate $G\alpha_{q/11}$ signaling, which might be expected to result in increased $G\alpha_{q/11}$ signaling. Excessive $G\alpha_{q/11}$ signaling is a factor which can lead to hypertension and eventually cardiac hypertrophy[13]. Knowing this allows our data to strengthen the hypothesis that RGS2 M5V may be associated with hypertension. Interestingly, there is a genetic defect called Bartter's/Gitelman's syndrome where individuals have the classic characteristics of hypertension (activation of the renin-angiotensin-aldosterone system, increased angiotensin II and aldosterone, hypokalemia, and sodium depletion), yet are normo/hypotensive due in part to increased cellular RGS2 levels[14]. Individuals carrying the RGS2 G23D mutation have a phenotypic profile including borderline IQ, hirsutism, increased bone alkaline phosphatase, and decrease platelet $G\alpha_s$ function, all characteristics seen in patients with a $G\alpha_s$ hypofunction condition known as Albright hereditary osteodystrophy (AHO)[3]. The authors concluded that this mutant RGS2 protein has an increased inhibitory effect on cAMP production due to a preference of the ribosomal machinery for translation initiation sites 1 and 5, which contain a putative adenylyl cyclase binding domain at amino acids 9-11[3]. Our data is consistent with the idea that RGS2 G23D will have an increased ability to inhibit Gα signaling but it may be more complex than the use of differential initiation sites. Based on the findings presented in this

thesis, we propose that RGS2 G23D has an increased half-life relative to the full-length wild-type form of RGS2 which will present a greater chance of RGS2 binding and inhibiting Gα-mediated signals; this phenomenon will only enhance the tendency towards decreased $G\alpha_s$ (and $G\alpha_{q/11}$) signaling in individuals who harbour the RGS2 G23D point mutation.

We also investigated two other RGS2 mutations with no known phenotypes, RGS2 R14I and RGS2 K18N (genecards.org). Our findings with these mutations reinforce our other findings which suggest that the half-life of an RGS2 protein is proportional to the ability to inactivate $G\alpha_{q/11}$ signaling. RGS2 R14I had a relatively short half-life of ~6 minutes. It would be interesting to determine if individuals with this mutation present any phenotypes associated with excessive Gα signaling, such as hypertension. RGS2 K18N on the other hand has a half-life comparable to full-length wild-type RGS2 and the only research done on this SNP shows it is not associated with hypertension[15]. This would seem reasonable as this mutation has a nearly identical ability to attenuate $G\alpha_{q/11}$ signaling as full-length wild-type RGS2. Overall, our data shows how important the N-terminus is within RGS2 and any manipulation in the amino acid sequence can affect how RGS2 is regulated and how it functions.

4.3 RGS2 IS DEGRADED VIA THE UBIQUITIN-PROTEASOME PATHWAY

Previous research has suggested that like RGS4 and RGS5, RGS2 may be degraded via the ubiquitin-proteasome pathway[10,16]. Our results propose that this hypothesis is correct at least for most variants of RGS2. Figure 1.5 shows the classical N-end rule pathway, where the amino acid next to the initiator methionine is imperative in predicting if, and how rapidly, the protein is ubiquitinated and degraded. It is unknown at what rate each process happens, for example, whether deamidation by NTAN Nt^N-amidase is quicker than NTAQ Nt^Q-amidase, or vice

versa. The overall process of degradation is likely due to the entire protein structure, but our results propose modifications to the classical pathway. Full-length wild-type RGS2 has a glutamine (Q) at amino acid position 2. Glutamine, according to the current formulation of the N-end rule pathway, is a tertiary destabilizing residue which must undergo deamidation to glutamic acid (E), arginylation to add a destabilizing arginine, and then recognition by N-recognins (E3's) and eventually degradation. Our results, as well as previous results[17], show full-length wild-type RGS2 to have a half-life of approximately 17.5 minutes. On the other hand, we found tM16 RGS2 to have a half-life of approximately 34.5 minutes, yet it contains a secondary destabilizing residue (aspartic acid, D) at amino acid position 2. Considering tM16 RGS2 has one less step than full-length wild-type RGS2 in the degradation pathway, it is realistic to assume that this form of RGS2 would be degraded more rapidly. To complicate things further, tM5 RGS2, which had an immeasurably short half-life in our hands (<5 min), and tM33 RGS2, which had a comparable half-life to the full-length RGS2 (17.3 min), both have primary destabilizing residues at amino acid position 2, phenylalanine (F) and lysine (K), respectively, yet different half-lives. This could indicate that there is a difference between Type 1 and Type 2 primary destabilizing residues of the N-end rule pathway. However, it is evident that the number of steps in the degradation pathway need not be proportional to the degradation rate.

Another potential contributor to the altered half-lives are the amino acid sequences further downstream of the initiator methionine. The N-end rule emphasizes the role of the second amino acid, however, the RGS2 G23D mutation has a substantial effect on the protein's half-life and moreover cellular levels are not affected by MG132 treatment, implying a lack of proteasomal degradation. Therefore, it is reasonable to assume that amino acids further

downstream of the initiation site can strongly influence proteasomal degradation. It is interesting to note that the full-length wild-type RGS2 and tM5 RGS2 only have a four amino acid difference yet such a large variability in half-lives. This could be in consequence of tM5 RGS2 having two primary destabilizing residues in a row (phenylalanine and leucine) whereas full-length RGS2 only has a tertiary destabilizing residue (glutamine) followed by a residue not associated with the N-end rule (serine), otherwise referred to as a stabilizing residue. Ultimately, our results indicate that the N-end rule is not a perfectly formulated pathway and even minor changes in the N-terminus of a protein can affect how rapidly it is degraded.

4.4 RGS2 MUTATIONS AS TARGETED GENE THERAPY

A common problem with hypertension and hypertrophic hearts, at least in animal models, is the expression and function of RGS2 is markedly decreased, which is accompanied with exacerbated $G\alpha_{q/11}$ signaling[18]. Previous research has shown RGS2 as an intrinsic suppressor of hypertension and cardiac hypertrophy[18,19]. Therefore, if the expression of RGS2 could be restored or if the GAP activity of RGS2 were to be increased, this could represent a promising direction in treating particular cardiovascular diseases such as hypertension[18]. An interesting new area of drug treatment is personalized medicine or gene therapy. Gene therapy is the therapeutic delivery of nucleic acid polymers via vectors into a patient's cells to treat disease by interfering with protein expression or possibly altering genetic mutations[20]. There is a lot of controversy and there are many unknowns about using gene therapy as a treatment for diseases, most notably cancer[20]. However, if gene therapy can alter genetic mutations, it would not be a stretch to assume we could alter wild-type genes to mutant forms. For instance, since RGS2 is markedly decreased in hypertensive patients, introducing the RGS2 G23D mutant into the cardiovascular system of a patient might allow for a functional form of RGS2 to be

produced while also attenuating $G\alpha_{q/11}$ to a greater degree than wild-type RGS2. Alternatively, phenotypes associated with low Gα protein activity, such as platelet $G\alpha_s$ hypofunction[3] or enhanced accumulation of glycogen and heat resistance[21], may benefit from introducing the RGS2 M5V mutation into the genome as opposed to the wild-type form. Undoubtedly, these are hypothetical treatment options. Nevertheless, altering a protein may limit adverse effects of commonly prescribed medications like angiotensin II receptor antagonists, as you are simply varying the amount of Gα signaling instead of completely impeding the action of a receptor. If these hypotheses are to be tested, the molecular mechanisms regulating RGS2 must first be uncovered. Our results provide evidence that RGS2 mutations can modify that rate of Gα signaling, hence it is important to know how a mutation effects the action of a protein before attempting to target it for therapeutic means.

4.5 RGS2 INITIATION VARIANTS

ATF4 is an example of a gene with multiple initiation sites, however, these initiator methionines are important for the action of the protein. Under basal, non-stressed conditions, two upstream open reading frames are translated which results in the exclusion of functional ATF4 due to an out of frame shift[22]. However, under stressful conditions (where initiation is delayed), the ribosome bypasses the upstream open reading frames and initiates translation at the ATF4 open reading frame. Therefore, ATF4 is increased in response to stress and can proceed to act as a transcription factor and attempt to mitigate the stressful event on the cell[22]. In contrast to ATF4, RGS2 has four initiator methionines[1] yet there is little or no understanding of why these alternative initiation variants, which appear to be conserved among multiple species, would need to exist. RGS2 does contain multiple domains including a GAP domain and a plasma membrane association domain[1], however both are downstream of each initiator

methionine suggesting all the RGS2 variants encompass both domains. It has been suggested that RGS2 has an adenylyl cyclase inhibitory domain between residues 9-11[1,3]. It has also been proposed that upon $G\alpha_s$-coupled receptor activation, the longest isoform of RGS2 is preferentially translated in order produce an RGS2 isoform with the adenylyl cyclase inhibitory domain[1]. The concern with this hypothesis is it remains controversial if RGS2 in fact binds adenylyl cyclase, $G\alpha_s$, or both, during its inhibitory effect on this signaling pathway. Previous research shows that RGS2 protein can be immunoprecipitated with purified or cellular $G\alpha_s$[23,24]. If RGS2 is able to impede $G\alpha_s$ signaling without binding to adenylyl cyclase, in other words, with the remaining residues downstream of methionine 33, the adenylyl cyclase inhibitory domain would appear to be a redundant mechanism. Another hypothesis for the four initiator methionines is that certain isoforms may be produced in times of stress. For instance, RGS2 is known to be able to arrest *de novo* protein synthesis during times of cellular stress[25]. As with other proteins that play a role in the stress response, downstream open reading frames of RGS2 (i.e., tM16 RGS2 and tM33 RGS2) may be more likely to be utilized by the ribosomal machinery during cellular stress. In the case of tM16 RGS2, this would result in a protein with a longer half-life. Again, this is simply a hypothesis and there still remains no convincing evidence for the need of the four initiator methionines.

Our results are consistent with the finding of another group, wherein under basal conditions, tM16 RGS2 was determined to be the most highly expressed initiation variant in cells transfected with the full RGS2 mRNA sequence (i.e., lacking any Kozak sequence)[1]. However in that study, RGS2 degradation was not considered and the present results suggest that slower degradation of tM16 RGS2 could also have contributed to its greater abundance. We hypothesize that this may be due to energy conservation. Simply by looking at the half-lives

of the RGS2 initiation variants we can see that RGS2 is a rapidly turned over protein. When there is limited GPCR activation, there is minimal need for RGS proteins. Therefore, it would be energy efficient for the cell to produce a shorter isoform of RGS2 and an isoform that was more stable. tM16 RGS2 maintains the ability to traffic RGS2 to the plasma membrane upon receptor stimulation[1] and is still able to attenuate $G\alpha_{q/11}$ signaling. The flaw with this hypothesis is tM33 RGS2 also maintains the ability to traffic RGS2 to the plasma membrane[1] and can attenuate $G\alpha_{q/11}$ signaling, but does not have as long of a half-life as tM16 RGS2. There may be a domain between M16 and M33 that remains critical to RGS2 that causes a preference of tM16 to be translated. Obviously, these concepts have not been tested and we cannot say for sure why tM16 RGS2 is preferentially translated as opposed to other RGS2 initiation variants.

4.6 FUTURE DIRECTIONS

Our research has revealed that variations in the N-terminus of RGS2 can affect both the stability of the protein and its inhibitory activity. Nonetheless, there remain mechanisms that need to be elucidated about RGS2 regulation and the activity of naturally occurring RGS2 variants. RGS2 is known to attenuate $G\alpha_s$ signaling[24] however, our research only focused on $G\alpha_{q/11}$-mediated signaling. Therefore, it would be prudent to perform cAMP experiments that will help determine the ability of RGS2 isoforms to inhibit $G\alpha_s$ signaling. Such experiments will help clarify multiple mechanisms including if the degradation rate of an RGS2 protein is proportional to the ability to attenuate $G\alpha_s$ signaling, and in the process would also likely determine if the putative adenylyl cyclase binding domain is actually necessary for this inhibitory function. There is some evidence supporting the presence of an adenylyl cyclase binding domain between residues 9-11 of RGS2. Thus, if tM16 RGS2 and tM33 RGS2 are

unable to reduce $G\alpha_s$ signaling, this would suggest the domain is essential. However, if both shorter isoforms of RGS2 are able to attenuate $G\alpha_s$ signaling, there are several possible explanations. First, an additional adenylyl cyclase binding domain may exist downstream of methionine 33. Second, RGS2 is able to bind to and inhibit both adenylyl cyclase and $G\alpha_s$[24]. Third, the RGS domain of RGS2 can bind and inhibit both $G\alpha_{q/11}$ and $G\alpha_s$. All these hypotheses are testable to determine which, if any, are correct. These experiments may also help strengthen the claim that the G23D mutation in RGS2 causes a relative preference for the use of the translation initiation sites at positions 1 and 5 compared to the wild-type sequence[3]. This would only become important if it was determined that the putative adenylyl cyclase binding domain of RGS2 does exist.

Further knowledge on the translational mechanisms of RGS2 could also be obtained in order to determine if one isoform of RGS2, i.e., tM16 RGS2, is translated preferentially over another isoform, i.e., tM5 RGS2, or if the difference we see if simply due to different rates of degradation. If a particular isoform of RGS2 is upregulated during times of stress, a simple experiment would be to stress cells known to contain wild-type RGS2, perform an immunoblot and compare stressed RGS2 initiation variants to a non-stressed control group. Since RGS2 is upregulated during times of stress[25], total RGS2 should increase. However, if a particular isoform increases relatively to total RGS2, it can be assumed that this initiation variant is preferentially translated times of stress. Ideally, these experiments would be executed using primary cells in order to determine if this translational effect happens *in vivo*.

Finally, to strengthen the argument that RGS2 is rapidly degraded via the ubiquitin proteasome pathway, two sets of experiments must be performed. First, RGS2-ubiquitin immunoprecipitation experiments should be done to ensure RGS2 is being ubiquitinated.

Previous research shows wild-type RGS2 to be ubiquitinated[26] but this experiment needs to be repeated for the other RGS2 variants. Second, cells transfected with RGS2 constructs should be treated with other inhibitors of both the ubiquitin-proteasome pathway and the lysosomal pathway. This should confirm that RGS2 is degraded via the ubiquitin-proteasome pathway and may help determine how RGS2 G23D is degraded. If RGS2 G23D cellular levels increase in the presence of a lysosomal inhibitor, it would suggest that certain RGS2 mutations may be regulated differently than wild-type RGS2.

4.7 CONCLUSION

Our results provide evidence for the importance of the N-terminus of RGS2 in regards to both stability and activity of the protein. Any variation within the N-terminus can affect how rapidly RGS2 is degraded and the ability of the protein to attenuate Gα protein signaling. RGS2 is an extremely important protein and varying its ability to function can lead to pathophysiological consequences, as indicated by the RGS2 G23D mutation[3]. Therefore, we have shown that naturally occurring N-terminal variants of RGS2 affect stability and activity *in vitro*.

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Chapter 5: Appendices

1 APPENDICES

5.1 RGS2-LUCIFERASE PROTEIN DEGRADATION

Originally, we wanted to take advantage of RGS2-luciferase fusion constructs to measure RGS2 stability and variability translation. The C-terminus of RGS2 constructs were fused with a renilla luciferase protein which, in theory, would emit light when RGS2 was synthesized. Thus, an increase in luminescence would be indicative of higher RGS2 levels. Unfortunately, luciferase has multiple initiator methionine residues which were preferentially chosen to initiate synthesis when mutations were introduced into the RGS2 protein. As seen in *Figure 5.1*, the majority luminescence we were detecting was simply luciferase itself, not fused with RGS2.

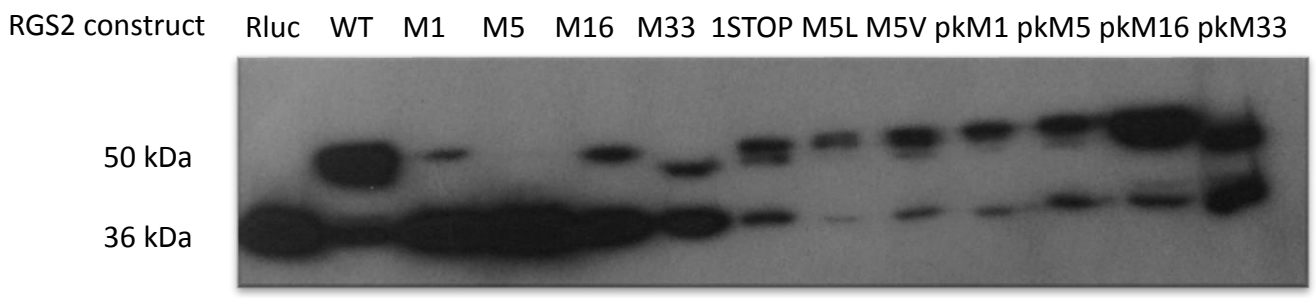

Figure 5.1. *RGS2-luciferase fusion proteins.* RGS2-luciferase fusion constructs were immunoblotted with anti-renilla luciferase. 50 kDa correlates to RGS2 and luciferase fusion and 36 kDa correlates to luciferase alone. Constructs include: Rluc = empty renilla luciferase control plasmid; WT = full-length wild-type RGS2; M1 = methionine 5, 16, and 33 were mutated to alanine; M5 = methionine 1, 16, and 33 were mutated to alanine; M16 = methionine 1, 5, and 33 were mutated to alanine; M33 = methionine 1, 5, and 16 were mutated to alanine; 1STOP = a stop codon was introduced between methionine 1 and 5 of RGS2; M5L = methionine 5 was mutated to leucine; M5V = methionine 5 was mutated to valine; pkM1 = pseudo-Kozak sequence was introduced downstream of methionine 1; pkM5 = pseudo-Kozak sequence was introduced downstream of methionine 5; pkM16 = pseudo-Kozak sequence was introduced downstream of methionine 16; pkM33 = pseudo-Kozak sequence was introduced downstream of methionine 33. Renilla luciferase protein = 36 kDa whereas RGS2-luciferase fusion protein = 50 kDa.

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