NOVEL MECHANISMS OF REGULATOR OF G PROTEIN SIGNALING 10 (RGS10) FUNCTIONS IN MICROGLIA

by

MOHAMMED ALI ALQINYAH

(Under the Direction of Shelley B. Hooks)

ABSTRACT

Microglia are immune cells that reside in the central nervous system and are essential in innate immunity, homeostasis, and development of the central nervous system. However, excessive and prolonged activation of these cells leads to an enhanced production of inflammatory cytokines, reactive oxygen species, and prostaglandins. These factors are neurotoxic and can cause significant neuronal death, and accordingly, are implicated in the pathogenesis of several neurodegenerative diseases. Regulator of G Protein Signaling 10 (RGS10) protein has been shown to negatively regulate inflammatory cytokine production from microglia and subsequent microglia-induced neurotoxicity. However, activation of microglia induces suppression of RGS10 expression in microglia, eliminating the neuroprotective effects of RGS10. In the first study, we aimed to determine the molecular mechanisms that regulate inflammation-induced suppression of RGS10 in microglia. Our data indicate that suppression of RGS10 in activated microglia is regulated by epigenetic mechanisms, mainly through histone deacetylase (HDAC)-induced repression of RGS10 gene transcription. HDAC inhibitors restored expression of RGS10 in activated microglia, indicating that it is feasible to utilize small molecules to manipulate RGS10 expression in microglia. Another aim of our study was to identify the molecular mechanisms that mediate the RGS10 anti-inflammatory function in microglia, which are currently unknown. Canonically, RGS10 negatively regulates G protein

signaling by acting as a GTPase-activating protein (GAP), thereby accelerating the inactivation reaction of G proteins. However, it has been reported that some RGS proteins can act in GAP-independent mechanisms. Here, we demonstrate for the first time that RGS10 suppresses inflammatory signaling in a G protein-independent mechanism that does not require its GAP activity. To further elucidate the nature of this mechanism, we conducted unbiased co-immunoprecipitation/mass spectrometry proteomics experiments to identify novel binding partners of endogenous RGS10 in microglia. In this study, we identified several novel partners of RGS10 in microglia associated with distinct cellular functions and localization. The findings of our study will expand our knowledge about the function and regulation of RGS10 in inflammatory signaling. Further, our study reports another GAP-independent function of RGS proteins, providing additional evidence that RGS proteins are much more than just GAPs.

INDEX WORDS: G proteins, Regulators of G protein signaling, microglia, neuroinflammation, Parkinson's, neuropathic pain, epigenetics, histone deacetylase, cycolooxygenase-2

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DEDICATION

I would like to dedicate this dissertation to my amazing parents: Ali and Hussah for their endless love, support and encouragement throughout my life. This dissertation is also dedicated to my brothers and sisters, thank you all for your help.

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CHAPTER 1

INTRODUCTION

G proteins

G proteins are molecular switches that initiate various signaling pathways upon activation (McCudden et al. 2005). There are two main classes of G proteins: heterotrimeric G protein complexes and monomeric small GTPases (Csepanyi-Komi, Levay, and Ligeti 2012; Oldham and Hamm 2008). Heterometric G proteins convey extracellular cues to activate intracellular pathways, thereby aiding cells in generating appropriate responses to external signals. In an inactive state, heterometric G proteins exist in a complex consisting of alpha (α), beta (β) and gamma (γ) subunits, where β and γ often forms a distinct βγ complex. The classic activation of this complex occurs via G protein coupled receptors (GPCRs) (Oldham and Hamm 2008).

In a general mechanism, an extracellular stimulant would induce a conformational change of the G protein coupled receptor (GPCR) allowing it to act as a guanine nucleotide exchange factor (GEF) that triggers a nucleotide exchange replacing the GDP bound to G protein to GTP. This provokes a dissociation of the G protein complex and a subsequent release of the now active Gα and Gβγ subunits that activate various signaling pathways through different mechanisms (Oldham and Hamm 2008).

Main types of Gα subunits include Gαs, Gαi/o, Gαq/11, and Gα12/13 (Cabrera-Vera et al. 2003). The presence of multiple types of G proteins allows for the activation of different pathways. For example, Gαs plays a stimulatory role by activating adenylate cyclase and the subsequent cAMP production, whereas Gαi exerts an opposite role and inhibits adenylate cyclase. cAMP is a second messenger that activates target effectors such as PKA and EPAC,

thereby resulting in the activation of several pathways (Cabrera-Vera et al. 2003). Gαq activate phospholipase C beta, which subsequently promotes the production of the second messengers IP3 and diacylglycerol (Cabrera-Vera et al. 2003). Gβγ subunits can also play molecular functions, such as the association and the activation of G protein-coupled inwardly-rectifying potassium channels (GIRK) (Logothetis et al. 1987). Since adenylate cyclase activity is crucial for multiple cellular functions, the regulation of this enzyme activity is controlled by two different G proteins that affect this enzyme in an opposing manner.

Naturally, due to the crucial functions of G proteins in cellular signaling, their activity is tightly regulated by several signaling molecules, including GPCRs and proteins called Regulators of G protein signaling (RGS) (discussed below) (Oldham and Hamm 2008). The upstream GPCR being stimulated determines which G protein type will be activated. Therefore, there are multiple receptors that activate the same downstream G protein type, providing the cells with several options to activate a certain pathway (Oldham and Hamm 2008). Interestingly, there are examples where a stimulant will activate GPCRs that couples to different G proteins, such as in the case of histamine. Histamine H1 receptor couples to Gαq, Histamine H2 receptor couples to Gαs, Histamine H3 & H4 receptors couples to Gαi (Hill et al. 1997). Therefore, the final response of the cell to histamine will be determined by which receptor is expressed or/and activated. Although this adds to the complexity of GPCR and G protein signaling, targeting this pathway is still an effective and common therapeutic approach. Historically, controlling G protein signaling was achieved by targeting GPCRs, making GPCRs a very common target for clinical therapeutic agents (Hauser et al. 2017). The enormous number of GPCR-targeted drugs is not surprising considering the essential roles of G proteins in several physiological processes, and the fact that dysregulation of G protein signaling is directly implicated in the pathogenesis of several disorders.

Small G proteins are similar to the Gα subunits and binds nucleotides but do not form heterometric complexes. They are also activated by binding to GTP and exerts different cellular

functions. Examples of small G proteins include Ras, which is involved in signal transduction and activate MAP-kinase pathways (Olson and Marais 2000), Rab, which regulate subcellular trafficking and localization (Pfeffer 1994), and Rho, that regulate cytoskeletal assembly (Hall 1998).

In conclusion, G proteins are crucial signaling molecules that have been studied intensively in the past. Yet, new findings continue to provide new information that sometimes challenge long-held presumptions, which is why G proteins remains an attractive area of research in science and drug discovery.

RGS proteins

G proteins are GTPases that hydrolyzes the GTP to GDP, resulting in an inactivation of the G protein (Cabrera-Vera et al. 2003). However, the rate of the hydrolysis mediated by G proteins in-vitro was too slow to account for the speed of deactivation observed in vivo, which points to the existence of accessory molecules that enhance the GTPase activity of G proteins. This led to the discovery of Regulators of G Protein Signaling (RGS) as GTPase-activating proteins. Several RGS proteins were identified by the presence of a shared “RGS” domain that is responsible for the GTPase-activating property of RGS proteins (Watson et al. 1996). This RGS domain directly interacts with switch regions of G proteins and stabilizes the transition state, which promotes GTP hydrolysis (Dohlman and Thorner 1997).

The RGS family can be divided into several subfamilies according to the homology of the RGS domain sequences and the presence of additional domains, including R4, R7, R12, and other subfamilies (Hollinger and Hepler 2002). The R4 subfamily members RGS1-5, 8, 13, 16, 18 and 21 contain a short C-terminal sequence and an amphipathic helix on their N-terminal domain in addition to the RGS domain (Bansal, Druey, and Xie 2007). Despite their small size, R4 RGS proteins play various essential functions and their dysregulation was implicated in several diseases (Bansal, Druey, and Xie 2007; Xie, Chan, and Druey 2016). For example, RGS1 and RGS13 regulate immune responses in different immune cells (Moratz et al. 2000;

Bansal, DiVietro, et al. 2008), RGS2 plays essential role in controlling blood pressure (Heximer et al. 2003), and RGS4 regulates serotonin signaling in the central nervous system (Gu, Jiang, and Yan 2007). This indicates that members of this family exert various cellular functions that takes place in different systems. Selectivity assays revealed that several R4 members (RGS1, RGS3, RGS4, RGS8, RGS16, and RGS18) interact with both Gαi and Gαq subunits in-vitro. On the other hand, RGS2 was selective for Gαq, possibly due to the presence of unique residues that prevents its interaction with Gαi (Heximer et al. 1999; Soundararajan et al. 2008b).

The R7 subfamily members RGS6, RGS7, RGS9, and RGS11 share a Gγ-like domain (GGL) domain that mediate the binding to Gβ5, which is a classic interacting partner of R7 subfamily members (Anderson, Posokhova, and Martemyanov 2009). DEP (disheveled Egl-10 pleckstrin) is another unique domain present in members of the R7 subfamily, which facilitates the interaction with the membrane proteins R7BP and R9AP (Anderson, Posokhova, and Martemyanov 2009). These domains and interacting partners are unique for this subfamily, as no other RGS members were shown to interact with Gβ5, R7BP or R9AP. Both the full length and the purified RGS domains of RGS6 and RGS7 displays specificity toward Gαi/o proteins in-vitro (Posner, Gilman, and Harris 1999; Soundararajan et al. 2008b).

RGS10, RGS12, and RGS14 belong to the R12 subfamily of RGS proteins and were shown to be selective for Gαi subunits (Soundararajan et al. 2008b). RGS12 and RGS14 share RBD (rab binding domain) that is essential in the ability of these RGS proteins to bind Ras and inhibit Ras-mediated signaling (Willard et al. 2007; Shu, Ramineni, and Hepler 2010). RGS12 and RGS14 also contain a GoLoco domain at their C-terminal, which enable them to act as GDP-dissociation inhibitors for Gαi (Kimple et al. 2001). This indicates that R12 members have the unique ability to inhibit Gαi signaling by both accelerating the hydrolysis of GTP and interfering with the dissociation of GDP. Furthermore, RGS12 contain PTB (phosphotyrosine-binding) domain that facilitate the binding to N-type calcium channel (Schiff et al. 2000).

The previous findings indicate the R12 subfamily members contain multifunctional domains that facilitate unique interactions and specific functions. However, RGS10 strikingly differs from its family members as it only contains an RGS domain and short N and C-terminal sequences (Hunt et al. 1996). Yet, RGS10 plays multiple roles and dysregulated expression of this small RGS protein was implicated in the pathogenesis of several disorders. For example, RGS10 protects against neuroinflammation-induced neurodegeneration by suppressing the release of inflammatory cytokines from activated microglia in the central nervous system (Lee et al. 2008; Lee, Chung, et al. 2011). RGS10 also inhibits M1 and promotes M2 phenotypes in the peripheral macrophage, providing further evidence that RGS10 acts as an anti-inflammatory protein in immune cells (Lee et al. 2013). In another system, RGS10 is needed for proper NFAT functions, adequate osteoclast differentiation and its loss is implicated in the development of osteopetrosis (Yang and Li 2007). In ovarian cancer cells, loss of RGS10 expression enhance the chemoresistance of ovarian cancer cells and enhance their viability (Hooks et al. 2010). RGS10 also play important functions in other cells, such as neurons (Lee, Chung, et al. 2012), T cells (Garcia-Bernal et al. 2011), cardiomyocytes (Miao et al. 2016), and platelets (Hensch et al. 2016). Interestingly, in many of these cells where RGS10 plays a role, RGS10 expression is often dysregulated. For example. RGS10 expression is suppressed by inflammatory stimulants in immune cells (Lee et al. 2008). Similarly, RGS10 expression is downregulated upon angiotensin II treatment in cardiomyocytes (Miao et al. 2016). In ovarian cancer cells, RGS10 expression is significantly lower in chemoresistance compared to chemoresensitive cells (Ali et al. 2013b). This indicates that restoring the expression of RGS10 may have beneficial effects in these systems. Another general observation that can be made regarding RGS10 functions in some of these systems is that the molecular mechanism governing RGS10 functions is poorly understood. This is particularly the case in the role of RGS10 in immune and ovarian cancer cells. Therefore, understanding the mechanisms mediating RGS10 regulation and functions

exerts may assist in developing therapeutic approaches to compensate for the loss of RGS10 in these cells.

Microglia

Microglia are cells from mesodermal/mesenchymal origins that travel to the central nervous system where they ultimately differentiate to microglia (Kettenmann et al. 2011). Microglia scavenges and clears the brain and the spinal cord from cell debris, pathogens, dead cells, and other materials (Kettenmann et al. 2011). In addition, microglia play a part in the development of the central nervous system and brain homeostasis (Michell-Robinson et al. 2015). During neuronal development, there is controlled and programmed cell death of neurons followed by removal of dead neurons, which is a main function of microglia. Abundant evidence demonstrates a critical role of microglia in apoptosis of different types of neurons during development. For example, it was demonstrated that nerve growth factor (NGF) released from microglia activates neurotrophin receptor p75 and subsequently induces neuronal death in retinas undergoing development (Frade and Barde 1998). Furthermore, selective removal of microglia from cerebellar slices significantly inhibited apoptosis of Purkinje cells (Marin-Teva et al. 2004). The suppressed apoptosis of Purkinje cells was mainly due to the loss of superoxide ions produced from microglia. The apoptotic functions of microglia were significantly reduced in microglia devoid of the integrin CD11b and the immunoreceptor DAP12, which are believed to be the receptors that recognize “eat me” signals that produced from neurons (Wakselman et al. 2008). Suppression of microglial activation inhibited neurogenesis during development, suggesting that microglia also play roles in neurogenesis (Shigemoto-Mogami et al. 2014).

Another important function of microglia during brain development is the assist in synaptic pruning, by removing excess synaptic projections during CNS development (Paolicelli et al. 2011). The complement C3 present in synaptic terminals attracts microglia through the microglial complement receptor 3 (CR3) for subsequent synaptic pruning (Schafer et al. 2012). Differentiation of some cell population in the CNS is also mediated by microglia. For example,

microglia facilitate the differentiation of basal forebrain progenitors to cholinergic neurons and embryonic neural precursor cells to astrocytes (Jonakait et al. 2000; Antony et al. 2011).

In the adult healthy brain, microglia maintain brain homeostasis by constantly being on the lookout for any disturbances in the central nervous system (Kettenmann et al. 2011). At this state, microglia are usually in ramified shape, and are maintained in this state by a constitutive function of certain molecules such as CX3CR receptor (Kettenmann et al. 2011). Microglia activation is accompanied by morphological, molecular, and functional changes, such as the enhancement of the expression of Toll Like receptors, chemokines receptors, and cytokine release (Kettenmann et al. 2011). These changes and others enable microglia to track and remove foreign components through phagocytosis (Fu et al. 2014a). Phagocytosis is often accompanied by inflammatory or anti-inflammatory cytokine production depending on the materials being engulfed and the receptors participating in the phagocytosis (Fu et al. 2014a).

Microglia have a unique ability of recognizing different types of pathogens, viruses, and other foreign entities due to the presence of multiple Toll Like receptors (TLRs) that recognize and interact with pathogen components or “pathogen-associated molecular patterns” (Kawai and Akira 2010). This large family of proteins enable microglia to recognize many different pathogens. For example, TLR1, TLR2, TLR4 and TLR6 senses lipids of different kinds of bacteria. TLR3 recognizes viral double-stranded RNA, whereas TLR7 and TLR8 recognize single-stranded viral RNA (Kawai and Akira 2010). Upon recognizing the foreign pathogen, microglia initiate downstream signaling pathways that results in phagocytosis accompanied by the release of inflammatory mediators such as TNF alpha and nitric oxide (NO) to remove the pathogen (Kawai and Akira 2010). Following removal of insult, microglia usually return to their surveillance state (Kettenmann et al. 2011).

Thus, microglia exert several important functions in the CNS development and innate immunity. However, abnormal and constant activation is harmful and is implicated in CNS-related diseases. Therefore, therapeutic approaches that aim to mitigate microglial functions

should consider that microglia still play numerous protective functions. An optimal approach is to suppress microglial functions enough to avoid harmful effects, but still maintain physiological functions of these immune cells.

Role of microglia in neuroinflammation-associated diseases

Depending on the type of stimulant, microglia can be activated into two distinct phenotypes, M1 and M2 (Tang and Le 2016). The M1 microglia are mainly pro-inflammatory by enhancing the secretion of inflammatory cytokines which leads to neuroinflammation. On the other hand, M2 microglia mainly are anti-inflammatory by producing proteins that are anti-inflammatory and help with repair and regeneration, thereby returning the normal homeostasis (Tang and Le 2016).

Due to the opposing functions of each phenotype, it is reasonable to expect that these distinct phenotypes of microglia play different roles in CNS diseases. Many studies have provided evidence that chronic neuroinflammation and elevated production of inflammatory mediators contributes to neurotoxicity and neurodegenerative diseases (Smith et al. 2012). Therefore, the loss of M2 microglia and the elevated number of M1 microglia can be a major contributing factor in these diseases. In Parkinson's disease, α-Synuclein aggregation polarizes microglia towards M1 phenotype, resulting in enhanced production of reactive oxygen species and subsequent dopaminergic neuronal death (Zhang et al. 2005). Activation of TLR4 by LPS, which strongly activate M1 phenotype, significantly induce dopaminergic neurodegeneration (Li et al. 2004). These findings suggest that M1 microglia contributes the Parkinson's disease etiology, which is expected as this type of microglial activation enhance the release of several proinflammatory cytokines and reactive oxidative species that are known neurotoxic factors. One of the hallmarks of Alzheimer's disease is the accumulation of Amyloid β peptides that contributes to the development of the disease (Bayer and Wirths 2010). Microglia are reported to clear Amyloid β peptides through phagocytosis (Koenigsknecht and Landreth 2004). However, LPS treatment and the subsequent production of inflammatory cytokines, which

induces M1 activation of microglia, impaired the microglial phagocytosis of Amyloid β (Koenigsknecht-Talboo and Landreth 2005). Conversely, inducing M2 activation by IL-4 or IL-10 enhanced microglial phagocytosis of Amyloid β (Koenigsknecht-Talboo and Landreth 2005; Michelucci et al. 2009). Further, Amyloid β can directly activate microglia and initiate inflammatory signaling by interacting with TLRs (Stewart et al. 2010; Liu et al. 2012), which may results in a vicious cycle of Amyloid β accumulation, enhanced M1 activation, and impaired phagocytosis. Since inflammatory mediators can also directly induce neuronal death (Guadagno et al. 2013), M1 activation of microglia may contribute to Alzheimer's disease by both accumulating Amyloid β and enhancing inflammation-induced neuronal death. On the other hand, M2 activation of microglia will enhance Amyloid β clearance and reduce inflammatory cytokines, which provide protective effects in Alzheimer's disease. These effects of M1 and M2 phenotypes are not exclusive to Parkinson's and Alzheimer's diseases, but also applies to multiple other neurodegenerative diseases that are influenced by neuroinflammation (Tang and Le 2016).

Ageing has been linked for a long time to be a main contributing factor that potentiate the harmful functions of microglia and at the same time dampen their protective processes. For example, the phagocytosis of microglia decreases with age and since many neurodegenerative diseases share a common property which is the accumulation of aggregated proteins such as alpha-synuclein in Parkinson's disease, the reduced ability of microglia to clear these aggregates with age can be a contributing factor to the development of these diseases (Koellhoffer, McCullough, and Ritzel 2017). Another prominent change that occurs in aged microglia is the elevated release of pro-inflammatory cytokines and reactive oxygen species (Ritzel et al. 2015; Koellhoffer, McCullough, and Ritzel 2017). Thus, age is a critical factor that shifts microglial phenotypes from M2 to M1, which may contribute to the onset or progression of neurodegenerative diseases.

In addition to their role in neurodegenerative diseases, microglia were also linked to neuropathic pain. Neuropathic pain is a disease characterized by a chronic pain that persists after the pain-inducing stimuli is vanished (Costigan, Scholz, and Woolf 2009). In neuropathic pain, neurons in the spinal cord becomes “sensitized”, which render them more active (Costigan, Scholz, and Woolf 2009). The sensitization process can occur in the peripheral and the central nervous system. Central sensitization, is a condition that leads to a persistent and an enhanced activity of neuronal fibers such as C-fibers. It is believed that central sensitization is the reason pain persists after the original pain stimuli vanishes (Woolf 2011). Microglia are well-established contributors to the central sensitization process that leads to neuropathic pain (Inoue and Tsuda 2009). Many inflammatory molecules produced by spinal microglia causes neuronal hypersensitivity and contribute to the development of neuropathic pain (Wen et al. 2011).

Overall, microglia play essential roles in maintaining normal brain homeostasis and are first-line defenders against pathogens, viruses, and other harmful materials. However, chronic activation of microglia induces neuroinflammation and is implicated in neuropathic pain and several neurodegenerative diseases. Developing therapeutic approaches to combat chronic microglial activation and the subsequent neuroinflammation will greatly assist in managing and treating neuroinflammation-associated diseases.

CHAPTER 2

REGULATING THE REGULATORS: EPIGENETIC, TRANSCRIPTIONAL, AND POST-TRANSLATIONAL REGULATION OF RGS PROTEINS[1]

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Abstract

Regulators of G-protein signalling (RGS) are a family of proteins classically known to accelerate the intrinsic GTPase activity of G-proteins, which results in accelerated inactivation of heterotrimeric G proteins and inhibition of G protein coupled receptor signaling. RGS proteins play major roles in essential cellular processes, and dysregulation of RGS protein expression is implicated in multiple diseases, including cancer, cardiovascular and neurodegenerative diseases. The expression of RGS proteins is highly dynamic and is regulated by epigenetic, transcriptional and post-translational mechanisms. This review summarizes studies that report dysregulation of RGS protein expression in disease states, and present examples of drugs that regulate RGS protein expression. Additionally, this review discusses, in detail, the transcriptional and post-transcriptional mechanisms regulating RGS protein expression, and further assesses the therapeutic potential of targeting these mechanisms. Understanding the molecular mechanisms controlling the expression of RGS proteins is essential for the development of therapeutics that indirectly modulate G protein signalling by regulating expression of RGS proteins.

1. Introduction

Regulator of G protein signaling (RGS) proteins control signaling through heterotrimeric G-proteins by accelerating the intrinsic GTPase activity of Gα subunits, typically resulting in an inhibition of downstream G protein signaling pathways (Koelle 1997; Watson et al. 1996). Due to the critical role of G-protein signaling pathways in diverse cellular functions, it is unsurprising that RGS proteins are also essential in maintaining normal physiological processes and that dysregulation of RGS proteins is implicated in many pathologies. Like the more extensively studied G protein coupled receptors (GPCRs), which activate heterotrimeric G proteins, RGS proteins have emerged as attractive therapeutic targets (Kimple et al. 2011). However, RGS protein activity is typically regulated by control of expression, stability and localization rather than ligand binding, so RGS proteins are not as amenable to direct small molecule regulation as GPCRs. Therefore, to exploit RGS protein regulation of G protein pathways as a therapeutic target, a comprehensive understanding of the mechanisms that regulate the expression of RGS proteins is critical. In this review, we discuss the molecular mechanisms governing the expression and stability of RGS proteins, and evaluate the therapeutic potential of targeting these mechanisms.

2. RGS proteins in Pathophysiology

The established role of G proteins and GPCRs in the central nervous system, cardiovascular system, and in cancer biology naturally led to exploration of the physiologic role of RGS proteins in these systems. In this section, we will briefly discuss evidence demonstrating the roles and the regulation of RGS proteins in normal physiology and disease states in these systems. Also, it should be mentioned that in addition to their roles in the central nervous system, cancer and cardiovascular system, RGS proteins have important roles in multiple other systems, such as the immune system (Xie, Chan, and Druey 2016).

2.1 RGS proteins in the Central Nervous System

RGS proteins participate in multiple processes in the central nervous system, including synaptic plasticity (Gerber, Squires, and Hepler 2016), memory (Evans, Dudek, and Hepler 2015), and vision (Chen 2015). Therefore, predictably, dysregulation of RGS protein expression is evident and implicated in several CNS disorders (Lee and Bou Dagher 2016; Neubig and Siderovski 2002). For example, RGS9 is a critical component of the phototransduction machinery of retinal neurons, and loss of RGS9 results in a visual disorder in which patients cannot adapt to changes in light (Chen et al. 2000; Nishiguchi et al. 2004); RGS7 and RGS9 critically regulate responses to dopamine (Rahman et al. 2003) and opiate receptors (Zachariou et al. 2003) and are implicated in the development of tolerance and addiction (Hooks, Martemyanov, and Zachariou 2008); and RGS14 has recently been identified as a critical control point for long term potentiation and memory (Evans, Dudek, and Hepler 2015). In many cases, multiple RGS proteins contribute to the same CNS-related pathology, as is the case in Parkinson's Disease (PD). RGS4 knockout animals display reduced motor symptoms in Parkinson's disease animal models (Lerner and Kreitzer 2012), and inhibition of RGS4 improves symptoms of Parkinson's disease (Blazer et al. 2015), (Ko et al. 2014) suggesting that RGS4 contributes to the pathology of this disease. On the contrary, RGS2 (Dusonchet et al. 2014), RGS6 (Bifsha et al. 2014), and RGS10 (Lee et al. 2008) protect dopaminergic neurons and delay Parkinson's progression. These opposing roles in PD do not simply correlate with distinct G protein selectivity of GAP function or the activity of domains outside of the RGS domain, since RGS4 and RGS10 are both small Gi/o selective GAPs. This suggests that each RGS protein is finely tuned to a specific response, and subtle differences in regulation are critical in determining the physiologic role of RGS proteins. In addition to acting as classic GAPs, some RGS proteins modulate the pathogenesis of these diseases by GAP-independent mechanisms. For example, RGS2 protects neurons by directly binding and inhibiting LRRK2 in a mechanism that does not require RGS2 binding to G proteins (Dusonchet et al. 2014). This indicates that targeting GAP-

independent function of RGS proteins can be a beneficial approach in the treatment of some CNS diseases.

A common mechanism for regulation of G protein pathways is the modulation of RGS expression by upstream receptor agonists, and this regulation can result in feedback inhibition or feedforward activation. The expression of many RGS encoding genes and protein levels is highly sensitive to CNS-targeted drugs, with distinct mechanisms and time courses. For example, examination of human tissues revealed that RGS4 protein level is increased in prefrontal cortex of long term opiate abusers with no change in short term users, while RGS10 protein level is decreased in short term opioid abuse but shows no change in long term users (Rivero et al. 2012). The increase in RGS4 expression is recapitulated in a rat model following chronic exposure to morphine (Rivero et al. 2012). Both RGS4 and RGS10 proteins have been shown to modulate μOR signaling, suggesting that μOR-induced regulation of RGS protein levels may mediate, at least partially, some tolerance to opioid agonists. Similarly, complex regulation of RGS expression occurs in psychosis and anti-psychotic treatment. RGS4 transcript levels are decreased in the prefrontal cortex of individuals with schizophrenia (Mirnics et al. 2001), and RGS4 immunoreactivity is higher in subjects treated anti-psychotics (Rivero et al. 2013). The antipsychotic drug olanzapine, which primarily targets 5-HT2A serotonin receptors, has been shown to increase RGS7 protein levels, and this effect is mediated by a Jak/Stat dependent pathway (Singh et al. 2007).Therefore, changes in RGS protein expression levels are associated with both the pathology and therapeutic responses in several CNS diseases.

2.2 RGS Proteins in Cancer

In the past two decades, the role of GPCRs and heterotrimeric G proteins in cancer initiation and progression has been established (Dorsam and Gutkind 2007), which has led to great interest in the regulatory role of RGS proteins in cancers (Hurst and Hooks 2009b). Studies have provided an abundance of evidence implicating RGS proteins in multiple cancers, where they may either promote or inhibit cancer progression, depending on the type of cancer

and RGS protein involved. For example, RGS2 (Lyu et al. 2015), RGS4 (Xie et al. 2009), RGS6 (Maity et al. 2013), and RGS16 (Liang et al. 2009) suppress various aspects of breast cancer progression, whereas RGS20 promotes breast cancer carcinogenesis (Yang, Lee, et al. 2016). Even the same RGS protein can have opposing effects on cancers derived from different tissue. RGS17 is associated with inhibited cell growth and improved responses to chemotherapeutic drugs in ovarian cancer cells (Hooks et al. 2010; Cao et al. 2006; Yang, Li, et al. 2016), while RGS17 has been shown to promote lung and prostate cancer growth (Bodle, Mackie, and Roman 2013). Given the diversity of effects on different cancer types, it is unsurprising that not all RGS proteins mediate their effects through a simple G protein GAP activity. For example, while RGS4 actions in breast cancer are mediated by classic GAP activity, RGS6 and RGS16 inhibit breast cancer via GAP-independent mechanisms (Maity et al. 2011). The fact that RGS proteins employ different mechanisms has therapeutic implications. For example, targeting the RGS-G protein interaction would selectively inhibit the GAP-dependent effects of RGS4 in breast cancer cells, while strategies targeting expression would impact both GAP-dependent and -independent functions of RGS4, RGS6 and RGS16.

Aberrant expression of RGS transcripts and proteins is also commonly observed in cancers. In breast cancer cells, RGS2, RGS4, and RGS6–which suppress growth–are down-regulated compared to normal cells (Lyu et al. 2015; Xie et al. 2009; Maity et al. 2013), while RGS20–which promotes growth–is up-regulated in cancer (Yang, Lee, et al. 2016). Thus, in both cases, the changes in RGS expression may contribute to progression of disease. This is also observed in prostate cancer cells, where RGS2–which suppresses prostate cancer cell growth–is reduced (Cao et al. 2006), while expression of RGS17–which promotes prostate cancer cells growth–is elevated (James et al. 2009; Wolff et al. 2012). Finally, RGS protein expression is also modulated by chemotherapeutic drugs (Huang et al. 2011; Liu et al. 2011; Hooks et al. 2010), suggesting that RGS regulation of cancer cell growth continues to be modified through disease progression and therapy. Together, these observations demonstrate

that RGS proteins are important regulators of cancer cell growth and survival, and dysregulation of RGS protein expression in cancer cells can modify disease progression.

2.3 RGS Proteins in Cardiovascular disease

Both GPCRs and G proteins are essential mediators of critical cardiovascular functions, and GPCRs are primary targets for many cardiovascular drugs (Belmonte and Blaxall 2012). RGS proteins also regulate multiple essential cardiac processes, and abnormal changes in their expression often results in cardiovascular system dysfunctions. For instance, loss of RGS2 amplifies angiotensin II (AngII) type 1 (AT_1) signaling, which leads to hypertension (Hercule et al. 2007), and cardiac remodeling is regulated by RGS2 (Zhang et al. 2006) and RGS14 (Li et al. 2016). RGS proteins also play important functions in heart failure and drug-induced cardiac injury, among other conditions (reviewed (Zhang and Mende 2011; Stewart, Huang, and Fisher 2012)).

Changes in RGS expression levels have been reported in cardiovascular disease, suggesting that abnormal expression of RGS proteins may contribute to pathogenesis. In particular, it appears that RGS proteins and GPCRs participate in bi-directional regulatory mechanisms, in which RGS proteins regulate GPCR activity and GPCR activation in turn alters the expression of RGS proteins. For example, AT1 receptor regulates the expression of RGS2 (Grant et al. 2000), RGS10 (Miao et al. 2016) and RGS14 (Li et al. 2016), which regulate AT1 receptor-induced effects. Similarly, the β1 and β2 adrenoreceptor agonist isoproterenol induces RGS5 expression (Jean-Baptiste et al. 2005), and RGS2 and RGS16 expression is regulated by Lysophospholipid Sphingosine 1-phosphate (S1P) receptor activation in vascular smooth muscle cells (Hendriks-Balk et al. 2009).

Based on these diverse studies that have defined the role of RGS proteins in various pathophysiologies and the dynamic regulation of RGS expression during disease progression and treatment, several common observations can be made. First, it is evident that RGS proteins are critically important regulators of physiology and disease in these systems. Second,

expression of RGS proteins is often dysregulated in disease states. Third, several drugs used in treatment of these diseases also alter the expression of RGS genes or protein levels. These observations suggest that changes in RGS expression may contribute directly to disease initiation, disease progression, treatment efficacy, tolerance, and unwanted side effects. Therefore, approaches targeted to manipulate the expression of RGS proteins can potentially be utilized for treating diseases in different systems, and also enhance the effectiveness or lower the toxicity of a number of drugs. To this end, understanding the molecular mechanisms that regulate the expression of RGS proteins will lay the groundwork for future development of effective and safe RGS-targeted therapies.

3. Mechanisms regulating RGS levels

RGS proteins are primarily regulated by mechanisms that control local concentration of the protein at the site of signaling, either by regulating subcellular localization, protein stability, transcriptional regulation or epigenetic regulation (**Figure 2.1**). These combined mechanisms allow acute and chronic regulation of RGS levels in response to multiple signals. They also provide multiple potential points of intervention.

3.1 Epigenetic Regulation of RGS expression

Epigenetic modifications regulate gene expression by altering the structure of chromatin, which consists of histone proteins tightly wrapped by DNA (Venkatesh and Workman 2015). Both DNA and histones can be epigenetically modified to influence the accessibility of transcription factors by loosening or tightening the chromatin complex, to ultimately activate or repress gene expression (Venkatesh and Workman 2015). DNA (de)methylation and histone (de)acetylation are classic examples of epigenetic modifications. DNA methylation at cytosine is mediated by DNA methyltransferase enzymes (DNMTs) and results in gene repression (Smith and Meissner 2013). Histones can be acetylated by Histone acetyltransferase (HATs) to activate gene expression, or deacetylated by histone deacetylases (HDACs) to repress gene expression (Eberharter and Becker 2002). DNA methylation and histone deacetylation are

critical epigenetic mechanisms that regulate RGS gene expression in cancer, central nervous system, and cardiovascular systems (**Figure 2.1**).

Several studies provide evidence of epigenetic-mediated regulation of RGS genes in cancer. An early example of epigenetic regulation of RGS expression was a report of an increase in the methylation of the RGS16 promoter in breast cancer tumors, which correlated with a reduction in RGS16 expression (Wiechec, Overgaard, and Hansen 2008). Similarly, epigenetic regulation of RGS2 expression by DNA methylation has been reported in prostate cancer, where the suppression of RGS2 in prostate tumors was accompanied by an increase in the methylation of its promoter (Wolff et al. 2012). Inhibition of DNA methylation restored the expression of RGS2 in prostate cancer, providing additional evidence that expression of RGS2 is reversibly regulated by methylation of the promoter (**Figure 2.1**) (Wolff et al. 2012). Similarly, enhancement of methylation at the RGS2 promoter by the multifunctional protein Ubiquitin-like with PHD and ring-finger domain 1 (UHRF1) suppressed RGS2 expression and induced progression of bladder cancer (Ying et al. 2015). In ovarian cancer, there is an increase in the methylation of RGS10 promoters in chemoresistant ovarian cancer cells, compared to their chemosensitive counterparts (Hooks et al. 2010). This hypermethylation, which was mediated by DNMT enzymes, correlated with suppressed expression of RGS10 in chemoresistant cells (Hooks et al. 2010; Ali et al. 2013b). However, in ovarian cancer cells, DNA methylation is not the only epigenetic mechanisms that have been shown to be involved in regulating RGS10 expression. Histone deacetylation, induced by HDAC enzymes, also mediates RGS10 suppression in chemoresistant cells (Cacan et al. 2014). Collectively, these studies demonstrate that epigenetic mechanisms contribute to the regulation of RGS genes in different cancers.

Several studies demonstrate that RGS genes can also be regulated by epigenetic mechanisms in the central nervous system. DNA methylation influences RGS expression in human neural progenitors (Tuggle et al. 2014). During neuronal progenitor cell differentiation, low levels of DNMT enzymes correspond with increased expression of RGS proteins, which

suggests that DNA methylation plays a role in regulating RGS protein expression in these cells (Tuggle et al. 2014). Indeed, inhibition of DNMT enhanced the expression of many RGS proteins in human neural progenitors, including RGS2 and RGS10 (Tuggle et al. 2014). In microglia, the resident macrophages of the central nervous system, inflammatory stimulators induce suppression of RGS10, a protein that has been shown to play a major anti-inflammatory function in these cells (Lee et al. 2008). Activation of Toll-like receptors 4 (TLR4) stimulated association of HDAC enzymes to RGS10 promoters, which resulted in deacetylation of RGS10 promoters (Alqinyah et al. 2017). The suppression of RGS10 by this HDAC-induced mechanism was blocked by the HDAC inhibitor TSA (Alqinyah et al. 2017). TSA is also shown to induce anti-inflammatory effects in microglia (Hsing et al. 2015); therefore, it will be of interest to test whether the anti-inflammatory effect of TSA is due to its ability to restore RGS10 expression in microglia. DNMT inhibition had no effect on TLR4-induced suppression of RGS10 in microglia, suggesting that DNA methylation does not play a major role in regulating RGS10 expression in microglia (Alqinyah et al. 2017). The fact that DNA methylation suppressed RGS10 expression in ovarian cancer cells and neuronal progenitors but not microglia suggests cell-type specific effects of epigenetic mechanisms in regulating RGS gene expression. Also, since HDAC enzymes are strongly implicated in mediated silencing of RGS10 during inflammation, it will be interesting to test whether HAT enzymes, which acetylate histones, would counteract HDAC and block suppression of RGS10.

Similar to the mechanisms described above, DNA methylation has also been implicated in regulating RGS5 expression in carotid arteries by enhanced methylation on CpG dinucleotides at its promoter (Zhang, Gu, et al. 2012), suggesting that epigenetic mechanisms contribute to the regulation of RGS genes in the cardiovascular system as well. Collectively, these studies provide evidence that RGS genes can be regulated in cancer, central nervous system and cardiovascular systems by epigenetic mechanisms (**Figure 2.1**).

3.2 Transcription Factors regulating RGS expression.

Detailed studies of RGS gene promoters have revealed binding sites for several transcription factors, indicating that multiple transcription factors regulate RGS transcription directly (**Table 2.1**). Isolation and characterization of the mouse RGS2 promoter revealed a highly conserved cAMP response element (CRE) site located at the RGS2 promoter (Xie et al. 2011). Mutation of the CRE site suppressed activation of the RGS2 promoter, suggesting that the CRE site is functionally required for efficient RGS2 transcription (Xie et al. 2011). CRE sites were also found at the promoters of RGS4 (Yang et al. 2010) and RGS5 (Seidl et al. 2015), indicating that this site is commonly participating in the transcription of multiple RGS genes. Interestingly, although CRE sites are present at the promoters of different RGS genes, they affect RGS transcription differently depending on the transcription factor occupying the CRE site. Binding of the transcription factor CRE-binding protein (CREB) to the CRE site in the RGS2 promoter activates RGS2 gene transcription (**Figure 2.2**), while binding of the related factor CRE-modulator (CREM) at the same site supresses RGS5 transcription, which suggests that CREB and CREM may compete and counteract each other at the CRE sites of RGS gene promoters to dynamically regulate expression.

In addition to the CRE site, promoters of RGS genes also harbour NF-κB (Li, Murthy, et al. 2010), AP-1 (Zhang, Li, et al. 2012) and P-53 binding sites (Iwaki et al. 2011), and these sites regulate transcription of RGS genes (**Table 2.1**). These sites can be present at the promoter of the same RGS gene allowing for several transcription factors to co-regulate the transcription of one RGS gene. For example, multiple transcription factors influence IL1β-induced upregulation of RGS4; NF-κB (Hu et al. 2008) and GATA-6 (Zhang et al. 2017) activate RGS4 transcription following IL1β treatment, whereas AP-1 suppresses RGS4 transcription in colonic muscle cells (Zhang, Li, et al. 2012). In addition to RGS4, other examples of RGS genes that are regulated by different transcription factors include RGS2, which is activated by both CREB (Xie et al. 2011) and STAT3 transcription factors (Yue et al. 2010), and RGS16, which is

regulated by both NF-κB (Xie, Li, et al. 2010; Li et al. 2001) and P-53 (Buckbinder et al. 1997). The participation of multiple transcription factors to regulate the same RGS gene allows for more complex regulation of expression in response to different physiologic signals, and provides multiple options in targeting these mechanisms to control the expression of RGS proteins. As the same RGS can be regulated by different transcription factors, the same transcription factor can also regulate different RGS genes. For example, NF-κB activates the transcription of both RGS16 (Xie, Li, et al. 2010) and RGS4 (Hu et al. 2008) while STAT3 enhances the transcription of RGS2 (Yue et al. 2010) and RGS7 (Singh et al. 2007).

Strikingly, the transcription factor P-53 increases the transcription of RGS16 in human EB1 colon cancer cells (Buckbinder et al. 1997) but acts as suppressor of RGS13 transcription in MCs and B lymphocytes (Iwaki et al. 2011), suggesting that the same transcription factor can regulate RGS genes transcription differently depending on the RGS gene being regulated and the type of cells involved. Transcriptional regulators typically function in multi-protein complexes to cooperatively regulate gene expression. Given that the expression of each component of these regulatory complexes may vary in different tissues and cell types, it is likely that there is significant variation in the effect of a specific transcription factor on RGS expression in different tissues. Further, many transcription factors regulate RGS gene transcription downstream of very specific and defined signalling pathways that tend to be active in a specific type of tissue. For example, the previous studies show that NF-κB, AP-1, and GATA-6 transcription factors acts on RGS4 transcription downstream of IL1β-induced signaling in colonic muscle cells, showing that this pathway in these cells is specifically regulated by multiple transcription factors. Such cell-type and pathway-specificity requires extensive investigation and characterization to fully exploit regulation of RGS expression, but also provides opportunities for more selective therapeutic approaches to regulate RGS gene expression with strategic pathway-specific approaches.

Evidence also suggests bi-directional regulation between RGS proteins and transcription factors, as several RGS proteins directly interact with transcription factors and regulate their

function. For example, RGS2 directly binds STAT3 and inhibits STAT3-mediated transcription (Lee, Park, et al. 2012). Similarly, RGS13 inhibits CREB-mediated transcription by translocating to the nucleus and forming a complex with CREB and CBP/P300 (Xie et al. 2008). Since both STAT3 and CREB directly regulate RGS gene transcription, it is possible that some RGS proteins participate in auto-regulatory transcriptional feedback mechanisms.

3.3 Regulation of RGS mRNA stability by miRNA and RNA binding proteins

MicroRNAs are a family of short endogenous non-coding RNA sequences ranging between 18-23 nucleotides in length that play an important role in posttranscriptional regulation of gene expression by targeting the 3' untranslated regions of mRNAs, which result in degradation of mRNAs and subsequent silencing of the encoded proteins (Nelson et al. 2003). Due to their crucial functions in many cellular processes, aberrant levels and/or mutations of microRNAs are implicated in various diseases (Nelson et al. 2003).

As the evidence of microRNA involvement in carcinogenesis accumulates, numerous studies have identified the target genes of these microRNAs. Among the target genes, several RGS transcripts have been identified. For example, RGS16 has been shown to be targeted by miR-181a in chondrosarcoma, a cancer of bones and joints (Sun et al. 2015). Genetically modifying miR-181a expression correlated with RGS16 mRNA expression in chondrosarcoma cell lines (Sun et al. 2015). Moreover, RGS16 overexpression blocked miR-181a-induced production of VEGF and MMP1, suggesting that miR-181a ability to promote angiogenesis and metastasis is mediated, at least partially, by silencing RGS16 (Sun et al. 2015). Activation of the G protein coupled receptor CXCR4 promotes production of VEGF, and it was previously shown that RGS16 regulates CXCR4 activity (Sun et al. 2013; Berthebaud et al. 2005). Therefore, a possible mechanism of miR-181a-induced production of VEGF is via RGS16 silencing and subsequent enhancement of CXCR4 signaling. The participation of CXCR4 signaling in microRNA-induced RGS16 regulation is also reported in apoptotic bodies, where miR-126

suppressed RGS16 expression and amplified CXCR4 activity (Zernecke et al. 2009). In addition to RGS16, miR-181a also targets RGS4 and induces its suppression during osteoplastic differentiation (Bhushan et al. 2013). Another example of miRNA-induced regulation of RGS proteins is the Hsa-miR-182-induced suppression of RGS17 in lung cancer cells (Sun et al. 2010). RGS17 overexpression blocked Hsa-miR-182-induced inhibition of lung cancer cells proliferation, suggesting that Hsa-miR-182 anti-proliferative actions are mediated through silencing of RGS17 (Sun et al. 2010). MiRNA profiling revealed that RGS17 is also a target of miR-363 (Mosakhani et al. 2013). Taken together, these studies provide evidence that RGS proteins can be regulated by miRNA in cancer (**Figure 2.1**), and that RGS silencing contributes to miRNAs actions. Whether the result of miRNA-induced silencing of RGS proteins is to promote or inhibit cancer progression depends on the cell type and the target RGS protein. Future studies that aim to understand the molecular mechanisms and specificity of miRNAs in cancer will potentially identify additional miRNA-regulated RGS proteins.

In addition to cancer, studies have also investigated whether RGS proteins are regulated by miRNAs in the central nervous system. In *in vitro* models of Huntington disease (HD), miR-22 induces RGS2 silencing and results in a neuroprotective effect (Jovicic et al. 2013). Interestingly, miR-22 (Muinos-Gimeno et al. 2011) and hsa-miR-4717-5p (Hommers et al. 2015) target RGS2 (**Figure 2.2**) and are associated with panic and anxiety related disorders. This suggests that RGS regulation by microRNA not only occurs in the central nervous system, but also plays a role in the etiology of CNS-related diseases. Attempts to therapeutically target microRNA-induced RGS protein regulation in the CNS should be preceded by comprehensive studies to evaluate the overall effects of this regulation in different brain regions that might result in unwanted CNS-related side effects.

Following transcription, mRNA stability is also controlled by specific RNA-binding proteins (Glisovic et al. 2008). For example, Ataxin-2 (ATXN2) binds and regulates steady-state levels of RGS8 mRNA (Dansithong et al. 2015). Furthermore, RGS4 mRNA is stabilized by

binding to human antigen R (HuR), which is required for IL1β-induced upregulation of RGS4 in colonic smooth muscle cells (Li, Hu, et al. 2010). IL1β also increases transcription of RGS4 via NF-κB, indicating that the same signal may employ multiple mechanisms to regulate the same RGS protein (Hu et al. 2008). In addition to HuR, RGS4 mRNA is also regulated by the splicing factor transformer-2β (Tra2β), which possibly mediates morphine-induced up-regulation of RGS4 in the brain (Li, Li, et al. 2013), and by the RNA-binding protein staufen2 (Stau2) in neurons (Heraud-Farlow et al. 2013). Taken together, these data demonstrate that RGS4 mRNA is a common target of RNA-binding proteins, and that mRNA stability of RGS proteins can be affected by both miRNAs and RNA-binding proteins (**Figure 2.1**).

To date, there are considerably fewer studies reporting regulation of RGS mRNA stability by miRNA or RNA binding proteins compared to regulation by other mechanisms such as protein degradation. However, due to growing evidence for key roles of RGS proteins, miRNAs, and RNA binding proteins, identifying additional mRNA-targeted mechanisms to control RGS expression in both cancer and the central nervous system is expected. Future studies should also be expanded to the cardiovascular system, where both RGS proteins and miRNAs play many crucial roles (Papageorgiou et al. 2012; Stewart, Huang, and Fisher 2012), to determine the mechanisms by which many important cardiovascular RGS proteins are regulated, and determine whether some of the miRNAs effects in the cardiovascular systems are mediated by targeting RGS proteins.

3.4 Protein Stability.

Degradation of proteins is an essential mechanism employed by cells to control the levels of stable and functional proteins. This degradation commonly occurs via either lysosomal proteolysis or the ubiquitin-proteasome pathway (Lecker, Goldberg, and Mitch 2006; Ciechanover 2005). Lysosomes engulf proteins and utilize digestive enzymes to induce proteolysis (Ciechanover 2005). The other pathway for protein degradation is the ubiquitin-proteasome pathway, where the target protein is polyubiquitinated (Lecker, Goldberg, and Mitch

2006). The polyubiquitinated proteins are recognized by the proteasome complex, which subsequently binds and eventually degrades the target protein (Lecker, Goldberg, and Mitch 2006). This process requires more energy compared to lysosomal degradation and is mediated by multiple enzymes, including ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3)(Myung, Kim, and Crews 2001).

Many studies have focused on RGS4 as a target for proteasomal degradation and the mechanisms have been well defined. RGS4 is targeted by the N-end rule pathway, a pathway that tags proteins for degradation based on the presence of certain residues at their N-termini (Davydov and Varshavsky 2000). Inhibitors of this pathway prevent degradation and ubiquitination of RGS4 in the reticulocyte lysate system (Davydov and Varshavsky 2000). In addition, the proteasome inhibitor MG132 blocked degradation and enhanced the levels of polyubiquitinated RGS4, suggesting that RGS4 is subject to ubiquitination and proteasome degradation in accordance to the N-end rule pathway (Davydov and Varshavsky 2000). Studies also revealed that the arginylation of the cysteine 2 residue (Cys2) at the N-terminus of RGS4 is the trigger for N-end rule pathway activation and subsequent degradation (Davydov and Varshavsky 2000). To determine whether this pathway targets RGS4 in intact mammalian cells, a subsequent study tested this mechanism in embryos and embryonic fibroblasts (EF) isolated from wild type or ATE1-/- animals (Lee et al. 2005). ATE1 encodes Arg-transferase, the enzyme that mediates arginylation resulting in RGS4 proteasome degradation (Lee et al. 2005). Proteasome inhibition or ATE1 depletion significantly increased levels of RGS4 in EF, indicating that arginylation and proteasome degradation regulates RGS4 levels in this cell model as well (Lee et al. 2005). Additionally, knockout of Ub ligases UBR1 and UBR2, which recognize and bind N-terminal Arginine, stabilized RGS4 expression, suggesting that UBR1 and/or UBR2 mediates ATE1-triggered degradation of RGS4 (Lee et al. 2005).

Nitric Oxide also contributes to RGS4 degradation by aiding in oxidizing the N-terminal cysteine residue required for arginylation (Hu et al. 2005). RGS4 proteasome degradation and

the crucial role of Cys2 in destabilizing RGS4 were also confirmed in HEK293 cells, in which using the proteasome inhibitor MG132 or replacing Cys2 with serine resulted in the stabilization of RGS4 (Bodenstein, Sunahara, and Neubig 2007). Interestingly, palmitoylation of Cys2 by acyltransferases protected RGS4 against proteasomal degradation, establishing this residue as an important determinant of RGS4 fate (Wang et al. 2010). An alternative pathway that exclusively targets cytoplasmic RGS4 for degradation and is mediated by the ubiquitin-like modifier activating enzyme UBA6 has also been described (Lee, Sowa, et al. 2011). In addition to RGS4, other RGS proteins are targeted by the N-end rule pathway, including RGS2, RGS5, and RGS16 (Lee et al. 2005; Hu et al. 2005; Bodenstein, Sunahara, and Neubig 2007).

In addition to N-terminal arginylation, the N-end rule pathway can also be initiated via N-terminal acetylation (Hwang, Shemorry, and Varshavsky 2010). In contrast to the RGS4 mechanism, RGS2 proteasomal degradation was found to be stimulated by N-terminal acetylation (Park et al. 2015). Furthermore, unlike RGS4, RGS2 proteasomal degradation does not require ATE-1 and depends on a complex of proteins consisting of cullin 4B (CUL4B), F-box 44 (FBXO44) and DNA damage binding protein 1 (DDB1) (**Figure 2.2**) (Sjogren, Swaney, and Neubig 2015). Therefore, proteasome degradation of RGS proteins can be mediated by different pathways, which provides opportunities to selectively target the degradation of RGS proteins.

RGS protein degradation and stability is regulated by multiple post-translational modifications and protein-protein interactions. Members of the R7 family of RGS proteins, including RGS9 and RGS7, uniquely depend on the binding partner Gβ5 for stabilization (Chen et al. 2003). Additionally, RGS9 Anchor Protein (R9AP) is another binding partner that is essential for RGS9-1-Gβ5 complex stability and membrane association in the retina (Keresztes et al. 2004). Similarly, R7 family binding protein (R7BP) binds and stabilizes RGS9-2 in striatal neurons (as well as other R7 family members)(Anderson, Semenov, et al. 2007). The association between R7BP and RGS9-2 prevents the binding of RGS9-2 and Hsc70 (Heat

shock cognate protein 70), a protein that mediates RGS9-2 degradation (Posokhova, Uversky, and Martemyanov 2010). Interestingly, cysteine protease inhibitors that block lysosomal degradation prevented RGS9-2 degradation, whereas proteasome inhibitors had no effect on RGS9-2 proteolysis, indicating that RGS9-2, unlike many other RGS proteins, is mainly regulated by lysosomal rather than proteasomal degradation (Anderson, Lujan, et al. 2007). Collectively, these findings indicate that association with different binding partners is a common method by which the stability of RGS7 family members is controlled.

Phosphorylation is a common post-translational modification that affects the activity, localization, and stability of many proteins, including RGS proteins. In fact, some RGS proteins are phosphorylated at multiple sites, leading to specific effects on the activity and stability of RGS proteins. For example, RGS16 is constitutively phosphorylated at serine 194 and is dynamically phosphorylated at serine 53 site by α2A-adrenoceptor activation. Both phosphorylation events result in the suppression of RGS16 GAP activity. On the other hand, phosphorylation at RGS16 Tyr 168 enhances both GAP activity (Derrien and Druey 2001) and stability (Derrien et al. 2003), indicating that RGS16 function and levels can be tightly regulated by phosphorylation at multiple sites. Moreover, the same kinase can induce multiple regulatory effects on RGS proteins. For example, protein-kinase A (PKA) was shown to trigger nuclear localization of RGS13, which facilitates the inhibitory effect of RGS13 on CREB-induced transcription in the nucleus (Xie et al. 2008). Additionally, PKA-induced phosphorylation at Thr residue (T41) inhibits the proteasome degradation of RGS13, which indicates that PKA influences both the nuclear function as well as the stability of RGS13 (Xie, Yang, and Druey 2010). Interestingly, RGS10 nuclear localization was also shown to be enhanced by PKA activation, indicating that PKA may affect other RGS proteins in a similar manner (Burgon et al. 2001). Finally, the cGMP-dependent protein kinase cGKIα regulates the stability, GAP activity, and localization of RGS2 (Osei-Owusu et al. 2007). In addition to phosphorylation, RGS

proteins have also been shown to undergo other post-translational modifications such as palmitoylation, but the effects on activity and stability are not fully defined (Hiol et al. 2003).

In summary, protein degradation is a critical regulatory mechanism utilized by cells to maintain physiological levels of RGS proteins (**Figure 2.1**), and degradation-mediated changes in RGS protein levels are implicated in the pathogenesis of several diseases. Further, the previous studies reveal that degradation of RGS proteins is the final result of a complex processes orchestrated by a network of enzymes, binding partners, and post-translational modifications offering multiple prospects for effective and selective therapeutic approaches to control RGS proteins levels.

4. Therapeutic Potential of targeting RGS expression

4.1 Why RGS proteins?

GPCRs are the most common molecular targets of clinically approved drugs, and these receptors can be modulated with extracellular agonists, antagonists, and biased agonists to carefully control downstream G protein pathways (Overington, Al-Lazikani, and Hopkins 2006). Given the enormous clinical success and versatility of therapeutics targeting GPCRs, it is reasonable to ask what benefits can be obtained by targeting RGS proteins that cannot be obtained by targeting GPCRs. A few reasons can be proposed based on the mechanistic differences between GPCRs and RGS proteins in regulating G protein signaling. First, it should be noted that the success of targeting GPCRs is not without limitations. Activation of GPCRs can trigger, in addition to G protein mediated pathways, other pathways mediated by β-arrestins that can induce unintended adverse effects (Shenoy and Lefkowitz 2011). Manipulating RGS protein activity or expression could be used as a strategy to differentially regulate G protein mediated versus G protein independent pathways downstream of GPCRs. For example, morphine-induced activation of G proteins downstream of opioid receptors produces analgesic effects, but morphine also activates the β-arrestin pathway causing desensitization and tolerance (Bohn et al. 1999; Bohn et al. 2000). RGS proteins selectively target G proteins

signaling downstream of opioid receptors, but not β-arrestin mediated signaling (Traynor 2012). Therefore, combining modest receptor agonist treatment with inhibition of RGS proteins would amplify G protein mediated analgesic effects with no added effect on the β-arrestin-mediated responses, which would theoretically allow for lower doses of morphine without compromising effectiveness. In contrast, in other systems, β-arrestin mediated signaling is actually desired, whereas G proteins mediate undesirable effects. For example, the Angiotensin II Type I or AT1 receptor improves cardiac function via β-arrestin-mediated signalling (Ryba et al. 2017), but also causes hypertension through G protein activation (Kawai et al. 2017). Loss of RGS2 causes an enhancement of AT1 receptor-induced G protein activation, resulting in hypertension (Hercule et al. 2007). Therefore, in theory, the combination of AT1 receptor agonists and induction of RGS2 expression should improve cardiac function while suppressing hypertension.

Second, many RGS proteins display highly tissue-specific expression patterns, such as RGS9-1 in the retina (Chen et al. 2000). This suggests that RGS modifying approaches could be coupled with sub-threshold doses of drugs that target more widely expressed GPCRs to achieve greater regional specificity of action and, again, reduce the required dose of the GPCR targeted drug and associated side effects. Finally, it is now well established that many RGS proteins are more than just GTPase-activating proteins (GAPs), which means that targeting RGS proteins can affect pathways that go beyond G protein signaling, providing yet another advantage over targeting GPCRs alone (Sethakorn, Yau, and Dulin 2010). These advantages have fuelled efforts to develop and identify small molecule inhibitors of RGS proteins (Kimple et al. 2011; Roman and Traynor 2011). Aside from directly targeting the activity of RGS proteins, an alternative approach is to target the expression and stability of RGS proteins, which would regulate the GAP-dependent and GAP-independent activities of RGS proteins. Unfortunately, the mechanisms that regulate RGS expression and post-translational stability are generally non-specific, so an ongoing challenge is to establish a therapeutic approach with sufficient specificity. Regardless of these challenges, multiple therapeutics targeting epigenetic,

transcriptional, and post-translational regulation have entered the drug discovery pipeline, some with surprising success.

4.2 Targeting Epigenetic mechanisms

Due to the role of epigenetic-mediated mechanisms in disease progression, efforts are underway in several disease models to therapeutically target epigenetic enzymes to restore the expression of dysregulated genes (Kelly, De Carvalho, and Jones 2010). In cancer, DNA methyl transferase enzymes (DNMTs) mediate silencing of RGS2 and RGS10 in prostate cancer and ovarian cancer, respectively. Restoring the expression of RGS2 or RGS10 can be beneficial, as the suppression of RGS2 is implicated in the progression of prostate cancer (Wolff et al. 2012), and RGS10 silencing contributes to the development of chemoresistance in ovarian cancer cells (Hooks et al. 2010). Inhibition of DNMT by 5-azacytidine successfully restored the expression of RGS2 in prostate cancer and 5-azacytidine-induced inhibition of prostate cancer growth was mediated partially be restoring the expression of RGS2 (Wolff et al. 2012). Likewise, both 5-azacytidine and the HDAC inhibitor TSA restored the expression of RGS10 in chemoresistant ovarian cancer cells (Cacan et al. 2014). These studies suggest that targeting epigenetic processes to restore the expression of RGS proteins may be a future therapeutic approach to slow or stop cancer progression.

Selectivity remains a major concern, as an epigenetic drug can potentially regulate multiple unintended genes that may produce side effects (Kelly, De Carvalho, and Jones 2010). Despite these concerns, a number of epigenetic drugs have been approved or reached clinical trials. The DNMT inhibitor azacytidine for Myelodysplastic syndrome (MDS) (Kaminskas et al. 2005) and additional drugs targeting DNA methylation have been developed and entered clinical trials to treat cancer (Cheng et al. 2003; Brueckner et al. 2005). HDAC inhibitors have also been intensely studied as a potential cancer therapy, and the HDAC inhibitor suberanilohydroxamic acid (SAHA) was FDA approved for cutaneous T-cell lymphoma (Duvic and Vu 2007). Combination therapy of DNMT and HDAC inhibitors is also being studied with encouraging

results (Pathania et al. 2016). Even with this success in developing effective epigenetic drugs, the side effects that they produce due to the dysregulation of unintended genes remain a disadvantage that necessitates better approaches (Heerboth et al. 2014). One approach is to carefully delineate which specific genes mediate the epigenetic drug's effects, and combining other non-epigenetic drugs that target these genes, which allows reducing the dose of the epigenetic drug without compromising the expression of the intended genes. This concept can be applied to RGS proteins as well, and future studies that gauge the extent of RGS protein contribution to the overall effects of epigenetic drugs should aid in developing more selective and safer epigenetic therapies.

4.3 Targeting Transcription Factors:

Traditionally, attempts to control the activity of transcription factors has included small molecules targeted against upstream proteins such as kinases to indirectly influence the activity of transcription factors **(**Kwiatkowski et al. 2014**)**, as well as directly preventing DNA binding of transcription factors to target transcription factors therapeutically (Leung et al. 2013). However, lack of specificity is a major barrier, given that one transcription factor can influence multiple target genes. An alternative approach is to target specific protein-protein interactions in transcriptional regulatory complexes (Reiter, Wienerroither, and Stark 2017). Targeting a transcriptional regulator's ability to bind to a specific complex rather than inhibiting a transcription factor activity altogether will potentially enhance specificity and minimize side effects. Employing small molecules to target protein-protein interactions in transcriptional complexes has shown promise in influencing RGS gene expression. For example, preventing the interaction between CREB and the co-factor P300 using the small molecule KG-501 suppressed CREB-induced RGS2 gene expression (**Figure 2.2**) (Best et al. 2004). Additionally, inhibiting CREB and the co-factor CBP interaction resulted in a reduction in RGS4 expression in cortical neurons (Chekler et al. 2015). These approaches provide proof of concept for targeting protein-protein interaction of transcription factors to control RGS protein gene expression. In

addition to CREB, protein-protein interactions of other transcription factors such as NF-κB, STAT3, and P53 have been targeted successfully by small molecules (reviewed in (Fontaine, Overman, and Francois 2015)); it remains to be determined, however, whether the expression of RGS proteins regulated by STAT3 or P53 is affected by these small molecules. Although promising, targeting protein-protein interactions is an extremely complex approach and much work remains to validate targets and establish this as a viable therapeutic strategy. Thus, more detailed structural studies and an overall better understanding of transcription factor interactions at RGS promoters are required for the development of effective and safe therapeutics to control the transcription of RGS genes.

4.4 Targeting mRNA stability:

The development of miRNA-based therapy is hampered by a plethora of specificity concerns as well as difficulties in ensuring the delivery of stable microRNAs to target tissues and cells (Li and Rana 2014).Yet, quite a few miRNA-targeted therapies are showing promise and some have reached clinical trials (Rupaimoole and Slack 2017). This success continuously ignites the drive to develop strategies to overcome challenging technical difficulties. The effectiveness of microRNA-based therapies, compared to traditionally targeting an individual protein, is attributed to the broad range of effects a single microRNA can have on several targets involved in the same disease. This is clearly demonstrated in different cancers as the expression of microRNAs influence many genes involved in regulating apoptosis and cell cycle, which effectively induces cell death (Garzon et al. 2009; Chang et al. 2007). Interestingly, microRNA-induced regulation of RGS proteins is often accompanied by regulating other relevant genes commonly implicated in the same disease. For example, in addition to RGS2, miR-22 down-regulates HDAC4 and Rcor1, and all three genes are implicated in Huntington's disease (Seredenina, Gokce, and Luthi-Carter 2011; Mielcarek et al. 2011; Zuccato et al. 2007; Jovicic et al. 2013). Similarly, miR-181a targets Tgfbi (Tgf-beta induced), TβR-I/Alk5 (TGF-β type I receptor) and Gata6 alongside regulating RGS4, which together promote osteoblastic

differentiation (Bhushan et al. 2013). This suggests that utilizing microRNAs to target RGS proteins for treatment dictates a more in-depth investigation of both the global effects of microRNAs as well as the role that the RGS protein plays within the network of targets implicated in the disease. Recently, an interesting study tested the effect of an antisense oligonucleotide (ASO7) targeted against ataxin 2 (ATXN2) in mouse models of spinocerebellar ataxia type 2 (SCA2) (Scoles et al. 2017). ASO7 suppressed ATXN2 mRNA and increased RGS8 mRNA and other genes implicated in SCA2, which significantly delayed the development of SCA2 (Scoles et al. 2017). This study provides additional evidence that RGS8 mRNA is targeted by the RNA-binding protein ATXN2, and further suggests that targeting this mechanism can be therapeutically beneficial. Overall, targeting stability of RGS mRNAs is indeed promising, but more studies to identify additional miRNAs and RNA-binding proteins that regulate RGS mRNAs are needed to seriously advance mRNA-targeted therapeutic approaches.

4.5 Targeting Protein Stability:

The N-end rule protein degradation pathway has been established as a key regulatory process of RGS4 expression, follow up studies have aimed to determine the effect of inhibiting RGS4 proteasomal degradation on cellular processes and/or disease progression. RGS4 suppresses breast cancer cell migration and invasion *in-vitro*, as well as tumor invasiveness *in vivo* (Xie et al. 2009). More importantly, RGS4 protein levels are suppressed in breast cancer cells and tissues compared to normal cells (Xie et al. 2009). Interestingly, the proteasome inhibitor MG132 inhibited breast cancer cell invasion and migration, but this inhibition was blocked by RGS4 knockdown, indicating that MG132 inhibition of breast cancer cell migration and invasion is due to its ability to block RGS4 degradation (Xie et al. 2009). A similar study also tested the effect of pristimerin, a natural compound that has been reported to inhibit proteasome, on breast cancer cell migration and invasion (Mu et al. 2012). Pristimerin inhibited breast cancer migration and invasion, and this inhibition was also lost by RGS4 knockdown (Mu et al. 2012). Similarly, blocking RGS4 proteasomal degradation also affects progression of renal

dysfunction; RGS4 KO animals display impaired renal functions and are more prone to renal injuries (Siedlecki et al. 2011). Treating the animals experiencing renal injury with the proteasomal inhibitor MG132 significantly improved renal functions in WT, and not RGS4 KO animals, again suggesting that inhibiting proteasomal degradation of RGS4 can influence disease pathogenesis (Siedlecki et al. 2011).

These studies provide encouraging results indicating that manipulating protein stability to restore dysregulated expression of RGS proteins can be considered as a viable therapeutic approach. However, proteasome degradation is a common regulatory mechanism for numerous proteins and it was unsurprising that the first FDA approved proteasome inhibitor caused dose-limiting side effects (Chen et al. 2011). Proteasome degradation of proteins is the final result of multiple events mediated by different proteins, which can be targeted for more selective approaches (Ravid and Hochstrasser 2008). In the case of RGS4, for example, instead of inhibiting global proteasome degradation to restore RGS4 expression, an alternative approach is to inhibit the N-end rule pathway that directly participates in the degradation of RGS4. Indeed, the N-end rule pathway inhibitor RF-C11 significantly stabilized RGS4 in mammalian cells with no significant cytotoxicity (Jiang et al. 2013). The substituted amphetamine compound para-chloroamphetamine has been shown to cross the blood brain barrier, where it inhibits arginylation and the N-end rule pathway, thus stabilizing RGS4 expression in the central nervous system (Jiang et al. 2014). Added selectivity can be achieved by identifying specific proteins that mediate degradation of only a specific subset of proteins that undergo N-end rule mediated degradation. An example is arginyltransferase (ATE-1), which mediates the degradation of RGS4 (Lee et al. 2005) but not RGS2 (Sjogren, Swaney, and Neubig 2015).Tannic acid and merbromin, small molecule inhibitors of ATE-1, stabilized RGS4 expression in cells, suggesting that selective inhibition of RGS protein proteasome degradation is possible (Saha et al. 2012). Taken together, the previous studies suggest that stabilizing RGS protein expression *in vitro* and *in vivo* by targeting the N-end rule pathway is not only

achievable, but also moderately safer and more selective compared to inhibiting global proteasome degradation. Future studies aiming to identify additional proteins that differentially regulate the RGS protein degradation, such as ATE-1, will potentially provide more targets for selective drugs.

Several drugs regulate RGS protein expression by influencing proteasome degradation, which can possibly mediate the drugs' intended or unintended effects. For example, opioid receptor agonists and subsequent $G\alpha_{o/i}$ activation down-regulate RGS4 (Wang and Traynor 2011) and RGS20 (Pagano et al. 2008) by activating the proteasome degradation process. Additionally, proteasome degradation of RGS proteins can be affected by several other stimulants, including inflammatory molecules (Benzing et al. 1999) and cardiotonic steroids (Sjogren et al. 2012). As discussed earlier, phosphorylation by some kinases, such as Src (Derrien et al. 2003) and PKA (Xie, Yang, and Druey 2010), also regulate proteasome degradation of RGS proteins. Therefore, inhibitors of these kinases can potentially be utilized to influence proteasome degradation of RGS proteins. Identifying drugs that control RGS protein expression by influencing their proteasome degradation will possibly provide more therapeutic options in conditions where controlling RGS protein expression is needed. Alternatively, recognizing that certain drugs influence RGS protein degradation might explain some of the drug's side effects and aid in developing approaches that mitigate these unwanted effects.

5. Summary and Conclusions

Targeting RGS proteins shows promise *in vitro* and *in vivo*, and RGS-targeted approaches can be used in combination with GPCR-targeted drugs or other drugs to minimize side effects or enhances effectiveness. However, the development of agents targeting RGS protein activity is understandably slow due to the difficulty of targeting RGS interactions with G proteins. Because the expression of RGS proteins is often dysregulated in diseases, an alternative approach is to manipulate the expression of RGS proteins, which allows for tuning their expression to the desired levels. Several epigenetic, transcriptional, and post-translational

mechanisms control the ultimate level of cellular RGS proteins, offering multiple opportunities for targeting (**Figure 2.1**). Indeed, many studies that tested the effect of targeting these mechanisms on RGS levels and disease progression reported promising results on both activity and safety. However, each of these mechanisms is inherently non-selective, and targeting any single regulatory mechanism is unlikely to provide sufficient selectivity to be therapeutically viable. Rather, strategies to regulate RGS expression and protein levels may be most effective when combined with complementary receptor-targeted approaches, or when multiple mechanisms targeting expression of a single RGS are simultaneously targeted for synergistic regulation. For example, the ultimate level of RGS2 protein in a cell reflects regulation by epigenetic enzymes, transcription factors, miRNAs, and proteasomal degradation (**Figure 2.2**). This indicates that targeting a combination of these mechanisms would enhance the effectiveness of regulating RGS2 levels, and simultaneously allow for lower doses, which will potentially reduce toxicity. Thus, it is essential to understand the network of regulatory mechanisms that ultimately control the expression of RGS proteins in disease states in order to design the appropriate interventions.

Table 2.1: Transcription factors shown to regulate RGS gene transcription

Transcription Factor	RGS gene Regulated	Cells	Type of Regulation	References
CREB	RGS2	vascular smooth muscle cells	Activation	(Xie et al. 2011)
	RGS4	Cortical neurons	Activation	(Chekler et al. 2015)
AP-1	RGS4	Colonic muscle cells	Repression	(Zhang, Li, et al. 2012)
NF-κB	RGS4	colonic muscle cells	Activation	(Hu et al. 2008)
	RGS16	B lymphocytes	Activation	(Li et al. 2001), (Xie, Li, et al. 2010)
STAT3	RGS2	cardiac myocytes	Activation	(Yue et al. 2010)
	RGS7	A1A1v cortical cells	Activation	(Singh et al. 2007)
P53	RGS16	human EB1 colon cancer cells	Activation	(Buckbinder et al. 1997)
	RGS13	B cells, mast cells	Repression	(Iwaki et al. 2011)
CREM	RGS5	vascular smooth muscle cells	Repression	(Seidl et al. 2015)
GATA-6	RGS4	Colonic muscle cells	Activation	(Zhang et al. 2017)
Phox2b	RGS4	cranial motor and sensory neurons	Activation	(Grillet et al. 2003)
YY1	RGS16	neonatal rat cardiac myocytes	Repression	(Stuebe et al. 2008)
Bcl6	RGS4	neuron-like PC6 cells	Repression	(Yang et al. 2010)
C/EBPβ	RGS4	neuron-like PC6 cells	Activation	(Yang et al. 2010)
NF-YA	RGS4	neuron-like PC6 cells	Activation	(Yang et al. 2010)

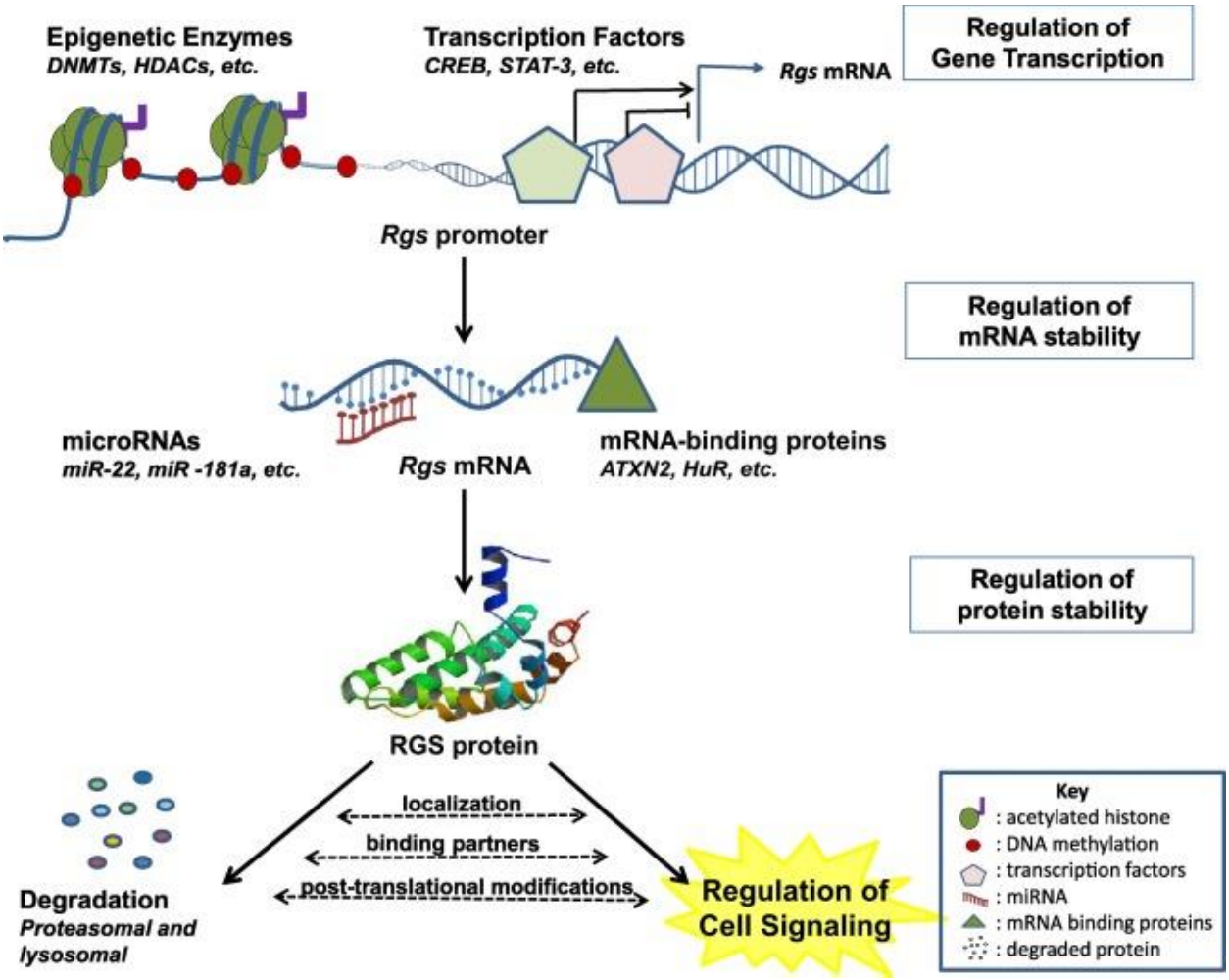

Figure 2.1: RGS gene expression and protein stability are regulated by multiple mechanisms.

Multiple regulatory mechanisms participate in determining the level of RGS proteins. Epigenetic modifications, mainly histone deacetylation and DNA methylation that are mediated by histone deacetylases (HDACs) or DNA methyltransferases (DNMTs), tighten the chromatin structure at RGS genes promoters, thereby obstructing the access of transcription factors and other proteins that are essential for transcription initiation, ultimately resulting in suppression of RGS gene expression. Multiple transcription factors directly bind RGS genes promoters to activate or repress transcription, adding another layer of regulation to the expression of RGS genes. In addition to transcription regulation, RGS mRNAs are targeted by both microRNAs and mRNA-binding proteins to either degrade or stabilize the respective mRNA, which critically determines the final levels of translated RGS proteins. Finally, active RGS proteins participate in various G protein dependant and independent signaling pathways in different cellular compartments. The activity and stability of RGS proteins is influenced by post-translational modifications such as phosphorylation, association with specific binding partners, and cellular localization of RGS proteins. Many RGS proteins undergo proteasomal degradation while some are degraded via lysosomal degradation. Regardless, this degradation is a critical step of regulation that ultimately governs the level of active cellular RGS proteins at a given time.

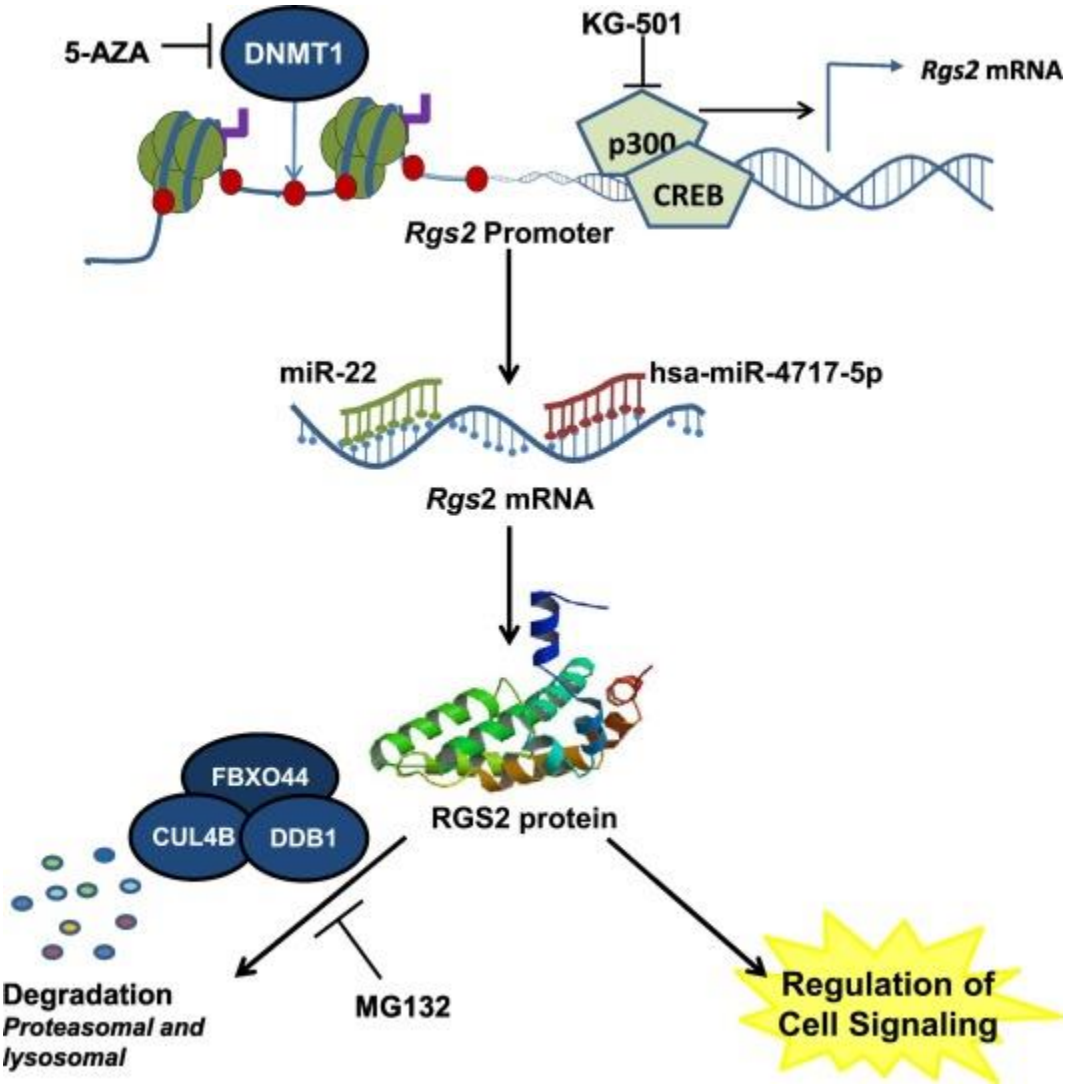

Figure 2.2: RGS2 protein levels are determined by multiple regulatory mechanisms, several of which can be manipulated by small molecules.

RGS2 transcription is suppressed by the DNA methyltransferase 1 (DNMT1) enzyme and activated by the transcription factor CREB. Accordingly, the DNMT1 inhibitor 5-AZA enhances RGS2 transcription, whereas inhibiting CREB-mediated transcription using the small molecule KG-501 results in suppressed transcription. In addition to transcriptional regulation, RGS2 is also regulated at the mRNA levels, by miR-22 and has-miR-4717-5p, and at the protein level, by proteasome-mediated degradation with the assistance of other proteins such as FOXO44, CUL4B, and DDB1. Because many of these regulatory mechanisms are usually not selective and can influence other proteins, manipulating RGS2 expression by combining drugs that targets multiple mechanisms of regulation is potentially more advantageous.

CHAPTER 3

REGULATOR OF G-PROTEIN SIGNALING 10 (RGS10) EXPRESSION IS TRANSCRIPTIONALLY SILENCED IN ACTIVATED MICROGLIA BY HISTONE DEACETYLASE ACTIVTIY[2]

[2] Alqinyah, M., N. Maganti, M. W. Ali, R. Yadav, M. Gao, E. Cacan, H. R. Weng, S. F. Greer, and S. B. Hooks. 2017., *Mol Pharmacol*, 91: 197-207.

Abstract

RGS10 has emerged as a key regulator of pro-inflammatory cytokine production in microglia, functioning as an important neuroprotective factor. While RGS10 is normally expressed in microglia at high levels, expression is silenced *in vitro* following activation of TLR4 receptor-activation. Given the ability of RGS10 to regulate inflammatory signaling, dynamic regulation of RGS10 levels in microglia may be an important mechanism to tune inflammatory responses. The goals of the current study were to confirm that RGS10 is suppressed in an *in vivo* inflammatory model of microglial activation and to determine the mechanism for activation-dependent silencing of *Rgs10* expression in microglia. We demonstrate that endogenous RGS10 is present in spinal cord microglia, and RGS10 protein levels are suppressed in the spinal cord in a nerve injury induced neuropathic pain mouse model. We show that the HDAC enzyme inhibitor Trichostatin A blocks the ability of LPS to suppress *Rgs10* transcription in BV-2 and primary microglia, demonstrating that HDAC enzymes are required for LPS silencing of *Rgs10*. Further, we used chromatin immunoprecipitation to demonstrate that H3 histones at the *Rgs10* proximal promoter are deacetylated in BV-2 microglia following LPS activation, and HDAC1 association at the *Rgs10* promoter is enhanced following LPS stimulation. Finally, we have shown that sphingosine 1-phosphate, an endogenous microglial signaling mediator which inhibits HDAC activity, enhances basal *Rgs10* expression in BV-2 microglia, suggesting that *Rgs10* expression is dynamically regulated in microglia in response to multiple signals.

Introduction

Microglia are central nervous system (CNS)-resident macrophages that serve protective functions to combat infection and clear cellular debris, as well as developmental functions including synaptic pruning (Gehrmann, Matsumoto, and Kreutzberg 1995; Stevens et al. 2007; Trang, Beggs, and Salter 2011). In addition to these normal physiologic functions, dysregulated microglial activation has been implicated in the initiation and progression of neurodegenerative disorders such as multiple sclerosis, Alzheimer's disease, and Parkinson's disease (Fu et al. 2014b) and in neuropathic pain (Trang, Beggs, and Salter 2011). Identifying signaling pathways regulating microglial functions bears significance in the development of strategies for the treatment of such neurological disorders.

Regulator of G-Protein Signaling 10 (RGS10)[1] has emerged as an important anti-inflammatory regulator in microglia. RGS10 is a member of the RGS superfamily of proteins that deactivate heterotrimeric G-proteins, with profound effects on G-protein coupled receptor (GPCR) signaling in neural diseases (Hurst and Hooks 2009a; Nishiguchi et al. 2004; Zachariou et al. 2003; Vellano et al. 2011; Okahisa et al. 2011). RGS proteins are a highly diverse group of proteins that regulate signaling pathways downstream of GPCRs. The classic role of RGS proteins is to regulate the duration and amplitude of G-protein signaling through their ability to function as GTPase activating proteins (GAPs) to accelerate the deactivation of G-proteins by increasing the rate of GTP hydrolysis (Posner et al. 1999). RGS10 selectively deactivates Gi family G-proteins (Hunt et al. 1996), and it is expressed at high levels in the brain (Gold et al. 1997) and immune tissues (Haller et al. 2002), with specific enrichment in microglia (Waugh et al. 2005).

Recent studies suggest that RGS10 protein in microglia serves to suppress microglial activation, proliferation, and Nuclear Factor-κB (NF-κB) activity downstream of Toll-like receptor 4 (TLR4) receptors, and loss of RGS10 enhances microglial mediated neuroinflammation and neurotoxicity. RGS10 knock-out mice display significantly more activated microglia in brain

tissue (Lee et al. 2008). Further, in a mouse model of Parkinson's disease, RGS10 knock-out animals display exacerbated dopaminergic neuron cell death compared to wildtype animals. The anti-inflammatory role of microglial RGS10 is also observed and consistently modeled in the mouse microglial cell line BV-2 (Henn et al. 2009). Knock-down of RGS10 in BV-2 cells enhances activity of the transcription factor NF-κB and increases expression of inflammatory cytokines including Tumor Necrosis Factor α (TNF-α) and Interleukin 1β (IL-1β) in response to the classic TLR4 activator lipopolysaccharide (LPS) (Henn et al. 2009). In complementary experiments, RGS10 overexpression suppressed microglial activation, pro-inflammatory cytokine release, and inflammatory neurotoxicity, and inhibited activation of microglial NF-κB (Lee et al. 2008; Lee, Chung, et al. 2011). Therefore, RGS10 is an important regulator of inflammatory signaling in microglia *in vivo* and *in vitro*, and changes in RGS10 levels have significant effects on microglial inflammatory signaling.

RGS10 is normally expressed in microglia at high levels, but Lee and colleagues have reported that RGS10 protein levels are markedly reduced following microglial activation by LPS or TNFα in microglia (Lee et al. 2008). Given the ability of RGS10 to regulate inflammatory signaling, understanding the mechanisms that control RGS10 levels in microglia may reveal new therapeutic strategies to address neuroinflammatory disease. The most commonly described mechanism for regulation of RGS protein abundance involves critical post-translational mechanisms that control protein stability (Raveh et al. 2014; Sjogren et al. 2012). However, our studies in ovarian cancer cells suggest that *Rgs10* is transcriptionally regulated by DNA and histone targeted epigenetic mechanisms (Ali et al. 2013b; Cacan et al. 2014). It is unclear whether and how expression of *Rgs10* in microglia is regulated via epigenetic mechanisms. Further, it is unknown whether *Rgs10* expression can be suppressed by pathological microglial activation and neuroinflammation *in vivo*.

The goals of the current study were to determine if RGS10 is suppressed in an *in vivo* neuroinflammatory model of microglial activation and to determine the mechanism for activation-

dependent silencing of *Rgs10* expression in microglia. We observed significant loss of RGS10 in the spinal dorsal horn following nerve injury-induced inflammation, and we define a role for Histone deacetylase (HDAC) regulation of histone acetylation at the *Rgs10* promoter following activation with LPS.

MATERIALS AND METHODS

Cells and Reagents

The murine microglial BV-2 cell line is a well-established model widely used to study microglial functions (Henn et al., 2009) and was a generous gift from Dr. George Hasko at University of Medicine and Dentistry of New Jersey. The BV-2 cell line was generated by infecting primary microglial cell cultures with a v-raf/v-myc oncogene carrying retrovirus (J2) (Blasi et al., 1990). BV-2 cells were maintained in Dulbecco's Modified Eagle's Medium (ATCC) supplemented with 10% FBS (PAA Laboratories, Inc.). Lipopolysaccharide (LPS), H89 dihydrochloride, Trichostatin A (TSA), and 5-Aza-2′-deoxycytidine were obtained from Sigma-Aldrich (St. Louis, MO). Sphingosine 1-phosphate was obtained from Avanti Polar Lipids (Alabaster, Alabama). TNF-α Antagonist III, R-7050 was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). For western blot analysis, the following antibodies were used: phospho p44/p42-ERK (Cell Signaling), total ERK(Cell Signaling), anti-RGS10 antibody (C10, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). For ChIP experiments, we used anti-histone H3 and anti-acetylated histone H3 (Millipore, Lake Placid, NY), and anti-HDAC1 (Santa Cruz Biotechnology, Santa Cruz, CA).

Quantitative real-time PCR

mRNA was isolated using Trizol reagent (Invitrogen/Life Technologies, Carlsbad, CA) and cDNA was synthesized from 2 µg of total RNA using the High Capacity Reverse Transcriptase cDNA kit (Applied Biosystems/ Life Technologies). Quantitative real-time

polymerase chain reaction was performed using Superscript III kit for RT-PCR (Invitrogen/Life Technologies, Carlsbad, CA) and Power SYBR Green reagent (Applied Biosystems). Reactions were normalized using the housekeeping gene (actin and/or GAPDH, as indicated) and calculations were performed according to the $2^{-\Delta\Delta CT}$ method. Fold change in expression was determined in triplicate in three independent experiments. Primers used were based on algorithm-generated sequences from Primer Bank (http://pga.mgh.harvard.edu/primerbank/). RGS10 Forward: CCT GGA GAA TCT TCT GGA AGA CC, RGS10 Reverse: CTG CTT CCT GTC CTC CGT TTT C, TNFα Forward: CCT GTA GCC CAC GTC GTA G, TNFα Reverse: GGG AGT AGA CAA GGT ACA ACC C, IL1β Forward: GAA ATG CCA CCT TTT GAC AGT G, IL1β Reverse: TGG ATG CTC TCA TCA GGA CAG, Actin Forward: GGC TGT ATT CCC CTC CAT CG, Actin Reverse: CCA GTT GGT AAC AAT GCC ATG T, GAPDH forward: TGG CCT TCC GTG TTC CTA C, GAPDH reverse: GAG TTG CTG TTG AAG TCG CA. All primers were purchased from Integrated DNA Technologies, IDT (Coralville, IA).

Western Blot Analysis

To evaluate protein expression in cell lines, 10^5 cells were lysed in SDS-PAGE sample buffer. The lysates were boiled for five minutes and analyzed using SDS-PAGE. Membranes were incubated with primary antibodies and appropriate HRP-conjugated rabbit secondary antibodies (ThermoScientific, Pierce) and visualized using ECL reagents (ThermoScientific, Pierce). Membranes were subsequently blotted with GAPDH antibodies (Millipore Technologies) as a loading control and quantified using ImageJ 1.46 software (NIH).

To analyze RGS10 protein expression in the spinal dorsal horn, mice were deeply anesthetized with urethane (1.3–1.5g/kg, i.p.). The L4 to L5 spinal segment was exposed by surgery and removed from the mice, and the animals were then euthanized by cervical dislocation. The dorsal quadrant of the spinal segment ipsilateral to the nerve injury site or sham operation site was isolated. The isolated tissues were quickly frozen in liquid nitrogen and

stored at −80 °C for later use. The frozen tissues were homogenized with a hand-held pellet in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% deoxycholic acid, 2 mM orthovanadate, 100 mM NaF, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, 100 IU ml^{-1} aprotinin) for 30 minutes at 37°C. The samples were then centrifuged for 20 min at 12,000 *g* at 4°C and the supernatants containing proteins were collected. The quantification of protein contents was made by the BCA method. Protein samples (40 μg) were electrophoresed in 8 % SDS polyacrylamide gels and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membranes were blocked with 5% milk and incubated overnight at 4°C with antibodies against RGS10 (1:1000, C10, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or β-actin (1:2000, Sigma-Aldrich, St. Louis, USA) as a loading control. The blots were then incubated for 1 hr at room temperature with the corresponding HRP-conjugated secondary antibody

1:5000; Santa Cruz Biotechnology, CA, USA), visualized in ECL solution (SuperSignal West Pico Chemiluminescent Substrate, Pierce, Rockford, IL, USA) for 1 min, and exposed onto FluorChem HD2 System. The intensity of immunoreactive bands was quantified using ImageJ 1.46 software (NIH). Results were expressed as the ratio of each marker over β-actin control.

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays were performed as described in Cacan et al., 2014 (Cacan et al. 2014). Briefly, BV-2 cells were plated at a density of 2.50 x 10^6 in 10 cm-tissue culture plates and the next day the cells were treated with vehicle or LPS (100ng/mL) for 24 hours. Cells were harvested and crosslinked with 1% formaldehyde at room temperature. The crosslinking reaction was stopped by the addition of 0.125 M glycine and cell nuclei were isolated and concentrated by lysing in fresh SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8.0, dH_2O) including protease inhibitors followed by flash freezing in liquid nitrogen. Nuclei were sonicated using a Bioruptor water bath sonicator to generate an average of 500-600 bp of sheared DNA. Sonication efficiency was confirmed by subjecting lysates to 1% agarose gel

electrophoresis. Sonicated lysates were then precleared with salmon-sperm/agarose beads (Upstate) and 5% of the total lysate was stored as input for normalization. Half of the remaining lysate was immunoprecipitated with 5µg of indicated antibody overnight at 4°C and the other half was immunoprecipitated with control antibody. Following an additional two hour immunoprecipitation with 60µl of salmon-sperm coated agarose beads, all samples were washed with each of the following buffers: low salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris pH 8.0, 150mM NaCl, dH2O), high salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris pH 8.0, 500mM NaCl, dH2O), LiCl (0.25M LiCl, 1% NP40, 1% DOC, 1mM EDTA, 10mM Tris pH 8.0, dH2O), and 1xTE. DNA was eluted with SDS elution buffer (1% SDS, 0.1M NaHCO3, dH2O). Following elution, cross-links were reversed overnight with 5M NaCl at 65°C and immunoprecipitated DNA was isolated using phenol:chloroform:isopropanol mix (Invitrogen/Life Technologies, Carlsbad, CA) as per the manufacturer's instructions. Isolated DNA was quantified by Real time PCR on an ABI prism 7900 (Applied Biosystems, Foster City, CA) using the following primers and probe for RGS10: forward, 5'-ACC CAA GTG TCG TCC AAG TTA-3', reverse, 5'-TGG AGC CTC TGC GGT TTC-3' and probe, 5'- TGC TGG CGC GCT CAG ATC CA -3'; and for GAPDH: forward, 5'- CCA TCC GGG TTC CTA TAA ATA CG -3', reverse, 5'- CGG CCG TCT CTG GAA CA -3' and probe, 5'-CTG CAG CCC TCC CTG GTG CTC TCT-3'. Values generated from Real time PCR reactions were calculated based on standard curves generated, were run in triplicate reactions, and were analyzed using the SDS 2.0 program. RT-PCR experiments were analyzed with both actin and GAPDH internal controls, with similar results.

Mice

C57BL/6J mice were obtained from Harlan laboratories. Male mice of 6–8 week old were used for partial sciatic nerve ligation and sham operation. Primary microglia cultures were prepared from cerebral cortexes of 2 day old neonatal mice pups. Protocols were approved by the Institutional Animal Care and Use Committee at the University of Georgia and were fully compliant with the National Institutes of Health Guidelines for the Use and Care of Laboratory Animals.

Primary microglia isolation

Total glial cultures were isolated from mouse pups at postnatal day 2 (P2) by dissecting total forebrain, removing meninges, dissociating with trypsin, and isolating individual cells by passage over a 40 µm filter. Total cell isolates were plated in T-75 flasks (~three brains per flask), and then the mixed cultures were grown for 14 days in Eagle's Mimimum Essential Medium (MEM, obtained from ATCC) supplemented with 10% FBS, L-glutamine (glutamax), 4.5 mg/mL glucose, and penicillin/streptomycin. Microglia were isolated by shaking the confluent mixed glial cultures at 250 rpm for 1.5 hours. The supernatant containing microglia was removed, passed through a 35 µm screen, and plated on poly-D-lysine coated 6 well plates (Levison and McCarthy 1991; Suzumura et al. 1987).

Immunofluoresence.

Mice were deeply anesthetized with urethane (1.3-1.5 g/kg,i.p.) and transcardially perfused with heparinized phosphate-buffered saline solution (0.1 M PBS) pH = 7.35 followed by a solution of 4% formaldehyde in (0.1 M PBS) pH = 7.35. The L4 and L5 spinal cord was removed and fixed for next 24 hours at 4 °C in fresh 4% formaldehyde. L4- L5 spinal lumbar region was dehydrated with gradient ethanol, and embedded in paraffin. The transverse sections of the spinal cord were sliced at a 10 µm thickness and mounted on microscope slides. After Paraffin-embedded sections were deparaffinized using xylene and ethanol washes, and

antigen retrieval treatment was performed by incubating slides in 50 mM sodium citrate (pH8.0) at 80ºC for 30 minutes. Slides were then allowed to cool to room temperature and washed in water and PBS. Tissue was blocked and permeabilized in 0.1X PBS containing 0.1% Triton, 0.5% BSA, and 5% horse serum for 2 hours at room temperature. Primary antibodies (RGS10-1:50; Iba1-1:250) were incubated with the tissue in 0.1X PBS containing 0.5% BSA and 5% horse serum overnight at 4ºC. Following washes in PBS, secondary antibodies (anti-goat 594-fluorophore and anti-rabbit 488-flurophore, both at 1:1000) were incubated with the tissue in PBS for 2 hours at room temperature. Following final washes in PBS, slides were mounted using ProLong Gold with Dapi (ThermoScientific). Slides were imaged using an Olympus U-CMAD3 camera and the Olympus-cell Sens Dimensions software. Exposure and contrast settings were held constant for all images. No manipulation of images was performed, except that the 10X image in figure 3.2A represents a composite of two images of the same section (one left, one right) to display the entire dorsal surface of the spinal cord.

pSNL animal model and behavioral tests.

8-week old male wild type C57BL/6J mice were subjected to either pSNL or SHAM surgery groups as described (Seltzer, Dubner, and Shir 1990) Briefly, the left sciatic nerve at the upper thigh was exposed and tightly ligated with 5-0 silk sutures to approximately one-third to one-half its original thickness, and the wound closed with muscle sutures and skin staples. The sham surgery group was subjected to surgical exposure of the sciatic nerve, but without ligation. Behavioral tests were used to determine the development of neuropathic pain in animals with pSNL. Briefly, behavioral tests were conducted in a quiet room with the room temperature at 22°C. To test possible changes in mechanical sensitivity, animals were placed on a wire mesh, loosely restrained under a plexiglass cage ($12 \times 20 \times 15$ cm^3) and allowed to acclimate for at least 30 min for rats and 1.5 h for mice. A series of von Frey monofilaments (bending force from 0.07 to 2.00 g) were tested in ascending order to generate response-frequency functions for each animal. Each von Frey filament was applied 5 times to the mid-plantar area of each hind

paw from beneath for about 1 s. The response-frequency [(number of withdrawal responses of both hind paws/10) × 100%] for each von Frey filament was determined. Withdrawal response mechanical threshold was defined as the lowest force filament that evoked a 50% or greater response-frequency. This value was later averaged across all animals in each group to yield the group response threshold.

Statistical Analysis

All quantitative data shown are compiled from three independent experimental repeats each performed in duplicate unless otherwise noted. Data were analyzed for statistical differences using an analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison test or Tukey's test between groups. *$p < 0.05$ **$p < 0.01$ and ***$p < 0.001$ indicate the levels of significance.

RESULTS

Suppression of RGS10 expression enhances GPCR signaling in BV-2 microglia

Previous studies have demonstrated the effect of RGS10 suppression on multiple inflammatory signaling pathways in microglia. To demonstrate the effect of RGS10 suppression on a classic GPCR signaling pathway, we treated BV-2 microglial cells with control or RGS10-targeted siRNA, and determined the effect of CXCL12/Sdf-1 stimulated ERK Map kinase phosphorylation. Knock-down of RGS10 by 70% had no effect on basal MAP kinase phosphorylation, but significantly ($p=0.045$) increased CXCL12 stimulated MAP kinase phosphorylation (**Figure 3.1**). This suggests that endogenous mechanisms that control the level of RGS10 expression may significantly impact the strength of GPCR signaling pathways.

LPS silencing of RGS10 transcript and protein expression in BV-2 microglia

RGS10 protein levels are reportedly suppressed in microglia following activation by LPS or TNF-α, but early studies suggested that the effect may not follow a typical dose-response (Lee et al. 2008), and the effect on transcription of *Rgs10* was not defined. To further characterize RGS10 suppression, we analyzed *Rgs10* transcript and RGS10 protein levels after treating mouse microglial BV-2 cells with increasing doses of LPS using qRT-PCR and western blotting, respectively. LPS treatment resulted in suppression of both *Rgs10* transcript (measured at 4 hours, **Figure 3.2A**) and RGS10 protein (measured at 24 hours, Figure 2B) in a dose-dependent fashion, with maximal effects observed at 1 µg/mL. We simultaneously measured LPS induced TNFα inflammatory cytokine transcript production to confirm robust microglial activation (**Figure 3.2A**, lower panel). The fold change and dose dependency of suppression of *Rgs10* transcript expression was very similar to protein expression changes, suggesting a primarily transcriptional mechanism of silencing. A time course of LPS treatment (100 ng/mL) showed *Rgs10* transcript was maximally inhibited at approximately 6 hours and remained suppressed up to 72 hours (**Figure 3.2C**). These results demonstrate activation of microglia by

LPS triggers transcriptional suppression of *Rgs10* while enhancing pro-inflammatory cytokine expression.

Silencing of endogenous RGS10 in an in vivo inflammatory model of microglial activation

Microglial activation and, specifically, endogenous activation of microglial TLR4 receptors are directly implicated in the development of pain sensitization in the mouse partial sciatic nerve ligation (pSNL) model of neuropathic pain (Raghavendra, Tanga, and DeLeo 2003; Bettoni et al. 2008). This suggests that the TLR4 mediated suppression of RGS10 we have observed *in vitro* may occur endogenously in the spinal cord following pSNL injury. To test this prediction, we first confirmed RGS10 levels in resident microglia of the spinal dorsal horn. Immunofluoresence staining with RGS10 and Iba1 microglial marker show robust RGS10 abundance in the spinal cord, and clear co-localization in Iba-1 positive cells with distinct microglial morphology (**Figure 3.3A**). We next performed SHAM or pSNL surgeries on three mice each, and assessed RGS10 protein levels in the spinal dorsal horn three days post surgery, at which time robust microglial activation occurs (Weng, Gao, and Maixner 2014). Baseline pain sensitivity was determined immediately prior to surgeries, and again three days after surgery immediately before sacrifice to confirm the development of pain sensitization in the pSNL animals (data not shown). The L4-L5 region of the spinal cord was isolated and total lysates were subjected to western blotting with RGS10 and β-actin control antibodies. Our results show an approximately 50% reduction in total RGS10 level in the spinal cord of pSNL animals, suggesting that RGS10 silencing occurs *in vivo* under conditions that trigger endogenous microglial activation (**Figure 3.3B**).

The role of DNMT and HDAC enzymes in LPS-mediated RGS10 silencing in microglia

We previously reported that the *Rgs10* gene is epigenetically suppressed in chemoresistant ovarian cancer cells via both DNA Methyl Transferase (DNMT) and Histone

Deacetylase (HDAC) activities (Hooks et al. 2010). However, the development of chemoresistance and the accompanying suppression of RGS10 reflects chronic culturing conditions that cause global gene expression changes, and it is therefore unclear whether the acute silencing of *Rgs10* observed in microglia in response to LPS may involve distinct mechanisms. To test whether *Rgs10* expression is regulated by DNMT enzymes in BV-2 cells following acute LPS treatment, we analyzed *Rgs10* transcript and RGS10 protein expression following pharmacological inhibition of DNMT enzymes. BV-2 cells were treated with vehicle or LPS (10 ng/mL) with or without co-treatment with 5-Aza (10 μM). In experiments measuring transcript, cells were treated with LPS for six hours; in experiments measuring protein expression, cells were treated with LPS for 48 hours. In both experiments, either 5-Aza or vehicle control was added to cells 1 hour before LPS and then maintained in media during the LPS treatment. Under these conditions, 5-Aza treatment did not alter basal or LPS-treated *Rgs10* transcript or RGS10 protein expression levels (**Figure 3.4A, B**), suggesting that DNMT enzymes do not play a major role in regulating *Rgs10* expression in BV-2 microglia under these conditions.

To test whether *Rgs10* expression is regulated by HDAC enzymes in BV-2 cells, we analyzed *Rgs10* transcript expression following pharmacological inhibition of HDAC enzymes using the HDAC inhibitor, trichostatin A (TSA). Basal *Rgs10* transcript expression was increased in BV-2 cells in a dose dependent fashion following 24 hour TSA treatment with maximal effect observed at 250 nM, suggesting that basal *Rgs10* gene expression is epigenetically regulated through HDAC activity (**Figure 3.5A**). Next, we tested whether TSA could block LPS-induced *Rgs10* suppression in BV-2 cells. To that end, we treated BV-2 cells with vehicle or LPS (10 ng/mL) for six hours following pre-treatment with TSA (100 nM) for one hour. TSA was also maintained in the cell media during the LPS treatment. We found that one hour pre-treatment increased basal activity and significantly blocked LPS-stimulated suppression of *Rgs10* transcript expression. To explore the ability of TSA to block the effect of

higher doses of LPS, we determined that a 20 hour pre-treatment with 250 nM TSA fully blocked the effect of 1 µg/mL LPS on *Rgs10* expression (**Figure 3.5C**). Finally, we also confirmed that the effect of TSA on *Rgs10* expression was not limited to transcript but resulted in restored RGS10 protein levels, as shown in Figure 5E. These results suggest that LPS-induced *Rgs10* suppression in BV-2 cells is epigenetically mediated via histone acetylation of the RGS10 promoter.

BV-2 cells were selected as a model system to study the mechanism of silencing because they recapitulate RGS10 silencing and they are a widely used reliable model of microglial signaling (Henn et al. 2009). However, it is possible that gene regulation in an immortalized cell culture model does not accurately represent regulation in primary microglia. To address this concern, we isolated primary microglia from neonatal mice brain tissue to assess regulation of *Rgs10* expression by LPS and TSA. Using immunostaining with Iba1 (microglia), GFAP (astrocytes), and NeuN (neurons), we confirmed our microglia-enriched cultures contain approximately 90-95% microglia, with a few astrocytes and no neurons detectable (data not shown). In these primary cultures, one hour pre-treatment with 100 nM TSA fully blocked silencing of *Rgs10* expression stimulated by LPS (10 ng/mL, 6 hours), consistent with the effects observed in BV-2 cells (**Figure 3.5D**).

Role of TNFα in LPS-stimulated RGS10 silencing

We also quantified *TNFα* transcript expression following LPS and/or TSA treatment using qRT-PCR, and found that the trend in *TNFα* transcript expression was opposite that of RGS10 expression; LPS significantly enhanced *TNFα* expression and HDAC inhibition blocked LPS-induced *TNFα* production (**Figure 3.6A**). Previous studies by Lee et al. show that TNFα as well as LPS can trigger suppression of RGS10 expression in microglia (Lee et al. 2008), suggesting that LPS-mediated suppression of *Rgs10* may, in part, be secondary to LPS-stimulated *TNFα* production and TNFα-mediated suppression of RGS10. Further, given that

HDAC inhibition by TSA suppressed *TNFα* as well as enhanced *Rgs10* expression, it is possible that the effect of TSA on *Rgs10* expression could also be secondary to effects on *TNFα* expression. To clarify the role of TNFα in LPS-mediated suppression of *Rgs10*, we determined the effect of inhibition of TNFα receptor activation on LPS-stimulated *Rgs10* suppression. Cells were treated with LPS as above with or without a one hour pre-treatment with 10 µM R-7050, a TNFα receptor inhibitor, and *Rgs10* transcript was quantified as above. TNFα receptor inhibition had no effect on LPS stimulated *Rgs10* transcript or protein suppression, indicating that TNFα receptor activity is not required for LPS effects on *Rgs10* (**Figure 3.6B, D**). The same treatment with R-7050 had a significant effect on LPS-stimulated IL-1β, confirming that the compound is active in the conditions tested (**Figure 3.6C**).

LPS effects on histone acetylation and HDAC1 recruitment at the RGS10 promoter in microglia

The ability of TSA to enhance *Rgs10* expression and block LPS-mediated silencing suggests a role for HDAC enzymes in controlling *Rgs10* expression, but TSA is not completely selective for HDAC enzymes and may have off-target effects. To directly test the role of histone acetylation in the regulation of *Rgs10* expression in BV-2 cells, we performed chromatin immunoprecipitation (ChIP) assays to quantify the amount of acetylated histones and HDAC1 enzyme bound to the proximal *Rgs10-1* promoter with and without LPS treatment (100 ng/ml, 24 hours). LPS treatment resulted in a nearly 5-fold increase in the association of HDAC1 at the *Rgs10-1* promoter (**Figure 3.7A**). The change in HDAC1 binding was accompanied by a marked decrease in acetylated histone H3 levels at the RGS10 promoter, while total histone H3 levels remained similar (**Figure 3.7B, C**). These results suggest the *Rgs10* gene is epigenetically silenced in microglia following LPS stimulation via HDAC1 recruitment and decreased histone H3 acetylation at the *Rgs10-1* promoter.

Sphingosine 1-phosphate enhances RGS10 expression and alters histone acetylation at *Rgs10* promoter

Our results suggest that activation of TLR4 receptors by LPS triggers a signaling cascade that culminates in recruitment of HDAC to *Rgs10* promoters, resulting in transcriptional silencing. However, the ability of TSA to dramatically enhance basal *Rgs10* expression (**Figure 3.5A**) suggests that even in the absence of an activation signal, HDAC activity suppresses *Rgs10* expression. This suggests that signaling pathways that inhibit HDAC activity in microglia may increase RGS10 expression in resting microglia. Sphingosine 1-phosphate (S1P) in an endogenous bioactive lipid mediator in neurons and glia, and S1P activates G-protein coupled receptors in microglia to regulate inflammatory signaling (Durafourt et al. 2011; Kimura et al. 2007; Nayak et al. 2010; Noda et al. 2013). S1P has also been shown to inhibit HDAC1 enzymes via receptor-independent nuclear mechanisms (Hait et al. 2009). Therefore, we tested the effect of S1P on *Rgs10* expression and histone modifications at the *Rgs10* promoter. BV-2 cells treated with S1P showed a five-fold increase in *Rgs10* transcript expression following 48 hours of S1P treatment (**Figure 3.8A**). To confirm that the ability of S1P to regulate *Rgs10* expression reflected a change in HDAC activity, we determined histone acetylation levels at the proximal *Rgs10-1* promoter following S1P treatment, and observed a significant increase in H3 histone acetylation, with no change in total H3 histone levels (**Figure 3.8B**). Together, our data demonstrate that histone acetylation of the *Rgs10* promotor is dynamically regulated in microglia by endogenous signals to increase or decrease expression of this critical regulator of inflammatory signaling.

DISCUSSION

Microglia, CNS resident macrophages, have been implicated in the initiation and progression of multiple neurological disorders including neurodegenerative disease and neuropathic pain (Schwartz et al. 2013). Microglia are activated by inflammatory agents such as LPS, resulting in activation of NF-κB transcriptional pathways and production of pro-inflammatory cytokines. RGS10 has emerged as an important regulator of pro-inflammatory cytokine production in microglia. Microglia from RGS10 knock-out mice show enhanced NF-κB reporter activity and higher expression of inflammatory cytokines including TNF-α and IL-1β following LPS stimulation, and restoration of RGS10 normalizes responses to LPS (Lee et al. 2008; Lee, Chung, et al. 2011). Further, RGS10 loss enhances LPS-induced dopaminergic neuronal death (Lee et al. 2008), while restoration of RGS10 rescues these neurons (Lee, Chung, et al. 2011). Importantly, siRNA-mediated reduction of microglial RGS10 expression by 50-75% was sufficient to significantly enhance cytokine levels (Lee et al. 2008). Our data also show that suppression of RGS10 by 70% significantly enhances chemokine signaling, demonstrating that even partial loss of RGS10 has an important impact on inflammatory signaling in microglia. While RGS10 is normally present in microglia at high levels, expression is silenced by activation of TLR4 or TNFα receptors. Following activation by the TLR4 activator LPS, microglial *Rgs10* transcript expression levels were reduced by up to 75%, comparable to the fold suppression sufficient to amplify NF-κB signaling and inflammatory cytokine production (Lee et al. 2008). This suggests that silencing of RGS10 is sufficient to amplify microglial inflammatory signaling in a feed-forward mechanism that may contribute to the dysregulation of inflammatory signaling in chronic neuroinflammation and disease. Understanding the molecular mechanisms of RGS10 suppression in microglia may reveal novel targets for therapeutic intervention in diseases with underlying chronic neuroinflammation. With this in mind, we sought to define the molecular mechanism of RGS10 suppression following TLR4 activation *in vitro*.

In this study we find that activation of microglia by LPS triggers a rapid and robust transcriptional silencing of *Rgs10*. Lee et. al previously reported that a low dose of LPS (10 ng/ml) decreased RGS10 protein in BV-2 cells, while a higher dose of LPS (1 μg/ml) did not change RGS10 protein level (Lee et al. 2008). Here we report that *Rgs10* transcript and protein levels were suppressed in BV-2 cells in a typical dose-dependent fashion. LPS-induced suppression of *Rgs10* transcript reflected and preceded the reduction in protein levels, which suggests that the reduction in RGS10 protein level observed following LPS treatment is mainly due to inhibition of transcription.

Previously, we reported an epigenetic regulation of *Rgs10* expression in chemoresistant ovarian cancer cells mediated by the combined activities of HDAC and DNMT enzymes. Here, we investigated whether *Rgs10* expression in microglia is regulated in a similar mechanism after acute LPS treatment. We found that the HDAC inhibitor TSA significantly enhances basal *Rgs10* transcript expression and blocks LPS-induced *Rgs10* suppression in microglia, while the DNMT inhibitor had no effect under the conditions tested. We further show that LPS increases HDAC1 association and decreases histone H3 acetylation at the *Rgs10* promoter, observations which strengthen the notion that *Rgs10* genes are epigenetically silenced via histone deacetylation in LPS-activated microglia. We also show that HDAC inhibition caused a decrease in LPS-mediated pro-inflammatory cytokine TNFα (and IL-1β, not shown) production in microglia. Consistent with our observations, HDAC inhibitors have been reported to possess anti-inflammatory effects by lowering pro-inflammatory cytokine production in microglia (Suh et al. 2010). The HDAC inhibitor SAHA has been shown to reduce the production of pro-inflammatory cytokines production in activated microglia (Faraco et al. 2009), while TSA reduces pro-inflammatory cytokine production in microglia and reduces brain injury in a model of LPS-sensitized neonatal hypoxic-ischemia (Fleiss et al. 2012).

The mouse *Rgs10* gene contains two transcriptional start sites, yielding two transcript isoforms with distinct first exons, and four common exons (Haller et al. 2002; Lee and Tansey 2015). Mouse transcripts *mRgs10-1* and *mRgs10-2* correspond to human transcripts with conserved exon structure and encoding highly conserved human RGS10 protein isoforms RGS10a (181aa) and RGS10b (167 aa). A third human transcript (173aa) has been proposed, resulting from an alternate transcriptional start site upstream of the first shared exon (Hunt et al. 1996), but the relevance of this transcript in mouse cells is unclear (Haller et al. 2002). We have shown that *Rgs10-1* is the predominant form expressed in human cancer cells, *Rgs10-1* is selectively silenced in chemoresistant cells, and that HDAC1 is recruited to the unique *Rgs10-1* promoter in these cells (Hooks et al. 2010; Ali et al. 2013a; Cacan et al. 2014; Ali et al. 2013b). Our studies and others have indicated that the long isoform (*Rgs10-1* → RGS10a) is also the predominantly expressed form in microglia and other immune cells (Lee and Tansey 2015). For these reasons, our studies in microglia focused on regulation of the mouse *Rgs10-1* transcript, and ChIP experiments were targeted to the mouse *Rgs10-1* proximal promoter.

Microglial activation in the spinal dorsal horn enhances nociception and is a causative factor in central sensitization that contributes to neuropathic pain (Zhuo, Wu, and Wu 2011; Raghavendra, Tanga, and DeLeo 2003). The pSNL model of injury-induced neuropathic pain results in robust microglial activation and spinal sensitization. During nerve injury-induced sensitization, endogenous mediators activate microglial TLR4 receptors, which trigger production of pro-inflammatory cytokines (Bettoni et al. 2008; Tanga, Nutile-McMenemy, and DeLeo 2005; Wu et al. 2010). Blocking either microglial activation or endogenous TLR4 receptors has been shown to attenuate neuropathic pain (Raghavendra, Tanga, and DeLeo 2003; Sorge et al. 2011; Saito et al. 2010; Yoon, Patel, and Dougherty 2012). Further, enhanced HDAC activity is observed in the spinal cord following nerve injury (Lv et al. 2011; Lu et al. 2013), and HDAC inhibitors reduce inflammatory cytokine release and neuroinflammation

(Blanchard and Chipoy 2005; Lv et al. 2011). Thus, the pSNL model of neuropathic pain is suitable for the in vivo study of molecular signaling pathways in microglia activated by TLR4.

We have demonstrated that RGS10 is abundant in the spinal cord, with confirmed localization in microglia. Non-microglial cell-types also stain positively for RGS10, consistent with reports of predominantly microglial and lesser neuronal expression in brain (Waugh et al. 2005). Following pSNL injury, we observe down-regulation of RGS10 protein expression by about 50% in spinal dorsal horn as measured by western blotting in total L4-L5 spinal dorsal horn tissue. Our observation that RGS10 is suppressed in the spinal cord in the pSNL model provides supporting evidence that the suppression observed in cultured BV-2 and primary microglia also occurs *in vivo*. However, our approach likely underestimates the cellular suppression of RGS10; given that RGS10 is predominantly expressed in microglia, the expansion of microglia during inflammation following pSNL likely masks the degree of RGS10 suppression in individual microglial cells. Further, the expression of RGS10 in neurons, although less than microglia, also limits quantitative interpretation of the degree of suppression in microglia. Thus, future studies should evaluate RGS10 levels in individual microglia isolated from *in vivo* neuroinflammation models. It will also be important for future studies to determine if HDAC inhibitors, which are known to inhibit inflammatory signaling, also restore *Rgs10* expression in the spinal cord, and if this effect contributes to their ability to impact inflammation. Tansey and colleagues have shown that re-introduction of RGS10 protein expression in microglia from knock-out animals normalizes inflammatory signaling and dopaminergic neuron cell death *in vivo (Lee, Chung, et al. 2011)*. Therefore, strategies that restore RGS10 levels in microglia may blunt neuroinflammation and associated diseases.

In recent years RGS proteins have emerged as promising novel drug targets in diverse disease states, but progress has lagged due to the lack of traditional small molecule ligand or substrate interaction sites. Strategies to target regulation of RGS protein expression have

focused on post-translational mechanisms that control protein stability (Raveh et al. 2014; Sjogren et al. 2012). In contrast, our data suggest predominantly transcriptional regulation of *Rgs10* expression. Our results demonstrate that *Rgs10* silencing is completely reversible with small molecule HDAC inhibitors and provide proof of concept for targeting RGS10 suppression in neuroinflammatory disease models. Multiple HDAC inhibitors are being assessed in clinical trials, but specificity, pharmacokinetic features and toxicity remain significant barriers to the use of broad spectrum HDAC inhibitors in CNS diseases. Thus, more selective pharmacological approaches are needed to target *Rgs10* silencing.

A potential alternative to targeting HDAC enzymes directly in order to regulate *Rgs10* expression is to target more selective pathways that may indirectly modulate HDAC activity. S1P activates a family of GPCRs, including several expressed in microglial cells, and S1P is a key regulator of microglial activity (Blaho and Hla 2014; Noda et al. 2013; Tham et al. 2003; Nayak et al. 2010). Our results here show that S1P may enhance *Rgs10* expression through regulation of HDAC. Notably, the time course of S1P effects on RGS10 expression (48 hours) was much longer than that of LPS effects on *Rgs10* expression (6 hours), and it remains to be determined if S1P receptor activation is sufficient to reverse LPS mediated silencing of RGS10. However, the involvement of GPCRs in regulating *Rgs10* expression offers another opportunity for therapeutic intervention, as GPCRs are relatively easy to target with reasonable selectivity compared to HDACs. Strikingly, the S1P receptor functional antagonist FTY-720 (Fingolimod, Gilenya) has been approved for use in relapsing-remitting multiple sclerosis (MS), and a recent study demonstrated that RGS10 knock-out animals exhibit blunted symptoms in the EAE model of MS (Lee et al. 2016). Given the role of microglial activation in MS pathology, it is tempting to postulate a connection between our observations connecting S1P receptors and RGS10 in microglia with these findings in models of MS. However, the predicted mechanisms of action of both FTY-720 and RGS10 in MS models are based on their effects on peripheral immune cells,

not microglia, so the relevance of the observed regulation in microglia to MS is unclear at this time.

Summary

In this study, we demonstrate dose-dependent suppression of *Rgs10* in response to LPS activation in microglia, demonstrate that the HDAC inhibitor TSA blocks the ability of LPS to suppress *Rgs10* expression, and demonstrate HDAC1 recruitment and H3 histones deacetylation at the *Rgs10-1* proximal promoter following LPS stimulation. These observations suggest that LPS-stimulated silencing of *Rgs10* expression is rapid, robust, and reversible in BV-2 microglial cells, and this model system reflects the mechanism observed in primary microglia. We further show that activation of microglial S1P receptors increases *Rgs10* expression, demonstrating that *Rgs10* expression may be precisely dialed up or down in microglia in response to multiple cues. Finally, we demonstrate that RGS10 is suppressed in an *in vivo* mouse model of injury-induced inflammation. Together, our results suggest that the ability of RGS10 to regulate microglial inflammatory pathways can be modulated by various pathways through HDAC regulation of *Rgs10* expression with significant implications on neuroinflammatory disease states.

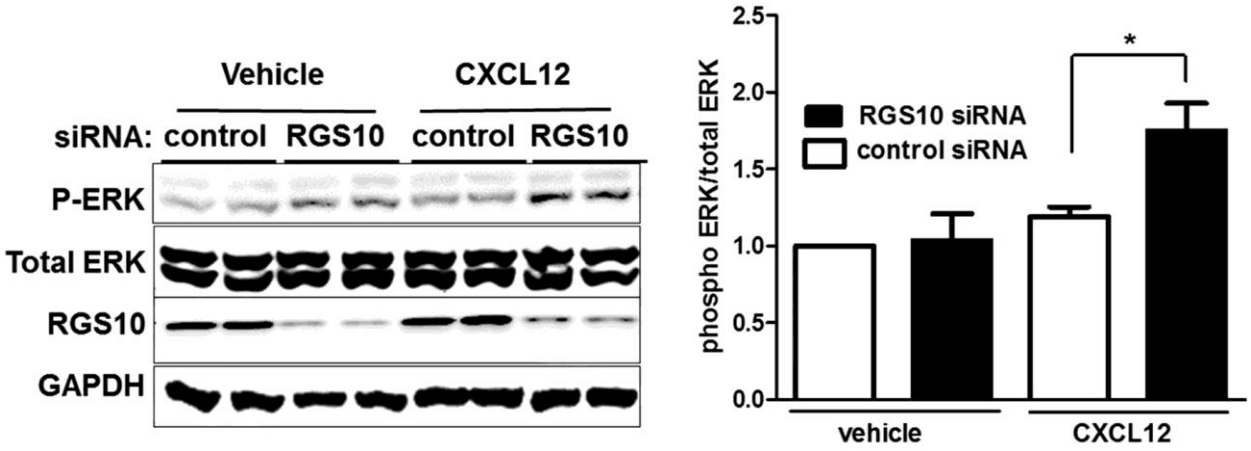

Figure 3.1: Suppression of RGS10 enhances CXCL12 signaling in microglia. BV-2 cells were treated with either scrambled small interfering RNA as negative control or RGS10 small interfering RNA for 48 hours. Cells were starved overnight prior to treatment with CXCL12 (100 ng/ml) for 20 minutes. Cells were lysed, and Western blotting was performed using specific antibodies against phospho-ERK (P-ERK), total ERK, RGS10, and GAPDH. Phospho-ERK and intensity were quantified and normalized to total ERK intensity, and statistical comparison was determined using an unpaired *t* test of compiled data from three independent experiments, each with duplicate samples (*P = 0.045).

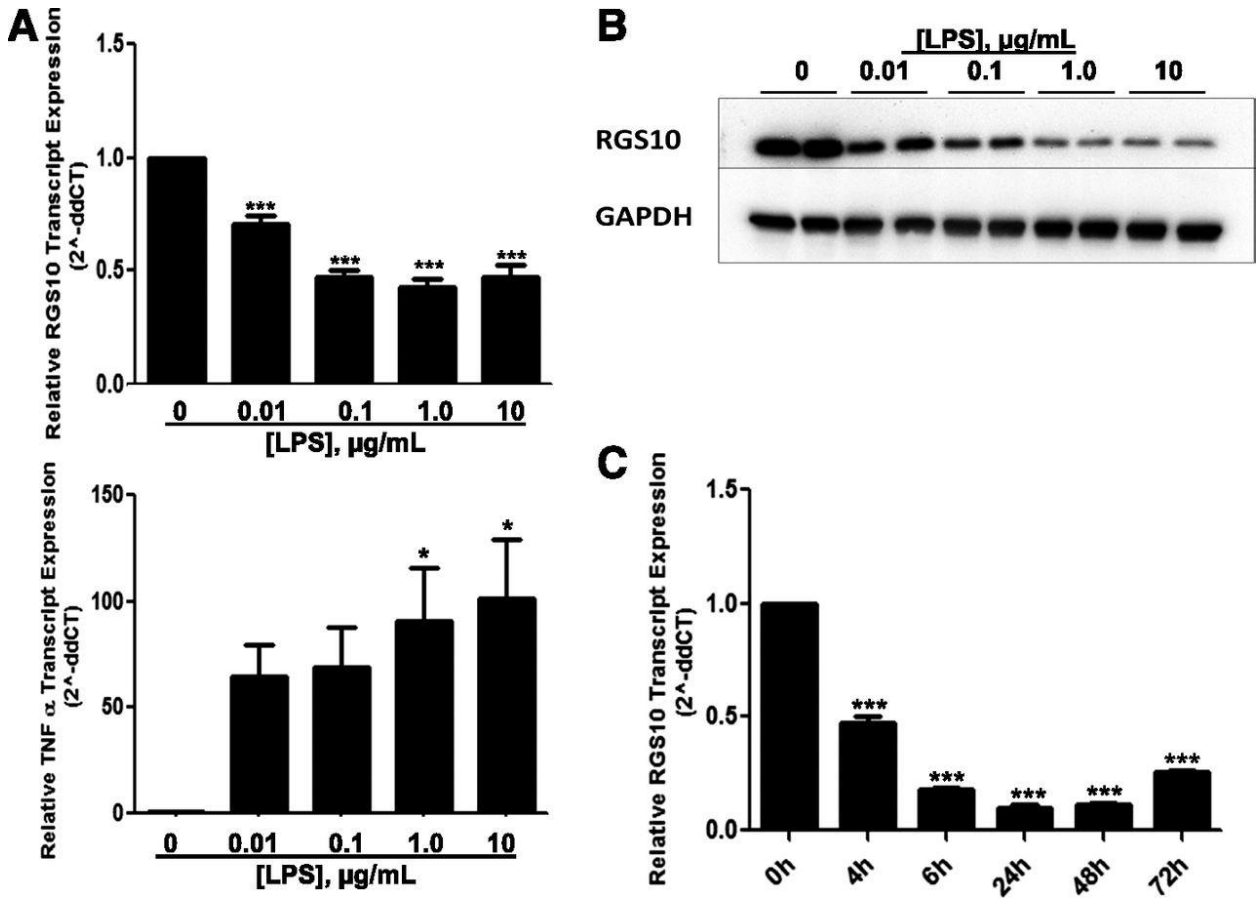

Figure 3.2: *Rgs10* transcript and RGS protein suppressed in BV-2 mouse microglial cells following LPS treatment in a dose-dependent fashion. (A) BV2 cells were treated with vehicle (serum-free media), 10 ng/ml, 100 ng/ml, 1 μg/ml, and 10 μg/ml LPS for 4 hours. Cells were harvested in TRIzol, and RNA was isolated. *Rgs10* (top panel) and *Tnf-α* (bottom panel) transcripts were quantified using SYBR Green RT-PCR reagents and normalized to the housekeeping gene actin ($2^{-\Delta\Delta CT}$). (B) BV-2 cells were treated with vehicle, 10 ng/ml, 100 ng/ml, 1 μg/ml, and 10 μg/ml LPS for 24 hours, and total cell lysates were collected in SDS sample buffer and assessed using Western blotting with RGS10 and GAPDH antibodies. (C) BV-2 cells were treated with 100 ng/mL LPS for the times indicated, and *Rgs10* transcript expression was determined using SYBR Green RT-PCR reagents and normalized to the housekeeping gene actin ($2^{-\Delta\Delta CT}$). Data are compiled from three independent experimental repeats, each performed in duplicate. Data were analyzed for statistical differences using an analysis of variance, followed by Tukey's test between groups. *$P < 0.05$; and ***$P < 0.001$ indicate the levels of significance. Similar RT-PCR results were obtained when normalized to GAPDH (data not shown).

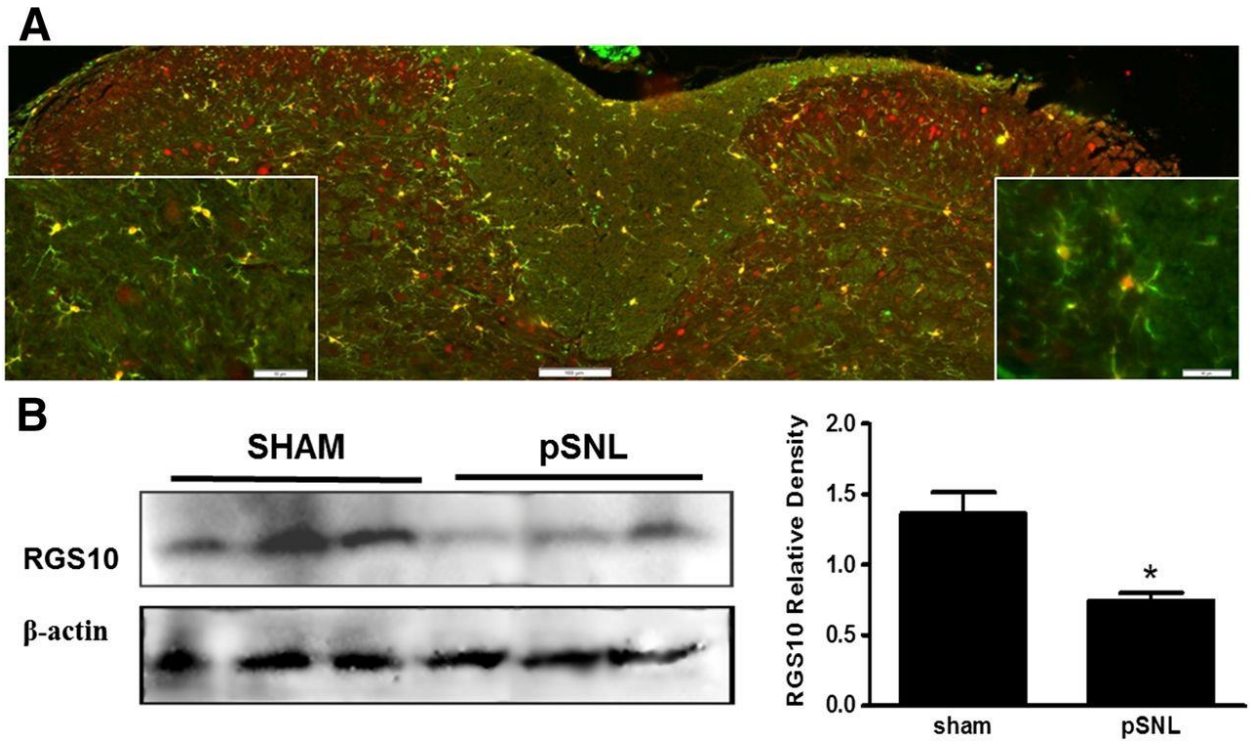

Figure 3.3: **Suppression of spinal RGS10 levels in pSNL model of inflammatory neuropathic pain.** (A) Tissue slices (10 μm) obtained the L4–L5 region of mouse spinal cord, and expression of RGS10 (red) and Iba1 (microglial marker, green) was visualized using immunofluorescence, as described in ***Materials and Methods***. Yellow indicates overlap of RGS10 and Iba1 expression. Center panel: original magnification, 10×. Left inset: original magnification, 20×. Right inset: original magnification, 40×. (B) Spinal dorsal horn (L4–L5) tissue from SHAM (n = 3) or pSNL (n = 3) mice was isolated 3 days after surgery, subjected to SDS-PAGE separation (one animal per lane), and analyzed using Western blotting with RGS10 and β-actin antibody. Data shown are average RGS10 levels normalized to β-actin, ±S.E.M. Data were normalized by dividing band intensity of RGS10 immunoblot by band intensity of β-actin immunoblot, and analyzed for statistical significance using an unpaired t test. *P < 0.05.

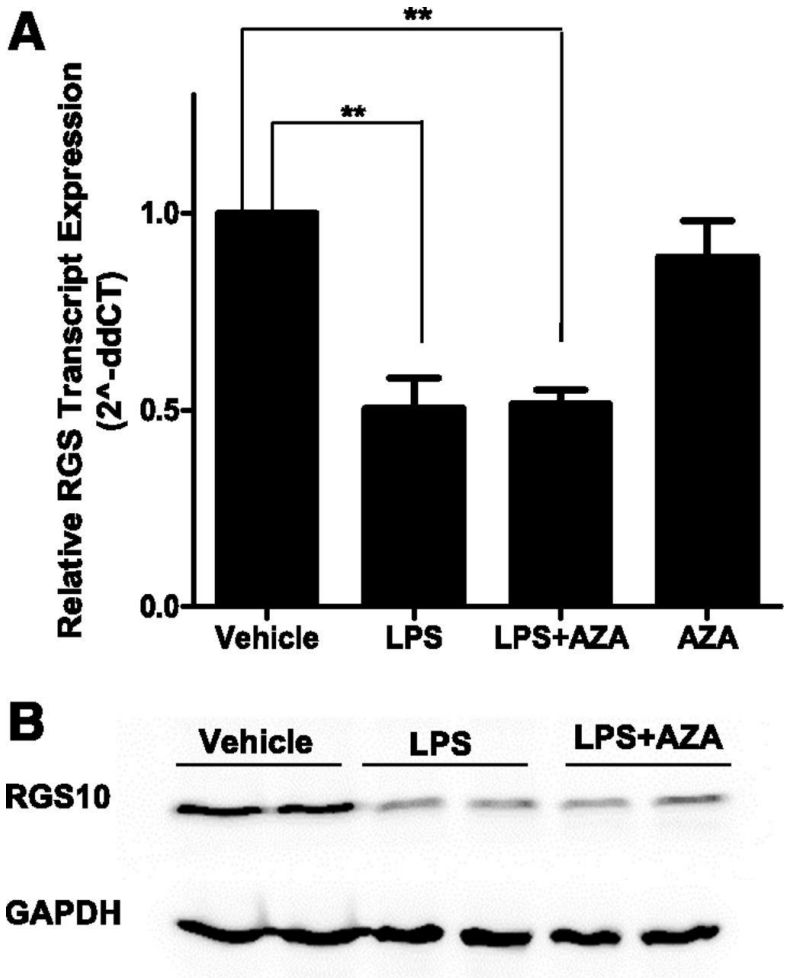

Figure 3.4: The 5-Aza does not affect LPS-induced suppression of *Rgs10* transcription. (A) BV-2 cells were plated in 12-well plate and allowed to adhere overnight. Cells were treated with vehicle (serum-free media) or LPS (10 ng/ml, for 6 hours) with or without 5-Aza (AZA, 10 μM). Cells were harvested in TRIzol, and RNA was isolated. mRNA transcript levels were measured by quantitative real-time PCR. Transcripts were normalized to the housekeeping gene actin. (B) BV-2 cells were plated in 24-well plate and allowed to adhere overnight. Cells were treated with either vehicle (serum-free media) or LPS (10 ng/ml) for 48 hours or with 5-Aza (AZA, 10 μM). Cells were harvested, and protein levels were assessed by Western blot analysis with RGS10 and GAPDH control antibodies. Data are compiled from three independent experimental repeats, each performed in duplicate. Data were analyzed for statistical differences using an analysis of variance, followed by Tukey's test between groups. $^{**}P < 0.01$ indicates the levels of significance.

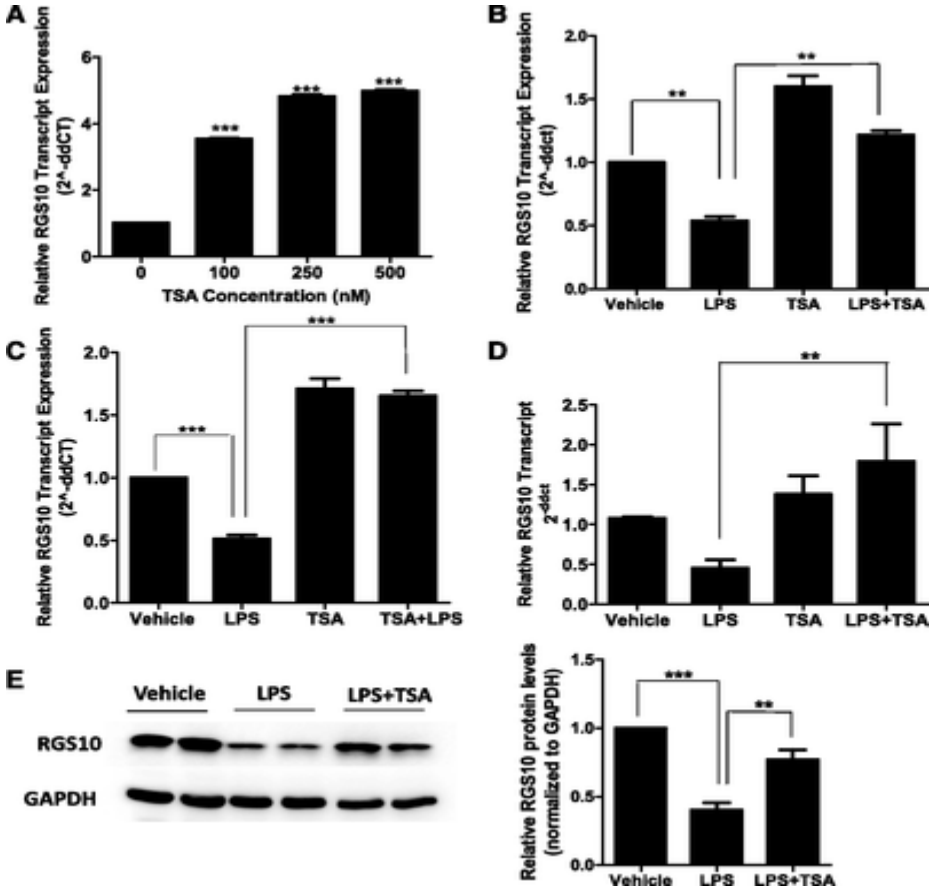

Figure 3.5: HDAC inhibitor TSA enhances basal *Rgs10* expression and blocks LPS-stimulated suppression in microglia. (A) BV-2 cells were plated in 12-well plate and allowed to adhere overnight. Cells were treated with vehicle, 100, 250, and 500 nM TSA for 24 hours. Cells were harvested in TRIzol, and RNA was isolated. *Rgs10* transcript was quantified using quantitative RT-PCR relative to actin. (B) BV-2 cells were treated with vehicle or 10 ng/mL LPS for 6 hours with or without 100 nM TSA, and *Rgs10* transcript was quantified relative to the actin. (C) BV-2 cells were treated with vehicle or 1 *μ*g/mL LPS for 4 hours with or without 250 nM TSA, and *Rgs10* transcript was quantified relative to the actin. (D) Primary microglia were isolated from 2- to 4-day-old mouse pups, as described in ***Materials and Methods***. Cells were treated with vehicle or LPS (10 ng/mL) for 6 hours with or without TSA (100 nM). *Rgs10* transcript expression was determined using quantitative RT-PCR relative to actin. (E) BV-2 cells were plated in six-well plate and allowed to adhere overnight. Cells were treated with either vehicle or LPS (10 ng/ml) for 48 hours with or without TSA (100 nM). Cells were harvested, and protein levels were assessed by Western blot analysis. Blot presented is a representative of three independent experiments. Densitometry values were normalized to

GAPDH loading control and then to vehicle-treated condition (right panel). Data in each graph are compiled from three independent experimental repeats, each performed in duplicate. Data were analyzed for statistical differences using an analysis of variance, followed by Tukey's test between groups. $^{**}P < 0.01$; and $^{***}P < 0.001$ indicate the levels of significance.

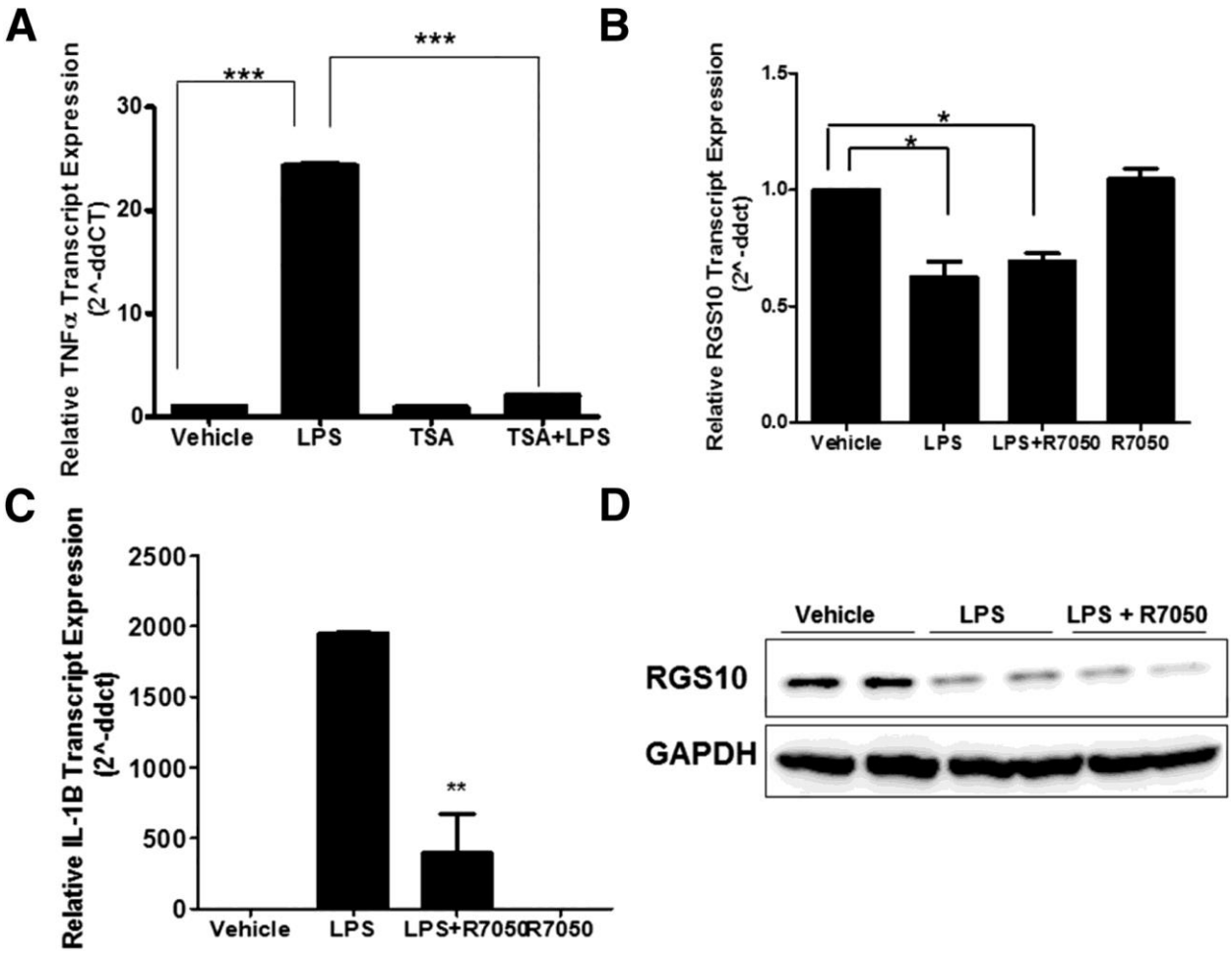

Figure 3.6: LPS effects on TNF-*α* do not mediate RGS10 silencing. (A) BV-2 cells were treated with LPS and/or TSA (as in **Fig. 5C**), and *Tnfα* transcript was quantified using SYBR Green RT-PCR reagents and normalized to actin control. (B and C) BV-2 cells were treated with vehicle or LPS (10 ng/ml, for 6 hours) with or without the TNF-*α* receptor antagonist, R-7050 (10 *μ*M). *Rgs10* (B) and *IL-1β* (C) transcript levels were measured by quantitative real-time PCR and normalized to the housekeeping gene actin. R-7050 treatment had no effect on *Rgs10* suppression, but robust effects on IL-1*β*confirm effective inhibition. (D) BV-2 cells were treated with either vehicle or LPS (10 ng/ml) for 48 hours with or without R7050 (10 *μ*M). Cells were harvested, and protein levels were assessed by Western blot analysis with RGS10 and GAPDH antibodies. Quantitative data in (A–C) are compiled from three independent experimental repeats, each performed in duplicate. Data were analyzed for statistical differences using an analysis of variance, followed by Tukey's test between groups. **P* < 0.05; ***P* < 0.01; and ****P* < 0.001 indicate the levels of significance. Blot presented in (D) is representative of two independent experiments.

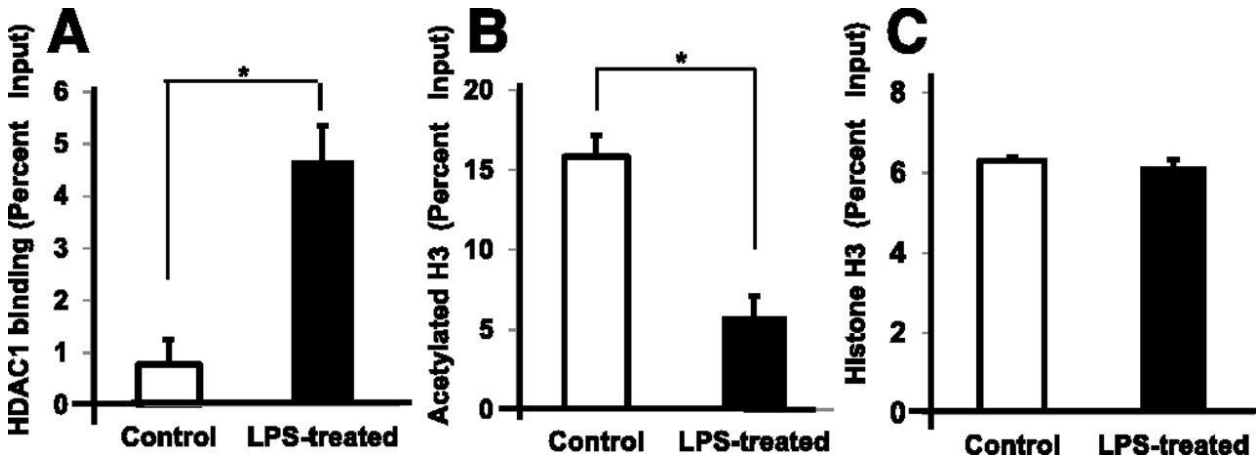

Figure 3.7: LPS increases HDAC1 binding and decreases histone H3 acetylation at the *Rgs10* promoter in BV2 cells. ChIP assays were carried out in LPS (100 ng/ml)-treated and vehicle-treated BV-2 cells. Lysates were immunoprecipitated with control, anti-HDAC1 (A), anti-acetyl histone H3 (B), or anti-histone H3 (C) antibodies. Associated DNA was isolated and analyzed via RT-PCR using primers and probes (see *Materials and Methods*) spanning the *Rgs10* promoter and *GAPDH* promoters. Real-time PCR values were normalized to the total amount of DNA added (input). Input values represent 5% of the total cell lysate. Values were normalized to *GAPDH* promoter and represent mean ± S.E.M. of two independent experiments, and RT-PCRs were run in triplicate reactions. Data were analyzed using the SDS 2.0 program, and significance was tested using a *t* test. **P* < 0.05.

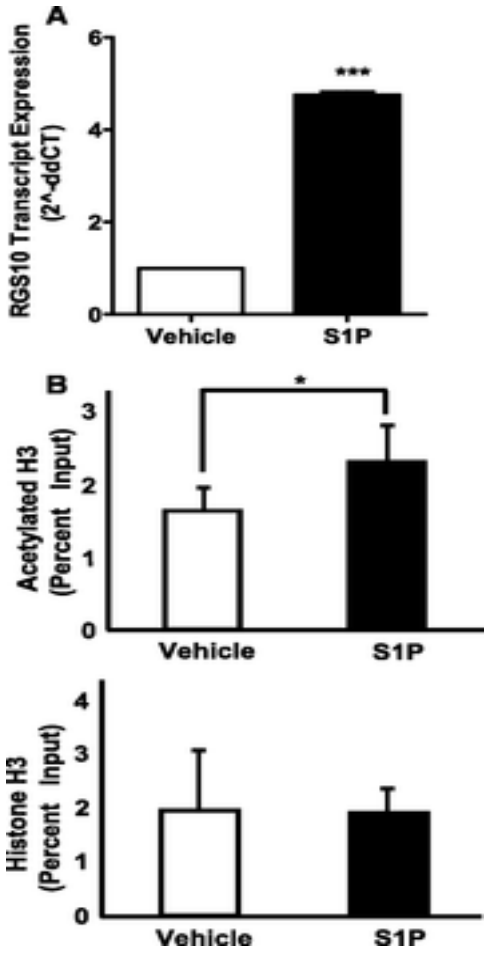

Figure 3.8: S1P upregulates *Rgs10* expression and histone acetylation at RGS10 promoters in BV-2. (A) BV-2 cells were serum starved overnight and treated with vehicle or S1P (10 μM) for 48 hours, and then *Rgs10* mRNA was isolated and assessed with RT-PCR relative to actin controls, as described. (B) Acetylated and total H3 Histone ChIP assays were carried out as described in BV-2 cells treated with vehicle or S1P (10 μM). Lysates were immunoprecipitated with control, anti-histone H3, or anti-acetyl histone H3 antibodies. Associated DNA was isolated and analyzed via RT-PCR using primers and probes spanning the *Rgs10* promoter and *GAPDH* promoter. Real-time PCR values obtained were normalized to the total amount of DNA added (input). Input values represent 5% of the total cell lysate. *Rgs10*promoter values were normalized to *GAPDH* promoter values and represent mean ± S.E.M. of two independent experiments, and RT-PCRs were run in triplicate reactions. Data were analyzed using the SDS 2.0 program, and significance was tested using a *t* test. **P* < 0.05; ***P* < 0.01; and ****P* < 0.001.

CHAPTER 4

NOVEL BINDING PARTNERS INFLUENCE G PROTEIN-DEPENDENT AND INDEPENDENT FUNCTIONS OF RGS PROTEINS

Introduction

Regulators of G protein singling (RGS) are a diverse family of proteins that share a conserved RGS domain that canonically accelerates the intrinsic GTPase activity of heterometric G proteins (Watson et al. 1996). This effect results in an accelerated inactivation of the active Gα subunit, thereby converting the G protein to the inactive form. RGS proteins exert this function by direct physical association with the target G protein through their RGS domains (Popov et al. 1997). Through this mechanism, RGS proteins critically regulate multiple signaling pathways. Dysregulation of RGS protein expression and/or activity is linked to several pathologies (Cho, Harrison, and Kehrl 2004; Jules et al. 2015; Xie, Chan, and Druey 2016). In addition to the RGS domain, many RGS proteins possess other multifunctional domains that do not directly participate in GTPase-accelerating activity (Martemyanov et al. 2003; Chatterjee, Liu, and Fisher 2003). Further, some members of this family localize in cellular compartments that are distant from the cell membrane where G proteins are localized (Chatterjee and Fisher 2000). These characteristics suggest that RGS proteins exerts GAP-independent functions. Indeed, many GAP-independent functions of RGS proteins were reported (Sethakorn, Yau, and Dulin 2010). Many of these functions are facilitated by direct interaction of RGS proteins with unique binding partners unrelated to the classic G protein interaction. These binding partners can influence GAP activity or GPCR selectivity of RGS proteins, and vice versa. The activity or function of these binding partners can be directly influenced by RGS proteins. Thus, RGS proteins can both affect or be affected by the interaction with these binding partners. Structurally, RGS proteins interact with these binding partners through both RGS and non-RGS

domains. Further understanding of these unique interactions will be beneficial in identifying additional roles of RGS proteins, and possibly aid in finding novel therapeutic approaches to target RGS GAP-independent functions. Here, we will discuss the physiological effects, structural requirements, regulation, and localization of these non-canonical interactions.

Binding partners that influence GAP activity of RGS proteins.

The RGS domain shared by all RGS proteins is responsible for binding G proteins, and mediating GAP activity (Popov et al. 1997). Generally, specific residues of the RGS domain directly interact with the switch regions in the Gα subunit, thereby stabilizing the transition state of G proteins, which accelerate the intrinsic GTPase-mediated hydrolysis of GTP (Tesmer et al. 1997; Soundararajan et al. 2008b). This GTPase-accelerating activity of RGS proteins is influenced by certain interactors that either inhibit or improve the interaction between RGS and G proteins, thereby altering the GAP activity.

14-3-3 is a prime example of such binding partners, which interact directly with RGS proteins and affect GAP activity. 14-3-3 was shown to bind the RGS domain of RGS7 and subsequently inhibit its GAP activity (Benzing et al. 2000). Interestingly, this interaction itself is regulated by phosphorylation of the RGS domain. External stimulants that alter the phosphorylation of that RGS domain, such as the protein kinase inhibitor Staurosporine (Benzing et al. 2000) or tumor necrosis factor-alpha (Benzing et al. 2002) inhibited the interaction between 14-3-3 and RGS7, thereby blocking the inhibitory effect on GAP activity. This suggests that the GAP activity of RGS7 can be indirectly regulated by kinase and phosphatase signaling. RGS3 GAP activity is also inhibited by the interaction with 14-3-3. However, there are conflicting reports about the specific domain that mediates this interaction, as one study suggests that 14-3-3 interacts directly with the RGS domain of RGS3 (Benzing et al. 2000), whereas another study mapped the interaction to the N-terminal domain (Niu et al. 2002). Nevertheless, both studies confirmed that the binding of 14-3-3 inhibits GAP activity of RGS3. Similarly, 14-3-3 interacts with the Gαo interacting site of RGS4 and RGS6, which

prevents the binding of Gαo with RGS proteins in a competitive manner (Abramow-Newerly, Ming, and Chidiac 2006). Additional studies are required to delineate the structural basis of this interaction and determine if this binding and mechanism are conserved across RGS domains.

Another binding partner that has been shown to affect GAP activity of RGS proteins is the calcium sensitive protein calmodulin. Calmodulin directly interacts with both the RGS domain and N-terminal of RGS4, and other RGS proteins (Popov et al. 2000). However, in a single turnover assay, the binding of calmodulin had no direct effect on GAP activity of RGS4. Instead, calmodulin influenced GAP activity indirectly by reversing the inhibitory effect of Phosphatidylinositol (3,4,5)-trisphosphate on RGS4 GAP functions. In addition to RGS4, calmodulin interacts with multiple RGS proteins in a calcium-dependent manner, including RGS1, RGS2, RGS10, RGS16, and RGS19. However, the strength of the interaction varies between RGS proteins (Popov et al. 2000). The RGS-CaM binding has a physiological relevance as it was shown that RGS4 ability to inhibit G protein-activated inward-rectifier K+ channels (GIRK) activity in cardiac atrial myocytes is inhibited by the binding of Phosphatidylinositol (3,4,5)-trisphosphate and was rescued by calmodulin binding (Ishii, Inanobe, and Kurachi 2002). Interestingly, it appears that this interaction may not be a simple turn-off regulatory mechanism, in which calmodulin or PIP3 mediate opposite effects on GAP activity of RGS proteins. Instead, it was suggested that a continuous cycle of RGS10 interaction with PIP3 and subsequent PIP3 detachment due to calmodulin binding causes [Ca2+]i oscillations and PLCγ activation to mediate osteoclasts differentiation (Yang and Li 2007). Therefore, interaction between RGS proteins and these binding partners not only affects RGS GAP activity but may also influence downstream signaling in unexpected ways.

Finally, Other interacting proteins influence GAP activity indirectly by altering the cellular localization of the interacting RGS protein. This is demonstrated by the enhancement of GAP activity of RGS9-1 and RGS7 via the interaction with R9AP and R7BP, respectively. R9AP and R7BP are membrane anchored proteins that bind the DEP domains of RGS9-1 and RGS7 and

localizes these RGS proteins at cell membranes for more effective GTPase-accelerating action (Hu, Zhang, and Wensel 2003; Drenan et al. 2006).

Attempts to manipulate RGS protein GAP activity have focused on the interaction between RGS and G proteins. This not an easy task, however, as the interactions between RGS and G proteins are transient and mediated by broad protein-protein interactions that are difficult to target. Therefore, identifying novel interacting proteins that regulate the interaction between RGS and G proteins and the subsequent GAP activity may provide new therapeutic avenues, especially when these novel interactions are regulated by cellular events such as phosphorylation and/or changes in calcium concentrations. This suggests that it is possible to manipulate RGS GAP activity indirectly by using exogenous agents that targets such events. Further, since some of these interacting proteins display more stable interaction with RGS proteins compared to the transient interaction with G proteins, it might be less difficult to target these interactions to influence GAP activity.

Specific interactions mediate RGS protein selectivity toward G proteins and GPCRs

Shortly after the discovery of RGS proteins as GAPs, it became evident that RGS proteins display selectivity towards the target G proteins and associated GPCRs. For example, studying the binding and selectivity of RGS proteins towards G proteins in-vitro revealed that members of the R12 family (RGS10, RGS12, and RGS14) selectively bind Gαi, whereas RGS2 was selective for Gαq (Soundararajan et al. 2008b). Interestingly, it was demonstrated that RGS2 can regulate Gαi activation in intact cells and tissues (Chakir et al. 2011) (Luessen et al. 2016), indicating that selectivity of RGS proteins is subjected to other influencing factors in-vivo, and findings obtained in biochemical and structural studies of isolated proteins may not necessarily reflect RGS function and selectivity in intact cellular systems. In addition to displaying specificity towards different types of G proteins, some RGS proteins can discriminate between different GPCRs that couple to the same G protein (Xu et al. 1999).

Understanding how this selectivity is achieved was and still is the focus of many studies (reviewed (Xie and Palmer 2007)). One factor that dictates selectivity of RGS proteins is the presence of structural determinants on either the RGS protein or the target G-protein that facilitates or hinders the binding, thereby resulting in different binding affinities of RGS members towards G-proteins (Kimple et al. 2009; Posner et al. 1999; Woulfe and Stadel 1999). Another factor that governs selectivity is simply the strategic presence of a given RGS protein at the right location and time to regulate the target G-protein. For example, RGS9-1 is exclusively expressed in the photoreceptor cells in the retina and specifically regulates the G protein transducin Gαt, which is also expressed in photoreceptor cells, thereby providing an opportunity for RGS9-1 to regulate the function of this G-protein in retina (Makino et al. 1999). Additionally, it was also discovered that RGS forms unique interactions that mediate their selectivity. One main finding that supports this notion is the identification of complexes linking RGS, G proteins, and GPCRs. The direct interaction of RGS proteins to GPCRs and G-proteins explains, at least in some cases, what drives the selectivity of RGS towards G proteins, and the ability of RGS proteins to distinguish between GPCRs coupled with the same type of G protein. Here, we will discuss examples of these interactions, structural requirements, and other interactors that influences these trimeric complexes.

One of the first reports of an RGS protein directly interacting with a GPCR was the interaction of RGS2 with M1 Muscarinic Acetylcholine Receptors (Bernstein et al. 2004). Structurally, this binding was mediated by the N-terminus of RGS2 and the third intracellular loop of the M1 Muscarinic Acetylcholine receptor. RGS2 also interacted with the active form of Gαq and formed a complex with the M1 mAChR. Interestingly, R4 family members differentially interact with M1 mAChR, as RGS2 and RGS4, but not RGS6, interacted with this receptor (Bernstein et al. 2004). In a very similar pattern, the N-terminal domain of RGS2 interacts with alpha1A-adrenergic receptor third intracellular loop to regulate Gαq/11 signaling (Hague et al. 2005).

Naturally, the discovery of direct interaction between RGS proteins and GPCRs prompted further investigation to identify other GPCR-RGS interactions. Indeed, additional RGS-interacting GPCRs were identified, such as opioid, melanin-concentrating hormone, dopamine, and protease-activated receptors (Georgoussi et al. 2006; Miyamoto-Matsubara et al. 2008; Min et al. 2012; Ghil, McCoy, and Hepler 2014). This indicates that this phenomenon is not unique to a few RGS members or GPCRs types, but rather a common event that link RGS proteins and GPCRs in a signaling complex. Notably, the RGS-interacting GPCRs are not coupled to a specific type of G protein, but rather couples to different types of G proteins. For example, M1 mAChR and alpha1A-adrenergic couples to Gαq and associates with RGS4 and RGS2 (Bernstein et al. 2004; Hague et al. 2005), whereas the same RGS proteins also associates with the Gαi-coupled D2R/D3R receptors (Luessen et al. 2016; Min et al. 2012). It was previously demonstrated that RGS2 is a weak GAP for Gαi-protein in-vitro (Soundararajan et al. 2008b). Yet, RGS2 regulates Gαi-mediated signaling initiated by D2R in neuroblastoma N2A cells (Luessen et al. 2016). This is perhaps possible by the direct association of RGS2, D2R and Gαi which places RGS2 at an optimal position that in some way compensate for the weak GAP activity and result in an inhibition of D2R-mediated Gαi signaling.

Studying RGS-GPCR interactions has revealed unique and common structural domains in both RGS proteins and GPCRs that mediate these interactions. The third intracellular loop domain of GPCRs is essential in mediating many GPCR-RGS interactions. In addition to mediating the interaction between muscarinic and alpha-adrenergic receptors binding to several RGS proteins, the third intracellular loop also mediate the association of Melanin-concentrating hormone receptor to both RGS2 and RGS8 (Miyamoto-Matsubara, Chung, and Saito 2010; Miyamoto-Matsubara et al. 2008), and the interaction of protease activated-receptor to RGS2 and RGS4 (Ghil, McCoy, and Hepler 2014). RGS8 also directly interacts with protease activated-receptor, but it is not confirmed yet whether it associates directly with the third intracellular loop (Lee and Ghil 2016). In contrast, many opioid receptors utilize different

structural domains, distinct from the third intracellular loop domain, to interact with RGS proteins. For example, mu-opioid and delta-opioid receptors specifically interact with RGS4 via their c-terminus domains (Georgoussi et al. 2006). Similarly, kappa-opioid receptors directly associate with RGS2 and RGS4 through the c-terminus domain (Papakonstantinou, Karoussiotis, and Georgoussi 2015). Another GPCR that was reported to interact with RGS proteins through the c-terminus was cholecystokinin receptor-2, which interacts with the RGS2 (Langer et al. 2009). As for the domains of RGS proteins that specifically interact with GPCRs, the N-terminal domains of many R4 family of RGS proteins were implicated as the mediators for this interaction. Indeed, RGS2, RGS4 and RGS8 utilized their N-terminal domains to interact with several GPCRs including M1 Muscarinic Acetylcholine, alpha1A-adrenergic receptors, dopamine, and Melanin-concentrating hormone receptors (Bernstein et al. 2004; Hague et al. 2005; Min et al. 2012; Miyamoto-Matsubara et al. 2008). Thus, it can be postulated that there is a common connection between the third intracellular loop and the c-terminus domains of GPCRs, and the N-terminal domains of RGS proteins that mediates the interaction. A few exceptions exist, for example, RGS7 and RGS9 interact with GPCRs through their unique DEP domain (Sandiford and Slepak 2009; Zheng et al. 2011). So far, it appears that the RGS domains are not as crucial as other domains in mediating interaction with GPCRs. Accordingly, by binding G proteins via the RGS domains, and GPCRs through the N-terminus, RGS proteins can simultaneously bind GPCRs and G proteins to form a GPCR-G protein-RGS complex. Overall, the previous studies indicate that RGS proteins and GPCRs interact via common domains, therefore, further understanding of these interactions will be advantageous in future GPCR and/or RGS-targeted therapeutic approaches.

Abundant evidence exists supporting the observation that interactions between GPCRs and RGS proteins can be regulated according to the activation status of the corresponding GPCR. Particularly, opioid receptor interaction with RGS is highly sensitive to agonist stimulation. Application of the mu-opioid receptor agonist DAMGO to co-immunoprecipitation

samples increased the binding between mu-opioid receptor to both RGS4 and Goα (Santhappan et al. 2015). In-vivo treatment of morphine caused various alterations in mu-opioid-RGS-G protein complexes in the mouse periaqueductal gray matter. 24 hour morphine treatment reduced the binding of mu-receptor to RGS4 and RGS9-2 and enhanced the association of RGSZ1 and RGSZ2 to the same receptor (Garzon, Rodriguez-Munoz, and Sanchez-Blazquez 2005). Interestingly, the RGS4 association with mu-opioid receptor was enhanced after 3 hours of morphine treatment. Conversely, RGS4 association with kappa-opioid receptors was not affected by the receptor activation, indicating that opioid receptor subtypes may have different mechanisms regulating the association with RGS proteins (Papakonstantinou, Karoussiotis, and Georgoussi 2015). Taken together, the previous findings indicate that the effect of agonist stimulation on GPCR-RGS interaction is dynamic and complex, which will require a more in-depth analysis under different conditions. These effects should be taking into consideration in opioid drugs discovery and development since agonist-induced alterations of GPCR-RGS-G protein interactions will have direct implications on the efficacy, onset and duration of action of opioid drugs. Furthermore, understanding how agonists induce changes in GPCR-RGS interactions will be applicable to other GPCRs as well, such as Sphingosine-1-phosphate (S1P) receptors which associates with RGS2 differently upon receptor stimulation (Kohno and Igarashi 2008).

Efforts to understand how RGS proteins recognize the interacting GPCRs led to the discovery of interacting proteins that critically affect the RGS-GPCR interactions. Spinophillin acts as a scaffold to both RGS proteins and GPCRs. Spinophilin binds the N-terminal domain of RGS2 and the third intracellular loop of alpha-adrenergic receptor and is required for RGS2-induced inhibition of α-adrenergic signaling, as RGS2 was rendered ineffective in inhibiting α-adrenergic-induced calcium signaling upon spinophilin knockdown in Xenopus laevis oocytes (Wang et al. 2005). This is also confirmed in prefrontal cortex neurons in which both RGS2 and RGS4 lost the ability to inhibit α1-adrenergic receptor-induced down-regulation of NMDAR-

mediated excitatory postsynaptic currents in neurons from spinophilin knockout mice (Liu et al. 2006). Similarly, but with another type of receptor, spinophilin is suggested to mediate the interaction between RGS4 and M(3) muscarinic acetylcholine receptors in β-cells, and the loss of this scaffolding protein enhanced M3R signaling and subsequent insulin release (Ruiz de Azua et al. 2012). Further, spinophilin potentially influences a broad range of RGS-mediated actions since it was demonstrated that it binds multiple RGS proteins, including RGS1, RGS2, RGS4, RGS8, RGS10, RGS16, RGS18 and RGS19 (Wang et al. 2005; Fujii et al. 2008; Ma et al. 2012). Although the previous findings collectively indicate that spinophilin functions to bring RGS proteins and GPCRs together for effective RGS function, this in not always the case. In platelets, spinophilin forms a unique complex with the tyrosine phosphatase SHP-1 and RGS proteins (RGS10 and RGS18), which sequesters RGS proteins away from the target GPCR. In this model, loss of spinophilin enhanced RGS functions and resulted in an accelerated deactivation of GPCR signaling (Ma et al. 2012).

The spinophilin homologue neurabin is also an RGS-interacting protein and its loss enhanced the binding of RGS2 to spinophilin, whereas spinophilin loss enhanced the interaction between RGS2 and neurabin (Wang et al. 2007). Unlike the interaction with spinophilin, the interaction between RGS2 and neurabin resulted in an inhibition of RGS inhibitory action on αAR (Wang et al. 2007). This indicates that while spinophilin interacts with RGS2 to link it to αAR and enhance its actions, neurabin sequesters RGS2 and prevents its binding to spinophilin, thereby inhibiting RGS2 action on αAR. However, in another model, neurabin behaved as a scaffold for RGS4 and adenosine A(1) receptor (A1R) and facilitated the inhibitory effect of RGS4 on the receptor (Chen et al. 2012). Thus, spinophilin and neurabin may regulate RGS-GPCR interactions and subsequent signaling differently depending on the specific GPCR type and/or RGS member forming the complex as well as the physiological background of this interaction.

Other scaffolding proteins that were reported to link RGS proteins to GPCRs include GIPC (GAIP-interacting protein, C terminus), which recruits RGS19 to dopamine D2 receptor (D2R), and β-Arrestin2, which interacts with both RGS9-2 and D(3)R (Jeanneteau et al. 2004; Zheng et al. 2011). However, whether these scaffold proteins affect other RGS-GPCR interactions is yet to be determined.

Overall, direct interactions between GPCR and RGS proteins commonly occurs, which confers RGS selectivity toward GPCRs and G proteins. The formation of this complex may explain why an RGS protein that displays a weak affinity for the target G protein in-vitro still enhances the GTPase activity of the same G protein in-vivo. These interactions are dynamically regulated by agonist stimulation or scaffold proteins and are mediated by specific domains on both GPCRs and RGS proteins.

RGS proteins interaction with unique binding partners mediate GAP-independent functions:

In addition to being classic GAPs for G proteins and inhibitors of GPCR signaling, it is established now that RGS proteins exerts other functions that are not related to this GAP activity. RGS proteins achieve these functions through interactions with a variety of binding partners that include ion channels, kinases, and transcription factors. RGS proteins associate with these partners in several subcellular locations. Here, we will discuss examples of these interactions, their physiological relevance, structural requirements, and localization.

RGS proteins directly associate with ion channels independent of G proteins

G proteins are considered classic modulators of ion channel activity. There are several ways by which G proteins alter the activity of ion channels. They can mediate this effect indirectly through different effectors (Breitwieser 1991). G protein subunits can also directly interact with ion channels, thereby regulating their opening (Breitwieser 1991). Accordingly, RGS proteins, via suppressing G protein activity, regulate the activity of ion channels. G protein gated inwardly-rectifying potassium (GIRK) channels and calcium channels are among classic

examples of ion channels that are regulated by RGS proteins through classic GAP activity on G proteins (Chuang and Chuang 2012; Han et al. 2006). Although many RGS proteins regulate ion channels via their classic GAP activity, it was also demonstrated that some RGS proteins directly interact and subsequently alter ion channel activity in a GAP-independent mechanism. In dorsal root ganglion neurons, RGS12 directly associated with N-type calcium channels and regulated GABAB-mediated inhibition of calcium currents (Schiff et al. 2000). In addition, the epithelial calcium channel TRPV6 directly associates RGS2 (Schoeber et al. 2006). Through this interaction, RGS2 suppressed calcium current of TRPV6. These findings indicate that RGS proteins can interact with different types of calcium channels and alter their activity.

RGS proteins form unique complexes with GIRK channels in neurons to regulate the channel currents. RGS4 formed a complex with GIRK channels and muscarinic m2 receptor which allowed RGS4 to effectively regulate the channel activity (Jaen and Doupnik 2006). Interestingly, comparing the potency of RGS4 to RGS3, which classically regulates GIRK channels but does not associate with it, revealed that RGS4 was significantly more effective (Jaen and Doupnik 2006). This indicates that RGS4 interaction with the GPCR-GIRK complex positions it in an optimal position, allowing a stronger effect on GIRK channels. Since GPCR forms complexes with GIRK, and it has been demonstrated that several RGS proteins directly associate with GPCRs, there is a possibility that other RGS-GIRK-GPCR complexes exit (Jaen and Doupnik 2006). The R7 family member RGS7 can directly associate with GIRK channels and alter their activity independently of specific GPCR interactions (Xie, Allen, et al. 2010; Zhou et al. 2012). However, this interaction requires the binding partners Gβ5 and R7BP. Since these binding partners uniquely interact with R7 family members, it is likely that this association with GIRK is specific to this family.

Notably, the direct association between RGS proteins and ion channels is mainly mediated by specific domains located outside the RGS domain. RGS12 interacts with N-type calcium channels through its unique PTB domain (Schiff et al. 2000), and RGS2-mediated

inhibition of TRPV6 channels requires its N-terminal domain (Schoeber et al. 2006). Similarly, the N-terminal domain of RGS4, and not the RGS domain, mediates the specific interaction with GIRK channels. RGS8 was shown to regulate GIRK channel activity via the N-terminal domain, whereas the RGS domain was dispensable (Jeong and Ikeda 2001). However, it is unknown yet if RGS8 regulate GIRK channels by direct association. The fact that RGS proteins tend to interact with ion channels via unique domains distinct from RGS domains indicate that RGS interaction with these channels is not a universal feature of RGS proteins, unlike the classic role of RGS proteins as GAP for G protein-induced modulation of ion channel activity. Therefore, mainly the presence of unique domains or the interaction with specific binding partners, as in the case of R7 family interaction with R7BP or Gβ5, can facilitate such direct association with ion channels.

In addition to the direct interactions with calcium channels discussed above, RGS proteins can classically influence calcium signaling by regulating the G protein-mediated modulation of calcium channels and/or by regulating GPCR/PLC/IP3 pathway (Hollinger and Hepler 2002). Further, RGS can directly interact with calcium-sensitive proteins such as calmodulin (Yang and Li 2007). This suggests a potential crosstalk between RGS proteins to cooperatively regulate calcium levels by playing different GAP-dependent and independent functions. Additional mechanistic studies are needed to decipher the source of calcium and the specific processes being regulated by RGS proteins.

RGS proteins regulate Kinase activity by direct interactions

G protein activation is established to be one of the main pathways that regulate activity of many kinases. Gαi inhibits adenylate cyclase-induced production of cAMP, resulting in PKA inhibition. Also, Gαq regulates PKC activity via the activation of the PLC/IP3/DAG pathway (Tuteja 2009).Therefore, RGS proteins, by acting as classic GAPs, can alter kinases activity. In addition to affecting kinases by acting on G protein, it has been demonstrated that some RGS

proteins directly interact with kinases and influence their activity in G protein-independent mechanisms.

In mast cells, RGS13 limits mast cell degranulation and anaphylactic reactions through a direct interaction with Phosphoinositide 3-kinase (PI3K), a kinase that plays pivotal roles in mediating allergic responses (Bansal, Xie, et al. 2008). This interaction was mediated by the N-terminal domain of RGS13, which interacted with the p85α regulatory subunit of PI3K resulting in blocking the recruitment of PI3K to essential signaling scaffolds needed for its activation (Bansal, Xie, et al. 2008). Interestingly, RGS13 also inhibits mast cell-mediated allergic responses triggered by several GPCR agonists such as adenosine, C5a, sphingosine-1-phosphate, and CXCL12 (Bansal, DiVietro, et al. 2008). Thus, RGS13 employs both G protein dependent and independent mechanisms to regulate similar physiological functions. Another RGS-PI3K interaction was also observed in breast cancer cells, where RGS16 interacted with PI3K and inhibited EGF-induced proliferation and subsequent activation of AKT (Liang et al. 2009). Unlike RGS13, however, the RGS domain of RGS16 was required for binding to PI3K. RGS16 interacted with the p85α subunit of PI3K and subsequently prevented the interaction between p85α and the adaptor Gab1, resulting in an inhibition of PI3K (Liang et al. 2009). The RGS16-PI3K interaction was regulated by tyrosine phosphorylation of p85α subunits, which is mediated by Src family kinases (Liang et al. 2009). This suggests that certain kinases can regulate the interaction of other kinases and RGS proteins. Notably, PI3K was shown to bind multiple RGS proteins in addition to RGS13 and RGS16, including RGS1, RGS2, and RGS5 (Liang et al. 2009). Since both PI3K and these RGS proteins play various roles in different systems, it is possible that PI3K-RGS interactions influence other signaling pathways. Further, it will be interesting to determine whether these RGS proteins interacts with PI3K via their N-terminal domains, like RGS13, or their RGS domains, like RGS16.

In another model, a unique interaction was identified between RGS2 and leucine-rich repeat kinase 2 (LRRK2) (Dusonchet et al. 2014). LRRK2 is strongly associated with

Parkinson's disease and mutations in this gene account for a large number of PD cases (Dusonchet et al. 2014). The interaction between LRRK2 and RGS2 was identified in HEK cells and in mouse striatal tissue, indicating that this binding occurs in-vitro and in-vivo. Furthermore, RGS2 interacted with WT LRRK2 and its Parkinson's disease-associated mutations (G2019S, R1441C and Y1699C) indiscriminately, suggesting that the RGS2-interacting domains in LRRK2 are not disrupted by these mutations. RGS2 lacking the N-terminal domain also interacted with LRRK2, suggesting a possible role of the RGS domain in this interaction. RGS2 inhibited both GTPase and kinase activity of LRRK2, and further limited LRRK2-induced neuronal toxicity, indicating that this interaction is physiologically relevant. Interestingly, RGS2 itself was found to be a substrate of LRRK2 (Dusonchet et al. 2014). This suggests that RGS-kinase interactions may affect the function of both RGS proteins and kinases. In addition, the fact that the RGS domain is possibly mediating the interaction between RGS2 and LRRK2 raises the possibility that other RGS proteins may also interact with LRRK2. GSK3β is another example of a kinase that directly interacts with RGS proteins. The kinase activity of GSK3β was inhibited by the direct interaction with PDZ-RGS3, which resulted in enhanced Wnt3a signaling (Shi, Huang, and Kehrl 2012).

In conclusion, direct interactions between RGS proteins and kinases exist, and these interactions play roles in physiological pathways and can produce distinct phenotypes. Since some of the kinases identified to interact with RGS proteins, such as PI3K and GSK3β, play various roles in different physiological systems, it will be interesting to assess whether RGS proteins interact with these kinases in specific cellular and tissue systems or interact in a broad manner. This can link the kinase-interacting RGS proteins to other signaling pathways that are regulated by the targeted kinase. Studying how the interaction between RGS proteins and kinases affect kinase activity should be accompanied by analyzing whether this interaction will also affect RGS proteins through phosphorylation. Furthermore, many kinases identified in the previous studies are also regulated by certain GPCRs, and since GPCRs are regulated by RGS

proteins, it is possible that some RGS proteins affect kinases directly by interactions and indirectly by altering GPCR signaling, thereby producing a possible synergistic effect. The fact that RGS proteins directly interact and influence kinase activity may encourage future attempts to manipulate kinase activity by targeting these interactions instead of traditionally targeting RGS-G protein interactions, which will be advantageous because it will bypass the GPCR/ G protein step and possibly avoid non-specific effects.

Nuclear binding partners mediate nuclear functions of RGS proteins

The localization of RGS proteins in the cytoplasm allows for rapid recruitment to the plasma membrane for subsequent deactivation of G proteins. Therefore, it was intriguing that many RGS proteins localize in the nucleus, either constitutively or upon stimulation by certain signaling pathways (Chatterjee and Fisher 2000; Burgon et al. 2001; Branch and Hepler 2017). An initial hypothesis suggested that the nuclear presence of RGS proteins is a regulatory mechanism employed by the cell to sequester RGS proteins away from the site of action (cytoplasm/membrane) to allow adequate G protein activation. In this model, the nuclear reservoir of RGS proteins would be exported to their site of action after G protein activation to inhibit the pathway in a timely manner. This is true in some cases, as it was demonstrated that activation of some GPCRs or G proteins triggers nuclear export of some RGS proteins and subsequent association with G proteins (Masuho et al. 2004; Roy, Lemberg, and Chidiac 2003). However, growing evidence suggests that RGS proteins actively play nuclear functions where they directly interact with nuclear binding partners and some of them are components of nuclear complexes.

Investigating the nuclear roles of RGS proteins revealed that several RGS proteins influence transcription by directly interacting with transcription factors. A first report linking RGS proteins to transcription machinery was the association of RGS6 with DMAP1 and the DMAP1-interacting protein DNMT1 (Liu and Fisher 2004). This interaction was identified in COS-7 cells and was shown to be mediated by the GGL domain of RGS6. Through this interaction, RGS6

inhibited DNMT1-induced transcription repression, which demonstrates a physiological relevance of this interaction. Intriguingly, in addition to inhibiting DNMT1-mediated transcriptional repression, RGS6 also enhanced the degradation of DNMT1 (Huang et al. 2014). This was facilitated by the interaction of RGS6 and the acetyltransferase Tip60, which triggered acetylation and subsequent degradation of DNMT1. Loss of RGS6 enhanced DNMT1-induced repression of tumor suppressor genes and promoted RAS-induced oncogenic cellular transformation (Huang et al. 2014). Unlike the interaction with DMAP1 which does not require the RGS domain, the interaction of RGS6 and Tip60 was mediated by the RGS domain of RGS6 (Huang et al. 2014). Since RGS6 influences DNMT1 activity and stability via different mechanism, it is possible that RGS6 is a universal regulator of DNMT1 transcription repression activity, which will have broad implications linking RGS6 to various physiological and pathological processes.

A common mechanism by which RGS proteins inhibits transcription is via disrupting transcriptional complexes that are critical for efficient transcription. RGS3, for example, interacts with Smad3 and Smad4 and blocks the formation of the Smad3/Smad4 complex, resulting in inhibition of smad-mediated transcription (Yau et al. 2008). Similarly, RGS13 interferes with the interaction between CREB and its cofactor CBP, thereby suppressing CREB-mediated transcription (Xie et al. 2008). The RGS domains of both RGS3 and RGS13 were not essential in these interactions, suggesting that these functions are possibly unique to these RGS proteins. Additional evidence of RGS proteins directly interacting with transcription factors was reported in Raw264.7 macrophages, where RGS2 directly interacted with STAT3 and inhibited STAT3-mediated transcription (Lee, Park, et al. 2012). Since the previous findings link RGS proteins directly to transcription machinery complexes, it is very possible that these RGS proteins play additional roles in other systems, as it is established that these complexes broadly influence transcription of several genes in multiple physiological models.

The nuclear localization and subsequent association of RGS proteins with transcription factors were found in some cases to be regulated by activation of upstream signaling pathways. Activation of PKA increased the nuclear translocation of RGS13 and the subsequent interaction with CREB (Xie et al. 2008). Similarly, DMAP1 triggered RGS6 nuclear translocation from the cytoplasm (Liu and Fisher 2004). In contrast, the association of RGS2 and STAT3 was reduced by TLR2 stimulation (Lee, Park, et al. 2012). This indicates the interaction between RGS proteins and transcription factors can be enhanced or repressed by upstream signaling pathways, offering a possible opportunity to target RGS-induced regulation of transcription factor activity by modifying these pathways. Since it is conceivable that these upstream pathways such as PKA, which is inhibited by the RGS-targeted Gαi, will also be affected by RGS proteins, there is a possibility that RGS protein direct interactions with transcription factors, which are GAP-independent, will be regulated by the GAP-mediated functions of other RGS proteins. As some nuclear partners and/or upstream pathways trigger nuclear localization, this may negatively regulate the classic GAP activity since it removes RGS from the cytoplasm, which is the optimal site for GAP. Indeed, PKA-induced nuclear translocation of RGS10 inhibited RGS10 GAP action (Burgon et al. 2001). On the other hand, GPCR activation exports some RGS from the nucleus, which may regulate their nuclear functions (Roy, Lemberg, and Chidiac 2003). Therefore, events that trigger cytoplasmic/nuclear localization of RGS proteins may have a double effect on the functions of RGS proteins.

The interaction between RGS proteins and transcription factors may have direct consequences on RGS gene transcription since many of these transcription factors directly participate in the transcription of several RGS genes. For example, DNMT1, which is regulated by unique interaction with RGS6, regulate the transcription of RGS2 and RGS10 (Cacan 2017; Cacan et al. 2014). CREB activation, which is suppressed by RGS13, promotes the transcription of RGS2 and RGS4 (Xie et al. 2011; Chekler et al. 2015). Interestingly, as RGS2 interacts and inhibits STAT3, RGS2 itself was shown to be regulated by activation of STAT3

(Lee, Park, et al. 2012), suggesting a bidirectional regulatory mechanism. This indicates that enhanced expression and/or activity of one RGS protein may directly regulate the levels of another RGS protein, which is possibly a mechanism utilized by cells to avoid abnormal RGS levels or activity.

The ability of RGS proteins to regulate transcription indirectly is not a new finding, since they can affect transcription through regulation of GPCR activity. The novelty arises from the fact that some RGS proteins directly interact with transcription factors and inhibit their transcription by blocking the formation of important transcriptional complexes. This suggests that RGS proteins will inhibit transcription regardless of which upstream GPCR is activated. In fact, this finding also indicates that RGS proteins will regulate transcription downstream of pathways distinct from GPCR activation, further expanding the effects of RGS proteins in cellular signaling. Clearly, this is not the case for the classic GAP functions of RGS proteins, where they influence downstream transcription according to the type of upstream GPCR and coupled G protein activated.

Collectively, the studies presented here demonstrate that nuclear localization of RGS proteins is not simply a mechanism for sequestering RGS proteins, and that RGS proteins perform several specific nuclear functions. The ability of RGS proteins to directly participate in nuclear functions is a very intriguing new aspect, which will possibly identify novel RGS functions and explain the mechanisms of some RGS-associated phenotypes. Moreover, this will lead to additional investigative studies on other RGS proteins that were shown to be localized to the nucleus such as RGS10, which travels to the nucleus upon PKA activation (Burgon et al. 2001), or RGS14, which was shown to localize in chromatin-rich regions in proximity to active RNA polymerase II (Branch and Hepler 2017). Intriguingly, RGS3 requires nuclear localization to induce apoptosis, clearly indicating a novel nuclear function of RGS3, which possibly involve interacting with nuclear binding partners. In addition, RGS12TS-S displays nuclear localization and suppresses transcription (Chatterjee and Fisher 2002). Therefore, continued investigation

of the nuclear functions of RGS proteins may reveal additional novel interacting partners and mechanisms. Since RGS proteins can still influence transcription via their classic GAP action on GPCRs, future studies reporting an RGS-mediated effect on transcription should add more efforts to distinguish whether RGS proteins affect transcription via GAP activity or in a more direct mechanism.

RGS proteins interact with specific binding partners in different cellular compartments

The cytoplasmic localization of RGS proteins near the cell membrane facilitates specific direct interactions with multiple membrane-associated proteins, including G proteins, GPCRs, and ion channels. This localization also allowed for other interactions with membrane-associated proteins, such as adenylyl cyclase (AC). RGS2 directly associates with several isoforms of AC and suppresses cAMP production in olfactory neurons (Sinnarajah et al. 2001). The RGS2-AC interaction suppresses AC activity but is distinct from the classic regulatory action of RGS proteins on AC, where they influence the enzyme activity by acting as GAPs. This interaction was mapped to the N-terminus of RGS2, whereas the RGS and C-terminal domains did not mediate the binding. Adenylyl cyclase is classically activated by Gαs, which is not classically targeted by RGS proteins (Yang, Lee, and Wand 1997). Thus, the ability of RGS2 to suppress AC directly will possibly enable RGS2 to act downstream of Gαs activation, a feature that is not common for RGS proteins. Interestingly, cAMP strongly activates RGS2 transcription and stimulates its expression. Therefore, the direct inhibitory effect of RGS2 on cAMP production may serve to regulate RGS2 expression in a feedback regulatory mechanism (Xie et al. 2011). In a similar manner, RGS9-1 interacted with retinal guanylyl cyclase through its N-terminal and inhibited cGMP production (Yu, Bondarenko, and Yamazaki 2001). These findings are further evidence demonstrating that RGS proteins can influence the functions of membrane-associated proteins directly through interactions involving non-RGS domains or indirectly by modulating G protein activity through RGS domains. In addition, some membrane-associated proteins directly regulate RGS protein localization and activity. For example, the

membrane adaptor R9AP recruits RGS9-1 to the plasma membrane and subsequently enhances its GAP activity (Hu, Zhang, and Wensel 2003).

Although RGS proteins canonically target heterometric and not small G proteins, Ras-binding domains (RBD) were identified in the R12 family members RGS12 and RGS14 (Snow et al. 1997). Via RBD domains, RGS12 interacted with H-Ras and B-Raf, whereas the PDZ domain mediated the interaction with MEK2 (Willard et al. 2007). RGS12 also interacted with TrkA receptors, thereby forming a complex consisting of TrkA, activated H-Ras, B-Raf, and MEK2, which facilitated the ability of RGS12 to regulate this pathway (Willard et al. 2007). This is an example of an RGS protein utilizing multiple domains to simultaneously interact with several components of the same pathway, thereby acting as a scaffold to coordinate pathway activation. RGS14 acted in a similar manner and associated with both H-Ras and Raf and inhibited PDGF-induced ERK activation (Shu, Ramineni, and Hepler 2010). Interestingly, activation of Gαi recruited RGS14 to the membrane and blocked the interaction between RGS14 and Raf, resulting in reversing the RGS14 inhibitory effect on ERK activation (Shu, Ramineni, and Hepler 2010). This indicates that different binding partners may compete to recruit RGS proteins to regulate a certain pathway in the expense of another.

In addition to their classic membrane/cytoplasmic localization, RGS proteins can also be found in several other cellular compartments (Chatterjee and Fisher 2000). This diverse localization allows RGS proteins to participate in multiple signaling events and exerts functions that are not necessarily GAP-mediated. Many of these functions involve location-specific interactions with binding partners. For example, both RGSZ1 and RGS6 interacted with the Golgi-localized protein SCG10 (superior cervical ganglia, neural specific 10) in PC12 (Nixon, Grenningloh, and Casey 2002; Liu, Chatterjee, and Fisher 2002). Interestingly, the interactions of RGSZ1 suppressed, whereas the interaction with RGS6 enhanced, SCG10-induced microtubule disassembly. RGS6 also enhanced NGF-induced neuronal differentiation, but whether RGSZ1-induced inhibition of SCG10 activity affected neuronal growth is yet to be

determined. RGS6 lacking GGL domain failed to interact with SCG10 or promote neuronal differentiation, indicating that this domain mediate RGS6 interaction and function in this model (Liu, Chatterjee, and Fisher 2002). The N-terminal domain of RGSZ1 (containing cysteine string motif) was required for the interaction with SCG10. In addition to RGSZ1, the other RZ family member GAIP also interacted with SCG10, suggesting that SCG10 may commonly interact with RZ family members (Nixon, Grenningloh, and Casey 2002). Treating PC12 with nerve growth factor triggered a co-localization of both RGSZ1 and SCG10 to the Golgi complex (Nixon, Grenningloh, and Casey 2002). This was not observed with RGS6-SCG10 interaction as NGF caused a co-localization of RGS6 and SCG10 in the perinuclear region (Liu, Chatterjee, and Fisher 2002). Collectively, the previous findings demonstrate that although both RGSZ1 and RGS6 interact with SCG10, they require different domains, produce opposite physiological effects, and do not display similar localization properties.

Another example of location-specific interaction of RGS proteins is the interaction of RGS14 with tubulin in microtubules, which promoted tubulin polymerization with an assist from Gαi1 (Martin-McCaffrey et al. 2005). Further emphasizing the role of RGS proteins in microtubule is the interaction of RGS2 with cell cycle-related kinase, Nek7 (de Souza et al. 2015). The localization of RGS2 to the mitotic spindle and its association with NeK7 was required for mitotic spindle organization (de Souza et al. 2015). Therefore, it can be concluded that RGS proteins can interact with specific localized binding partners, causing location-specific physiological effects.

cellular localization of RGS proteins is a critical determinant of their function, therefore, binding partners that influence the trafficking of RGS proteins will naturally impact how RGS proteins functions. Therefore, as more studies are beginning to investigate how specific interactions of RGS proteins and binding partners in different cellular compartments influence the function of these interactors, a parallel attention should be focused on studying how these interactions influence RGS GAP-dependent and independent functions.

Conclusions

The classic interaction between RGS and G proteins mediate the canonical GAP activity of RGS proteins. However, the GAP activity of RGS proteins is influenced by other binding partners that can form more stable interactions and are regulated by external agents. These qualities may encourage further studies to assess the suitability of targeting these interactions as an alternative approach to control GAP activity. Besides, the direct interactions of RGS proteins with several non-G protein binding partners mediate various GAP-independent functions such as regulating the activity of kinases, transcriptional machinery, membrane-associated enzymes and ion channels. These functions are facilitated by distinct structural domains of RGS proteins that specifically interacted with unique binding partners. Further, these interactions take place in different cellular locations and often trigger RGS localization. These findings clearly indicate that RGS proteins are much more than just cytoplasmic GAP molecules. In fact, it is expected that revisiting some phenotypes previously believed to be exclusively mediated by classic GAP activity may reveal some GAP-independent mechanisms as well. More universal interactome profiling studies of RGS proteins in different physiological models will possibly identify additional novel binding partners. This should critically aid in discovering previously unknown functions of RGS proteins and/or identify molecular mechanisms of RGS proteins that remains elusive.

CHAPTER 5

RGS10 REGULATES THE EXPRESSION OF CYCLOOXYGENASE-2 AND TUMOR NECROSIS FACTOR ALPHA THROUGH A G PROTEIN-INDEPENDENT MECHANISM[3]

[3] Mohammed Alqinyah, Faris Almutairi, Menbere Y Wendimu, and Shelley Hooks. Submitted to Molecular Pharmacology, January 2018.

Abstract

The small RGS protein RGS10 is a key regulator of neuroinflammation and ovarian cancer cell survival; however, the mechanism for RGS10 function in these cells is unknown and has not been linked to specific G protein pathways. RGS10 is highly enriched in microglia, and loss of RGS10 expression in microglia amplifies production of the inflammatory cytokine TNFα and enhances microglia-induced neurotoxicity. RGS10 also regulates cell survival and chemoresistance of ovarian cancer cells. Cyclooxygenase-2 (COX-2) mediated production of prostaglandins such as PGE2 is a key factor in both neuroinflammation and cancer chemoresistance, suggesting it may be involved in RGS10 function in both cell types, but a connection between RGS10 and COX-2 has not been reported. To address these questions, we completed a mechanistic study to characterize RGS10 regulation of TNFα and COX-2 and to determine if these effects are mediated through a G protein dependent mechanism. Our data show for the first time that loss of RGS10 expression significantly elevates stimulated COX-2 expression and PGE2 production in microglia. Further, the elevated inflammatory signaling resulting from RGS10 loss was not affected by Gαi inhibition, and a RGS10 mutant that is unable to bind activated G proteins was as effective as wildtype in inhibiting TNFα expression. Similarly, suppression of RGS10 in ovarian cancer cells enhanced TNFα and COX-2 expression, and this effect did not require Gi activity. Together, our data strongly indicate that RGS10 inhibits COX-2 expression by a G protein independent mechanism to regulate inflammatory signaling in microglia and ovarian cancer cells.

Introduction

Regulators of G-protein signaling (RGS) are a family of proteins that classically act as activators of the intrinsic GTPase activity of heterotrimeric Gα subunits (Watson et al. 1996). Owing to this GTPase-accelerating protein (GAP) activity and inhibition of signaling initiated by G-protein coupled receptors, RGS proteins play numerous roles in physiological and pathological conditions in diverse systems. However, multiple studies have revealed actions of RGS proteins that are independent of GTPase-accelerating activity (recently reviewed in: (Sethakorn, Yau, and Dulin 2010)). These non-canonical functions of RGS proteins can affect a variety of targets, including GPCRs, kinases, and transcription factors (Sethakorn, Yau, and Dulin 2010). Therefore, to investigate the molecular mechanism of specific RGS protein actions, a critical initial question to answer is whether the RGS protein is acting in a classic GAP-dependent or non-canonical GAP-independent mechanism.

The small RGS protein RGS10 regulates inflammatory and survival signaling in multiple cell types (Lee et al. 2013; Lee, Chung, et al. 2011; Hooks et al. 2010), and has been proposed as a potential drug target for neuroinflammatory disease and ovarian cancer. However, the mechanisms by which RGS10 affects inflammatory and survival signaling are undefined, hampering the development of RGS10-targeted therapeutic strategies. RGS10 is the smallest member of the R12 RGS subfamily with no functional domains outside of the RGS domain. RGS10 has been shown to selectively target Gαi family G proteins via classic GAP activity (Hunt et al. 1996), and is highly enriched in immune cells, including peripheral macrophages and microglia (Lee et al. 2008; Lee et al. 2013). Loss of RGS10 in microglia amplifies production of inflammatory cytokines, such as TNFα, and enhances microglia-induced neurotoxicity (Lee, Chung, et al. 2011). In addition to its anti-inflammatory role in microglia, RGS10 also regulates survival of ovarian cancer cells, and loss of RGS10 induces chemoresistance in ovarian cancer cells (Ali et al. 2013b; Hooks et al. 2010). Cyclooxygenase-2 (COX-2) mediated production of

prostaglandins such as PGE2 is also a key factor in both neuroinflammation and cancer chemoresistance (Minghetti 2004; Bijman et al. 2008), suggesting that this pathway may also be related to RGS10 effects, but the effect of RGS10 on COX-2 function is not known.

The goal of the current study was to test the hypothesis that RGS10 regulates TNFα and COX-2 through a GAP-dependent mechanism. Our data show for the first time that RGS10 deficiency significantly elevates LPS-stimulated COX-2 expression and release of PGE2 from microglia. Furthermore, the elevated inflammatory signaling resulted by RGS10 loss was not affected by Gαi inhibition, and a RGS10 GAP-deficient mutant was as effective in inhibiting TNFα expression as wild type RGS10, suggesting that the anti-inflammatory functions of RGS10 are not mediated by its classic GAP activity on G-proteins. We also show for the first time that RGS10 regulates TNFα and COX-2 expression in ovarian cancer cells through a Gi-independent mechanism. Identification and delineation of the G-protein independent RGS10 binding partners and mechanisms will facilitate strategic targeting of RGS10 function to develop novel therapeutics for inflammatory disease.

Materials and Methods

Cells and Reagents

Murine BV-2 microglia cell line was a gift from G. Hasko at University of Medicine and Dentistry of New Jersey (Newark, NJ) and was previously generated previously (Blasi et al. 1990). BV-2 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (VWR) supplemented with 10% Fetal Bovine Serum (ThermoFisher Scientific). HEK-Blue™ hTLR4 cell line was purchased from Invivogen and was maintained in Dulbecco's Modified Eagle's Medium (DMEM) (VWR) with 10% Fetal bovine serum and HEK-Blue™ Selection antibiotics (Invivogen) to selectively maintain cells over-expressing TLR4. Human SKOV-3 cell line was purchased from ATCC and was maintained in McCoy’s 5A, 1X (Iwakata & Grace Mod.) medium (Corning) supplemented with L-glutamine and 10%FBS.

Lipopolysaccharide was purchased from Sigma-Aldrich (St. Louis, MO), recombinant mouse CXCL12/SDF-1 alpha protein was purchased from R&D systems (Minneapolis, MN), Pertussis Toxin was obtained from Tocris (Pittsburgh, PA) and LPA was from Avanti Polar Lipids (Alabaster, AL).

siRNA and plasmid transfection

Mouse and human siRNA duplexes were purchased from Santa Cruz Biotechnology, and Lipofectamine-LTX with PLUS reagent was purchased from Thermo Fisher Scientific. The final concentration of siRNA in the culture medium was 60 nM, and the transfection was performed according to the manufacturer's protocol. Following transfection, cells were cultured an additional 48 hours in an antibiotic free culture medium before assessing expression or function.

RGS10-2 DNA plasmids were purchased from cDNA resource center (Bloomsburg University). The E52K mutation was generated with the QuikChange site directed mutagenesis kit (Stratagene) using primer sequences:(forward: 5'-TA AAA AAG GAA TTC AGT GAA **A**AA AAT; reverse: 5'-GC TAG CCA AAA CAA AAC ATT TT**T** TTC A). Mutagenesis resulted in a single nucleotide change from G□A, corresponding to codon GAA□AAA, Glutamic acid□ lysine at position 52 of human RGS10-2.

For transfection, 0.5 µg of plasmid DNA was used per well of 24 wells plate or scaled up or down appropriately for different size wells or plates. DNA plasmids were added to cells with Lipofectamine reagent according to the manufacturer's instructions, and cells were cultured an additional 48 hours in antibiotic free medium prior to assessing function.

Western Blot Analysis

Cells were lysed in SDS-PAGE sample buffer (0.5 M Tris pH 6.8, 10% SDS, Glycerol, βmercaptoethanol, bromophenol blue) and samples were subjected to SDS-PAGE using standard protocols followed by transfer to nitrocellulose membranes. Primary antibodies for

RGS10, COX-2, P65, and GNAI3 were purchased from Santa Cruz Biotechnology (Dallas, TX). P-ERK, ERK, P-P65, P-AKT, AKT were obtained from Cell Signaling Technology (Danvers, MA), and GAPDH was purchased from Millipore Technologies (Temecula, CA). Following primary antibody incubation, the suitable secondary HRPconjugated antibodies were used to incubate the membranes: donkey anti-goat IgGHRP (Santa Cruz), Goat Anti-Rabbit IgG-HRP (Millipore), and Goat anti-Mouse IgGHRP (Bethyl). The membranes were visualized utilizing an enhanced Chemiluminescent substrate for detection of HRP (ThermoScientific), and quantified using Flourchem HD2 software. The values were normalized to endogenous control GAPDH.

Quantitative Real-Time Polymerase Chain Reaction

Isolation of mRNA was performed using TRIzol reagent (Invitrogen/Life Technologies, Carlsbad, CA) and cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Actin and/or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as housekeeping genes for normalization.

Calculating the fold difference was performed using the $2^{-\Delta\Delta CT}$ method. Mouse actin and COX-2 mouse and human primers were purchased from Sigma. Mouse TNFα primers and human GAPDH and TNFα primers were obtained from Integrated DNA Technologies, IDT. Mouse primers used: actin forward 5′-GGC TGT ATT CCC CTC CAT CG-3′, actin reverse 5′-CCA GTT GGT AAC AAT GCC ATG T-3′, Cox-2 forward 5'-TGCAAGATCCACAGCCTACC-3', Cox-2 reverse 5'- GCTCAGTTGAACGCCTTTTG-3', TNF-α forward, 5′-CCT GTA GCC CAC GTC GTA G-3′; TNF-α reverse, 5′-GGG AGT AGA CAA GGT ACA ACC C-3′. Human primers used: GAPDH forward, 5′-GCCAAGGTCATCCATGACAACT-3', GAPDH reverse, 5′-GAGGGGCCATCCACAGTCTT-3', TNF-α forward, 5′-CTCTTCTGCCTGCACTTTG-3', TNF-α reverse, 5′-ATGGGCTACAGGCTTGTCACTC-3', Cox-2 forward 5'-CCCTTGGGTGTCAAAGGTAA-3', Cox-2 reverse 5'-GCCCTCGCTTATGATCTGTC-3'.

Co-immunoprecipitation

For Co-immunoprecipitation experiments in BV-2 microglia, cells were plated in 15 cm dishes and then lysed with 1.5 ml of a modified lysis buffer (50 mM Tris HCL,150 mM NaCl, 6mM MgCl2, 1% NP 40) containing protease/phosphatase inhibitor cocktail (Cell Signaling). Cell lysate was left for 30 minutes on ice and subsequently centrifuged at 27,216 G (15,000 RPM) for 10 Minutes at 4°C. Cell lysates were incubated with either GDP (10 µM) alone or with GDP + $AlCl_3$ (100 µm) + NaF (10 mM) at 20 °C with gentle shaking for 30 minutes. Next, 2 µg of Gαi3 antibody (Santa Cruz) or normal rabbit IgG (Santa Cruz) was added. For Co-immunoprecipitation experiments in HEK-hTLR4293 cells were transfected prior to the co-immunoprecipitation with 0.25 µg of RGS10 WT, RGS10 E52K, and Gαi3. Following transfection, the co-immunoprecipitation was performed as described above.

PGE2 Measurement

To quantify the levels of PGE2 in culture medium, BV-2 microglia culture medium was collected and centrifuged at 1,500 rpm for 10 min at 4°C. Enzyme-linked immunosorbent assay (ELISA) was performed using PGE2 ELISA kit (Enzo) to measure PGE2 levels in media following manufacturer's protocol.

Experimental rigor and statistical analysis

All qRT-PCR data presented were performed in experimental and technical triplicates, except where indicated in Figure legends. All western images were quantified using densitometry of raw, unmodified image data, including background subtraction. Some images of western data have been modified slightly to adjust brightness, as indicated in legends. For analyzing data for statistical differences between groups, data were analyzed using analysis of variance (ANOVA) test followed by Tukey's multiple comparison tests. Statistical significance cutoff ranges indicated in figures correspond to: *$p < 0.05$ **$p < 0.01$ and ***$p < 0.001$.

Results

RGS10 knockdown elevates LPS-induced production of COX-2 and PGE2 in BV-2 microglia

RGS10 has been shown to regulate expression of multiple inflammatory mediators following activation with lipopolysaccharide (LPS), but an effect on COX-2 has not been reported. Due to the essential roles of COX-2 in maintaining inflammation in microglia, we aimed to test whether LPS-stimulated COX-2 is also affected by RGS10 loss. To test this hypothesis, we treated BV-2 microglia with RGS10-targeted siRNA or control siRNA for 24 hours before stimulating with LPS (10 ng/ml) for an additional 24 hours. RGS10 knockdown significantly enhanced LPS-induced upregulation of COX-2 mRNA (**Figure 5.1A**). Similarly, LPS treatment resulted in higher levels of COX-2 protein in BV-2 cells, and this effect was significantly enhanced in cells transfected with RGS10 siRNA compared to control siRNA cells, suggesting that endogenous RGS10 suppresses COX-2 protein expression in BV-2 microglia (**Figure 5.1B, see also Figure 5.3B)**. One of the essential functions of COX-2 is the production of prostaglandins including PGE2, which is a critical mediator of neuroinflammation (Andreasson 2010). Therefore, we tested the effect of RGS10 loss on LPS-induced production of PGE2 in BV-2 microglia. RGS10 knockdown enhanced LPS-stimulated release of PGE2 into microglia culture medium by approximately 25% (**Figure 5.1C**). Taken together, the results indicate that endogenous RGS10 regulates the expression of COX-2 and significantly suppresses PGE2 release from microglia following LPS stimulation. Importantly, the magnitude and duration of RGS10 suppression induced by transient siRNA transfection recapitulates that observed during suppression of RGS10 expression induced by endogenous activation of microglia (Alqinyah et al. 2017), suggesting that the effects observed here will serve to amplify the magnitude of COX-2 and PGE2 signaling during microglial activation.

RGS10 acts as a classic GTPase-activating protein in microglia, but does not affect TLR4-induced acute signaling

RGS10 deactivates Gαi signaling, and signaling cross-talk has been reported between Gαi pathways and LPS-stimulated toll-like receptors (Fan et al. 2007; Marty and Richard 2010). Thus, we sought to determine whether the ability of RGS10 to regulate LPS-stimulated inflammatory signaling in microglia is mediated by RGS10 regulation of Gαi. First, we aimed to confirm that RGS10 expressed in BV-2 microglia can act as a Gαi GAP by assessing binding between RGS10 and transition state Gαi. This conformation of Gα subunits is the high affinity substrate for RGS domain GAP activity and is mimicked and stabilized in the presence of GDP with aluminum fluoride (AlF_4^-), while Gα bound to GDP alone has low affinity for RGS domains (Berman, Kozasa, and Gilman 1996; Tesmer et al. 1997). Multiple studies have described strong Gαi-selectivity of the RGS10 RGS domain (Ajit and Young 2005; Taylor, Bommarito, and Tesmer 2016); however, it has also been reported that RGS10 can display weak interactions with Gαq *(Soundararajan et al. 2008a)*. To determine if endogenous microglial RGS10 interacts with Gαq, we also assessed interaction between RGS10 and Gαq in BV-2 cells. BV-2 cell lysates were immunoprecipitated with Gαi3 or Gαq targeted antibody or control IgG in the presence of GDP or GDP plus AlF_4^-. Our results revealed a strong and distinct binding between RGS10 and Gαi3-AlF_4^-, with no detectable binding between RGS10 and Gαi3-GDP or between RGS10 and either form of Gαq **(Figure 5.1A)**. Therefore, consistent with the literature, RGS10 appears to function as a Gi-selective GAP in BV-2 microglia.

GPCRs and TLR4 receptors both activate ERK and AKT kinase cascades as well as the transcription factor NFκB, and these pathways facilitate signaling crosstalk between the two receptor classes and downstream inflammatory signaling (Fan et al. 2004; Dauphinee et al. 2011; Ye 2001). Given the ability of RGS10 to deactivate Gαi and the ability of Gαi pathways to activate ERK, AKT, and NFκB, we predicted that RGS10 regulation of TLR4-induced COX-2 and PGE2 production may be mediated by RGS10 deactivation of Gαi proteins that impact the

ability of TLR4 to initiate these signaling pathways. To explore this hypothesis further, we tested LPS-induced phosphorylation of p42/44 ERK with or without RGS10 knockdown. RGS10 knockdown had no effect on ERK phosphorylation following 20 minutes of LPS treatment (**Figure 5.2C**). Similarly, acute LPS treatment stimulated phosphorylation of AKT and p38 in BV-2 microglia, but RGS10 knockdown had no effect on these responses (data not shown). NFκB phosphorylation on subunit p65, an early step indicating NFκB activation, was also significantly enhanced by LPS treatment, but this response was not affected by RGS10 knockdown (**Figure 5.3C**). Therefore, endogenous RGS10 appears to function as a Gαi GAP, but does not regulate acute LPS-stimulated signaling pathways that are co-regulated by Gαi.

The effect of Gαi inhibition on RGS10 knockdown-induced enhancement of TLR4 signaling

To further explore the role of Gαi in RGS10's effects on inflammatory signaling, we next sought to determine whether enhanced inflammatory signaling resulting from RGS10 knockdown is the result of an amplified Gαi signaling. If the primary mechanism by which RGS10 regulates LPS stimulated inflammatory signaling is through regulation of Gαi GAP activity, then the effects of RGS10 knockdown should be reversed by inhibition of Gαi. To test this prediction, we measured the expression of COX-2 mRNA after LPS treatment and RGS10 knockdown with or without pretreatment with the Gi/o family inhibitor pertussis toxin (Ptx). Surprisingly, the RGS10 siRNA-mediated increase in LPS-stimulated COX-2 mRNA production (**Figure 5.3A**) or protein levels (**Figure 5.3B**) was completely resistant to pertussis toxin pretreatment. Since RGS10 loss has also been shown to enhance the production of the inflammatory cytokine TNFα, we further tested whether RGS10 knockdown-induced increase in TNFα expression was sensitive to Gαi inhibition. Like COX-2, the increase in LPS-stimulated TNFα expression mediated by RGS10 knockdown was not affected by Ptx (**Figure 5.3C**). To ensure that the dose of Ptx used is fully efficacious in BV-2 cells, we confirmed that Ptx completely blocked CXCL12 stimulated AKT phosphorylation, which has been shown to be

mediated by Gαi signaling (Kumar et al. 2012). (**Figure 5.3D**). Collectively, these data suggest that RGS10 knockdown-mediated amplification of inflammatory signaling is not mediated by an enhanced Gαi signaling.

Evaluating HEK293-hTLR4 cells as a model to study the effect of RGS10 on TLR4-induced inflammatory signaling

To more directly test the possibility that RGS10 regulates LPS-stimulated inflammatory signaling through a GAP-independent mechanism, we sought to assess whether RGS10 GAP activity is required for this effect by comparing the effects of exogenous wildtype and GAP-deficient RGS10. BV-2 microglia cells are not an appropriate model for this experiment due to the difficulty of transfecting BV-2 cells with RGS10 plasmids and the high levels of endogenous RGS10 normally present in these cells. As an alternative, we utilized HEK293-hTLR4 cells because they are easy to transfect, express low endogenous RGS10 levels, and stably overexpress TLR4 receptors. To confirm that this cell line is responsive to LPS treatment, we treated HEK293-hTLR4 cells with LPS (10 ng/ml), a dose that potently activates TLR4 signaling in BV-2 microglia. LPS caused a dramatic increase in TNFα mRNA levels (**Figure 5.4**), and activated NFκB and MAPK pathways (data not shown). In addition, we confirmed that HEK293-hTLR4 cells express high levels of exogenous RGS10 following transient transfection. Next, we determined the effect of wildtype RGS10 over-expression on LPS-induced signaling in HEK293-hTLR4 cells. RGS10 overexpression suppressed LPS-induced production of TNFα after 24 hours of LPS treatment (**Figure 5.4).** In contrast, RGS10 overexpression had no effect on acute LPS-induced activation of ERK and NFκB (data not shown). These data suggest that HEK293-hTLR4 cells behave similarly to BV-2 microglia after LPS treatment and manipulation of RGS10 expression.

The effect of RGS10 WT and E52K mutant on TLR4-induced signaling in HEK293-hTLR4 cells

We generated a single amino acid mutation E52K in RGS10 corresponding to a previously characterized GAP-dead mutation in the RGS domain of RGS12 (Sambi et al. 2006). To confirm that the mutation did not affect expression or protein stability, we compared RGS10 protein levels 48 hours after transient transfection with plasmid encoding RGS10 WT or RGS10 E52K in HEK293-hTLR4 cells. Our results indicate that the RGS10E52K mutant was expressed equally to the wild type (**Figure 5.5A**). To confirm that RGS10 E52K mutant is indeed unable to bind the active form of G-proteins, we transfected HEK293-hTLR4 cells with wildtype and E52K RGS10 and performed co-immunoprecipitation to compare their G-protein binding abilities. Only RGS10WT, and not E52K mutant, was able to bind active Gαi3 (**Figure 5.5B**), which confirms that RGS10 E52K mutant has deficient GAP activity. Next, we aimed to determine whether the RGS10 GAP-deficient mutant lost the ability to suppress LPS-induced TNFα mRNA in these cells. The results showed that overexpression of both RGS10 WT and E52K mutant resulted in a similar inhibition of LPS-induced production of TNFα (**Figure 5.5C**). These data provide further compelling evidence that RGS10 inhibits TLR4 signaling in a mechanism independent of its GTPase accelerating activity.

Loss of RGS10 enhances TNFα and COX-2 expression in ovarian cancer cells, and this effect is mediated via a Gαi-independent mechanism

The previous data demonstrate an inhibitory effect of RGS10 on inflammatory cytokines and the inflammatory enzyme COX-2 in microglia. However, RGS10 has also been shown to play important roles in other physiological systems, most notably in ovarian cancer cells (Hooks et al. 2010; Ali et al. 2013b). RGS10 suppresses survival signaling and maintains sensitivity to chemotherapeutic induced cell death in these cells, but the mechanism is unknown and has not been directly linked to a Gαi mediated pathway. Inflammatory signaling including COX-2 and TNFα mediated pathways are directly linked to ovarian cancer chemoresistance (Symowicz et

al. 2005; Gu et al. 2008). Based on our previous results demonstrating that RGS10 regulates the expression of both COX-2 and TNFα in microglia, we aimed to test whether RGS10 also regulates inflammatory mediators in ovarian cancer, and if so whether RGS10 also functions in a GAP-independent mechanism in ovarian cancer cells. We transfected SKOV-3 ovarian cancer cells with RGS10 siRNA or control siRNA in the presence or absence of Ptx and assessed mRNA levels of COX-2 and TNFα. SKOV-3 cells transfected with RGS10 siRNA produced significantly higher levels of COX-2 (**Figure 5.6A**) and TNFα (**Figure 5.6B**) mRNA compared to cells transfected with control siRNA. SKOV-3 cells did not require stimulation to reveal an effect of RGS10 knockdown, and these cells did not respond to LPS treatment with further COX-2 or TNFα expression. As observed in microglia, Ptx treatment had no effect on RGS10 siRNA-mediated upregulation of COX-2 and TNFα expression, demonstrating that RGS10 regulates inflammatory signaling in cancer cells through a G protein independent mechanism. Successful knockdown of RGS10 following siRNA transfection in SKOV-3 cells was confirmed by RT-PCR (**Figure 5.6C**), and we confirmed that the dose of Ptx used in the experiment was sufficient to fully inhibit LPA-mediated ERK phosphorylation in SKOV-3, an established Gαi-mediated event (**Figure 5.6D**) (Hurst et al. 2008). These results demonstrate for the first time that RGS10 regulates inflammatory signaling pathways in ovarian cancer cells and demonstrate that RGS10 anti-inflammatory actions and mechanisms are not exclusive to immune cells but extend to other models. These findings provide new insight in understanding the mechanisms by which RGS10 suppresses chemoresistance and survival of ovarian cancer cells.

Discussion

RGS10 regulates cellular physiology and fundamental signaling pathways in microglia (Lee, Chung, et al. 2011; Lee et al. 2008), macrophages (Lee et al. 2013), T-lymphycytes (Lee et al. 2016), neurons (Lee, Chung, et al. 2012), osteoclasts (Yang et al. 2013; Yang and Li 2007; Yang et al. 2007), cardiomyocytes (Miao et al. 2016), platelets (Hensch et al. 2016; Hensch et al. 2017) , and cancer cells (Hooks and Murph 2015; Cacan et al. 2014; Hooks et al.

2010; Ali et al. 2013b). However, despite its small size and seemingly simple function as a G protein GAP, the molecular mechanisms accounting for RGS10 effects have not been defined. The results presented here significantly expand our understanding of the scope and mechanism of RGS10 regulation of inflammatory signaling. First, we show for the first time that RGS10 regulates COX-2 expression and subsequent prostaglandin production. Given the essential role of COX-2 and PGE2 in the physiology of diverse systems, this finding expands the potential relevance of RGS10 in physiology and disease. Further, our results provide the first evidence of regulation of inflammatory signaling pathways in cancer cells by RGS10, establishing a potential explanation for the effect of RGS10 on ovarian cancer cell survival. Finally, and most importantly, our results demonstrate that the effect of RGS10 on inflammatory signaling in both microglia and ovarian cancer cells cannot be explained by the ability of RGS10 to suppress Gαi signaling. We further show that exogenously expressed GAP-deficient RGS10 suppresses LPS-stimulated TNFα to the same extent as wildtype RGS10. This surprising result suggests novel RGS10 mechanisms and potentially novel binding partners linking RGS10 to inflammatory signaling mediators TNFα and COX-2. Because RGS10 is the smallest member of the RGS protein family, with no defined functional domains outside of the RGS domain, it is possible that this novel mechanism is conserved across the RGS protein family, with major implications on our understanding of RGS protein function.

RGS10 is highly expressed in microglia and RGS10 has profound effects on microglial function; thus, we performed most of our experiments in a mouse microglial cell line. Microglia are immune cells that specifically reside in the central nervous system and normally function to eliminate foreign bodies and neutralize toxins (Kettenmann et al. 2011). However, chronic activation of these cells leads to an amplified production of inflammatory cytokines, prostaglandins, and other molecules that are harmful to neuronal cells (Lull and Block 2010). The persistence of microglial activation and enhanced levels of neurotoxic factors ultimately results in a significant neuronal death and is implicated in many neurodegenerative diseases

(Lull and Block 2010). RGS10 suppresses the release of TNFα and other inflammatory cytokines following inflammatory triggers (Lee et al. 2008) and plays a protective role against microglia-induced neurodegeneration of dopaminergic neurons (Lee, Chung, et al. 2011). However, microglial activation by either LPS or TNFα suppresses the expression of RGS10 and reduced RGS10 protein levels by approximately 75% for approximately 48 hours (Alqinyah et al. 2017). Transient siRNA transfection achieves comparable magnitude and duration of RGS10 protein suppression, and this level of RGS10 suppression dramatically increases inflammatory signaling. Thus, endogenous RGS10 silencing mechanisms likely serve to amplify and enhance inflammation in a feed-forward mechanism that sustains a continuous cycle of RGS10 suppression and enhanced production of inflammatory cytokines (Lee et al. 2008; Alqinyah et al. 2017).

To further assess inflammatory pathways regulated by RGS10 in microglia, we tested the effect of RGS10 loss on the expression of COX-2 and production of PGE2. COX-2 enhances neuroinflammation and is implicated in microglia-induced neurotoxicity. RGS10 knockdown enhanced LPS-induced production of COX-2 and the subsequent release of PGE2 from BV-2 microglia. Since PGE2 is only one product produced by COX-2, it is reasonable to assume that PGE2 is not the only COX-2-mediated inflammatory mediator affected by RGS10, and that subsequent studies will potentially identify additional COX-2 controlled inflammatory mediators that are regulated by RGS10. These findings confirm the previous reports demonstrating the anti-inflammatory roles of RGS10 in microglia, and further expands the scope of this effect. Several studies provided evidence of the deleterious roles of COX-2 and PGE2 in enhancing neurodegeneration of dopaminergic neurons (Teismann et al. 2003; Sanchez-Pernaute et al. 2004). Therefore, it is possible that the effect of RGS10 on COX-2 and PGE2 production accounts, fully or partially, for the neuroprotective properties of RGS10 on dopaminergic neurons. Since COX-2 and PGE2 play diverse roles in the central nervous

system, it is likely that RGS10 serves additional and previously unidentified functions in the brain and spinal cord related to COX-2 regulation.

Remarkably, despite the many anti-inflammatory and neuroprotective roles of RGS10 presented here and from other studies, the mechanisms by which RGS10 exerts these actions have not been defined. Therefore, a main goal of this study was to delineate the molecular mechanisms mediating the anti-inflammatory activities of RGS10. As their name implies, RGS proteins classically function to accelerate the inactivation of heterotrimeric G-proteins, thereby inhibiting G-protein-mediated signaling pathways (Watson et al. 1996). Indeed, RGS10 has been shown to function as a bona fide GAP with specificity to Gαi subunits (Hunt et al. 1996). Gαi has been shown to enhance LPS-induced activation of multiple acute signaling pathways, including AKT and ERK kinase cascades (Fan et al. 2004; Dauphinee et al. 2011), as well as NFκB signaling, which is an essential pathway regulating the production of inflammatory cytokines (Ye 2001). Therefore, our initial hypothesis was that RGS10, via its classic GTPase accelerating activity on Gαi, would suppress these acute signaling pathways following LPS treatment. However, RGS10 knockdown had no effect on LPS-induced acute activation of ERK, AKT, or NFκB. This suggested the possibility that RGS10 may function in a mechanism that is independent of its effect on Gαi. Although there are no previous studies reporting GAP-independent functions of RGS10, it is well established that multiple other RGS proteins function in non-canonical mechanisms that are independent of their GTPase-activating properties (Sethakorn, Yau, and Dulin 2010).

Our data revealed that the amplified TNFα and COX-2 production observed with RGS10 knockdown were not altered by Gαi inhibition with pertussis toxin. While Ptx is an established and commonly used inhibitor of Gαi, it can produce G-protein-independent effects (Mangmool and Kurose 2011). To more rigorously assess the role of Gαi in RGS10 function, we created a RGS10 mutant E52K, based on a previously characterized GAP-dead RGS12 construct (Sambi et al. 2006). Overexpression studies comparing RGS10 E52K and wildtype RGS10 were

performed in HEK293-hTLR4 cells, which stably express TLR4, MD-2 and CD14 co-receptor genes to recapitulate functional TLR4 signaling. Indeed, we confirmed that HEK293-hTLR4 cells show dramatically enhanced TNFα expression and activation of NFκB and ERK phosphorylation in response to LPS treatment with similar dose response as BV-2 cells. However, COX-2 expression was not affected by LPS treatment in these cells, demonstrating that not all downstream TLR4 signaling pathways observed in BV-2 microglia will be recapitulated in HEK293-hTLR4 Cells. Nonetheless, the cells provided a suitable platform to assess the effect of exogenous RGS10 on LPS-induced production of TNFα. We demonstrated that overexpression of GAP-deficient mutant RGS10 suppressed LPS-induced TNFα to the same degree as wild type, suggesting that Gαi interaction is not required for the ability of RGS10 to inhibit TLR4 signaling. These data, combined with the observation that RGS10 regulation of LPS stimulated TNFα and COX-2 is Ptx insensitive in BV-2 microglia cells, strongly suggest that RGS10 anti-inflammatory effects are mediated by G protein-independent mechanisms. Further, we have validated the RGS10E52K mutant as a valuable tool to assess the role of RGS10 GAP activity in mediating the overall functions of RGS10.

In addition to its anti-inflammatory effects in microglia and macrophages, RGS10 has also been shown to regulate ovarian cancer cell survival and chemosensitivity. We have previously postulated that the ability of RGS10 to regulate cell survival in ovarian cancer cells was based on its ability to regulate signaling through Gi-coupled receptors for growth factors such as lysophosphatidic acid (LPA), a well-established autocrine survival factor that is upregulated in ovarian cancer (Hurst et al. 2008). However, while RGS10 suppression enhanced cell survival and basal AKT signaling, it did not enhance LPA-stimulated AKT or ERK signaling in our previous studies (Hooks et al. 2010). Our current observations demonstrate that RGS10 suppression strongly enhances basal TNFα and COX-2 expression in SKOV-3 ovarian cancer cells, indicating that RGS10 suppresses the production of inflammatory mediators in both immune and non-immune cells. As observed in BV-2 microglia, Gαi inhibition

had no effect on RGS10 knockdown-induced upregulation of COX-2 and TNFα in SKOV-3. TNFα and COX-2 are strongly implicated in the development of chemoresistance and control of cell survival of many cancer cells, suggesting that these pathways may mediate the effect of RGS10 on chemoresistance (Kulbe et al. 2007; Greenhough et al. 2009). Further studies are warranted to determine if the ability of RGS10 to regulate inflammatory signaling in cancer cells fully accounts for its effects on chemoresistance, and if this mechanism extends to other cancer types.

The notion of GAP-independent mechanisms for RGS proteins is not new, and has been described in multiple RGS family members (see review: (Sethakorn, Yau, and Dulin 2010)). However, many of these RGS proteins possess, in addition to RGS domains, multifunctional additional domains that mediate some of these GAP-independent functions. For example, RGS12 and RGS14, the RGS family members most closely related to RGS10, interact with RAS and MEK2 proteins via RBD and PDZ domains respectively to perform multiple GAP-independent functions (Willard et al. 2007). In contrast, RGS10 contains only the conserved RGS domain and short N- and C-terminal extensions containing sites for regulatory modifications, but no defined functions. This suggests that the RGS domain itself may mediate the GAP-independent function of RGS10. Structural studies have identified modest differences in the structure and flexibility of the RGS10 RGS domain, compared to other family members, which may allow distinct binding interactions (Soundararajan et al. 2008a). Indeed, G-protein independent interactions and associations have been mapped to the RGS domains of multiple RGS proteins (Popov et al. 2000; Nguyen et al. 2009; Sethakorn, Yau, and Dulin 2010), supporting the notion that the RGS domain is much more than just a GAP for G-proteins. Interestingly, RGS10 and other RGS proteins are frequently localized to the nucleus and other sub cellular compartments, rather that the plasma membrane where heterotrimeric G protein signaling canonically occurs. Therefore, we predict that G protein-independent mechanisms and binding partners facilitate a significant subset of RGS protein functions, including those

described here for RGS10. Defining these molecular mechanisms is a critical next step in targeting RGS10 in the diverse pathologies in which its function is implicated.

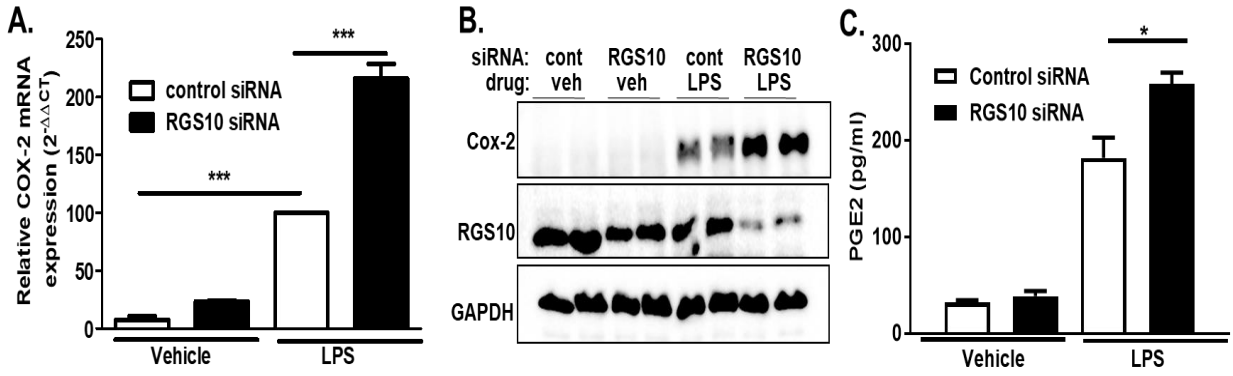

Figure 5.1: RGS10 Knockdown enhances LPS-stimulated COX-2 expression and PGE2 production in BV-2 microglia

A. BV-2 microglia cells were plated in 6 well plates and simultaneously transfected with either control or RGS10 siRNA. Cells were left for 24 hours and then incubated with vehicle or LPS (10 ng/ml) for 24 hours. RNA extraction, cDNA synthesis, qRT-PCR were performed as described in Methods. Expression of COX-2 mRNA was normalized to the endogenous control Actin, and relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method. Data are analyzed from two independent experiments. The difference between groups was analyzed by ANOVA test followed by Tukey's test. Data are presented as Mean ± SEM where *p < 0.05 **p < 0.01 and ***p < 0.001. **B.** BV-2 microglia were plated in 24 well plates and transfected with control or RGS10 siRNA for 24 hours and then treated with vehicle or LPS for an additional 24 hours. Cells were lysed, and SDS-PAGE was performed followed by immunoblotting using specific antibodies against COX-2, RGS10, and the loading control GAPDH. The image is representative of three independent experiments. Quantification of the same conditions shown in Figure 3B. **C.** BV-2 microglia cells were plated in 6 well plates and simultaneously transfected with either control or RGS10 siRNA. Cells were left for 24 hours and then incubated with vehicle or LPS (10 ng/ml) for an additional 24 hours. Culture medium was collected and PGE2 levels were measured using ELISA. Conversion of raw absorbance values to pg/mL concentration was conducted using a standard curve following manufacturer's protocol. Data are analyzed from four independent experiments and difference between groups was analyzed by ANOVA test followed by Tukey's test. Data are presented as Mean ± SEM where *p < 0.05.

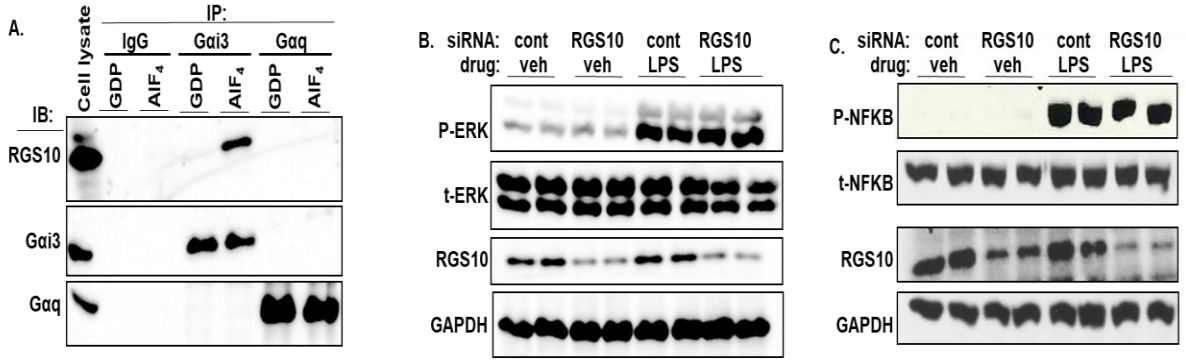

Figure 5.2: Endogenous RGS10 associates with the active form of Gαi, but doesn't regulate ERK or NFKB phosphorylation

A) BV-microglia cells were plated in 15 cm dishes and then lysed using modified RIPA lysis buffer as described in Methods. Cell lysates were incubated with GDP (10 μM) alone or with AlF_4^- for 30 minutes at 25 °C. Co-immunoprecipitation was performed using Gαi3, Gαq, or control IgG antibodies. Western blot analysis was conducted to probe for RGS10, Gαi3, or Gαq. **B-C)** BV-2 microglia cells were plated in 24 well plates and transfected with control or RGS10 siRNA before incubation with vehicle or LPS for 20 minutes. Cells were lysed, and SDS-PAGE was performed followed by immunoblotting using specific antibodies against **(B.)** phospho-ERK, total ERK, RGS10, and the loading control GAPDH, and **(C.)** phospho-P65, total P65, RGS10 and GAPDH.

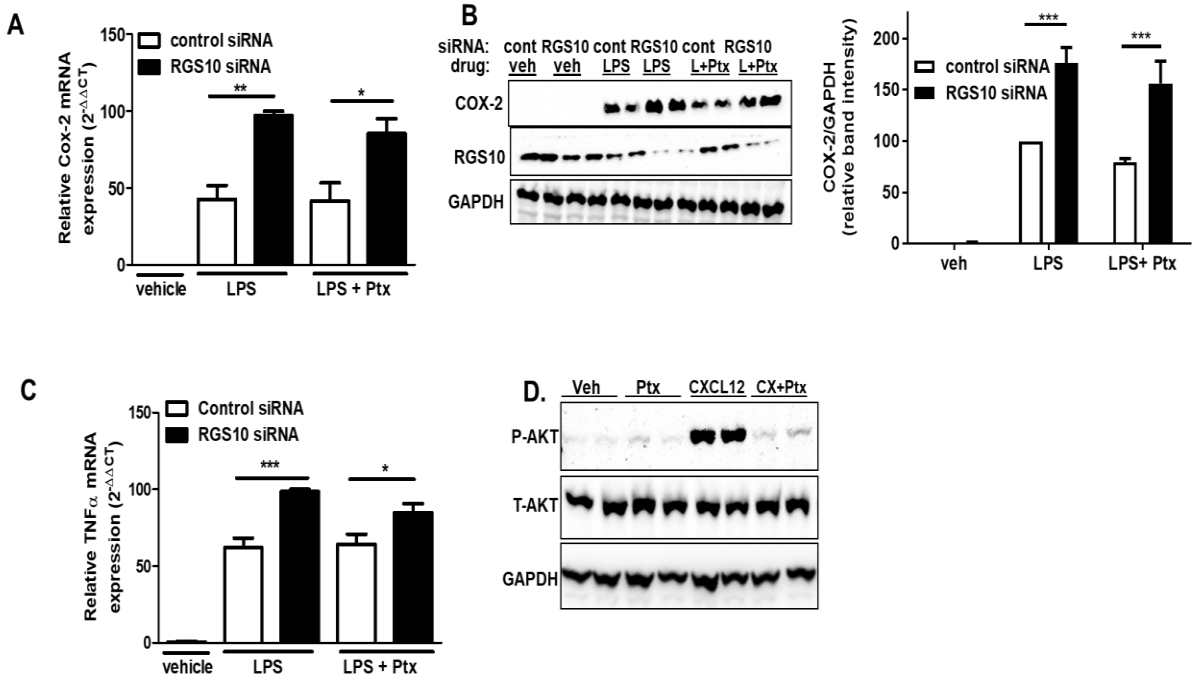

Figure 5.3: PTX pretreatment does not affect RGS10 knockdown-induced increase of LPS-stimulated COX-2 and TNFα

A) BV-2 microglia cells were plated in 6 well plates and transfected with either control or RGS10 siRNA. After 24 hours, cells were incubated with vehicle or LPS (10 ng/ml) for 24 hours with or without a pretreatment of pertussis toxin (100 ng/ml) for 4 hours. Expression of COX-2 mRNA was normalized to the endogenous control Actin, and relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method. Data are analyzed from four independent experiments and difference between groups was analyzed by ANOVA test followed by Tukey's test. Data are presented as Mean ± SEM where *p < 0.05 **p < 0.01 and ***p < 0.001. **B)** BV-2 microglia cells were plated in 24 well plates and transfected with control or RGS10 siRNA. 24 hours after transfection, cells were treated with vehicle or LPS for an additional 24 hours with or without pertussis toxin (100 ng/mL) pretreatment for 4 hours. Cells were lysed, and SDS-PAGE was performed followed by immunoblotting using specific antibodies against COX-2, RGS10, and the loading control GAPDH. Band intensity was analyzed from three independent experiments and difference between groups was analyzed by ANOVA test followed by Tukey's test. Data are presented as Mean ± SEM where *p < 0.05 **p < 0.01 and ***p < 0.001.**C)** BV-2 microglia cells were plated in 6 well plates and transfected with either control or RGS10 siRNA. After 24 hours, cells were incubated with vehicle or LPS (10 ng/ml) for an additional 24 hours with or without a pretreatment of pertussis toxin (100 ng/ml) for 4 hours. Expression of TNFα mRNA was normalized to the endogenous control Actin, and relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method. Data are analyzed from five independent experiments and difference between groups was analyzed by ANOVA test followed by Tukey's test. Data are presented as Mean ± SEM where *p < 0.05 **p < 0.01 and ***p < 0.001. **D)** BV-2 microglia were plated in 24 well plates and then treated with vehicle or CXCL12 (200 ng/ml) for 5 minutes with or without a

preincubation with pertussis toxin (100 ng/ml) overnight. Following incubation, cells were lysed, and SDS-PAGE was performed followed by immunoblotting using specific antibodies against phospho-AKT, total AKT, and GAPDH. The experiment is a representative of two independent experiments.

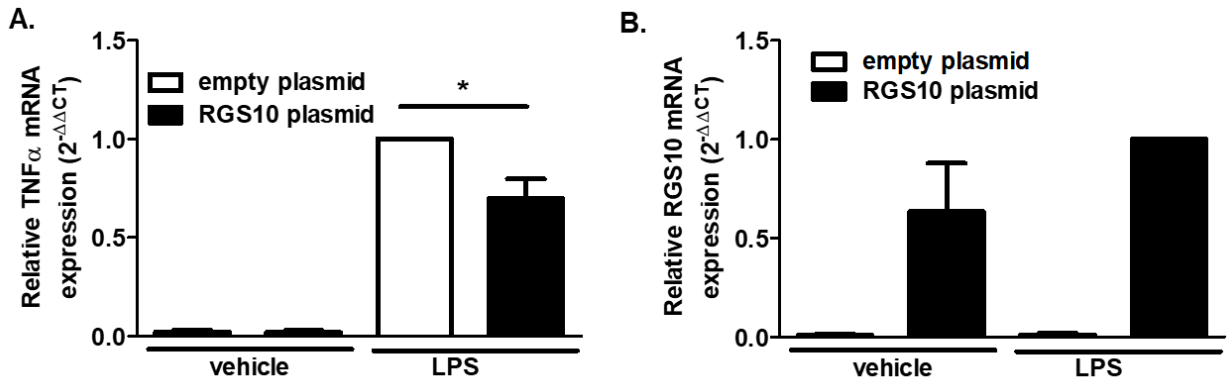

Figure 5.4: HEK293-hTLR4 cells express TNFα in response LPS and express high levels of exogenous RGS10

HEK-293-hTLR4 cells were plated in 24 wells plate and allowed to reach ~ 80% confluency. Next, cells were transfected with either empty vector or RGS10 plasmids for 48 hours. Cells were treated with vehicle or LPS 24 hours after transfection. mRNA expression of TNFα **(A)** and RGS10 **(B)** were normalized to the endogenous control Actin, and relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method. Data are analyzed from three independent experiments each with technical duplicates and difference between groups was analyzed by ANOVA test followed by Tukey's test. Data are presented as Mean ± SEM where *p < 0.05 **p < 0.01 and ***p < 0.001.

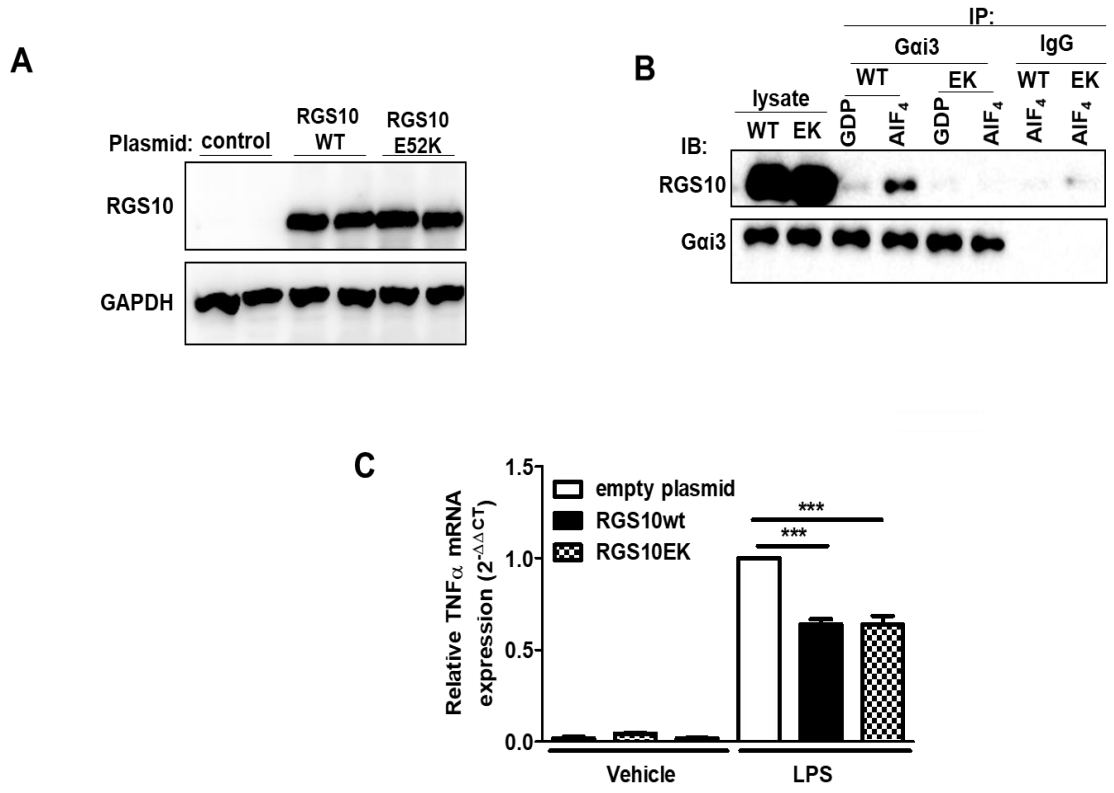

Figure 5.5: Overexpression of wildtype and "GAP-dead" RGS10 equally suppress LPS-induced TNFα expression

A) HEK-293-hTLR4 cells were plated in 24 wells plate and allowed to reach ~ 80% confluency before transfection with 0.5μg of RGS10 WT or RGS10 E52K plasmids. 48 hours after transfection, cells were lysed, and SDS-PAGE was performed followed by immunoblotting using specific antibodies against RGS10 and GAPDH. **B)** HEK-293 TLR4 cells were plated in 10 cm dishes and transfected with pcDNA encoding Gαi3 with either RGS10 WT or RGS10 E52K plasmids. 48 hours after transfection, cells were lysed with modified lysis buffer and cell lysates were incubated with GDP (10 μM) alone or with AlF_4^-. Co-immunoprecipitation was performed using Gαi3 or control IgG antibodies. Western blot analysis was conducted to probe for RGS10 and Gαi3. **C)** HEK-h293 TLR4 cells were plated in 24 well plates and transfected with RGS10 WT or RGS10 E52K plasmids for 48 hours. Cells were incubated with LPS (10 ng/ml)) for 24 hours. mRNA expression of TNFα was normalized to the endogenous control Actin, and relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method. Data are analyzed from two independent experiments each with technical duplicates and difference between groups was analyzed by ANOVA test followed by Tukey's test. Data are presented as mean ± SEM where *p < 0.05 **p < 0.01 and ***p < 0.001.

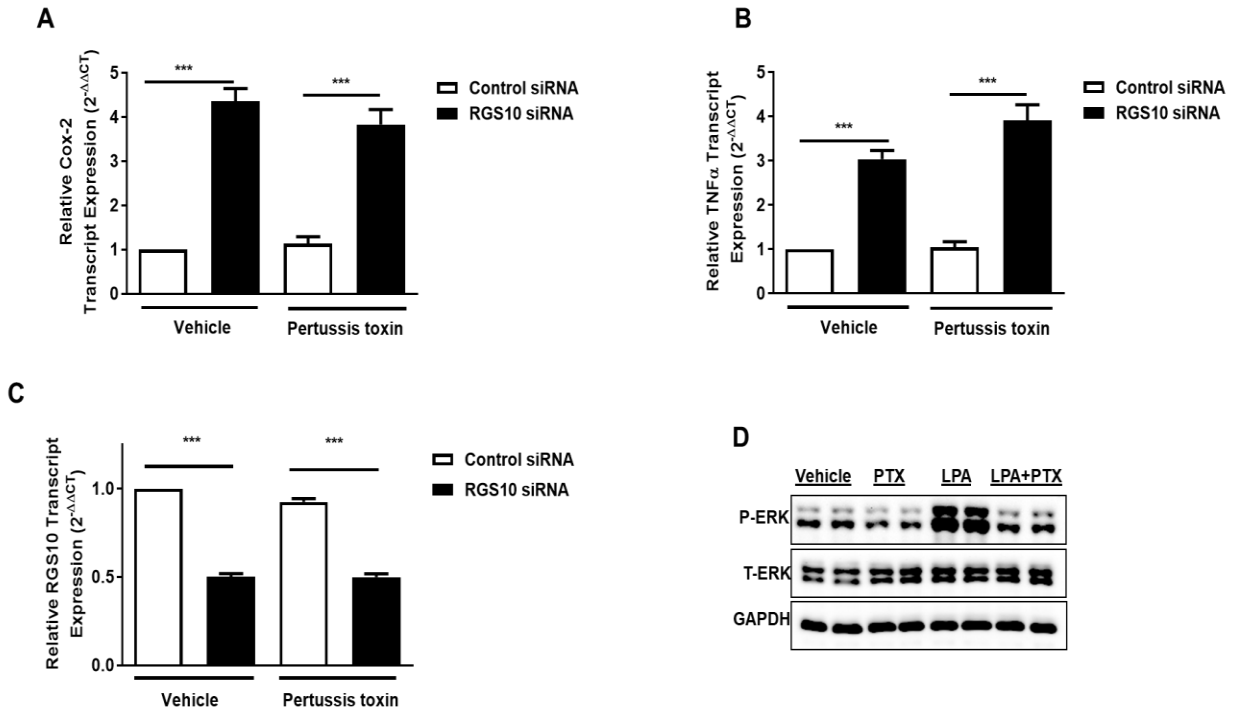

Figure 5.6: Knockdown of RGS10 significantly increases expression of COX-2 and TNFα via a Gi-independent mechanism in SKOV-3 ovarian cancer cells.

SKOV-3 cells were plated in a 6 well plate and transiently transfected with control or RGS10-targeted siRNA constructs. 20 hours after transfection, cells were treated with vehicle or Ptx (100ng/ml) for an additional 28 hours. RNA was isolated from cells using TRIzol reagent and cDNA was synthesized from the extracted RNA. COX-2 **(A)**, TNFα **(B)**, and RGS10 **(C)** transcript levels were quantified using quantitative RT-PCR and normalized to the housekeeping gene GAPDH. The relative expression was calculated by the $2^{-\Delta\Delta CT}$ method. Data in **(A-C)** are compiled from three independent experimental repeats. Data were analyzed for statistical differences using an analysis of variance (ANOVA) followed by Tukey's test between groups. Data are presented as Mean ± SEM where *: P<0.005, **: P<0.01, and ***: P<0.001 indicate the levels of significance. **(D)** SKOV-3 cells plated in 24 wells plate were serum-starved overnight with or without Ptx (100ng/ml) prior to treatment with vehicle or LPA (10 µM) for five minutes. Cells were lysed and subjected to SDS-PAGE and western blotting was performed using specific antibodies against phospho-ERK, total ERK, and GAPDH.

CHAPTER 6

IDENTIFYING NOVEL BINDING PARTNERS OF RGS10

Introduction

Regulators of G protein signaling (RGS) are a family of proteins that classically acts as GTPase-activating proteins (GAPs) for heterometric G proteins (Watson et al. 1996). This GAP activity accelerates the hydrolysis of the G protein bound GTP to GDP, which deactivate G proteins. To functions as GAPS, the RGS domain of RGS protein directly interacts switch regions of the substrate G protein (Popov et al. 1997). In addition to acting as GAPs, it is now established that RGS proteins exert multiple GAP-independent functions (Sethakorn, Yau, and Dulin 2010). These functions are mediated by direct interactions of RGS proteins with various targets, including membrane ion channels (Schiff et al. 2000), kinases (Liang et al. 2009), and transcription factors (Xie et al. 2008). While the classic GAP activity of RGS proteins often occurs at cell membrane, RGS GAP-independent functions take place in several cellular compartments including the cell membrane (Zhou et al. 2012), Golgi (Nixon, Grenningloh, and Casey 2002), and the nucleus (Liu and Fisher 2004). Therefore, identifying interacting partners of RGS proteins will potentially aid in identifying novel RGS GAP-independent functions.

RGS10 is a member of the R12 family, including RGS10, RGS12, and RGS14 and all three members have been shown to specifically target Gαi via classic GAP activity (Hunt et al. 1996; Soundararajan et al. 2008b). However, unlike the other R12 members which possess multifunctional domains, RGS10 is consists of only an RGS domain and short N and C-termini (Hunt et al. 1996). Unsurprisingly, multiple interacting partners have been identified for RGS12 (Schiff et al. 2000; Willard et al. 2007) and RGS14 (Shu, Ramineni, and Hepler 2010; Martin-McCaffrey et al. 2005) that do not bind RGS10. RGS10 is highly expressed in immune cells and has been shown to regulate inflammatory processes in these cells. RGS10 suppressed the

expression of inflammatory cytokines (Lee, Chung, et al. 2011), COX-2, and PGE2 release from activated microglia (chapter 5). Further, RGS10 protected against microglia-induced neurodegeneration (Lee, Chung, et al. 2011). Interestingly, microglial activation suppresses the expression of RGS10 in microglia, thereby exacerbating inflammatory conditions (Lee, Chung, et al. 2011). Despite these crucial functions, the mechanisms of the anti-inflammatory and neuroprotective functions of RGS10 are unknown. Recently, our work revealed that RGS10 suppresses inflammatory singling in a GAP-independent mechanism (chapter 5). Therefore, identifying the interacting partners of RGS10 in microglia may aid in identifying the GAP-independent mechanism mediating the anti-inflammatory effect of RGS10. Further, studying RGS10 interactions in microglia could reveal additional novel functions of RGS10 in microglia and/or other cells. Therefore, the main aim of this study was to identify binding partners of RGS10 in microglia using a co-immunoprecipitation technique followed by mass spectrometry. The mass spectrometry analysis revealed several interacting partners of endogenous RGS10 in BV-2 microglia. We further validated key hits by performing co-immunoprecipitation followed by western using specific antibodies.

Materials and methods.

Cells and Reagents

All experiments were conducted on Murine BV-2 microglia cells. We obtained this cell line from G. Hasko at University of Medicine and Dentistry of New Jersey (Newark, NJ) as a generous gift. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (VWR) supplemented with 10% Fetal Bovine Serum (ThermoFisher Scientific). The lipopolysaccharide used in this study was purchased from Sigma-Aldrich (St. Louis, MO).

Co-immunoprecipitation, elution, and protein precipitation

To prepare co-immunoprecipitation samples for mass spectrometry analysis, BV-2 microglia cells were plated in 6 15 cm dishes. Cells from each plate were washed with PBS twice and then lysed with 1 ml of modified lysis buffer (50 mMTris HCL,150 mM NaCl, 6mM MgCl2, 1% Nonidet P-40, 0.5% sodium deoxycholate). Cell lysate was left for 30 minutes on ice and subsequently centrifuged at 27,216 G (15,000 RPM) for 10 minutes at 4°C. Cell lysates was incubated with agarose-conjugated RGS10 antibody (Santa Cruz, C-20) or agarose-conjugated goat IgG antibody (Santa Cruz) at 2 µg/ml concentration overnight at 4°C. The samples were centrifuged (2000 RPM for 5 minutes), and the pellets were washed 3 times with wash buffer (50 mMTris HCL,150 mM NaCl, 6 mM MgCl2). At this point, pellets were either incubated with elution buffer (for mass spec) or SDS-PAGE sample buffer (for western blot application). If samples were to be analyzed by mass spectrometry, 100 µL of elution buffer (0.15 M Glycine PH 2.6) was incubated with the pellets for 10 minutes at room temperature with shaking. The samples were centrifuged again (2000 RPM for 5 minutes) and supernatant was collected and neutralized with an equal volume of 1 M Tris HCl, pH 8. Next, acetone precipitation method was used to precipitate proteins by adding acetone in 4 times the volume of the sample. After the addition of acetone, samples were incubated at – 20 °C overnight before centrifugation at (16,000 G, 10 minutes, 4 °C). The pellet was washed with acetone one time before centrifugation again. The acetone was then removed, and pellets were left to dry for 30 minutes.

mass spectrometry analysis

Samples were digested by the sequential addition of lys-c and trypsin proteases and then analyzed by LC-MS/MS on a Thermofisher Q-Exactive mass spectrometer as described (Wohlschlegel 2009; Kelstrup et al. 2012). MS/MS data was analyzed using the ProLuCID and DTA Select algorithms (Tabb, McDonald, and Yates 2002; Xu et al. 2015). The SAINT algorithm was used to identify interacting proteins statistically enriched relative to control purifications (Choi et al. 2012)

Western Blot Analysis

SDS-PAGE sample buffer (containing Tris-HCl, SDS, β-mercaptoethanol, and glycerol) was used to lyse cells. Next, SDS-PAGE was conducted using standard protocols followed by transfer to nitrocellulose membranes. Primary antibody for RGS10 was purchased from Santa Cruz Biotechnology (Dallas, TX). Antibodies against STIM2, PDE4A and Gαi3 were purchased from Proteintech (Rosemont, IL). Following primary antibody incubation, the membranes were incubated with the appropriate secondary HRP-conjugated antibodies, which were donkey anti-goat IgG-HRP (Santa Cruz), Goat Anti-Rabbit IgG-HRP (Millipore). The membranes were then visualized utilizing Chemiluminescent substrate (ThermoScientific).

Results

Optimizing CO-Immunoprecipitation conditions

To determine the efficiency of our co-immunoprecipitation technique, we ran a trial experiment to assess the ability of the antibody we used to precipitate endogenous RGS10 from BV-2 microglia cell lysates and to test whether the elution buffer is sufficient to elute RGS10 protein from the pellet containing the agarose beads. The first elution step recovered most of the RGS10 in the beads and repeating the elution did not recover additional RGS10 protein, indicating that the first elution step is enough to elute RGS10 from the agarose-conjugated antibody (**Figure 6.1**). The supernatant samples were devoid of RGS10 after incubation with RGS10 antibody, indicating that the antibody used is sufficient to precipitate essentially all RGS10 present in the lysates. Further, RGS10 was not detected after eluting beads from goat IgG-treated samples, indicating that the pulldown was specific, and the control IgG used does not precipitate any RGS10 (**Figure 6.1**). These results indicate the conditions of the experiment produces adequate RGS10-concentrated samples, which is needed to run a mass spectrometry analysis.

Endogenous RGS10 interact with multiple binding partners in microglia

Mass spectrometry analysis of the RGS10 immunoprecipitated samples detected an enrichment of RGS10, whereas the IgG control samples did not contain RGS10, indicating that the Co-immunoprecipitation was successful and specific (**Table 6.1**). After excluding proteins that were equally detected in both RGS10 and control IgG, and proteins that were not interacting in different independent repeats, we obtained a final list of a total of 21 interacting proteins that were statistically significant (**Table 6.1**). The analysis revealed the presence of classic RGS-interacting proteins, Guanine nucleotide-binding protein G(i) subunit alpha-2 and Guanine Nucleotide-Binding Protein G(K) Subunit Alpha (aka Gαi3) but neither Gαi1 or Gαq subunits were detected (**Table 6.2**). In addition to the classic G protein partners, the list of interacting proteins detected by our mass spectrometry analysis contained various novel interacting proteins with different molecular functions (**Table 6.2**). Protein classification according to the main molecular functions revealed that the interacting proteins were involved in a variety of functions. The proteins were involved in metabolism, protein degradation, mitochondrial functions, ion channel functions, intracellular trafficking, and one chromatin-associated protein (**Table 6.2**).

To assess the interaction of RGS10 according to the location of the interacting protein, we classified the interacting proteins depending on their cellular localization. The interacting proteins localize in different cellular compartments, including the cytoplasm, nucleus, and mitochondria (**Table 6.3**). We also identified three membrane-associated and three Endoplasmic reticulum-associated proteins (**Table 6.3**). Further, some proteins identified are localized in the Golgi and perinuclear region (**Table 6.3**). Collectively, our results indicate that endogenous RGS10 interact with multiple novel binding partners in BV-2 microglia that differs in their functions and localization.

Validation of mass spectrometry results

To validate the data obtained from the mass spectrometry analysis, we conducted a co-immunoprecipitation experiment followed by western blot analysis. The same RGS10 antibody, cell line, and conditions used in preparing the samples for the mass spectrometry analysis were used in the validation experiment. In this experiment, we used specific antibodies against the following proteins: Gαi3, STIM2, and PDE4A. Probing samples obtained from RGS10 immunoprecipitation with these antibodies detected a presence of a specific band at predicted molecular weights of Gαi3, STIM2, and PDE4A (**Figure 6.2**). Further, we did not detect any of these proteins in the control IgG samples after we incubated the samples with the same antibodies (**Figure 6.2**). These results validate the data we obtained from the mass spectrometry analysis. Interestingly, treating BV-2 microglia with lipopolysaccharide enhanced the interaction between RGS10 and Gαi3. Similarly, LPS enhanced the interaction between RGS10 and PDE4A. However, we did not observe significant changes in the interaction between RGS10 and STIM2 (**Figure 6.2**). These results indicate that LPS may differently influence RGS10 interactions in BV-2 microglia.

Discussion

Regulator of G protein signaling 10 (RGS10), which belongs to the R12 family of RGS proteins, is a small GTPase-activating protein (GAP) that was shown to play crucial functions in microglia (Lee, Chung, et al. 2011), macrophages (Lee et al. 2013), osteoclasts (Yang and Li 2007), cardiomyocytes (Miao et al. 2016), neurons (Lee, Chung, et al. 2012), and ovarian cancer cells (Hooks et al. 2010). Although many functions of RGS10 have been identified in these systems, there is still a gap in understanding the exact molecular mechanism governing these functions. In microglia, loss of RGS10 enhanced cyclooxygenase-2 expression, and the release of PGE2 (Chapter5) and inflammatory cytokines following TLR4 stimulation (Lee, Chung, et al. 2011). Further, RGS10 knockout animals experienced greater microglia-induced neurotoxicity (Lee, Chung, et al. 2011). We previously demonstrated that RGS10 exerts these

anti-inflammatory effects in a GAP-independent mechanism (chapter 5). To begin investigating the nature of this mechanism, we aimed to identify novel binding partners of RGS10 in microglia. Identifying these interactors will also be beneficial in possibly finding previously unidentified functions of RGS10 in microglia.

Here, we show that endogenous RGS10 interacts with multiple partners in BV-2 microglia. These binding partners include classic G proteins and non-classic proteins that differ in their molecular functions and subcellular localization.

In this study, we identified that endogenous RGS10 interacts with Gαi2 and Gαi3 in microglia. Although these proteins are considered classic binding partners of RGS proteins, for RGS-G protein interaction to occur, it requires stimulation by either upstream GPCR agonists (Garcia-Bernal et al. 2011) or by in-vitro incubation with aluminum tetrafluoride (Bosch et al. 2012). Since we performed the experiment without using agonists or aluminum tetrafluoride, it was surprising to detect such strong interaction between RGS10 and G proteins. This may suggest that there is a basal G protein activity in BV-2 microglia that induce the interaction with RGS10. Another possibility is that RGS10 is a part of a complex consisting of the partner G proteins, which has been reported for other RGS proteins (Bernstein et al. 2004). Unlike Gαi2 and Gαi3, we did not detect an interaction between RGS10 and Gαi1 or Gαq. This is expected as Gαi1 is not highly expressed in BV-2 microglia compared to Gαi2 and Gαi3 (Atwood et al. 2011), and we previously demonstrated that RGS10 does not interact with Gαq in BV-2 microglia (Chapter 5).

The main aim of this study is to identify RGS10 binding partners that play roles in inflammatory signaling. This can be the first step in ultimately identifying the GAP-independent mechanism of RGS10 anti-inflammatory actions. Here, we identified, and validated, stromal interaction molecule 2 (STIM2) as a binding partner of endogenous RGS10 in BV-2 microglia. STIM2 is a calcium sensor that localizes in the endoplasmic reticulum and mediate store-operated Ca2+ entry (SOCE) upon calcium depletion (Soboloff et al. 2012). STIM2 is required

for adequate LPS-induced production of inflammatory cytokines in macrophages (Sogkas et al. 2015). We also observed that STIM2 knockdown inhibited LPS-induced production of COX-2 in microglia (data not shown). Since LPS-induced inflammatory signaling in immune cells is differentially affected by RGS10 and STIM2, and these proteins interact in BV-2 microglia, it will be interesting to assess whether RGS10 anti-inflammatory effects are mediated by influencing STIM2. Further, STIM2 plays additional roles in microglia, such as mediating nucleotide-induced migration and phagocytosis (Michaelis et al. 2015). Therefore, studying the effect of RGS10 on STIM2-mediated enhancement of LPS-induced production of inflammatory mediators should be expanded to assess whether RGS10 also affects additional functions of STIM2.

In microglia, the expression of RGS10 is suppressed by inflammatory stimulants (Lee, Chung, et al. 2011). Since RGS10 plays anti-inflammatory and neuroprotective roles in these cells, it is crucial to understand the regulatory mechanisms governing the expression of RGS10. We previously reported that RGS10 expression was silenced by LPS treatment via epigenetic mechanisms (Alqinyah et al. 2017). However, it is not known whether mechanisms affecting the stability of RGS10 participates in controlling basal levels or non-LPS-induced changes in RGS10 expression in microglia. Controlling the stability of RGS proteins is a central regulatory mechanism employed by the cells to maintain physiological levels of several RGS proteins (Alqinyah and Hooks 2018). Such stability-modifying mechanisms for RGS10 are not identified yet. Here, we demonstrate that two E3 ligase proteins: E3 ubiquitin-protein ligase RNF146 and STIP1 homology and U box-containing protein 1 (STUB1) interacts with RGS10 in BV-2 microglia. Therefore, it is possible that affecting the stability of RGS10 is one of the mechanisms that regulate RGS10 expression in microglia, and that these E3 ligase enzymes play roles in affecting the stability of RGS10 in BV-2 microglia. Since RGS10 expression is also often altered by different signaling pathways in other cells, such as cardiomyocytes and ovarian cancer cells (Miao et al. 2016; Hooks et al. 2010), the E3 ligases identified in this study may possibly play a role in the regulating RGS10 expression in these cells as well.

Nuclear localization of RGS10 has been reported in different cell lines including microglia (Lee et al. 2008). This localization was shown to be enhanced by PKA activation in HEK293 cells and LPS treatment in BV-2 microglia (Burgon et al. 2001; Lee et al. 2008). Despite these findings, the nuclear function of RGS10 is still unknown. Some nuclear-localized RGS proteins were shown to exert unique nuclear functions by interacting and influencing the activity of nuclear proteins (Liu and Fisher 2004; Xie et al. 2008; Yau et al. 2008). Here, we identify several RGS10-interacting proteins that display nuclear localization. One interesting protein is the Dual specificity tyrosine-phosphorylation-regulated kinase 1A (Dyrk1a). Among its various functions, it was demonstrated by several studies that this kinase regulates the activity and the expression of the transcription factor NFATc (Arron et al. 2006; Gwack et al. 2006; Grebe et al. 2011; Stefos et al. 2013; Booiman et al. 2015; Liu et al. 2017). Interestingly, NFATc expression and activity are significantly altered in RGS10 knockout osteoclasts (Yang and Li 2007). Future studies investigating the physiological function of RGS10 and Dyrk1a interaction may reveal a novel link between RGS10, NFAT, and Dyrk1a. An interesting and novel nuclear function of some RGS proteins is the direct interaction with transcriptional machinery components and participation in regulating transcription (Xie et al. 2008; Lee, Park, et al. 2012). In this study, we identify Chromodomain-helicase-DNA-binding protein 1-like (Chd1l) as an interacting partner of RGS10. Chd1l can act as a transcription factor and was shown to bind promoters of several genes (Chen et al. 2010; Chan et al. 2012; Li, Chen, et al. 2013). This may link RGS10 directly to the transcription machinery, which will be novel and previously unidentified role of RGS10. In addition to the nuclear proteins, we identified multiple mitochondrial proteins interacting with RGS10. Some of these proteins, such as NOA1 and Ndufa10, were shown to play roles in mitochondrial respiration and apoptotic processes (Tang et al. 2009; Heidler et al. 2011; Morais et al. 2014; Hoefs et al. 2011). A mitochondrial-specific function of RGS10 has not been identified before, thus, it will be interesting to assess whether RGS10 influences mitochondrial functions in microglia.

In addition to microglia, the findings of this study will potentially assist in further understanding of RGS10 functions and mechanism in other systems. For example, RGS10 is crucial for osteoclast differentiation and its loss leads to osteopetrosis (Yang and Li 2007). Similarly, several studies demonstrate a critical role of anion exchange protein 2 (Slc4a2) in osteoclast functions and differentiation, and further implicated this protein in osteopetrosis (Coury et al. 2013; Meyers et al. 2010; Wu, Glimcher, and Aliprantis 2008). We show here a possible interaction between RGS10 and Slc4a2 in microglia, which are closely related to osteoclasts. Applying the data presented here in osteoclasts may reveal a connection between RGS10 and Slc4a2 in mediating osteoclasts functions. In ovarian cancer cells, RGS10 suppresses the chemoresistance of ovarian cancer cells and enhances their death (Hooks et al. 2010). However, the molecular mechanisms mediating these functions are not yet fully understood. We identified Chromodomain-helicase-DNA-binding protein 1 (Chd1l) as a possible interactor of RGS10. This protein is of interest because it was established to be an oncogene that promotes the progression of many cancers (Wu et al. 2014; Ji et al. 2013; Cheng, Su, and Xu 2013; Liu et al. 2016). Additionally, it is overexpressed in ovarian cancers and its expression is associated with reduced patient survival (He et al. 2012). Moreover, this protein was also detected as an interactor of RGS10 in a mass spectrometry analysis we conducted on RGS10 immunoprecipitation samples obtained from the ovarian cancer cell line, SKOV-3 (data not shown). Therefore, it will be interesting to determine whether RGS10 interaction with Chd1l is of physiological relevance in ovarian cancer cells. Combined, the previous examples illustrate that findings of this study may potentially aid in investigating the roles and functions of RGS10 in multiple systems.

As observed here, other RGS proteins have been shown to interact with several proteins, including kinases (Liang et al. 2009), transcription factors (Liu and Fisher 2004), and ion channels (Schiff et al. 2000). However, a large portion of these interaction is mediated via unique domains that often distinct from the RGS domain. RGS10 is a small protein consisting

only of the RGS domain and short N and C-terminal sequences (Hunt et al. 1996). This suggests that a significant subset of the interacting proteins identified in this study interact with the RGS domain of RGS10. Thus, it possible that some of these binding partners may also interact with other RGS proteins since all RGS proteins share an RGS domain. Further, this will expand our knowledge about the functions of the RGS domain, which was believed for a long time to only mediate the GAP activity of RGS proteins. Prior to this study, it was reported that RGS10 may interact with common binding partners that usually interact with several members of RGS proteins, such as spinophilin and calmodulin (Yang and Li 2007; Ma et al. 2012). However, we identified multiple and possibly specific interacting partners of RGS10 in microglia. Therefore, this study will crucially assist future efforts to delineate the function, regulation, and mechanism of RGS10 in different physiological systems. Furthermore, these findings are further evidence that RGS proteins are not just GAPs, but rather multipurpose proteins that mediate many GAP-independent functions in addition to their classic GAP activity.

Table 6.1. List of interacting proteins of RGS10 in microglia (Mass spectrometry conducted at Dr. James Wohlschlegel laboratory)

UniProtKB	Protein Name	Average Spectral Count	
		RGS10	Control IgG
Q9CQE5	Regulator of G-protein signaling 10	75.2	0
Q8QZR5	Alanine aminotransferase 1	33.7	0
P08752	Guanine nucleotide-binding protein G(i) subunit alpha-2	52.2	2.2
P83093	Stromal interaction molecule 2	30.7	3.5
Q9DCL9	Multifunctional protein ADE2	32.2	5.5
P13808	Anion exchange protein 2	26.2	1
Q9DC51	Guanine nucleotide-binding protein G(k) subunit alpha	18.5	1
O89084	cAMP-specific 3',5'-cyclic phosphodiesterase 4A	16	1.5
Q06180	Tyrosine-protein phosphatase non-receptor type 2	12	0
Q99LC3	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10	14.2	0
Q9JJG9	Nitric oxide-associated protein 1	14.2	0
Q9CXF7	Chromodomain-helicase-DNA-binding protein 1-like	8	0
Q61214	Dual specificity tyrosine-phosphorylation-regulated kinase 1A	8.5	0
Q8K1E0	Syntaxin-5	7.2	0
Q9CZW6	E3 ubiquitin-protein ligase RNF146	9	0
Q99J25	rRNA methyltransferase 1	5.5	0
Q9WUD1	STIP1 homology and U box-containing protein 1	7	0
Q3USJ8	F-BAR and double SH3 domains protein 2	5.2	0
Q7TQC7	G patch domain-containing protein 2	4.7	0
Q91XA2	Golgi membrane protein 1	6	0
Q99J39	Malonyl-CoA decarboxylase	4.2	0

Table 6.2. Classification of interacting proteins according to main molecular functions/cellular process

Molecular Functions	Protein Names
G proteins and Effectors	Guanine nucleotide-binding protein G(i) subunit alpha-2 Guanine nucleotide-binding protein G(k) subunit alpha cAMP-specific 3',5'-cyclic phosphodiesterase 4A
Metabolism	Alanine aminotransferase 1 Multifunctional protein ADE2 Malonyl-CoA decarboxylase
Mitochondrial Functions	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10 Nitric oxide-associated protein 1 rRNA methyltransferase 1, mitochondrial
Ion channel Functions	Anion exchange protein 2 Stromal interaction molecule 2 Methylosome subunit pICln
Intracellular Trafficking	Syntaxin-5 Golgi membrane protein 1 F-BAR and double SH3 domains protein
Protein Degradation	E3 ubiquitin-protein ligase RNF146 STIP1 homology and U box-containing protein 1
Phosphorylation/ dephosphorylation	Tyrosine-protein phosphatase non-receptor type 2 Dual specificity tyrosine-phosphorylation-regulated kinase 1A
Chromatin Remodeling	Chromodomain-helicase-DNA-binding protein 1
Possible RNA-binding Protein	G patch domain-containing protein 2

Table 6.3. Classification of interacting proteins according to cellular localization.

Cellular Compartment	Protein Names
Cytoplasm	Alanine aminotransferase 1 Multifunctional protein ADE2 E3 ubiquitin-protein ligase RNF146 Methylosome subunit pICln Malonyl-CoA decarboxylase STIP1 homology and U box-containing protein 1
Nucleus	Tyrosine-protein phosphatase non-receptor type 2 (isoform 2) Chromodomain-helicase-DNA-binding protein 1 Dual specificity tyrosine-phosphorylation-regulated kinase 1A E3 ubiquitin-protein ligase RNF146 Methylosome subunit pICln STIP1 homology and U box-containing protein 1 cAMP-specific 3',5'-cyclic phosphodiesterase 4A (perinuclear region)
Mitochondrion	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10 Nitric oxide-associated protein 1 rRNA methyltransferase 1 Malonyl-CoA decarboxylase
Plasma Membrane	Guanine nucleotide-binding protein G(i) subunit alpha-2 Guanine nucleotide-binding protein G(k) subunit alpha Anion exchange protein 2
Endoplasmic Reticulum	Stromal interaction molecule 2 Tyrosine-protein phosphatase non-receptor type 2 (isoform 1) Syntaxin-5
Golgi Apparatus	Golgi membrane protein 1 Syntaxin-5
Endosome	F-BAR and double SH3 domains protein 2

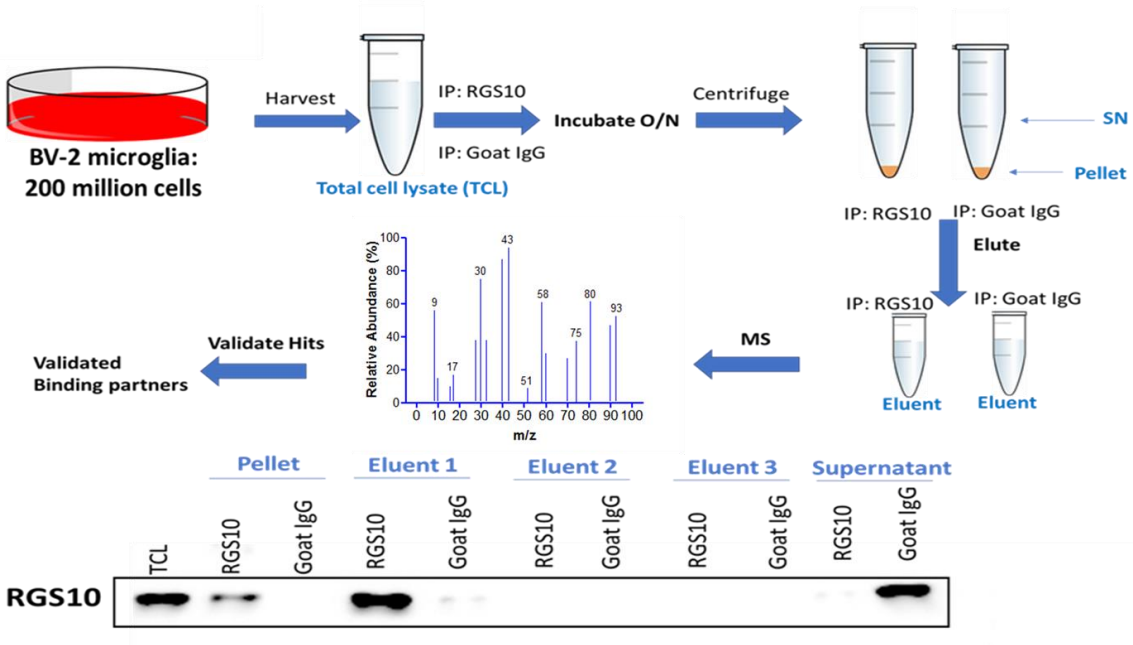

Figure 6.1: Co-immunoprecipitation/Mass Spectrometry workflow and optimization. This Figure illustrates the workflow of the co-immunoprecipitation/mass spectrometry experiment conducted in this aim. The western blot analysis was conducted to track RGS10 throughout the steps of the of the experiments. (cell dish and tube illustrations were free to use and obtained from clipart-library.com and clker.com)

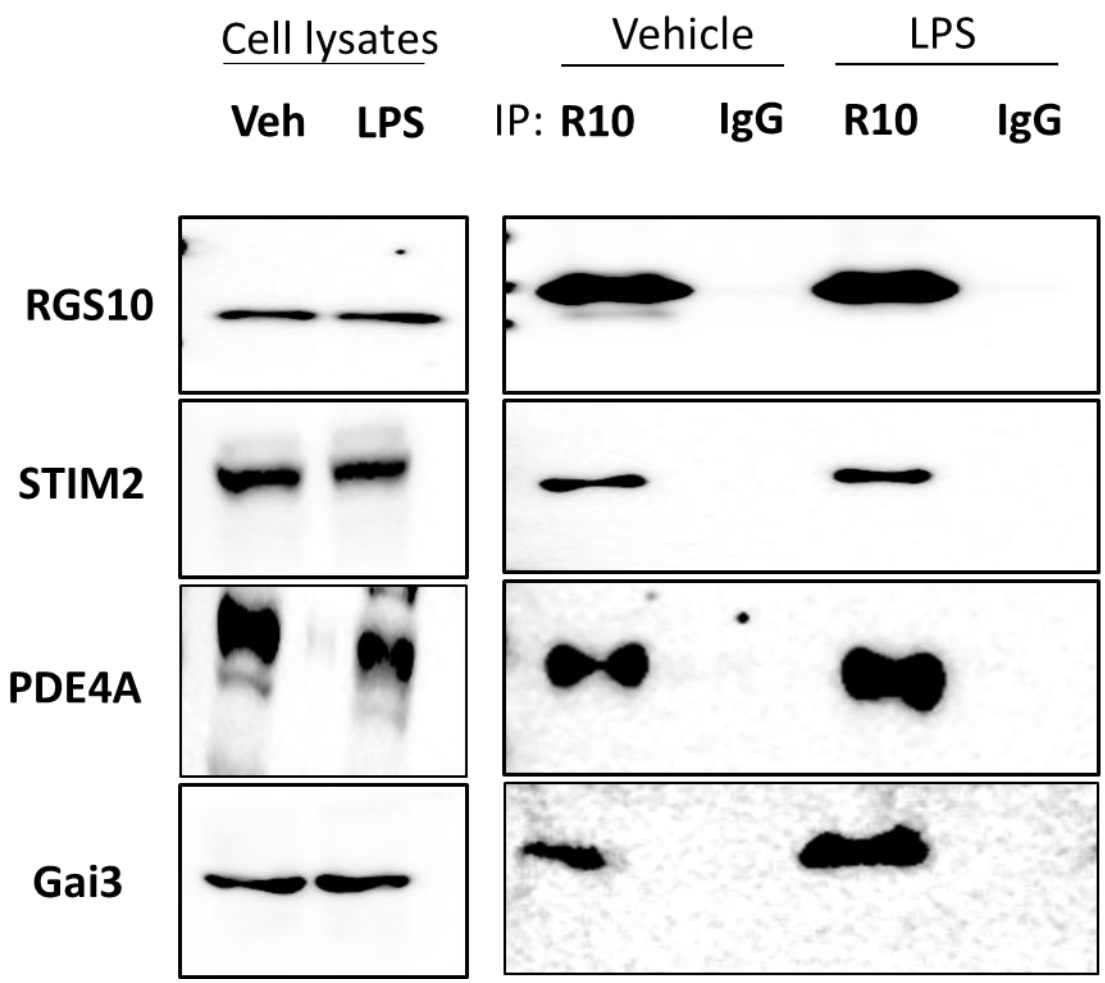

Figure 6.2: Validation of STIM2, PDE4A, Gai3. BV-2 microglia cells were plated in 150 mm dish. Cells were treated with LPS (10 ng/ml) or vehicle (Serum-free media) for 3 hours. After treatment, cells were lysed and specific RGS10 antibody was used to pulldown RGS10 from the cell lysate. Pellet containing the immunoprecipitated RGS10 was washed twice before SDS sample buffer (2X) was added. Next, SDS-PAGE was performed followed by immunoblotting using specific antibodies against RGS10, STIM2, PDE4A, and Gai3.

CHAPTER 7

SUMMARY AND FUTURE WORK

The first goal of our study was to understand the mechanisms governing the regulation of RGS10 expression in inflammatory signaling. Previously, it was demonstrated that activation of microglia by inflammatory stimulants causes RGS10 silencing in-vitro by unidentified mechanisms (Lee et al. 2008). In this study, we demonstrate that RGS10 expression is silenced in activated microglia epigenetically via histone deacetylase (HDAC) enzymes. We demonstrated that LPS induce an association of HDAC to the promoter of RGS10 in microglia, triggering histone deacetylation and subsequent RGS10 gene repression. We were able to restore RGS10 expression by treating activated microglia with the HDAC inhibitor Trichostatin A (TSA), which provide an approach to prevent the reduction of RGS10 expression in inflammation. We further showed for the first time that RGS10 expression is silenced when inflammation is induced in animal models. This was illustrated by comparing the expression of RGS10 in control and animals experiencing neuropathic pain, a condition characterized by enhanced neuroinflammation in the spinal cord (Ellis and Bennett 2013). We found that spinal cords isolated after inducing neuropathic pain in animals express significantly lower levels of RGS10 compared to control animals.

Potential future studies following our work should assess whether HDAC inhibitors will restore the expression of RGS10 in neuropathic pain animal models, and whether restoration of RGS10 will affect the progression and/or intensity of neuropathic pain in these animals. Further, identifying other compounds that restore RGS10 expression will be beneficial as HDAC inhibitors may cause several non-specific side effects. Since RGS10 expression is also dysregulated in other cells, such as neurons (Lee, Chung, et al. 2012) and cardiomyocytes

(Miao et al. 2016), future studies in other cells may identify similar mechanisms regulating the expression of RGS10.

The second aim of our study was to further investigate the anti-inflammatory function and mechanism of RGS10 in microglia. Previously, it was shown that RGS10 suppresses the release of inflammatory cytokines following inflammatory stimulants in microglia and macrophages (Lee, Chung, et al. 2011; Lee et al. 2013). Here, we also demonstrate for the first time that RGS10 regulates the mRNA and protein expression of the inflammatory enzyme cyclooxygenase-2 (COX-2) and suppress the release of Prostaglandin E2 (PGE2) from microglia. We also investigated whether RGS10 regulate inflammatory signaling in ovarian cancer cells, and we found that RGS10 suppresses COX-2 and TNFα expression in these cells as well, indicating that RGS10 inhibits inflammatory signaling in both immune and non-immune cells. The molecular mechanisms mediating RGS10 anti-inflammatory functions are still unknown. The classic function of RGS proteins is by accelerating GTPase activity of G proteins (Watson et al. 1996). However, it is established now that many RGS proteins can exerts function in G protein-independent mechanisms (Sethakorn, Yau, and Dulin 2010). Therefore, we recognized that identifying whether RGS10 inhibits inflammation in G protein dependent or independent mechanism will be a crucial initial step in understanding the anti-inflammatory mechanisms of RGS10. Our data indicate that RGS10 ability to suppress the expression of COX-2 and TNFα does not require its GAP activity. We reached this conclusion because the enhanced COX-2 and TNFα production resulting from RGS10 knockdown was not influenced by the Gαi inhibition. We also showed that RGS10 GAP-dead mutant that is incapable of inhibiting G proteins was as effective in suppressing TNFα production as the wild type, providing yet another evidence that RGS10 GAP function is dispensable for its anti-inflammatory effect. We also confirmed that RGS10 ability to reduce COX-2 and TNFα in ovarian cancer cells is not influenced by Gαi inhibition. Although it was previously shown that some RGS exerts GAP-independent mechanisms, we are the first to report a GAP-independent function of RGS10.

Identifying the nature of the GAP-independent mechanism of RGS10 will naturally be the next logical step in future studies. However, this will not be an easy task, as it requires an intensive analysis of RGS10 structure, localization, and interacting partners in microglia to gain insights about the molecular mechanisms that mediate RGS10 actions. Another potential future direction is studying the effect of RGS10 in conditions where COX-2 plays major roles. For example, COX-2 and PGE2 induce neuronal death in-vitro and in-vivo (Sanchez-Pernaute et al. 2004; Xia et al. 2015). This combined with the findings demonstrating that RGS10 protects dopaminergic neurons (Lee et al. 2008; Lee, Chung, et al. 2012), should promote future studies to assess whether the effect of RGS10 on COX-2 mediates its neuroprotective effect. In ovarian cancer, our laboratory reported that RGS10 suppress chemoresistance of ovarian cancer cells, but the exact mechanism was not identified (Hooks et al. 2010). Since we identified that RGS10 regulates inflammatory signaling in ovarian cancer cells, and it was previously demonstrated that inflammation induces chemoresistance in ovarian cancer, a future work can investigate whether RGS10 ability to suppress chemoresistance is due to its anti-inflammatory role in ovarian cancer cells (Maccio and Madeddu 2012).

Many RGS proteins interact with unique binding partners that mediate several GAP-dependent and independent functions (Sethakorn, Yau, and Dulin 2010). By conducting co-immunoprecipitation and mass spectrometry analysis, we identified multiple novel binding partners of endogenous RGS10 in microglia that differs in their functions and localization. We further validated selected hits (STIM2, PDE4A, and Gαi3) by performing co-immunoprecipitation followed by western blot analysis utilizing specific antibodies against STIM2, PDE4A, and Gαi3. We believe that findings of this study will greatly assist in investigating the functions and mechanisms of RGS10 in microglia and other cells as well. Some of the interacting partners plays a role in the inflammatory signaling in microglia, making them candidate mediators of RGS10 anti-inflammatory actions. Additionally, some of these interacting partners plays a role in different microglial functions, such as mitochondrial respiration and chemotaxis. Therefore,

future studies should assess whether RGS10 plays other roles in microglia in addition to its anti-inflammatory action. Since some of the interacting partners are known to function in other cells, such as osteoclasts and ovarian cancers, the findings of this study may apply in studying the role and function of RGS10 in other cellular models, further expanding the significance of our study. Future studies can also aim to determine which domain mediate these interactions. Since RGS10 contain only the RGS domain and small N and C-terminals, we predict that future studies will demonstrate that the RGS domain mediates large subset of these interactions, which is interesting as the RGS domain was thought for a long time as mainly a mediator of G protein interactions.

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