EXPLORING CHROMATIN INTERACTIONS OF REGULATOR OF G-PROTEIN SIGNALING 10 (RGS10) WITHIN THE NUCLEUS OF MICROGLIAL CELLS

by

VICTORIA NELSON HOLT

(Under the Direction of Shelley Hooks)

ABSTRACT

RGS10 plays an important role in regulating microglia activation; however, the mechanism of RGS10 is poorly understood. RGS10 has the unique ability to translocate to the nucleus upon PKA phosphorylation. Inflammatory cytokine production is increased upon loss of RGS10, suggesting that RGS10 plays an important anti-inflammatory role. Additionally, literature suggests RGS10 augments anti-inflammatory CREB signaling via interactions with PKA. When stimulated by PKA, CREB binds to the CRE consensus sequence on the promoters of inflammatory cytokines. First, we hypothesize that RGS10 is indirectly associating with chromatin or in a complex with other DNA binding proteins at the promoters of inflammatory cytokines. Secondly, we hypothesize that the PKA phosphorylation site on RGS10 is critical to its regulatory activity within the nucleus.

INDEX WORDS: Regulator of G-protein Signaling, Microglia, Inflammatory Cytokines, Promoters.

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The University of Georgia
August 2016

## DEDICATION

I dedicate this manuscript to my endearing husband, Graham Holt, my inspiring parents, Bradley and Kathy Nelson, and my encouraging brothers, Matthew and Jeremy Nelson. Without them, I would not be where I am today. Through laughs, smiles, tears, & tantrums, they have encouraged me to persevere and be the absolute best that I can be.

ACKNOWLEDGEMENTS

First, I would like to give all honor and glory to my creator, the Lord Jesus Christ, for His grace and gifts to me. He has been by my side, guiding my steps, this entire journey.

Next, I would like to thank my major professor Dr. Shelley Hooks for her support and guidance. I am thankful for her acceptance of me into her lab and her mentorship and friendship along the way. Additionally, I would like to thank all the members of Dr. Hooks's lab, particularly Mohammed Alqinyah and Andrew Webster. They taught me the techniques I needed to know to be successful in the Hooks lab with patience and persistence.

I would also like to thank Drs. George Zheng and Han-rong Weng for serving on my advisory committee, and for providing guidance and words of wisdom.

Finally, I owe many thanks to my husband, my family, and my friends for their encouragement and confidence in me.

# INTRODUCTION

RGS10 is highly expressed in the brain and the immune system, and is known to play an important role in regulating microglia activation; however, the mechanism of RGS10 is poorly understood. Like other RGS proteins, RGS10 has the canonical role of accelerating the inactivation of the Gα subunits of G-protein coupled receptors (GPCRs). Evidence suggests RGS10 also has the ability to translocate to the nucleus upon PKA phosphorylation. Some preliminary data suggests that RGS10 possess a non-canonical role in the nucleus that remains to be elucidated. It has been shown that inflammatory cytokine production is increased upon loss of RGS10, suggesting that RGS10 plays an anti-inflammatory role. The transcription factor CREB is an important negative regulator of the inflammatory transcription factor NFκB and lies downstream of Protein Kinase A (PKA). Literature suggests that RGS10 augments CREB signaling via interactions with PKA in the cytoplasm. When stimulated by PKA, CREB binds to the cyclic AMP response element (CRE) sequence on promoters of inflammatory cytokines. We hypothesize that RGS10 is indirectly associating with chromatin or in complex with other DNA binding proteins at the promoters of inflammatory cytokines. First, we aim to probe for direct RGS10 binding at the CREB binding site. Secondly, we aim to take a more global look at the direct binding sequences of RGS10 in the nucleus. Additional studies involve employing an RGS10 mutant that is unable to be phosphorylated by PKA to determine if the phosphorylation site on RGS10 that signals translocation to the nucleus is critical to its regulatory activity within the nucleus.

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# CHAPTER 1

# INTRODUCTION AND LITERATURE REVIEW

G-protein Coupled Receptors (GPCRs) are among the most diverse and most important groups of receptors in eukaryotic organisms (Tuteja, 2005). These seven-transmembrane receptors are coupled to heterotrimeric G-proteins at the plasma membrane. When a ligand binds to the GPCR on the outside of the cell, the receptor undergoes a conformation change inside the cell that results in a GDP → GTP nucleotide exchange on the associated G-protein alpha subunit (Tuteja, 2005). When bound to GTP, the heterotrimeric G-protein becomes active, promoting the activation of a variety of downstream effector pathways, such as the pathway described in detail in Figure 3.

Like any signaling molecule, GPCRs and their G-proteins need to have the ability to be turned off quickly. Proteins known as Regulator of G-protein Signaling (RGS) proteins do just that. The RGS protein family consists of more than 30 members (Hollinger *et al.*, 2002). RGS proteins canonically act as GAP proteins, or GTPase-accelerating proteins. They bind directly to activated G-proteins and hydrolyze the activating GTP back to a deactivating GDP in order to turn off signaling (Elliot *et al.,* 2000; Willars, 2006). All RGS proteins have a conserved RGS domain that provides them with their GAP activity.

Work in the Hooks laboratory is currently focused on a 20 kDa RGS protein known as RGS10. RGS10 is a member of the R12 family of RGS proteins and is primarily responsible for inactivating the G-protein, Gαi (Sierra *et al.,* 2002). RGS10 is highly expressed in the immune system and in the brain and has been shown to regulate microglial activation (Lee *et al.,* 2008). Microglia are brain macrophage cells important in the immune response. Microglia become activated in response to several neuronal assaults including injury, infection, and environmental toxins and are important in neutralizing those assaults (Ferrer *et al.,* 1990; Lee *et al.,* 2011). Chronic activation of microglia leads to an overproduction of pro-inflammatory cytokines such as TNF-α and IL-1β, resulting in neuroinflammation (Calcia *et al.,* 2016; Lee *et al.,* 2008). Neuroinflammation is associated with several neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, and Multiple Sclerosis (Crowley *et al.,* 2016).

When stimulated with lipopolysaccharide (LPS) or Tumor Necrosis Factor (TNF) to induce inflammatory conditions, expression of RGS10 is suppressed. Western blot analysis of BV-2 cells that have been stimulated with LPS or TNF reveals that RGS10 protein expression is reduced (Lee *et al.,* 2008). Furthermore, in ELISA data from primary microglia isolated from RGS10 knockout mice, after treatment with LPS for 24 hours, production of inflammatory cytokines is increased, specifically TNF-α and IL-1β (Lee *et al.,* 2012).

A loss of RGS10 leads to dysregulation of pro-inflammatory cytokine gene expression, suggesting that it plays an important regulatory role in inflammation (Lee *et al.,* 2008). RGS10 knockdown increases the microglial burden in the

Central Nervous System, leading to dopaminergic neuron degeneration in animal models of chronic inflammation (Lee *et al.,* 2008). The suppression of RGS10 augments the neuroinflammatory phenotype by removing the neuronal protection that RGS10 provides. Most convincingly, when RGS10 is reintroduced into an RGS10 knockdown system, the neuroprotective effects are reestablished (Lee *et al.,* 2011). Restoring RGS10 expression to a neuroinflammatory disease states, such as those of Alzheimer's Disease, Parkinson's Disease, and Multiple Sclerosis hold potential therapeutic advantages. Additionally, elucidating RGS10's anti-inflammatory mechanism could lead to novel therapeutic targets that could be explored for the management or treatment of these devastating diseases.

RGS10 acts as a canonical RGS protein at the plasma membrane by accelerating the hydrolysis of GTP to GDP to terminate G-protein signaling. RGS10 specifically inactivates the G-protein, Gαi (Sierra *et al.,* 2002). Gαi inhibits Adenyl Cyclase (AC). As shown in the proposed model described in Figure 3, RGS10 releases the breaks on Gαi's inhibition of AC. Adenyl Cyclase then is able to stimulate cyclic-AMP (cAMP) which then stimulates Protein Kinase A (PKA) downstream. PKA has many substrates, such as the transcription factors CREB and NFκB, which will be discussed in detail later in this thesis.

Interestingly, researchers have begun to uncover GAP-independent, non-canonical roles of RGS proteins (Willars, 2006). Our laboratory generated a "GAP-dead" E66K mutant version of RGS10 that is unable to bind to Gαi and consequently unable to perform its GTPase function. As shown in Figure 1,

HEK293 cells transfected with wildtype RGS10 reduces TNF-α mRNA. Most surprisingly, TNF-α mRNA is also reduced when the RGS10 E66K mutant is transfected, suggesting that RGS10 reduces TNF-α expression independently of its canonical role. These results suggest that RGS10 is playing a non-canonical, GAP-independent role in regulating TNF-α expression.

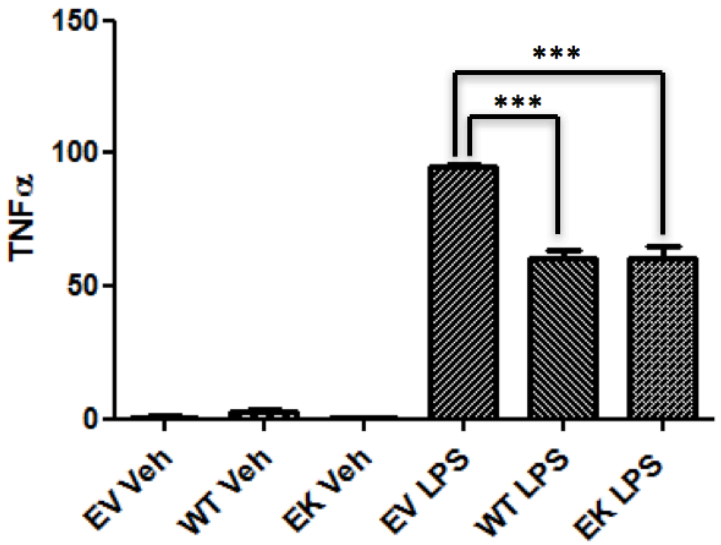


**Figure 1.** RGS10 reduces TNF-α expression independent of its canonical GAP function. TNF-α mRNA is significantly reduced when HEK293 cells are transfected with wildtype (WT) RGS10 and mutant GAP-dead RGS10 (EK) after stimulation with 10 ng/ml of LPS for 24 hours.

In 2001, Burgon *et al.* showed that RGS10 translocates to the nucleus upon phosphorylation of its serine-168 residue by Protein Kinase A (PKA) (Burgon, *et al.,* 2001; Lee *et al.,* 2008). Forskolin stimulates cAMP, which causes downstream stimulation of PKA. Upon phosphorylation by 25 microMolar (uM) Forskolin for 2 hours, RGS10 can be seen translocating to the nucleus in *stably* transfected HEK293 cells. In RGS10 harboring a point mutation of Serine168→alanine, RGS10 is unable to be phosphorylated by PKA at the S168 residue. Burgon *et al.* demonstrated that this mutant form of RGS10 is unable to translocate to the nucleus upon FSK treatment (Burgon *et al.,* 2001). Waugh *et al.* also explored RGS10 localization, particularly in a variety of both rat and mouse brain cells. RGS10 was observed localized to the nucleus, specifically to the euchromatin compartment of nuclei and other transcriptionally active regions of the nucleus, in both microglia and neurons (Waugh *et al.,* 2005). These results suggest that RGS10 plays a role in modulating inflammatory cytokine gene expression within the nucleus.

Unlike some RGS proteins that are sequestered in the nucleus as a means of signaling regulation (Burchett, 2003; Chatterjee *et al.,* 2000), preliminary data from our laboratory suggests that RGS10 is acting in the nucleus to mediate its effects on inflammation. Chromatin Immunoprecipitation (ChIP) data displayed in Figure 2 suggests that RGS10 is interacting with the chromatin of promoters of genes implicated in inflammation, specifically TNF-α and IL-1β. RGS10 can be seen on the promoter of TNF-α and IL-1β before exposure to inflammatory factor lipopolysaccharide (LPS), while the important

inflammatory transcription factor NFκB is bound to the promoters of TNF-α and IL-1β after 3-hours and 6-hours of LPS treatment as expected (Yabe *et al.,* 2005). From this data, we suspected RGS10 may be binding at the same site on the promoter as NFκB. To explore this idea further, our lab conducted a gel super shift assay (data not shown) to determine if RGS10 is binding on the NFκB binding site on DNA. We concluded that RGS10 is *not* binding at the NFκB binding site, as no supershift band was present; however, RGS10 is indeed negatively regulating NFκB and appears to be binding to or associating with other proteins that are binding to an unknown site on the TNF-α and IL-1β promoters.

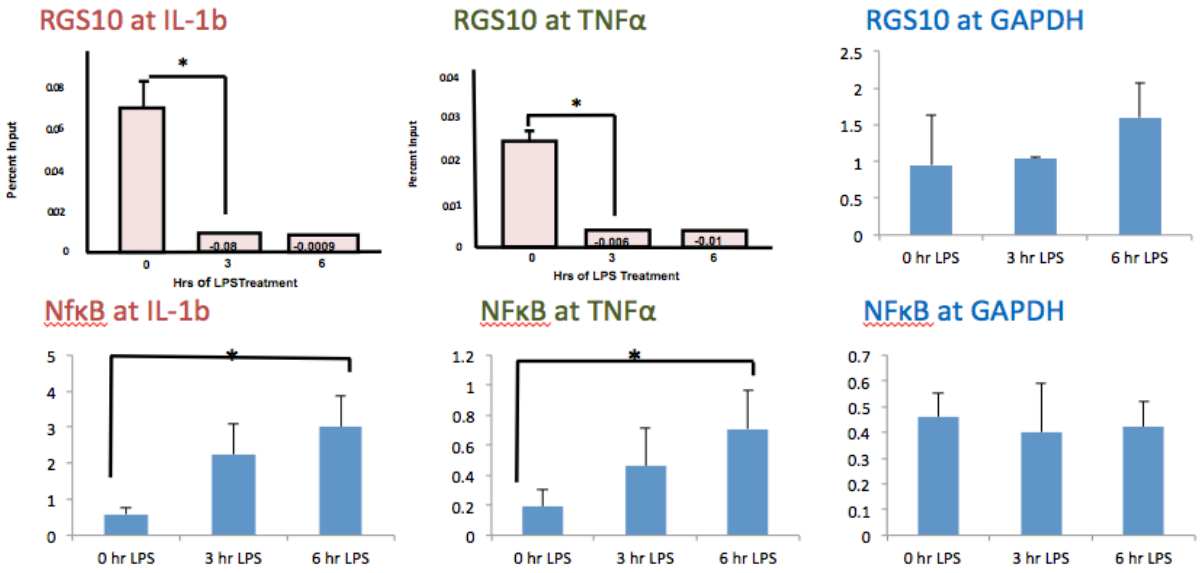


**Figure 2**. Ch-IP experiments in BV-2 cells on the promoters of TNF-α and IL-1β followed by PCR quantification after treatment of vehicle or LPS (3 hours or 6 hours). RGS10 is found bound to the promoters of TNF-α and IL-1β before LPS stimulation. RGS10 dissociates upon LPS treatment, while NFκB is observed binding to the TNF-α or IL-1β promoter after LPS treatment. Data generated in collaboration with Dr. Nagini Maganti, Georgia State University.

From the evidence described, it is suggested that RGS10 is translocated to the nucleus for a GAP-independent, anti-inflammatory purpose. We have evidence that RGS10 is interacting with the chromatin or chromatin associated proteins at the promotors of inflammatory cytokine genes, suggesting that RGS10 regulates inflammation at the transcription level from within the nucleus. It is also suggested that the serine-168 residue on RGS10 is critical for its phosphorylation by PKA and subsequent nuclear localization. As shown in Figure 3, RGS10 may act both upstream and downstream of PKA: upstream by inactivating the inhibition of Adenyl Cylase and downstream by acting as a PKA substrate. The research described in this thesis explores two hypotheses encompassed in the proposed pathway displayed in Figure 3. First, we hypothesize that the PKA phosphorylation site on RGS10 is critical for its nuclear localization and anti-inflammatory effects. This hypothesis will be tested by achieving Aim 1: employ the use of a PKA-deficient RGS10 mutant and explore its effects on nuclear localization and inflammatory cytokine expression. The second hypothesis that will be tested is with regards to RGS10's nuclear role. We hypothesize that nuclear RGS10 is associating with chromatin at the promoters of inflammatory cytokine genes. This hypothesis will be tested by Aim 2A and Aim 2B. Aim 2A will probe for association with specific promoter elements, and Aim 2B will globally assess RGS10 association with genomic DNA.

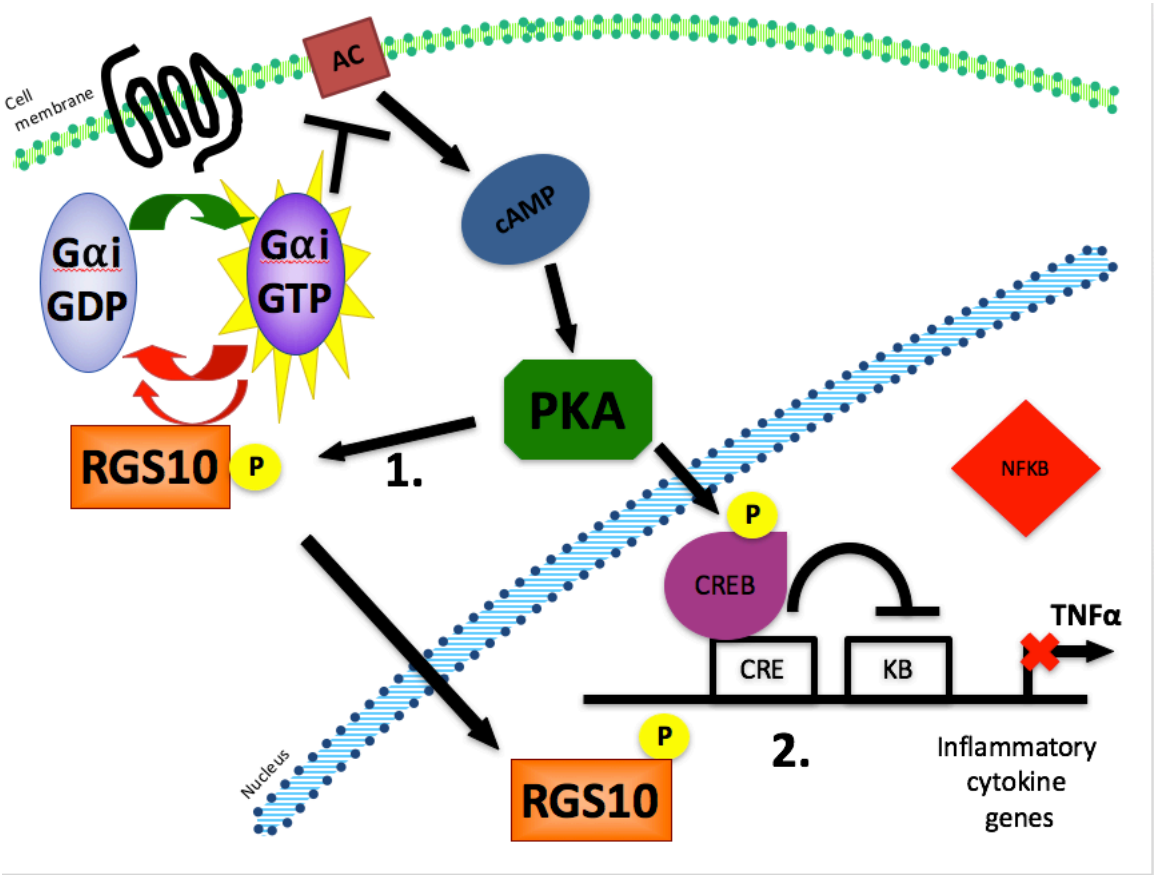


**Figure 3**. An overview of the proposed pathway tested in Aims 1 and 2 (labeled 1 and 2). In the proposed model, RGS10 inactivates Gαi. Gαi inhibits AC. AC stimulates cAMP, which stimulates PKA. PKA phosphorylates RGS10 and CREB. RGS10 phosphorylation leads to subsequent nuclear localization. CREB phosphorylation leads to active CREB that is able to bind to CBP and to the CRE site on the promotor of TNF-α, for example, inhibiting NFκB binding at the κB site, ultimately reducing the amount of TNF-α expression.

# CHAPTER 2

# DESCRIPTION OF RGS10 MUTANTS AND PRELIMINARY EXPERIMENTS

## 2.1 Introduction

Preliminary experiments exploring the canonical impact of RGS10 on PKA activity and phosphorylated CREB levels are explored herein, in light of the proposed mechanism in Figure 3. Additionally, two RGS10 mutant constructs are explained in detail in this chapter that were used in the experiments presented in the remaining chapters. The Hooks laboratory uses a human WT RGS10 construct incorporated into a pcDNA3.1 parent vector. Both of the mutant constructs described in this chapter have also been incorporated into a pcDNA3.1 parent vector. These constructs are able to be transfected into our HEK293 cell system, allowing us to harvest those cells and observe a variety of cellular responses.

## 2.2 Preliminary Experiments

### 2.2.1 RGS10 effect on PKA activity

As described previously, RGS10 has the canonical role of inactivating Gαi (Sierra *et al.,* 2002). By inactivating Gαi, RGS10 releases the breaks on Adenyl Cyclase (AC). AC then goes on to stimulate cyclic-AMP (cAMP) which then stimulates Protein Kinase A (PKA). To test whether or not an effect on PKA activity can be observed by simply overexpressing RGS10 in an HEK293 cell system, wildtype RGS10 transfections followed by Western Blot Analyses were conducted.

HEK293 cells were chosen as a model cell system for this experiment as they are easily transfected and have very low basal levels of endogenous RGS10. Because the HEK293 cells have low levels of endogenous RGS10, the effects observed with RGS10 overexpression can be attributed to the RGS10 proteins present in the RGS10 transfected cells compared to the empty vector overexpression. The HEK293 cell system used in these experiments described in this thesis express Toll-like Receptor 4 (TLR4) receptors. Activation of TLR4 receptors by lipopolysaccharide (LPS) leads to the activation of NFKB, which results in the transcription of inflammatory cytokines produced during an immune response. For these reasons, HEK293 cells make a sufficient and reliable model for the scope of this project.

The HEK293 cells were plated at 100,000 cells per well in a 24-well plate. Cells were given 48 hours to adhere to the plate and then transfected in penicillin/streptomycin-free media with empty pcDNA3.1 or wildtype RGS10 overexpression vector at a concentration of 500 nanograms (ng) per well. Invitrogen's Lipofectamine and Plus Reagent kit was used for the transfection following manufacturer's protocol. After 48 hours of incubation in transfection media, the cells were harvested in 100µl of lysis buffer (2M Tris-HCL, 4% SDS, 10% glycerol, Bromophenol blue, and .5% 2-Mercaptoethanol).

Samples were then boiled in a water bath and centrifuged briefly in a benchtop quick spin centrifuge. Samples were loaded into a 15% polyacrylamide gel. Samples were run on the gel at 100 Volts for 1.5 hours. The gel was then transferred to a nitrocellulose membrane (ThermoScientific) and then blocked in 5% milk for 1 hour. A wash in 0.5% TBST-tween for 10 minutes while shaking followed the blocking step. The washed membrane was then placed in primary antibody at 4$^{o}$C overnight. A 1:1000 RGS10 antibody (SantaCruz) was used to blot for RGS10 protein. A 1:500 PKA Substrate (Cell Signaling) was used to detect proteins that have been phosphorylated by PKA. A 1:1000 GAPDH antibody (Ambion) was used to detect GAPDH, a loading control.

After incubation in primary antibody, the membranes were washed 3 times for 10 minutes each in .5% TBST-tween. Secondary antibody incubation for 1 hour with shaking followed. After incubation in secondary antibody, the membranes were washed 3 times for 10 minutes each in 0.5% TBST-tween. The membrane was then placed into Ehnhanced Chemoluminescence Substrate

(ThermoScientific) for 5 minutes prior to imaging in a Flourochem imager. The data were analyzed on the Fluorochem machine and normalized to GAPDH protein levels. A two-tailed t-test was used to determine significance.

Figure 4 shows that RGS10 was successfully overexpressed and has a significant increase on PKA activity on a protein or protein complex of approximately 180 kDa, quantified in Figure 4B. As expected, RGS10 appears to differentially regulate PKA activity. As PKA has many substrates, and RGS10's canonical role is to release the breaks on upstream players of PKA activity, a global impact of RGS10 on PKA activity was not expected.

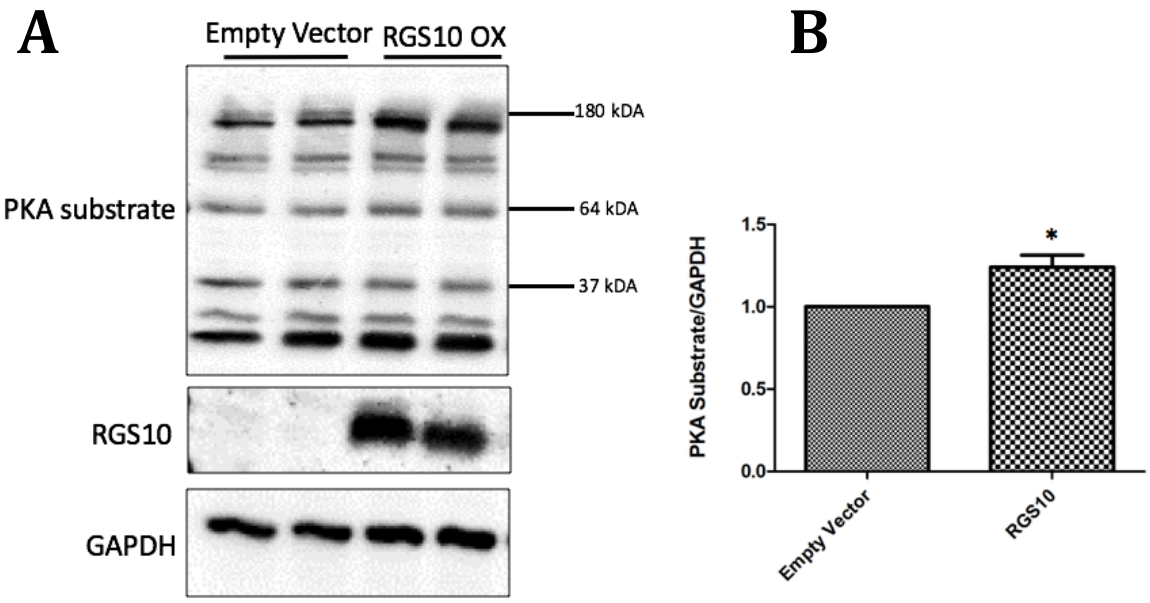


**Figure 4**. A.) RGS10 overexpression in HEK293 cells appears to promote PKA activity, specifically for a protein or protein complex of approximately 180 kDa, when normalized to GAPDH. B.) The 180 kDa band was quantified in panel B. P-value = .0456. RGS10 significantly increases PKA activity at the 180kDA substrate, but differently regulates PKA activity as a whole. RGS10 can be seen significantly overexpressed. GAPDH acts as a loading control.

### 2.2.2 RGS10 effect on phosphorylated CREB

PKA has many important substrates. One important PKA substrate, and one that will be discussed in detail in chapter 5, is the transcription factor CREB. To test whether or not RGS10 overexpression has an effect on increasing the levels of phosphorylated CREB, western blot experiments were conducted in the same manner described previously. A 1:1000 phospho-CREB antibody (Cell Signaling) was used to blot for phosphorylated CREB protein. A 1:1000 GAPDH antibody (Ambion) was used to detect GAPDH as a loading control. The membranes were quantified and normalized to GAPDH. A two-tailed t-test was used to determine significance.

Figure 5 shows that RGS10 was successfully overexpressed and appears to increase the amount of phosphorylated CREB. Although not significant (p-value = .0558), there does appear to be a trend. This is expected as phosphorylated CREB is significantly downstream of canonical RGS10 activity at the plasma membrane.

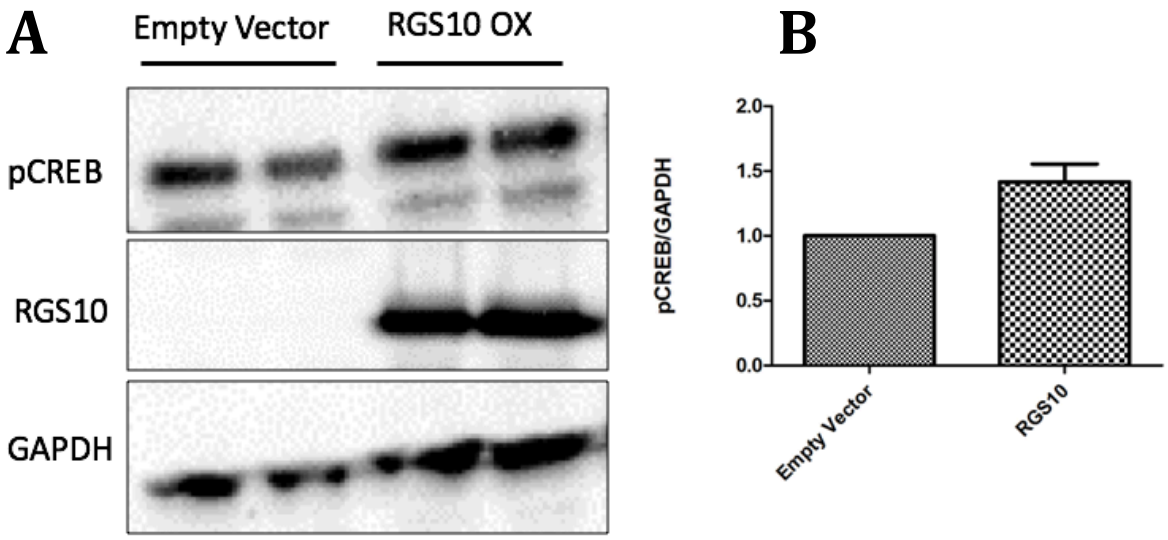


**Figure 5**. A.) RGS10 overexpression in HEK293 cells tends to increase levels of phosphorylated CREB (pCREB) when normalized to GAPDH. B.) Quantification of pCREB from Panel A. GAPDH acts as a loading control.

## 2.3 Generation and Validation of Mutant Constructs

Two mutants are used in the work described herein. First a “PKA-deficient mutant” of RGS10, referred to as “S168A mutant”, or simply “SA” has been generated that contains a point mutation (G→T), which causes an amino acid change from serine to alanine at residue 168, represented by Figure 6. The alanine residue is not able to accept the phosphoryl group from PKA, making RGS10 unable to be phosphorylated at that specific residue previously determined to be important to RGS10’s subsequent nuclear localization. This mutant has been confirmed by sequencing. Additionally, a preliminary western blot experiment was conducted to determine what concentration of SA mutant resulted in comparable expression to wildtype (WT) RGS10 after transfection. It was determined that 200 ng of SA mutant DNA should be transfected to achieve similar RGS10 expression from a transfection of 500 ng of WT RGS10. The western blot data can be seen in Figure 7.

Linearized SA mutant was gifted to our lab by Dr. Jae-Kyung Lee. The SA mutant resides in a parent pcDNA3.1 vector. The linearized plasmid was re-ligated using “Quick Ligase” enzyme from NEB. The ligation reaction was set up following NEB’s Quick Ligase protocol. The ligation reaction was then transformed into XL Blue cells (ThermoFisher) according to the manufacturer’s protocol. The SA mutant plasmid is resistant to ampicillin; therefore, transformed cells were plated on Terrific Broth (Fisher BioReagents) agar plates containing 100 µg/mL of ampicillin. Plates were left at $37^0$C overnight. Colonies were then

selected and grown in Terrific broth up to 200 mL. A Polymerase Chain Reaction (PCR) was done to confirm the presence of a complete SA mutant plasmid. For the PCR, 2 µl of broth was added to a PCR reaction consisting of an RGS10 forward primer and a BGH reverse primer. The presence of a band at 6.4 Kb suggested a positive clone. Plasmid DNA was then isolated by an Endotoxin-free Maxiprep kit from Omega Bio-tek. The Maxiprep kit yielded SA mutant plasmid at a concentration of 0.16 µg/µl. The plasmid DNA was then sent for sequencing to the University of Georgia's Genomics Facility to confirm that only the G→T mutation was present in the plasmid. The SA mutant plasmid DNA was confirmed by sequencing, allowing overexpression experiments with the SA mutant plasmid to proceed.

The SA mutant plasmid from Dr. Jae-Kyung Lee's laboratory does not contain the HA tag that is present at the N-terminus of our lab's wildtype RGS10. This explains why when WT RGS10 compared to SA RGS10 in Figure 7, appears to be approximately 26 kDa while the SA mutant RGS10 appears to be approximately 20 kDa.

Additionally, a series of pull-down experiments need to be conducted to confirm that the SA mutant RGS10 acts as a functional GAP protein. To this point, no experiments have been conducted to confirm that the SA mutant RGS10 protein is an active and functional protein. The possibility exists that the SA mutant RGS10 protein is nonfunctional. This is not expected to be the case, however, as the only residue altered in the SA mutant is at residue 168. If alteration at residue 168 results in a non-functional RGS10 protein, the residue

could be considered more critical to the protein's function than previously understood. In order to determine whether or not the SA mutant plasmid has GTPase-accelerating protein activity, a pull down assay should be conducted similar to what was conducted with the E66K GAP-dead mutant. Aluminum-tetrafluoride (AlF4) treatment would be used to stabilize Gαi in its RGS protein bound state. Either Gαi antibody or IgG antibody would be used for the immunoprecipitation of RGS10.

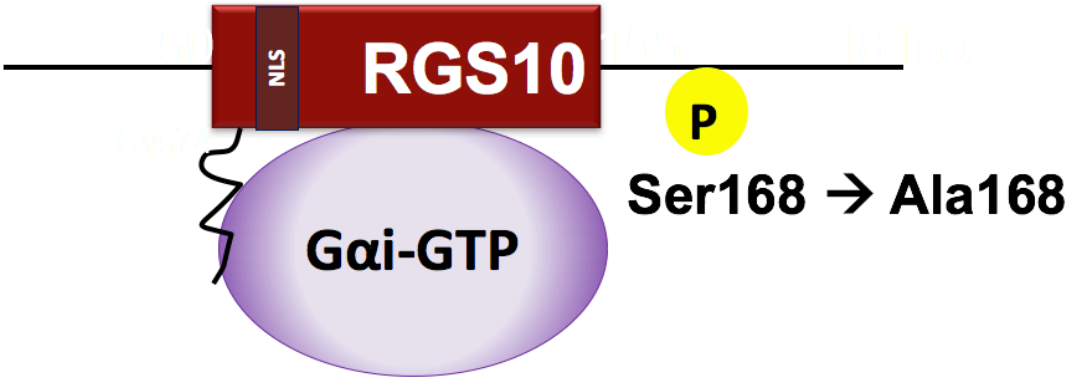


**Figure 6**. Representation of the PKA-deficient S168A RGS10 mutant. The SA mutant has a mutation that results in a serine → alanine amino acid change at residue 168. This mutation inhibits PKA from phosphorylating (P) RGS10 at residue 168. NLS represents the nuclear localization sequence of RGS10.

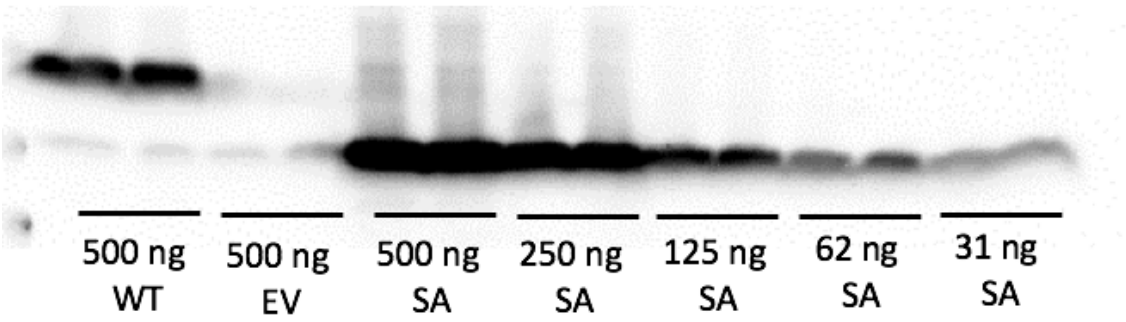


**Figure 7**. Western blot data of a step-wise transfection of SA mutant plasmid compared to WT RGS10 plasmid and Empty pcDNA3.1 (EV). 500ng of WT RGS10 was transfected in the first two lanes. 500ng of empty pcDNA3.1 parent vector was transfected in lanes 3 and 4. SA mutant plasmid was transfected at 500ng, 250ng, 125ng, 62ng, and 31ng to determine which concentration yielded comparable expression to WT RGS10. 200ng was determined to be most comparable to 500ng WT RGS10.

The second mutant used for this thesis work is a “GAP-dead mutant” of RGS10 that was generated in our laboratory. This GAP-dead mutant (shown in Figure 8), also referred to as “EK mutant” or “EK,” has a point mutation that changes glutamic acid to lysine at residue 66. Lysine at this residue results in the inability of RGS10 to bind to Gαi, resulting in a functionally GAP-dead RGS10 protein. This mutant was confirmed by a co-immunoprecipitation experiment shown in Figure 9. In this experiment, when HEK293 cells transfected with the mutant are stimulated with aluminum-tetrafluoride (AlF4), a compound that stabilizes Gαi in its transient form bound to RGS10, little to no RGS10 immunoprecipitating with Gαi is observed in the RGS10 GAP-dead mutant, confirming the GAP-dead nature of this mutant. The E66K mutant is unable to bind to Gαi and consequently unable to perform its GTPase-accelerating protein function. Transfecting 500 ng of EK mutant results in similar expression as transfecting 500 ng of WT RGS10.

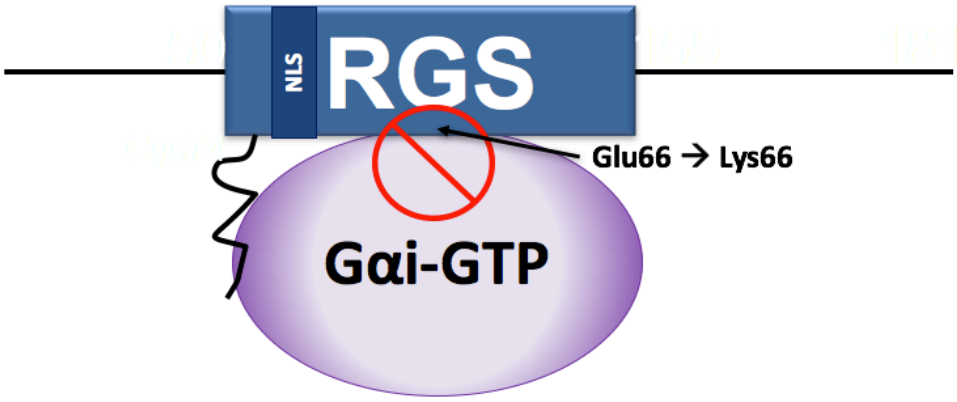


**Figure 8**. Representation of the GAP-dead E66K RGS10 mutant. The EK mutant has a mutation that results in a glutamic acid → lysine amino acid change at residue 66. This mutation blocks RGS10 binding to Gαi-GTP. NLS represents the nuclear localization sequence of RGS10.

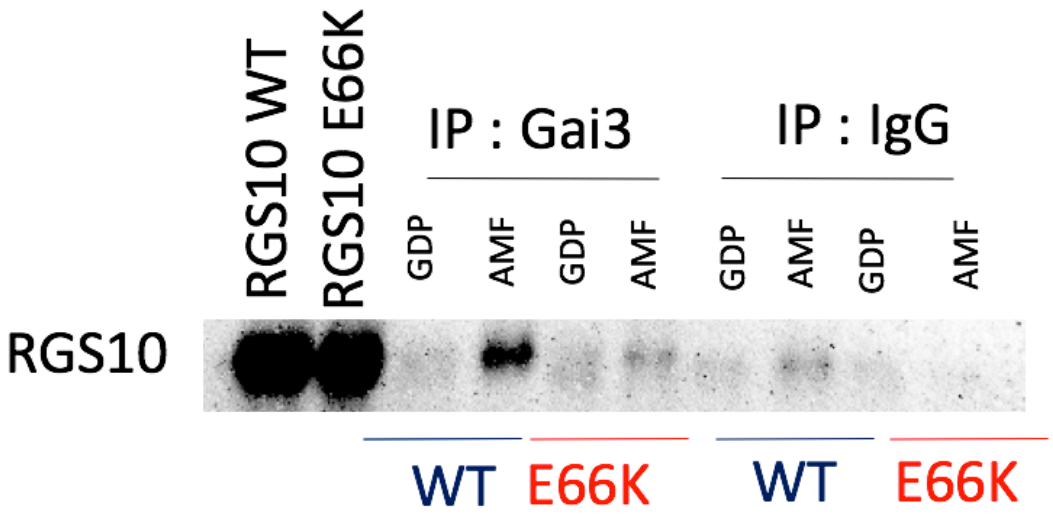


**Figure 9**. Co-IP experiment results confirming the absence of GTPase activating activity of the RGS10 E66K mutant. AlF4 treatment stabilizes Gαi in its RGS protein bound state. Either Gαi antibody or IgG antibody was used for the immunoprecipitation. RGS10 antibody was used for the western blot. IgG acts as a control.

# CHAPTER 3

# THE PKA PHOSPHORYLATION SITE ON RGS10 IS CRITICAL FOR ITS NUCLEAR LOCALIZATION.

## 3.1 Introduction

It has been previously reported that upon phosphorylation by PKA at the serine 168 residue, RGS10 translocates to the nucleus (Burgon *et al.,* 2001). However, this report and conclusion needed to be confirmed. Closer inspection of the original images did not fully support the conclusion. Further, the experiment compared the activity of stably expressed wildtype RGS10 with transiently transfected expressed SA mutant RGS10. The difference in transfection methods may have caused global differences in the HEK293 cells.

## 3.2 Materials and Methods

Before beginning this experiment, circular glass cover slides were primed in HEK293 media overnight in a 6-well plate. The following day, HEK293 cells were plated on top of the glass slides at a density of 100,000 cells per well in a 6-well plate. Cells were given 48 hours to adhere to the slides and then transfected in penicillin/streptomycin-free media with wildtype RGS10 overexpression vector

or PKA-deficient SA mutant RGS10 overexpression vector at a concentration of 500ng per well and 200ng per well, respectively. Invitrogen's Lipofectamine and Plus Reagent kit was used for the transfection following manufacturer's protocol. After 36 hours, transfection media was removed and the cells were treated with either vehicle (serum-free media) or 25μg of Forskolin (FSK) for 2 hours.

After the 2-hour treatment of either vehicle or FSK, cells were fixed in ice cold methanol for 10 minutes. Following fixation, cells were blocked and permeabilized in blocking solution (PBS++, 1% BSA, 0.2% Triton-100) for 30 minutes while shaking. Next, cells were incubated in primary antibody (PBS++, 1% BSA, 0.5% Tween-20, 1:1000 RGS10 Antibody) for 1 hour while shaking. Three 15 minute washes in 0.5% TBST-tween followed. Cells were then incubated in secondary antibody (PBS++, 1% BSA, 0.5% Tween-20, 1:1000 Anti-goat Antibody) for 1 hour while shaking, followed by three 15 minute washes. Cells were mounted onto the slide with ProLong Gold Antifade Mountant with DAPI (ThermoFisher) and allowed to dry for 30 minutes before being stored at $4^{o}$C. Slides were imaged using Olympus BX43 microscope with an Olympus U-CMAD3 camera. Images were processed using Olympus-cellSens Dimensions. Both 20x and 40x magnification was used to generate the figures shown below.

## 3.3 Results and Discussion

### 3.3.1 Results

As expected, wildtype RGS10 is observed localized to both the cytoplasm and the nucleus after treatment with vehicle (serum-free media) in Figure 10 at 20x magnification and in Figure 11 at 40x magnification. The HEK293 cells represented by these figures have been transiently transfected with WT RGS10 construct. RGS10 can be seen in red, and the nuclei of cells in blue.

In Figures 12 and 13, WT RGS10 is observed in both the cytoplasm and the nucleus of HEK293 cells transiently transfected with wildtype RGS10 and treated with FSK. Figure 12 is an image of representative cells taken at 20x magnification, in which RGS10 (shown in red) can be seen in both the cytoplasm and in the nucleus (shown in blue). Figure 13 is at 40x magnification. Again, RGS10 is observed in both the cytoplasm and the nucleus even after Forskolin treatment.

In Figure 14 at 20x magnification, SA RGS10 can be observed predominately in the cytoplasm, leaving a blue nuclear center. Representative HEK293 cells transiently transfected with PKA-deficient SA Mutant RGS10 and treated with vehicle appear to have less RGS10 localized in the nucleus compared to the HEK293 cells transiently transfected with WT RGS10.

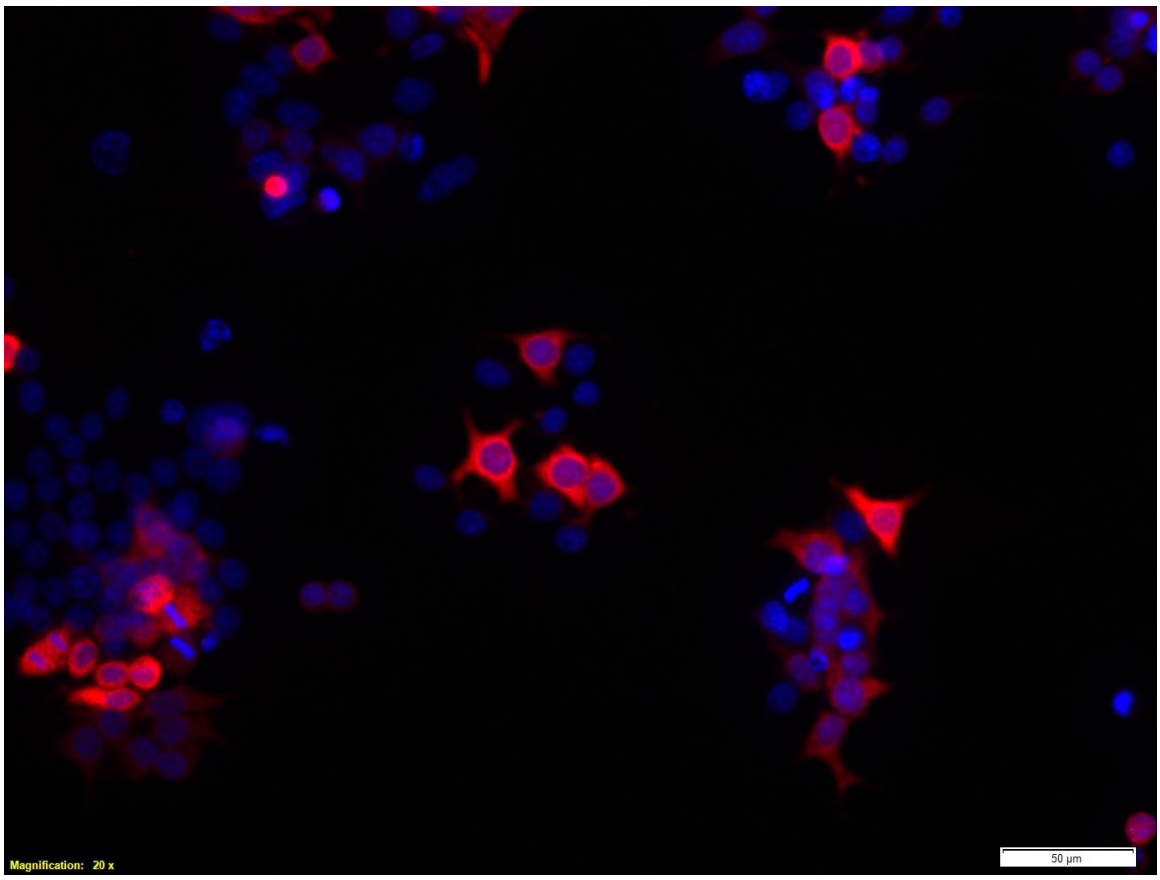


**Figure 10**. Image taken at 20x magnification of HEK293 cells transiently transfected with WT RGS10 and treated with vehicle for 2 hours. RGS10 (red) can be observed in both the cytoplasm and the nucleus. The nucleus is stained with DAPI (blue). Overlay appears purple. Un-transfected cells appear as only DAPI stained nuclei and can be used as negative controls.

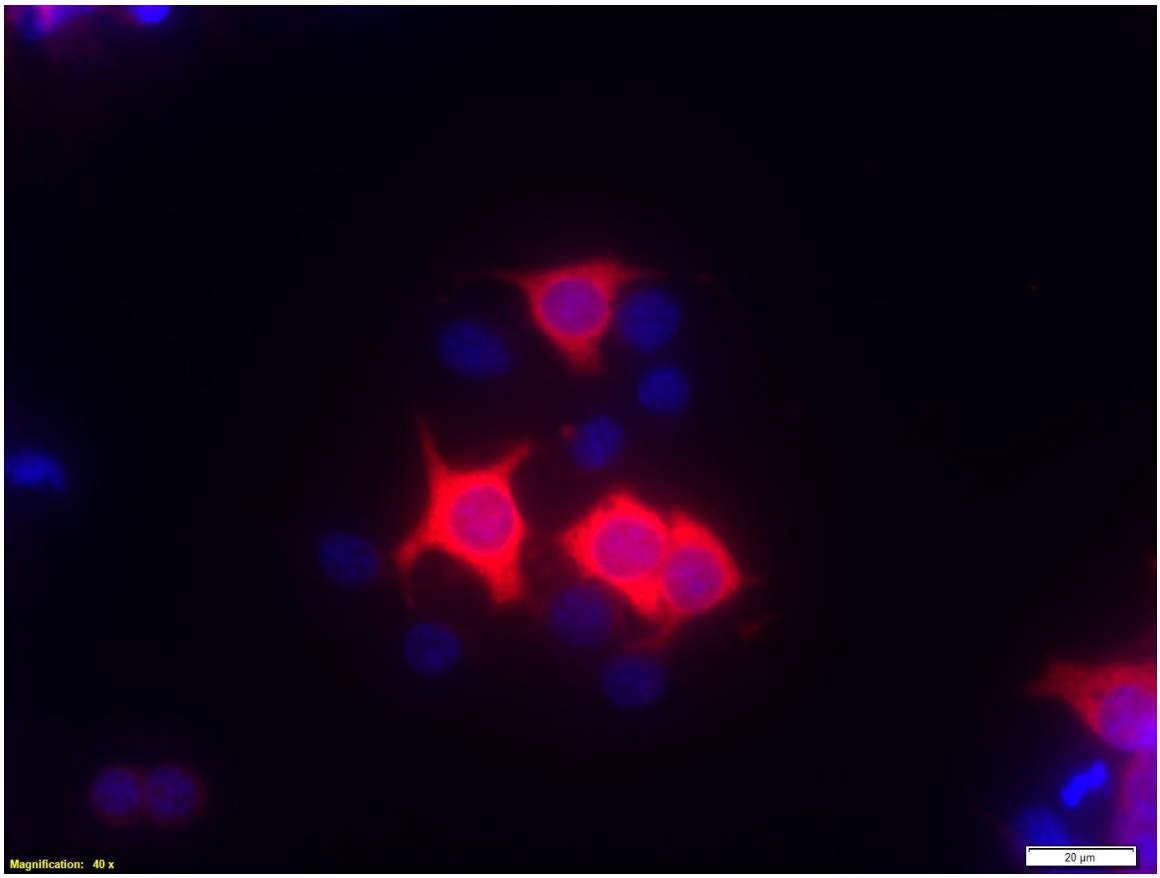


**Figure 11**. Image taken at 40x magnification of HEK293 cells transiently transfected with WT RGS10 and treated with vehicle for 2 hours. RGS10 (red) can be observed in both the cytoplasm and the nucleus. The nucleus is stained with DAPI (blue). Overlay appears purple. Un-transfected cells appear as only DAPI stained nuclei and can be used as negative controls.

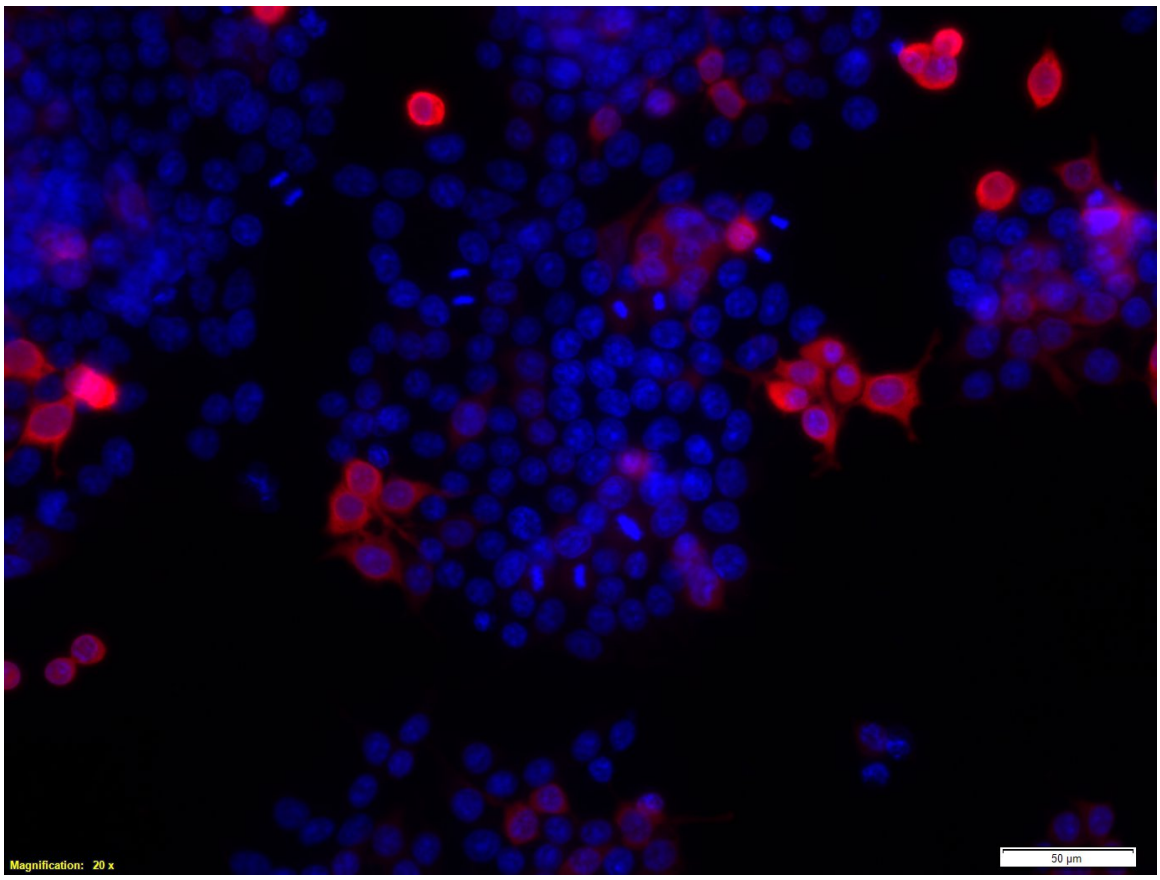


**Figure 12**. Image taken at 20x magnification of HEK293 cells transiently transfected with WT RGS10 and treated with 25µg of Forskolin for 2 hours. RGS10 (red) can be observed in both the cytoplasm and the nucleus. The nucleus is stained with DAPI (blue). Overlay appears purple. Un-transfected cells appear as only DAPI stained nuclei and can be used as negative controls.

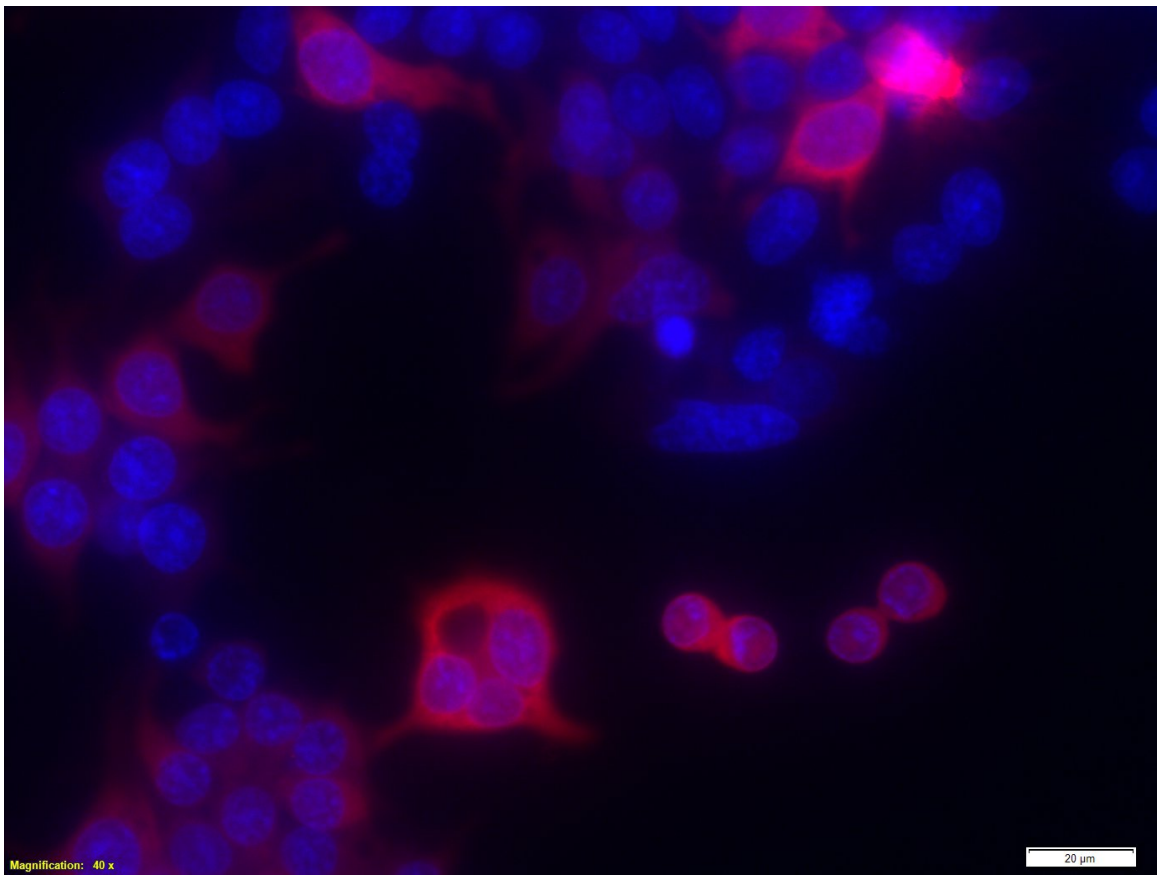


**Figure 13**. Image taken at 40x magnification of HEK293 cells transiently transfected with WT RGS10 and treated with 25µg of Forskolin for 2 hours. RGS10 (red) can be observed in the both the cytoplasm and the nucleus. The nucleus is stained with DAPI (blue). Overlay appears purple. Un-transfected cells appear as only DAPI stained nuclei and can be used as negative controls.

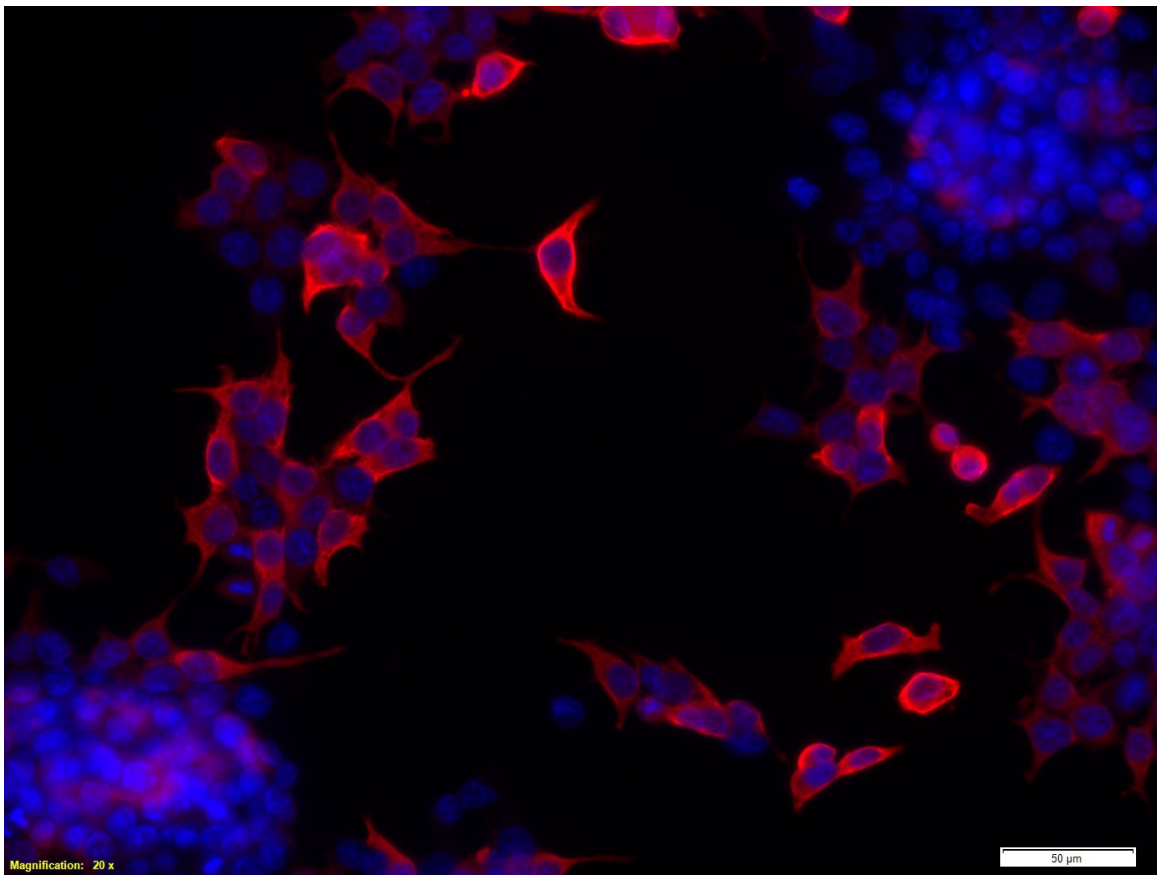


**Figure 14**. Image taken at 20x magnification of HEK293 cells transiently transfected with SA mutant RGS10 and treated with vehicle for 2 hours. RGS10 (red) can be observed predominately in the cytoplasm. The nucleus is stained with DAPI (blue). Un-transfected cells appear as only DAPI stained nuclei and can be used as negative controls.

### 3.3.2 Discussion

The observation by Burgon *et al.* that WT RGS10 translocation to the nucleus occurs upon PKA phosphorylation driven by 2-hour treatment of 25μg of Forskolin was not able to be confirmed. When transiently transfected cells with WT RGS10 were stimulated with FSK, no significant subsequent translocation to the nucleus can be seen in either Figure 12 or Figure 13 compared to transiently transfected WT RGS10 treated with vehicle in Figure 10 or Figure 11. Because wildtype RGS10 translocation was not observed, the effects seen with the SA mutant become moot. As seen in Figure 14, the transiently transfected SA mutant can be seen predominately in the cytoplasm, leaving a clear blue nucleus in the center of the cell. The transiently transfected SA mutant HEK293 cells treated with Forskolin looked nearly identical to the cells treated with vehicle (data not shown).

There are many possible reasons this experiment was unsuccessful in observing RGS10 translocation to the nucleus upon PKA stimulation. First, our wildtype RGS10 expression vector has an N-terminal HA-tag. This tag could be interfering with translocation machinery, thus inhibiting RGS10 translocation to the nucleus. For equal comparisons, a WT RGS10 expression vector needs to be generated that lacks the HA-tag on the N-terminus. Secondly, the transfection process might affect the cells in such a way that renders translocation no longer possible. This experiment should to be repeated using a transiently transfected non-HA-tagged wildtype RGS10 overexpression construct.  For this reason, it

cannot be concluded that the phosphorylation at the serine 168 residue is critical for subsequent RGS10 translocation to the nucleus. Additionally, future experiments should seek to use confocal imagery to obtain these images in order to confirm true translocation into the nucleus of the cell.

Although we were unable to observe WT RGS10 translocating to the nucleus in our HEK293 cell system, it has been clearly shown that RGS10 is localized in the nucleus of cells, particularly brain cells such as microglia in both rat and mouse brains (Burchett, 2003; Waugh *et al.,* 2005). For this reason, the inability to observe RGS10 translocation did not affect the following series of experiments in which only the presence of RGS10 in the nucleus is necessary.

# CHAPTER 4

# THE PKA PHOSPHORYLATION SITE ON RGS10 IS CRITICAL FOR ITS ANTI-INFLAMMATORY EFFECTS.

## 4.1 Introduction

RGS10 is suppressed under inflammatory conditions (Lee *et al.,* 2008). Additionally, a loss of RGS10 leads to dysregulation of pro-inflammatory cytokine gene expression (Lee *et al.,* 2012). These data suggest RGS10 plays an important regulatory role in inflammation (Lee *et al.,* 2008). As seen in Figure 15, HEK293 cells transfected with wildtype RGS10 are able to significantly reduce TNF-α expression compared to empty vector after 10ng/mL treatment of LPS for 12 hours. Similarly, as described earlier, TNF-α expression suppression is achieved independently of RGS10's GTPase-accelerating protein activity. The GAP-independent mutant also significantly reduces TNF-α expression. As previous research has suggested that the phosphorylation site at residue 168 is critical for RGS10's subsequent translocation to the nucleus, we wanted to test whether the same phosphorylation is required for RGS10's anti-inflammatory effects. This was done by conducting a real-time PCR experiment with HEK293 transfected with either empty vector, wildtype RGS10, SA mutant RGS10, or EK mutant RGS10.

## 4.2 Materials and Methods

HEK293 cells were plated in a 24-well plate at 100,000 cells per well. Cells were given 48 hours to adhere to the plate and then were transfected in penicillin/streptomycin-free media with either empty vector (negative control), wildtype RGS10 overexpression vector, GAP-dead EK mutant RGS10 overexpression vector, or PKA-deficient SA mutant RGS10 overexpression vector at a concentration of 500ng per well of EV, WT, and EK vectors and 200ng per well of SA vector. Invitrogen's Lipofectamine and Plus Reagent kit was used for the transfection following manufacturer's protocol.

After 48 hours, the cells were harvested in 200μL of TRIzol (Ambion). RNA extraction using TRIzol was conducted to isolate mRNA from the cells following manufacturer's protocol. RNA concentrations were determined using a NanoDrop machine, and concentrations were brought to 0.2μg of RNA into 10μL of Diethylpyrocarbonate (DEPC) water. A High Capacity cDNA Reverse Transcription Kit from Applied Biosystems was used to generate cDNA from the isolated mRNA. The cDNA PCR reaction was run according to manufacturer's protocol. The cDNA then was used in the real-time PCR reaction.

The real-time PCR reaction was set up using SYBR Green Mastermix (ThermoFisher). The Mastermix, cDNA, and desired primers were added to a 384 well plate. The primers used for this reaction were human RGS10 pooled forward and reverse primers, human TNF-α pooled forward and reverse primers, and human Actin pooled forward and reverse primers. The real-time PCR

reaction was run on a 7900HT Fast Real-Time PCR System from Applied Biosystems. CT values were extracted and analyzed. All values were normalized first to the housekeeping gene, Actin. The fold change was calculated and set on a 0 to 100 scale to normalize across experiments. The results can be seen in Figure 15.

## 4.3 Results and Discussion

### 4.3.1 Results

As seen in Figure 15, both WT RGS10 and EK RGS10 are able to significantly reduce the amount of TNF-α expression after LPS treatment, compared to the pcDNA3.1 empty vector (EV). Most interestingly, SA RGS10 loses the ability to reduce TNF-α expression after LPS treatment, suggesting that the S168A site on RGS10 is critical for its regulation of TNF-α expression.

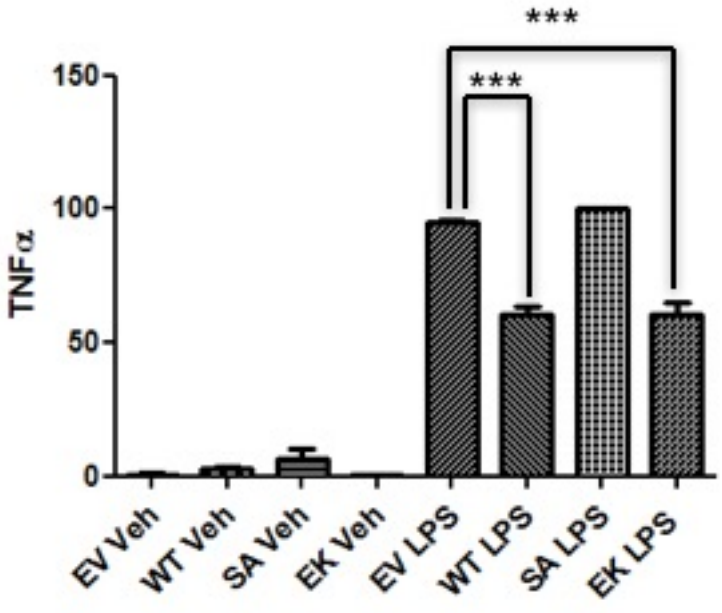


**Figure 15**. Real-time PCR data showing the effect of empty vector (EV), wildtype RGS10 (WT), PKA-deficient mutant RGS10 (SA), or GAP-dead mutant RGS10 (EK) transfection on TNF-α expression after treatment of either vehicle or 10ng/mL LPS for 12 hours. The significant decrease in TNF-α expression that is observed with both WT and EK RGS10 overexpression is lost in cells transfected with SA mutant. P-value = 0.0456.

### 4.3.2 Discussion

As expected, wildtype RGS10 and GAP-dead mutant RGS10 significantly reduced TNF-α expression ($P = 0.0456$). Interestingly, PKA-deficient S168A mutant lost the ability to reduce TNF-α expression. This data suggests that phosphorylation of the serine 168 residue of RGS10 is critical for RGS10's ability to regulate TNF-α expression.

This data is interesting as it suggests that PKA phosphorylation of RGS10, particularly at residue 168, plays a significant role in RGS10's non-canonical regulation of TNF-α expression. PKA phosphorylation at residue 168 seems to be an important player with regards to RGS10's anti-inflammatory activity. As described previously, RGS10 also seems to differentially regulate PKA activity establishing a signaling feedback loop with PKA important to both RGS10's canonical and non-canonical function.

# CHAPTER 5

# PROBE FOR ASSOCIATION WITH SPECIFIC PROMOTER ELEMENTS: GEL SHIFT ASSAY STUDIES ON THE CREB BINDING SITE, CRE

## 5.1 Introduction

PKA has many important substrates. One of those substrates is the transcription factor CREB. When phosphorylated by PKA, CREB binds to its cofactor CBP and then to its DNA binding element, the cAMP Response Element (CRE) in promoter regions of DNA (Spooren *et al.,* 2010). Evidence suggests that CREB competes with another transcription factor, NFkB for the co-activator CBP (Parry *et al.,* 1997). NFkB is an important inflammatory transcription factor responsible for the transcription of inflammatory cytokines (Yabe *et al.,* 2005). NFkB can be seen binding to the promoter of proinflammatory cytokines upon the introduction of an inflammatory mediator such as LPS (Beg *et al.,* 1993; Dai *et al.,* 2011; Verma *et al.,* 1995).

Literature suggests that RGS10 augments CREB signaling via interactions with PKA in the cytoplasm during inflammatory stress in dopaminergic neuron-like cells. Cells that overexpress RGS10 have significantly increased levels of phosphorylated CREB (Lee *et al.,* 2012). As described earlier, CREB lies downstream of PKA and is implicated in several important pathways. Because of

the apparent competition between CREB and NFkB, and preliminary data that suggests RGS10 augments CREB signaling in the cytoplasm via PKA, as well as the finding that RGS10 is not associating with the NFkB binding site on the DNA, we hypothesized that the CREB binding sequence, CRE, may be a target for RGS10 interaction. Discovering a connection between RGS10 and CREB, specifically the CREB binding sequence would unlock several new and significant therapeutic options for a variety of inflammation-based diseases.

## 5.2 Materials and Methods

Electrophoretic Mobility Shift Assays (EMSAs), commonly referred as gel-shift assays, were conducted to probe for direct interactions between RGS10 and the CREB binding site, CRE. BV-2 microglia cells were used for all EMSA experiments. First, BV-2 cells were treated with either vehicle or 10μg of FSK for 1 hour. Next, nuclear extracts were isolated from total cell lysates using a Nuclear Extraction Kit from Active Motif. Samples were then prepared for the EMSA reaction. A critical component to an EMSA experiment is a labeled probe comprised of the sequence being tested. For these experiments a Licor CRE probe labeled with an infrared tag was used. An EMSA kit (Licor) was used to conduct the EMSA reactions. Reactions were set up following manufacturer's protocol. Nuclear protein was allowed to incubate with the infrared-CRE probe for 10 minutes at room temperature.

A competition experiment, seen in Figure 17, was completed to confirm specificity of the bands to the infrared-CRE probe. In this experiment, unlabeled CRE probe of identical sequence to the infrared labeled CRE probe was added 1000x more than the infrared labeled probe to the reaction mixture.

For Supershift Assays, RGS10 antibody (SantaCruz) or CREB antibody (Cell Signaling) was added to the protein + probe reaction mixture and allowed to incubate at room temperature for 10 minutes. Samples were run on a 6% DNA retardation gel. Gel was pre-run for 45 minutes prior to loading and running samples. Gels were run for either 1 hour or 3 hours at 4$^{o}$C. Gels were imaged on a Licor Odyssey FC imager.

## 5.3 Results and Discussion

### 5.3.1 Results

Free probe and gel-shift bands can be observed in Figure 16, after addition of BV-2 nuclear lysate to infrared labeled CRE probe. If a protein or protein complex within the nuclear lysate binds to the probe, a shift upward of the band is observed compared to free-probe. Several bands can be observed when nuclear extract is added to the probe compared to the blank, suggesting that proteins within the nucleus bind to the CRE probe. Unbound probe can be seen accumulated at the bottom of the Blank lane. This unbound probe will no longer

be seen in subsequent images, as gels were run until the unbound probe had run off the end of the gel.

Figure 17 represents a competition experiment in which excess unlabeled CRE probe was added to the reaction mixture to confirm specificity of binding to the CRE sequence. Approximately five bands are observed consistently in each EMSA experiment using BV-2 nuclear extracts and the infrared labeled CRE probe. The five bands disappeared upon the addition of the unlabeled probe, suggesting that the bands observed are specifically binding to the CRE sequence.

In Figure 18, CREB antibody was added in an effort to establish a positive control. Forskolin treatment was used to stimulate PKA phosphorylation, leading to more CREB being present in the nucleus. An antibody-dependent loss of the three lower bands was observed when CREB antibody was added to the reaction mixture.

In Figure 19, RSG10 antibody was added to determine if RGS10 is binding to, or in a complex with, other proteins binding to the infrared labeled CRE probe. Neither an antibody-dependent loss or shift of any of the bands was observed, suggesting that RGS10 does not bind directly to, or in a protein complex with, proteins that bind to the CREB binding site, CRE.


**Figure 16**. An EMSA reaction was carried out using infrared labeled CRE probe incubated with BV-2 nuclear lysate and run on a 6% DNA retardation gel. In lane one labeled “Blank,” only free probe was added. In lane two labeled “Nuclear Extract,” nuclear lysate was incubated with infrared CRE probe. Bands can be observed, representing binding of a protein or protein complex to the free probe. Gel was run for 1 hour.

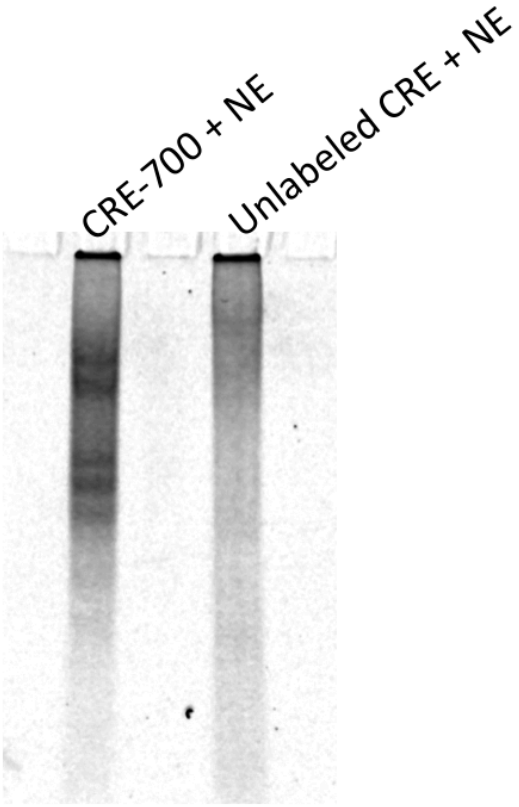


**Figure 17**. An EMSA reaction was carried out using infrared labeled CRE probe and 1000x more unlabeled CRE probe, both incubated with BV-2 nuclear lysates and run on a 6% DNA retardation gel. Bands disappear upon addition of unlabeled probe suggesting the five bands consistently observed are specific to the CRE sequence. Gel was run for 3 hours.

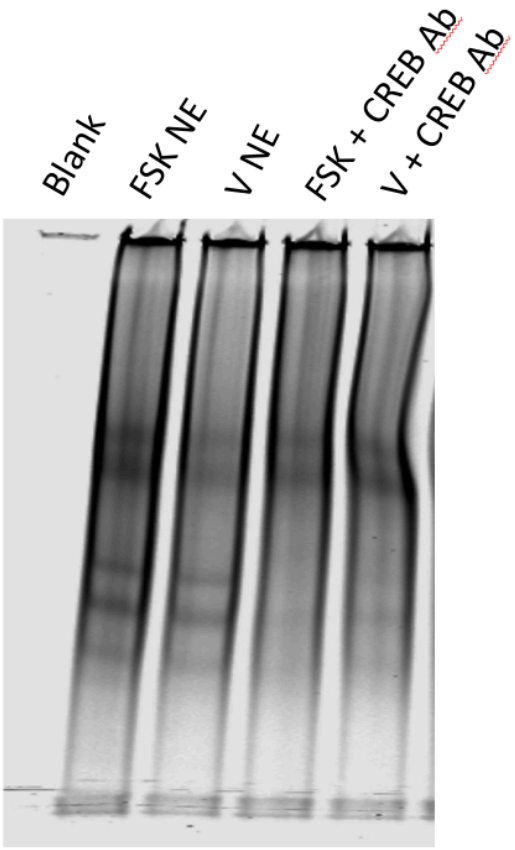


**Figure 18**. An EMSA reaction was carried out using infrared labeled CRE probe incubated with 1-hour treatment of either vehicle (V) or Forskolin (FSK) treated BV-2 nuclear lysates and run on a 6% DNA retardation gel. In lanes four and five, a CREB antibody was added for a supershift experiment. An antibody dependent loss of the lower three bands was observed. Gel was run for three hours.

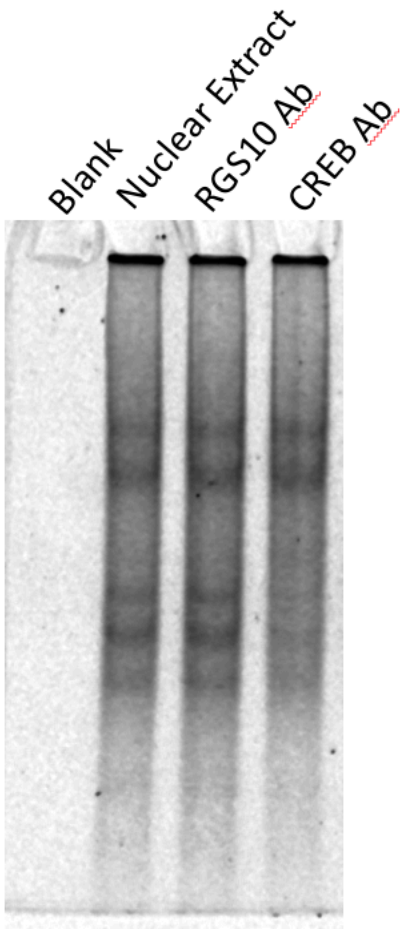


**Figure 19**. An EMSA reaction was carried out using infrared labeled CRE probe incubated with BV-2 nuclear lysates and run on a 6% DNA retardation gel. In lanes three and four, an RS10 antibody or a CREB antibody was added for a supershift experiment. No antibody dependent shift or loss of bands was observed when RGS10 antibody was added to the reaction. Gel was run for 3 hours.

### 5.3.2 Discussion

Previous evidence suggests that RGS10 is not binding to the NFkB binding site, nor does the evidence presented here suggest that RGS10 is binding to the CREB binding site in the nucleus of BV-2 cells. No antibody-dependent shift or antibody-dependent loss of bands was observed, suggesting that RGS10 is not binding to, or in a complex with other proteins that bind to the CRE binding sequence.

Before any additional sequences (probes) are tested for interactions, a positive control that causes an antibody-dependent upward shift of a band, as opposed to the antibody-dependent loss of bands observed in these experiments should be optimized. We hypothesize in these experiments that the proteins or protein complexes are becoming aggregated with the addition of the CREB antibody, inhibiting them from exiting the well, thus causing the antibody-dependent loss of specific bands observed. Once a positive control has been established, additional promoter elements can be tested for direct chromatin interaction.

Our previous results suggest that RGS10 is interacting at the promoters of genes implicated in inflammation, particularly TNF-α and IL-1β, before stimulating with inflammatory factor LPS. There are many consensus sequences at the promoters of inflammatory cytokines that RGS10 could be associating with. For this reason, a more global approach was taken to determine what RGS10 is associating with on genomic DNA.

# CHAPTER 6

# GLOBALLY ASSESS RGS10 ASSOCIATION WITH GENOMIC DNA: CHIP-SEQ ASSAY

## 6.1 Introduction

Our preliminary data in Figure 2 of Chapter 1, suggests that RGS10 interacts with chromatin or chromatin associating proteins at specific sequences on the promoters of the inflammatory cytokines TNF-α and IL-1β. Upon LPS treatment, the association at these promoters is lost. Additionally, previous research has determined that RGS10 is not directly binding to the CRE or the KB consensus sequences on the DNA. To determine what exactly RGS10 is associating with in the nucleus, a more global look at the genomic DNA associations of RGS10 was taken by employing a Chromatin Immunoprecipitation experiment followed by Next Generation Sequencing.

## 6.2 Materials and Methods

First, BV-2 cells were plated at a density of $2x10^6$ in a 24-well plate. Cells were treated with LPS or vehicle for 0 and 6 hours. Next, cells were scraped off of the plates and added to conical tubes for cross-linking. The DNA is cross-linked with 37% formaldehyde at a 1:100 ratio. After 8 minutes incubation at

room temperature, glycine is added to stop the cross-linking reaction. Samples were centrifuged for 5 minutes and washed with PBS. Supernatant was removed and the pellet was re-suspended in cell lysis buffer (5 mM PIPES pH8, 85mM KCl). After 15 minute incubation on ice, samples were centrifuged for 15 minutes at 2100RPM to pellet nuclei. Pellet was re-suspended in SDS lysis buffer (1% SDS, 10mM EDTA, 50 mM Tris-HCl) and diluted with ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH8, 167 mM NaCl). Samples were then flash frozen in liquid nitrogen. After being flash frozen, the samples were then subjected to three 10-minute rounds of sonication in a BioRuptor  to shear the cross-linked DNA.SDS Elution Buffer was used to reverse the crosslinking. Samples were spun down at 13000RPM for 10 minutes to pellet cell debris. The samples were precleared by adding salmon sperm DNA/protein A agarose beads (EMD Millipore). Beads were pelleted and re-suspended in ChIP dilution buffer.  RGS10 antibody or IgG control antibody were added and incubated overnight.

A pull down assay was conducted to precipitate the protein-DNA complexes. First, salmon sperm/protein A agarose beads were added to the samples and allowed to incubate for 1 hour at 4$^{o}$C. Beads were then pelleted and washed in the following sequence of buffers:

1. Low salt wash buffer: (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH8, 150 mM NaCl).
2. High salt wash buffer: (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH8, 500 mM NaCl).

3. LiCl wash buffer: (0.25 M LiCl, 1% NP40, 1% DOC, 1mM EDTA, 10mM Tris-Hcl pH8).
4. 1 x TE: (10mM Tris-HCl pH8, 1mM EDTA).

After washing in 1 x TE, SDS elution buffer (1% SDS, 0.1 M $NaHCO_3$) was added. Beads were pelleted and re-suspended in ChIP dilution buffer. NaCl was added to the samples, followed by hybridization at 65$^{o}$C overnight. After incubation with Proteinase K for 1 hour, DNA was isolated by standard phenol-chloroform DNA extraction. The obtained ChIP DNA was then sent off for sequencing to BGI Americas. BGI Americas will be assisting us in sequencing the DNA and conducting the statistical analysis on the samples. Unfortunately, the data has not been returned to us yet and, therefore, is not discussed in this thesis.

## 6.3 Expected Results

When BV-2 cells are treated with vehicle, we expect to see RGS10 associated with chromatin of genes important in the inflammatory response. We also expect to observe those associations changing or become lost when the BV-2 cells are treated with LPS. Overall, we expect to observe RGS10 associating with the promoter regions of genes implicated in inflammation.

# CHAPTER 7

# CONCLUSIONS AND FUTURE STUDIES

In conclusion, the data in this thesis suggests that the PKA phosphorylation site at RGS10's serine 168 residue is critical for the ability of RGS10 to regulate TNF-α expression. When this residue is removed, RGS10 loses the ability to reduce TNF-α expression after stimulation with inflammatory factor LPS. Unfortunately, we are presently unable to confirm the importance of the phosphorylation of the serine 168 residue for subsequent RGS10 translocation to the nucleus, as described by Burgon *et al.* Additional experiments need to be conducted before any conclusions can be drawn.

The nuclear role of RGS10 remains poorly understood. Although preliminary data showed that RGS10 is associating with chromatin in the nucleus of BV-2 microglia cells, the location of the association remains to be elucidated. The work in this thesis determined that RGS10 is not associating with the CRE or KB consensus sequences in gene promoters, two candidate locations that are strongly linked to inflammatory signaling pathways. The ChIP-sequencing experiment conducted as part of this thesis should provide future researchers continuing this project more direction when probing for direct interactions between RGS10 and the chromatin or chromatin associating proteins of genes implicated in inflammation.

Although RGS10 through its canonical GAP activity can have an effect on PKA activity and the levels of phosphorylated CREB, which can indeed contribute its own anti-inflammatory effects, the significant reduction in TNF-α expression that results from RGS10 overexpression has been shown to be GAP-independent. Because RGS10 can be localized to the nucleus via its nuclear localization site, combined with our preliminary work showing that RGS10 associates with the promoters of TNF-α and IL-1β, it likely that RGS10 provides its neuroprotective, anti-inflammatory effects at a transcriptional level from within the nucleus. Previous work that we were unable to confirm suggests that phosphorylation of the serine 168 residue of RGS10 results in its subsequent nuclear localization. Through employing the PKA-deficient S168A mutant construct of RGS10, the importance of RGS10's serine 168 residue was able to be explored with regards to its translocation and ability to regulate transcription. The serine 168 residue appears to be essential in the GAP-independent anti-inflammatory function of RGS10. Unfortunately, further experiments need to be conducted to confirm that wildtype RGS10 is indeed sent to the nucleus upon phosphorylation of its serine 168 residue.

The discovery of a small RGS protein possessing a non-canonical, GAP-independent role of regulating an important biological process such as inflammation within the nucleus of cells would be a significant discovery in the field of RGS proteins. Elucidating the mechanism behind RGS10's neuroprotective, anti-inflammatory effects would provide additional therapeutic

treatment options to explore for devastating diseases such as Parkinson's disease, Alzheimer's disease, and Multiple Sclerosis.

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