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Role of RGS2 in Cellular Stress

Chang-Hui Wang, *The University of Western Ontario*

Supervisor: Dr. Peter Chidiac, *The University of Western Ontario*

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

ROLE OF RGS2 IN CELLULAR STRESS

(Thesis format: Monograph)

by

Chang-Hui Wang

Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment
of the requirements for the degree of
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The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

Abstract

Stresses from the external environment can disrupt cellular processes and result in damaging effects, such as the misfolding of proteins, which have been linked to several diseases. Regulator of G protein signalling 2 (RGS2) is upregulated by several forms of stress and can inhibit protein synthesis, an established response to stress typically achieved via the phosphorylation of the initiation factor, eIF2, to conserve energy and resources. Under reduced translation, some factors are selectively expressed via alternative translation mechanisms and these factors consequently may promote apoptosis. The molecular mechanisms mediating such opposing responses to stress are not well understood. Here, we suggested that RGS2 may be an important regulatory component in the cellular stress response and we hypothesized that RGS2 contributes to the response of cells to stress through its translational control abilities. Previously, we have shown that RGS2 can interact with the translation initiation factor, eIF2B, and inhibit *de novo* protein synthesis. Here, we demonstrated that the expression of RGS2 decreased total protein levels and significantly increased levels of factors linked to stress-induced apoptosis such as ATF4 and CHOP. Interestingly, expression of the eIF2Bε-interacting domain of RGS2 ($RGS2^{eb}$) alone resulted in a 20-fold increase in caspase 3 activation which was not seen with full-length RGS2. Furthermore, we showed that these effects are translationally regulated and independent of eIF2 phosphorylation. Thus, we present a novel mechanism in the regulation of stress response by RGS2. These results also suggest that RGS2 may be pro-apoptotic and may potentially be an important target in stress-related pathologies.

Keywords

Regulator of G protein signalling 2 (RGS2), cell stress response, translation, eukaryotic initiation factor 2 (eIF2), ATF4, CHOP, apoptosis

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Lastly, to all who may be reading this in the future, keep an open mind and be curious, reach for your goals, don’t give up!

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List of Abbreviations

ATF4	Activating transcription factor
ATF6	Activating transcription factor 6
Bcl-2	B cell lymphoma-2
BiP/GRP78	Binding immunoglobulin protein/Glucose regulated protein 78
bZIP	Basic-region leucine zipper
C/EBP	CCAAT-enhancer binding protein
cAMP	Cyclic adenosine monophosphate
CHOP/GADD153	C/EBP homologous protein/Growth arrest and DNA damage-inducible gene 153
CRE	cAMP responsive element
CREB	cAMP response element binding protein
CSR	Cellular stress response
DMSO	Dimethyl sulfoxide
eIF2	Eukaryotic initiation factor 2
eIF2B	Eukaryotic initiation factor 2B
eIF2Bε	Eukaryotic initiation factor 2B epsilon
eIF2α	Eukaryotic initiation factor 2 alpha
eIF2β	Eukaryotic initiation factor 2 beta
eIF5	Eukaryotic initiation factor 5

ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation
ERK1/2	Extracellular signal regulated kinase 1 and 2
ERSE	ER stress response element
GADD34	Growth arrest and DNA-damage inducible protein 34
GAP	GTPase accelerating protein
GCN2	General control non-derepressible 2
GEF	Guanine nucleotide exchange factor
GlcNAc	N-acetylglucosamine
GPCR	G protein coupled receptor
GPT	GlcNAc phosphotransferase
HRI	Heme-regulated inhibitor
HSE	Heat shock element
HSF	Heat shock factor
HSP	Heat shock protein
IRE1	Inositol requiring enzyme 1
IRES	Internal ribosomal entry site
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
Met-tRNAi	Initiator methonine

MOI	Multiplicity of Infection
ORF	Open reading frame
p-eIF2α	Phosphorylated eukaryotic initiation factor 2 alpha
PERK	PKR-like endoplasmic reticulum kinase
PKB/Akt	Protein kinase B
PKR	Protein kinase RNA-activated kinase
PP1	Protein phosphatase 1
qPCR	Quantitative polymerase chain reaction
RGS	Regulator of G protein signalling
RGS2	Regulator of G protein signalling 2
$RGS2^{eb}$	RGS2 eIF2Bε-binding domain
RT-PCR	Reverse transcriptase polymerase chain reaction
ST	Staurosporine
TH	Thapsigargin
TM	Tunicamycin
uORF	Upstream open reading frame
UPR	Unfolded protein response
XBP1	X-box binding protein 1

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Chapter 1

1 Introduction

1.1 Cellular responses to stress: Cell survival and cell death

One of the major features of cells is the maintenance of intracellular levels of important ions, metabolites, and biomolecules such as lipids, polysaccharides, nucleic acids, and proteins, to sustain proper cellular function and homeostasis. At the same time, cells are exposed to a constantly changing environment which may include stressful stimuli that can damage or cause imbalances in such macromolecules. These include stresses such as nutrient deprivation, temperature fluctuations, hypoxia, oxidative damage, exposure to toxins or ultraviolet radiation, mechanical damage, and viral or bacterial infections. Appropriate responses to stress therefore must be in place to adapt to changes in the physiological environment to prevent or ameliorate aberrant functions. The cellular stress response (CSR) is a highly conserved mechanism coordinating gene expression and protein translation to serve as an adaptive response to alleviate the stressful state[1,2]. Features of the CSR include increases in the expression of proteins involved in reparative, restorative, or pro-apoptotic effects, where such changes are mediated through several processes and pathways including heat shock response, mitogen-activated protein kinase signalling, proteasomal systems, and the unfolded protein response[1,2]. While initial responses toward cell survival via repair and recovery pathways are expected, it is understood that cell death may be preferable to remove dysfunctional cells should recovery be unsuccessful. Whether cells pursue a protective or destructive stress response may be dependent on the nature and severity of the stress as well as the cell type. Studies have shown selective responses *in vitro* and *in vivo* that are dependent on the type of stress[3,4], however the molecular mechanisms regulating the switch between survival or death of a cell is not well understood.

1.2 Cellular stress pathways: Endoplasmic reticulum (ER) stress

The endoplasmic reticulum is a major organelle in the cell that regulates several important cellular activities. These activities include protein synthesis and post-translational quality control processes such as protein folding, modification, and trafficking; calcium homeostasis; lipid and steroid metabolism; and drug detoxification[5–8]. The ER also modulates the degradation of misfolded proteins via a process known as the ER-associated degradation (ERAD) pathway which involves recognition and targeting of nascent misfolded proteins in the ER for retro-translocation to the cytosol for disposal by the ubiquitin-proteasomal degradation pathway[9,10]. The recognition step includes the detection of exposed hydrophobic regions (*e.g.*, by BiP chaperones), broken cysteine bonds (*e.g.*, by protein disulfide isomerases), or improper glycan attachments (*e.g.*, by lectins such as calnexin, calreticulin, or other glycosyltransferases) of misfolded proteins[9]. After cycles of refolding and re-glycosylation, terminally misfolded proteins are recognized by HRD E3 ubiquitin ligase complexes located within the ER membrane, which serve to translocate peptides to the cytosol where they are ubiquitinated and degraded by 26S proteasomes[9]. Stressors such as heat shock, oxidative stress, ischemia, and pharmacological agents such as tunicamycin (an N-glycosylation inhibitor) and thapsigargin (a sarco-endoplasmic reticulum calcium-ATPase inhibitor) can lead to improper protein folding or unfolding and disrupt the integrity of the ER, thereby inducing ER stress and associated pathphysiological states[11–16].

1.2.1 Proteotoxic stress and associated diseases

Proteins are one of the main biomolecules involved in virtually every living process whose functions are determined by their structural integrity and functional properties. Dysregulation in protein synthesis, quality control processes, or damages in protein structure by external perturbations can cause proteins to misfold or unfold and become dysfunctional. Aggregation of such misfolded proteins and impairments in proteasomal pathways that function to ubiquitinate, degrade, and remove such proteins can lead to ER stress and a number of disorders and pathologies[17,18]. A growing body of work shows that ER and proteotoxic stress are linked to various diseases such as diabetes, inflammation,

metabolic and cardiovascular diseases, cancer, and neurodegenerative disorders[11]. Impairments in lipid metabolism due to the disruption of lipid metabolizing enzymes or secretory pathways involved in cholesterol efflux can lead to lipotoxicity[19] and impaired hepatic function[20]. Nonetheless, it is important to note that levels of protein synthesis are under constant flux depending on the current physiological needs of the cell; hence, regulation of the pathways within the ER is essential for its integrity and functionality. Understanding the molecular pathways regulating protein translation and activation of specific pathways in response to ER stress may reveal important therapeutic targets to combat ER stress-related diseases.

1.2.2 The unfolded protein response (UPR)

Cells respond to stress through either of two major pathways: the upregulation of molecular mechanisms involved in cell recovery and survival[14,15], and intrinsic signalling cascades leading to apoptosis (programmed cell death)[21–24]. During times of ER stress, a set of signaling pathways known as the unfolded protein response (UPR) is activated, and this response involves the change in expression and/or activity of several molecular components that functions to alleviate stress in the ER and/or induce apoptosis. The UPR functions in three major ways to decrease the accumulation of misfolded proteins via: i) increased protein degradation through ubiquitin-proteasomal ERAD[9,25] or lysosomal[25] pathways to reduce protein overload, ii) increased protein folding capacity via upregulation of molecular chaperones to assist in protein refolding[1,26], and iii) transient inhibition of protein translation to reduce protein load and allow for recovery and refolding pathways to "catch up"[22,27–29]. Notably, recent studies have also shown stress-activated changes of the ER itself to alleviate stress. This includes greater lipid biosynthesis to increase the size of the ER, which studies show that this is mediated by both UPR-dependent and independent signalling[30]. Increase in ER size allows for the accommodation of higher protein load within the lumen of the ER and heightened stress-adaptive functions such as chaperone-mediated folding during ER stress[30].

The maintenance of proper protein folding involves a lumenal ER chaperone known as binding immunoglobulin protein or the 78 kilodalton glucose-regulated protein (BiP/GRP78), which functions to ensure proper protein folding of nascent peptides.

Under quiescent conditions, the majority of the available molecules of this chaperone are bound to and repress the activity of three ER transmembrane sensors: PKR-like ER kinase (PERK), inositol requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6)[11,25,26]. Upon an increase in unfolded or misfolded proteins during ER stress, BiP translocates from these sensors and binds to hydrophobic regions of the misfolded proteins to facilitate folding via ATP-dependent processes[11]. Dissociation of BiP leads to the activation of the sensor proteins, each shown to activate particular sets of downstream effectors, resulting in different cellular outcomes to remediate ER stress[13,22,24,27,31] (Fig. 1.1 A, B). Furthermore, structural studies on the lumenal domain of IRE1 show that it is involved in the recognition and binding of misfolded proteins as part of its ER stress-sensing functions[9,32].

Derepression of PERK due to increased BiP association with misfolded proteins in the ER leads to its activation involving homodimerization and autophosphorylation of its cytoplasmic serine/threonine kinase domain[33]. Activated PERK subsequently phosphorylates the alpha subunit of the eukaryotic initiation factor 2 (eIF2α) at serine 51, which interferes with the formation of the 43S translation initiation complex[11,34]. This results in transient global inhibition of protein synthesis at the initiation stage of translation to prevent further protein load in the ER[13,16,35,36]. Interestingly, the decrease in translation leads to preferential expression of particular factors involved in stress response, such as activating transcription factor 4 (ATF4), whose protein levels are present at very low amounts during non-stressed states[37] and substantially increase during ER stress[33,38–40]. In addition to decreasing protein synthesis to reduce ER load, the PERK-mediated phospho-eIF2α pathway, along with the two other branches of the UPR, induces gene expression of several targets to mount a response against stress[41].

Similar to PERK, activation of IRE1 upon the release of BiP involves homodimerization and autophosphorylation through its cytoplasmic kinase domain[25]. Unlike PERK, the cytoplasmic C-terminal portion of IRE1 also contains an endoribonuclease domain that is shown to alternatively splice the mRNA of X-box binding protein 1 (XBP1), resulting in a transcription factor (XBP1s) that increases the transcription of several stress response genes[11,25,42]. The resulting IRE1-XBP1s pathway leads to reduced protein load and

removal of misfolded proteins via increased transcription of genes involved in ERAD[11]. Additionally, XPB1s has been linked to greater protein folding capacity through increased expression of ER chaperones such as BiP, and is also involved in the expression of proteins involved in lipid synthesis and ER biogenesis[43–46]. A process known as the regulated IRE1-dependent decay (RIDD) of mRNA is also involved in response to cell stress, where IRE1 degrades mRNA localized to the ER via its RNase activity to decrease the amount of translation and therefore reduce ER protein load[43]. Several substrates targeted by the RNase activity of IRE1 have also been identified to result in a global decrease in protein synthesis, such as the cleavage of ribosomal 28S rRNA[43,47], to alleviate ER stress.

Unlike the two previously described ER transmembrane sensor proteins, activated ATF6 is itself a transcription factor that goes on to activate many UPR target genes[42,48,49]. The cytoplasmic portion of ATF6 consists of a DNA-binding domain containing a basic-leucine zipper motif (bZIP) that is involved in regulation of gene expression[11]. Upon ER stress, dissociation of BiP reveals the lumenal domain of ATF6 that contains a Golgi-localization signal, thereby allowing it to translocate to the Golgi apparatus. From there, ATF6 is sequentially cleaved by site 1 (S1P) and site 2 proteases (S2P), resulting release of a 50 kilodalton cytoplasmic fragment of ATF6 that translocates to the nucleus and activates the transcription of ER chaperone genes such as BiP, GRP94, and calreticulin[11,50].

The highly involved processes within the UPR are therefore important to coordinate the appropriate response to ER stress. In fact, disruption of the activities of the UPR, such as in the PERK, IRE1, or ATF6 pathways, can prevent an appropriate ER stress response, thereby further exacerbating the pathophysiological state[12]. This has been seen in neurologic diseases such as Alzheimer's and Huntington's disease[5,11,51], cardiovascular diseases[13,21–24,52], metabolic and liver disease[11,53], pancreatic inflammation and diabetes[8,54–56], and more recently in cancer[7,57]. ER stress has been associated with hypertrophic myocardium in humans and mice, and targeted increase or decrease in the activity of the UPR has been shown to lead to different physiological outcomes of the heart[27].

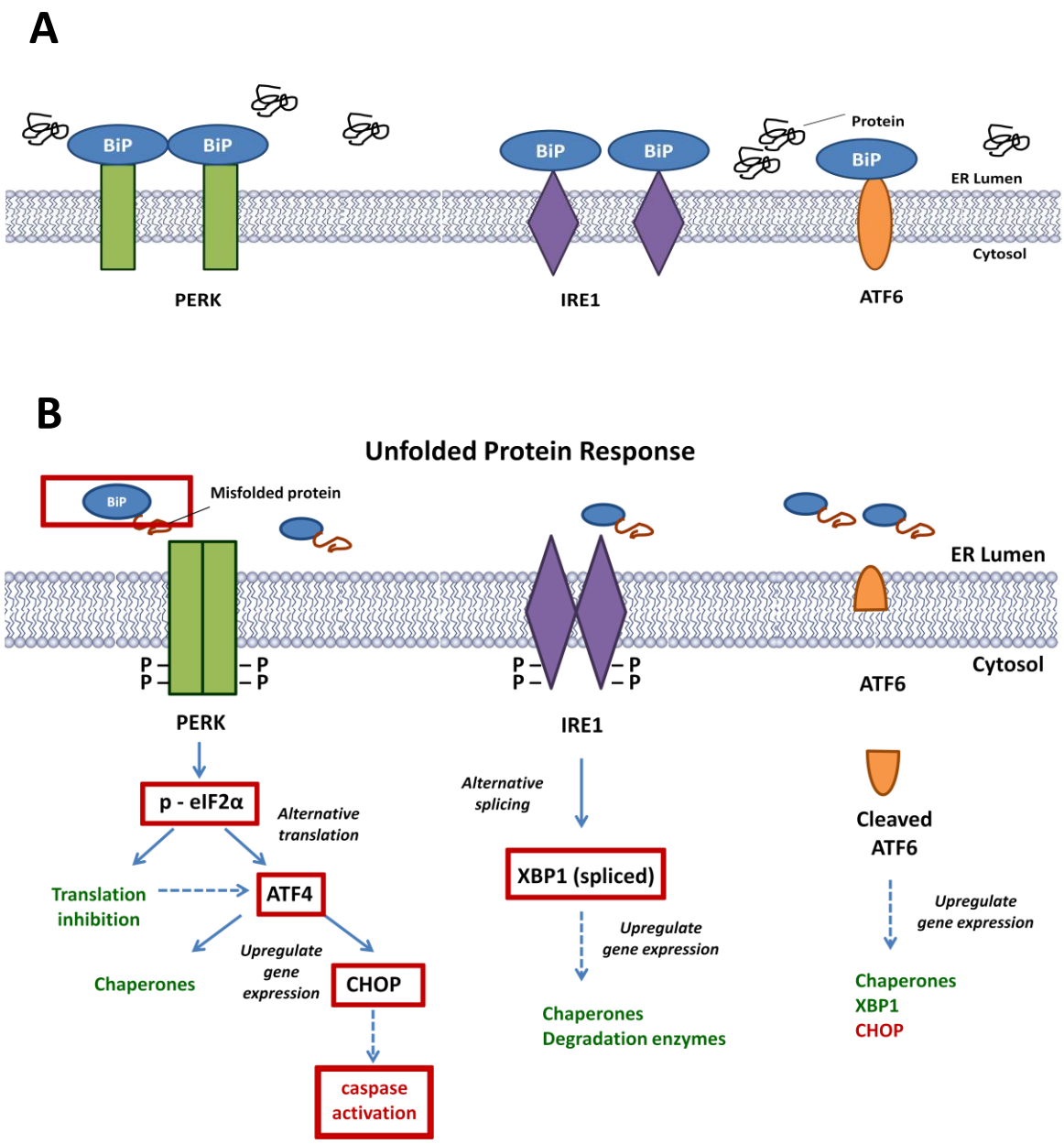


Figure 1.1. Molecular pathways involved in ER stress and the unfolded protein response (UPR). (**A**) Under non-stressed conditions, the majority of BiP proteins are bound to ER transmembrane sensor proteins PERK, IRE1, and ATF6, leading to the repression of transmembrane sensor functions. (**B**) BiP is depleted upon the accumulation of misfolded proteins leading to differential activation of signalling cascades to reduce protein overload or the induction of apoptotic pathways (caspase activation).

1.2.3 ER stress induced apoptosis and diseases

Paradoxically, some ER stress-activated factors involved in the UPR can promote both cell survival and apoptosis[58]. Little is known about the regulatory mechanisms mediating such opposing endpoints, although it is expected that upon irreversible damage, pathways leading to apoptosis are initiated to alleviate further dysfunction. ER stress-activated apoptotic pathways have been shown to be active in neurodegenerative diseases, atherosclerotic lesions, metabolic disorders, and heart failure[21,27,57]. Several studies have shown the increase in gene and protein expression of ER stress-induced apoptotic factors linked to such diseases[27].

1.2.3.1 ATF4-CHOP mediated apoptosis and diseases

As mentioned earlier, phosphorylation of eIF2 leads to the general inhibition of protein synthesis at the initiation step of mRNA translation and upregulates specific stress response effectors such as ATF4 through alternative translational mechanisms[59–64]. ATF4 is a stress-activated nuclear transcription factor belonging to the activating transcription factor/cAMP response element binding protein (ATF/CREB) family of proteins. Members of this protein family are involved in regulating the transcription of pro-survival and pro-apoptotic targets that contain CCAAT-enhancer binding protein (C/EBP)-ATF response elements in their genes[3,62,65,66]. Levels of ATF4 are shown to increase in response to several stressful stimuli including ER and oxidative stress, hypoxia, and amino acid deficiency[3,67–69]. ATF4 mRNA contains three initiator methionine residues, only one of which yields a functional protein. Translation of ATF4 mRNA is thus regulated by two upstream open reading frames (uORFs) and is dependent on the efficiency of the translational machinery (Fig. 1.2A)[34,63]. Initiation and translation using the first ATF4 uORF (uORF1), gives rise to a short non-functional peptide and facilitates ribosomal re-initiation at the downstream ATF4 uORF2. This second uORF of the ATF4 transcript is inhibitory as it overlaps with the start codon of the actual ATF4 ORF but is out of frame[39,63,64]. During non-stressed states, levels of activated eIF2-GTP-Met-tRNAi (initiator methionines) are plentiful. When scanning ribosomal pre-initiation complexes reach ATF4 uORF2 and are able to acquire an initiator methionine, initiation occurs at uORF2 and therefore translation of the ATF4 ORF is prevented (Fig. 1.2B).

Under states of stress leading to the phosphorylation of eIF2, decreased abundance of eIF2-GTP-initiator methionine results in the delay of scanning ribosomes becoming competent, bypassing the inhibitory ATF4 uORF2, and instead facilitate ribosome initiation at the ATF4 ORF to produce functional ATF4[34,63,70].

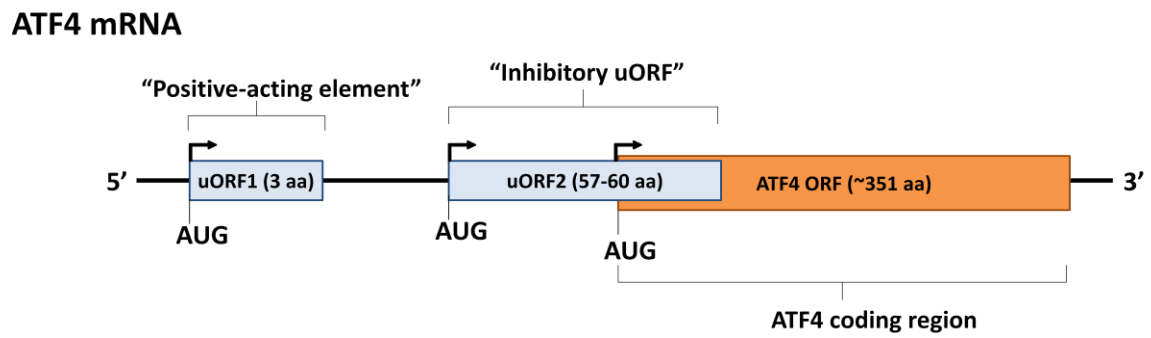
Pro-survivial functions of ATF4 include the increased expression of molecular chaperones and antioxidant species to reduce ER stress[21,62] and increased nutrient uptake during starvation or amino acid deficiency by increasing gene expression of amino acid transporters[3,41,69,71]. Conversely, ATF4 is shown to increase the gene expression of downstream pro-apoptotic factors such as the C/EBP homologous protein (CHOP), also known as growth arrest and DNA damage-inducible gene 153 (GADD153)[39,72,73]. CHOP is a 29 kilodalton transcription factor consisting of a bZIP DNA binding motif[74] that regulates a variety of genes involved in immune functions, cell differentiation, proliferation, and apoptosis[72]. The upregulation of CHOP leads to differential expression of effectors involved in apoptosis, namely those within the B cell lymphoma-2 (Bcl-2) family of proteins that consists of pro- and anti-apoptotic members[75], as well as other apoptotic targets[76,77]. CHOP increases the transcription of pro-apoptotic factors such as BIM and PUMA during ER dysfunction[78–81], and down-regulates the expression of anti-apoptotic proteins such as Bcl-2[13,80]. This in turn leads to ER stress-activated caspase cascades such as the cleavage of executioner caspases including caspases 3 and 12 that ultimately lead to cell death[21].

ATF4-CHOP mediated apoptosis is linked to a number of diseases such as Parkinson's disease, through increased neuronal death[82], and diabetes[83]. In addition, ATF4 is observed to be present at much higher levels in tumors[62] and CHOP expression has been associated in the development of sarcomas[84–86], increasing the complexity of the physiological functions mediated by these two factors. While inhibition of translation during ER stress can lead to the activation of the ATF4-CHOP pathway, less is known about the molecular switches involved in the destiny of cells towards recovery or death. Thus, the response to stress is multifaceted and may involve other molecular components and pathways yet to be elucidated.

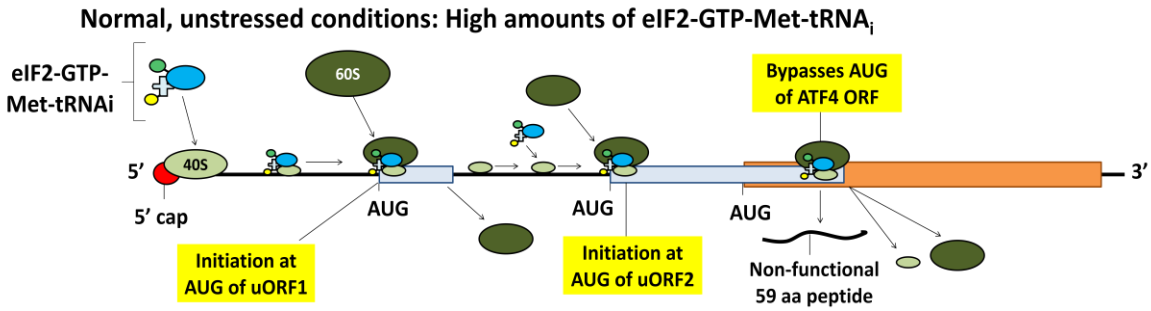
As mentioned previously, the IRE1 branch of the UPR has stress-adaptive functions, such as increase in chaperone expression to drive greater protein folding and/or the degradation of misfolded proteins to reduce ER protein load. However, IRE1 has also been shown to regulate apoptosis through its interaction with cytoplasmic partners and kinases to alter cellular levels of anti- and pro-apoptotic factors. Several studies show this primarily occurs through the formation of a multi-component complex with IRE1, ultimately leading to the activation of executioner caspases that carry out the destruction of cells. Interaction of the cytoplasmic domain of IRE1 with the adaptor protein, tumor necrosis factor receptor-associated factor 2 (TRAF2), leads to IRE1 coupling with the plasma membrane death receptor tumor necrosis factor receptor 1 (TNFR1) and apoptosis signal-regulating kinase 1 (ASK1). This complex then activates c-Jun N-terminal kinases (JNK), leading to downstream activation of caspases and results in cell death[11,13,43]. Furthermore, the RIDD activity of IRE1 has been found to reduce levels of micro-RNA precursors that normally function to increase the expression of respective micro-RNAs that are responsible for repressing the translation of caspases[47]. Thus, the activities of IRE1 provide another mechanism in addition to the PERK-ATF4-CHOP mediated apoptotic pathway that may contribute to a pro-apoptotic response during cell stress.

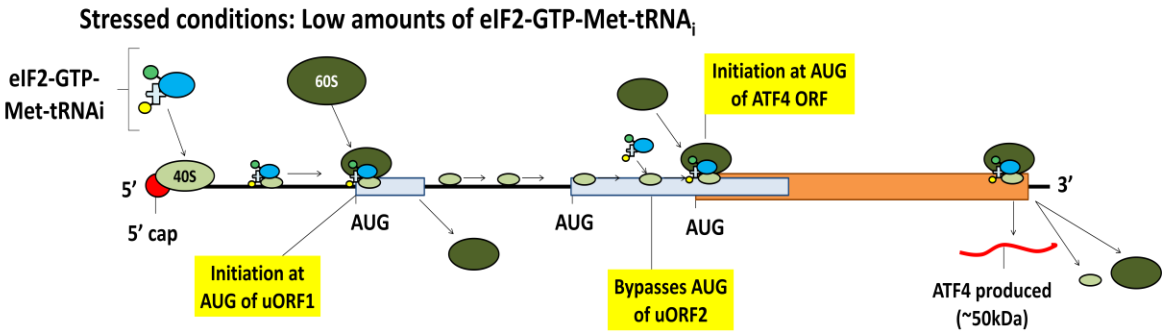
Figure 1.2. Regulation of ATF4 mRNA translation. (**A**) Diagram of the mammalian ATF4 mRNA transcript showing the sequence motifs involved in regulating the production of functional ATF4 encoded by its open reading frame (ATF4 ORF). ATF4 has three initiation start sites, represented by the black arrows. Translation of ATF4 is mainly regulated by the second upstream open reading frame (uORF2) as it overlaps with the start codon (AUG) of the main coding region of ATF4 and is out of frame. (**B**) Under non-stressed conditions where normal eIF2-eIF2B guanine nucleotide exchange activity occurs, once scanning ribosomes reach the AUG of uORF2 and acquire an eIF2-GTP-Met-tRNA$_i$ to initiate there, it bypasses the initiation start site of the ATF4 ORF, and therefore ATF4 translation is prevented. Under stressed conditions with lowered levels of activated eIF2, scanning ribosomes do not acquire an eIF2-GTP ternary complex in time and downstream initiation sites are preferentially used, such as the ATF4 ORF, resulting in the increased levels of ATF4 translation.

A


ATF4 mRNA
"Positive-acting element"
"Inhibitory uORF"
5'
uORF1 (3 aa)
uORF2 (57-60 aa)
ATF4 ORF (~351 aa)
3'
AUG
AUG
AUG
ATF4 coding region

B


Normal, unstressed conditions: High amounts of eIF2-GTP-Met-tRNA$_i$
eIF2-GTP-
Met-tRNAi
60S
Bypasses AUG
of ATF4 ORF
5'
40S
3'
5' cap
AUG
AUG
AUG
Initiation at
AUG of uORF1
Initiation at
AUG of uORF2
Non-functional
59 aa peptide


Stressed conditions: Low amounts of eIF2-GTP-Met-tRNA$_i$
eIF2-GTP-
Met-tRNAi
60S
Initiation at AUG
of ATF4 ORF
5'
40S
3'
5' cap
AUG
AUG
AUG
Initiation at
AUG of uORF1
Bypasses AUG
of uORF2
ATF4 produced
(~50kDa)

1.3 Other cellular stress pathways and *in vitro* models of stress

As mentioned previously, a wide range of stressful stimuli exists that can be damaging and destructive to cells. Often these stresses converge to affect the integrity and functionality of macromolecules such as proteins and DNA or cause imbalance of important ions and organic compounds such as lipids, co-factors, essential amino acids, and metabolites. Experimentally induced forms of cellular stress that interfere with these macromolecules or cellular processes have been widely studied through the use of physical, biological, and pharmacological stressors that model stress-related pathological states and diseases.

Oxidative stress for example is a well-studied form of stress that occurs as a result of an increase in reactive oxygen species (ROS). These include damaging radical species such as hydroxyl radicals (•OH) and superoxide radicals ($O_2•^-$) that result from the homolytic covalent bond cleavage of oxygen and peroxides that exists in cells[87]. These radicals can then go on to damage biomolecules such as proteins[88], lipids[89], and nucleic acids[90], resulting in cellular stress and dysfunction. Consequences of oxidative stress have been linked to several diseases such as hyperglycemia and diabetes, Alzheimer's, and cancer[87,91]. Imbalances in pro-oxidant and anti-oxidant species, such as the major anti-oxidant glutathionine, can also lead to the production of such damaging molecules and disrupt the redox status of cells[87]. This can be imposed by toxins found in the environment such as cigarette smoke or deprivation of minerals or vitamins as a result of malnutrition or starvation that are needed as cofactors for the activity of enzymes to prevent the production of ROS[91]. *In vitro* models of oxidative stress often use pharmacological methods such as treatment with hydrogen peroxide[87], arsenite[92], and rotenone[93] to induce the production of ROS and study downstream effects.

Stimuli leading to the disruption of protein synthesis, structure, and function represent another widely studied model of cellular stress. Thermal stress via heat shock can denature proteins that can lead to toxic aggregates if not recognized and removed by degradation machinery[94–96]. Treatment with tunicamycin has been used in several studies to prevent the glycosylation of essential proteins within the endoplasmic reticulum (ER)

and Golgi apparatus to induce protein stress[97]. It does so by inhibiting the enzyme N-acetylglucosamine (GlcNAc) phosphotransferase (GPT) to prevent the transfer of a GlcNAc-1-phosphate on a UDP-GlcNAc molecule to a dolichol phosphate, thereby blocking the linkage of glycan molecules to asparagine residues of proteins[98–100]. The formation of disulfide bond linkages and the glycosylation of proteins are highly important with respect to the stabilities of proteins likely to be exposed to harsher extracellular conditions, such as secretory and cell surface proteins[9]. Stresses such as ischemia and malnutrition can affect proper glycosylation of proteins in the ER, leading to ER stress[101]. ER stress as a result of the disruption in protein glycosylation has been linked to diseases such as diabetes[100,102], development of receptor-mediated carcinomas[103,104], muscular dystrophies[105], and a set of pathologies grouped under congenital disorders of glycosylation (CDGs)[102,106]. Thus the use of tunicamycin within the experiments presented in this thesis allows us to model physiological stresses linked to disruption in protein glycosylation and study stress response in cells.

Other stressors often used to experimentally induce cellular stress and disrupt functions of the ER include thapisgargin, which is a sarcoplasmic-endoplasmic reticulum calcium-ATPase inhibitor that raises cytosolic calcium ions concentrations by blocking the re-uptake of these ions back into the ER[107]. This would result in ER stress and the depletion of intracellular calcium stores leading to downstream deficiency of calcium ions[108]. Furthermore, the maintenance of high cytosolic concentrations of calcium can lead to aberrant cellular signalling mediated by calcium-dependent pathways, many of which regulate proper cardiovascular physiology[13,23,24]. Lastly, staurosporine is also used to induce stress in various cell lines as a potent non-specific inhibitor of protein kinases[109] and is known to activate apoptosis through both caspase-dependent and caspase-independent mechanisms[110]. These methods to experimentally induce cellular stress are invaluable as they allow us to gain a better understanding of the biochemical processes and the molecular components involved in stress and stress response.

1.4 Regulation of protein synthesis: Translational control at initiation

The process of protein synthesis, or translation of an mRNA transcript, is a highly energetic process that involves three major steps: initiation, elongation, and termination. Regulation of protein synthesis is important to prevent aberrant protein load. Translation is principally regulated at the initiation stage to allow rapid, spatial control of gene expression[34]. Initiation is the rate-limiting step in protein synthesis and is affected by properties of the mRNA transcripts themselves, the ribosomal machinery, several different enzymes and a family of proteins referred to as eukaryotic initiation factors (eIFs)[111–113], where the dyregulation in any of these components can contribute to diseases related with protein stress[59,60].

The majority of proteins are translated via a "scanning" mechanism that begins at the most proximal region of an mRNA via recruitment of the 43S pre-initiation complex, composed of a 40S small ribosomal subunit and eIFs, to the 5' guanosine cap. In mammals, nine different eIFs are required to begin translation, and importantly, activated eIF2 brings the initiator methionine to the start codon, which is usually the first AUG of an mRNA transcript[34]. As the ribosomal pre-initiation complex moves along and scans the transcript in 5' to 3' fashion, acquisition of an eIF2-GTP-Met-tRNA$_i$ base pairs with the triplet codon sequence within the open reading frame of the target gene. This is followed by the recruitment of a 60S large ribosomal subunit to form a complete, elongation-competent, 80S ribosomal complex that initiates and proceeds to translate the mRNA[34,63,70]. Addition of sequential amino acids to the growing peptide via specific tRNA molecule anti-codon base pairing to codons of the mRNA sequence occurs during elongation. This continues until a stop codon is reached, terminating protein synthesis and the dissociation of the ribosomal components to release the formed peptide chain.

1.4.1 Translational control by eukaryotic initiation factor 2 (eIF2)

As mentioned previously, protein translational control can occur as a result of the phosphorylation of eIF2. eIF2 is a heterotrimeric GTPase that is made up of an α, β, and γ-subunit[111]. The γ-subunit contain the guanine nucleotide binding domain and has also

been shown to be the site of binding for initiator methionine (Met-tRNA$_i$)[111]. Both the β-subunit and the α-subunit of eIF2 are sites that are involved in the regulation of initiation of protein synthesis. At the end of initiation, GTP bound to eIF2 is hydrolyzed to GDP and eIF2-GDP is released from the ribosome. Another eukaryotic initiation factor, eIF2B, is a heteropentameric guanine nucleotide exchange factor (GEF) that catalyzes the exchange of GDP for GTP on eIF2, thereby reconstitutes eIF2-GTP that is capable for another round of translation initiation (Fig. 1.3)[113,114]. The interaction between eIF2 and eIF2B occurs at the C-terminus of the β-subunit of eIF2 (eIF2β) and the ε-subunit of eIF2B (eIF2Bε)[111]. Serine residue 51 of the α-subunit of eIF2 (eIF2α) is the site of phosphorylation targeted by various stress-activated kinases. During ER stress, the phosphorylation of eIF2α (p-eIF2α) by activated PERK increases eIF2 binding affinity to eIF2B, thereby blocking its GTP-exchange activity[35,115–118]. Furthermore, the stable complex between eIF2 and eIF2B reduces the amount of available eIF2B for the reformation of eIF2-GTP and thereby impedes initiation, resulting in global translation inhibition (Fig. 1.3)[113]. In mammals, three other kinases activated by different stress stimuli converge to phosphorylate eIF2α. These include the general control non-derepressible 2 (GCN2) kinase, activated upon deficiency in essential amino acids[69,119]; protein kinase RNA-activated (PKR) kinase, activated during the presence of double-stranded RNA from viral infection[118,120]; and heme-regulated inhibitory (HRI) kinase, activated during heme deficiency, oxidative stress, osmotic and heat shock[120,121]. Regardless of the type of stress initially imposed, the fact that they all converge to phosphorylate eIF2α demonstrates the importance in the regulation of initiation of translation in response to stress.

The transient inhibition of protein synthesis can be considered beneficial during times of stress as it allows for the conservation of energy and the allocation of resources focusing on recovery pathways for cell survival[27,29,122]. However, as discussed above, the inhibition of initiation as a result of reduced eIF2-GTP can lead to the upregulation of factors such as ATF4 that mediate apoptosis. Additionally, prolonged inhibition of translation can lead to the depletion of necessary proteins for other physiological functions or maintenance of cell integrity. The growth arrest and DNA damage-inducible

protein (GADD34) associates with protein phosphatase 1 (PP1) to promote dephosphorylation of eIF2α[123] and re-establish normal translational activity.

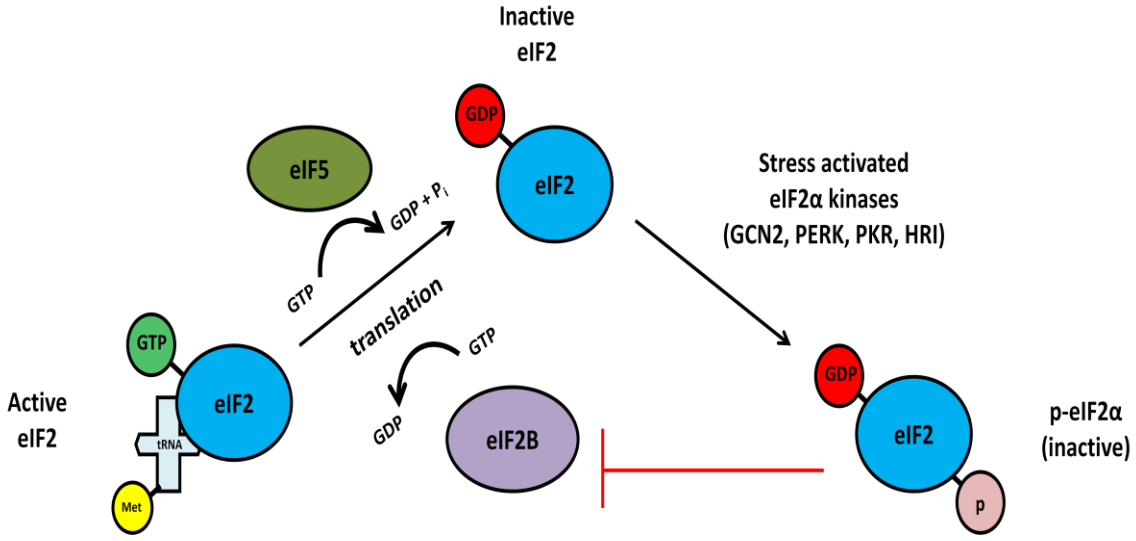


Figure 1.3. Factors involved in regulating translation. Activated eIF2 (eIF2-GTP-Met-tRNA$_i$) brings the initiator methionine to the start codon of an mRNA transcript, a requisite step to initiate translation, followed by GTP hydrolysis catalyzed by eukaryotic initiation factor 5 (eIF5) to begin the process of protein synthesis. Activity of the guanine nucleotide exchange factor, eukaryotic initiation factor 2B (eIF2B), reconstitutes eIF2-GTP to continue the cycle of translation. During times of stress, the α-subunit of eIF2 gets phosphorylated by various stress activated kinases which leads to greater binding affinity of eIF2 to eIF2B. eIF2 becomes a competitive inhibitor of eIF2B, preventing its GTP exchange activity, thereby inhibiting translation initiation as a result of reduced amounts of activated eIF2.

1.4.2 Alternative translational mechanisms

In addition to a regular translational start site, some transcripts contain additional AUG sequences upstream of the open reading frame of the protein coding region. These may facilitate or repress subsequent re-initiation at downstream AUG sequences, depending on the efficiency in the formation of functional ribosomal initiation complexes[113]. In mammals, about 45-50% of genes encode mRNAs that have at least one short upstream open reading frame that typically reduces translation at the main open reading frame[34], and lead to various functional isoforms of a protein. In addition, eukaryotic initiation factor 5 (eIF5) catalyzes GTP hydrolysis of eIF2 only when it is bound to a ribosomal initiation complex to initiate translation, and therefore could also be a site to regulate initiation[111,124]. Other ways that the processes of translation of target genes can be regulated include the modification of the 5' cap structure or polyadenylation tail of mRNAs, which are normally strong promoters for the recruitment of eukaryotic initiation factors to initiate translation.

During reduced translation as a result of eIF2α phosphorylation under times of stress, the increased synthesis of particular mRNAs can occur through alternative translation mechanisms, such as the use of internal ribosome-entry sequences (IRESs), which mediate 5' cap-independent translation initiation. IRESs within mRNA directly recruit ribosomes and bypass the need to acquire all the initiation factors normally required to start scanning-dependent initiation at the cap[28,113,125]. Leaky ribosome scanning is another alternative way for translation to occur, where this usually happens when an mRNA molecule has a poorly defined start site, such as the lack of proper Kozak sequence, and contain multiple AUG sequences along the transcript where a ribosome can initiate when it acquires a Met-$tRNA_i$[126]. Regardless of the mechanism, these pathways provide an alternative way for protein expression of selective factors during times of reduced translation in response to stress.

1.5 G protein coupled receptor (GPCR) mediated signalling and function in cellular stress

Receptor-mediated stress signalling is known to drive both pathways in cell recovery and programmed cell death by regulating multiple response pathways that in turn determine cellular function and outcome[127–129]. Unregulated signals are also linked to pathologies and the progression of disease. G protein coupled receptors (GPCRs) are the largest family of transmembrane receptors involved in virtually every physiological process[130,131]. As such, it is well known that aberrant GPCR signalling can lead to a variety of disease states, and perturbation upstream or downstream of GPCRs and their signalling pathways can exacerbate pathophysiological states[128,131–135].

GPCRs serve a wide array of physiological and pathological roles mediating the transduction of extracellular signals into intracellular effector pathways upon activation by various ligands including hormones, neurotransmitters, chemokines, and pharmacological compounds[136]. Intracellular responses include the activation of G proteins and their target effector proteins which in turn control intracellular levels of ions and second messengers[137]. The specific effects resulting from the activation of a GPCR are largely dependent on the heterotrimeric GTP binding proteins (G proteins) to which it is associated. Heterotrimeric G proteins consist of three subunits: α, β, and γ, each with several different functional isoforms identified[138]. There have been 23 different Gα subunit isoforms identified and these are grouped into four subfamilies: Gαs, Gαi/o, Gαq/11, and Gα12/13[136,138]. Gαs stimulates the activity of adenylyl cyclases (AC) to increase intracellular cyclic adenosine monophosphate (cAMP) levels, which is an important second messenger that acts to amplify the signal and can regulate the activity of downstream proteins in the signalling cascade such as kinases and ion channels[139]. In contrast to Gαs, Gαi/o inhibits AC and thereby decreases cAMP levels[139], as well as regulating other effectors. The primary effect of Gαq/11 activity is the activation of the enzyme phospholipase Cβ, which leads to the increase of intracellular inositol triphosphates (IP3) and diacylglycerol (DAG) upon cleavage of the membrane-bound phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2). This further acts to regulate Ca^{2+} levels and the activation of protein kinase C[140]. Studies show that Gα12/13 regulates

cellular processes through the use of guanine nucleotide exchange factors such as Rho proteins that further activate Rho-dependent kinases and play a role in cytoskeleton remodelling and cell migration[141].

As mentioned above, dysfunctional GPCRs are linked to diseases such as retinitis pigmentosa, nephrogenic diabetes insipidus, obesity, cardiovascular disease, asthma, and several others[128]. Thus, the regulation of GPCR and G protein activities and functions are essential to maintain proper organismal physiology and survival.

1.6 Regulator of G protein signalling (RGS) proteins

Several accessory proteins have been identified that modulate GPCR signalling and function[142–146], one of which is a family of small proteins known as the regulator of G protein signalling (RGS) proteins. RGS proteins are a family of GTPase accelerating proteins (GAPs) that contain a conserved 120 amino acid RGS domain that decreases GPCR-mediated signalling by increasing the rate of hydrolysis of GTP bound to Gα subunits, thereby inhibiting signaling and activation of downstream effectors (Fig. 1.4)[142,143]. To date, twenty distinct genes for RGS proteins have been identified in mammals (RGS1 through 21, with the exclusion of 15), and these are further categorized into four subfamilies (R4/B, RZ/A, R7/C, and R12/D)[142,147]. In addition, there are approximately twenty related "RGS-like" proteins that are structurally diverse and have some GAP functions[147]. All RGS proteins serve as GAPs for Gαi/o while some can also act on Gαq/11 proteins[130,148]. So far, none of these RGS proteins appear to affect the rate of GTP hydrolysis of either Gαs or Gα12/13 subfamily of proteins[130], however there is evidence for the mediation of Gαs signalling by RGS proteins through affecting its effector adenylyl cyclases and/or direct binding to Gαs[149–153]. Furthermore, different RGS proteins have been shown to have selective GAP activity to different Gα isoforms. In particular, regulator of G protein signalling 2 (RGS2) has been shown to have low affinity for Gαi/o inhibition and thus preferentially acts on Gαq/11[154–156]. A number of studies also show that RGS proteins can attenuate GPCR signalling via interaction with downstream effectors and other regulatory components[142,149], and have roles outside of their effects on G protein signalling[142,157–160], further demonstrating their importance in cellular physiology.

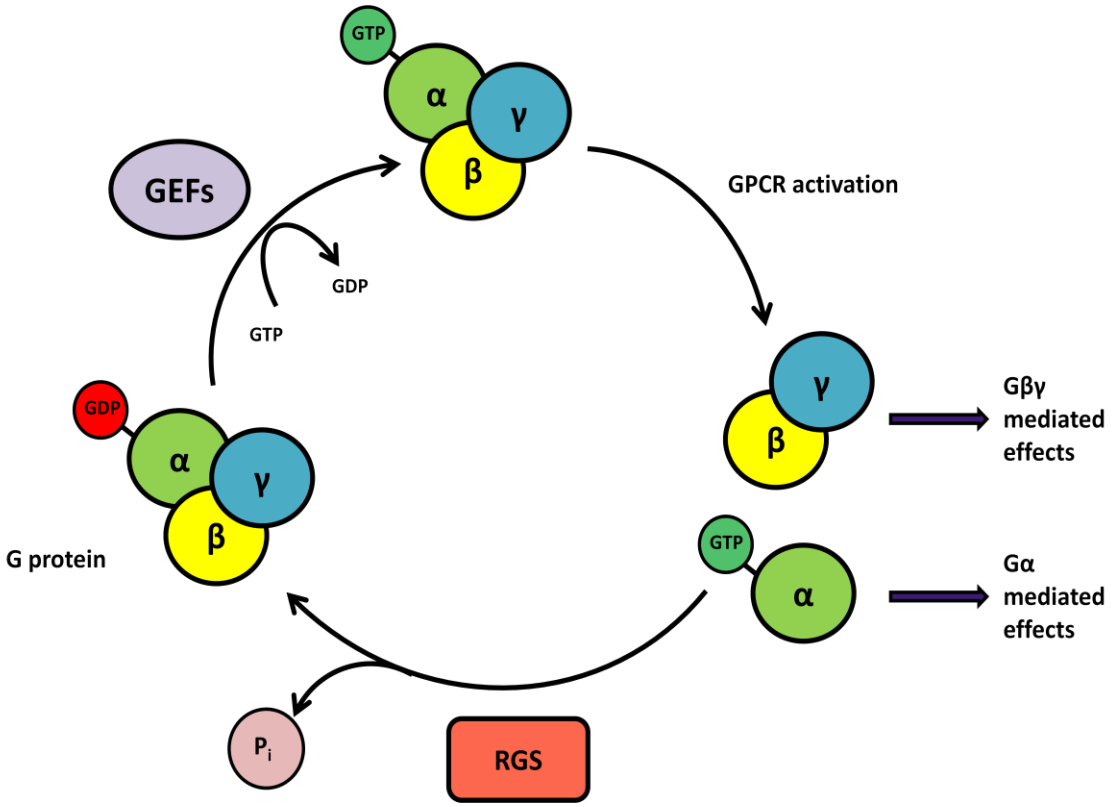


Figure 1.4. Regulation of G protein coupled receptor (GPCR)-mediated activity by regulator of G protein signalling (RGS) proteins. Heterotrimeric G proteins govern the specific cellular effects upon activation of GPCRs. RGS proteins attenuate GPCR signalling by binding to Gα subunits and increasing the intrinsic GTP hydrolytic activity of Gα. Hydrolysis of GTP inactivates Gα and is thought to promote re-association with Gβγ, thereby turning off both Gα and Gβγ signalling effects. A set of proteins known as guanine nucleotide exchange factors (GEFs) mediate the GDP-GTP exchange on Gα leading to its reactivation and allowing receptor-mediated signalling and functions.

1.7 Regulator of G protein signalling 2 (RGS2)

1.7.1 Cellular functions of RGS2 and modulation of GPCR signalling

As mentioned previously, the regulation of protein synthesis and folding is important for their functionality and to prevent cell stress and associated pathologies. RGS2 is a member of the RGS family of proteins that appears to play an important role in cellular stress responses[159]. RGS2 belongs to the R4/B subfamily of RGS proteins which consists of small (20-30 kilodalton) proteins with short, simple N- and C-termini flanking the conserved RGS domain (Fig. 1.5A)[142,160,161]. The N-terminal domain of RGS2 has been shown to be important in the recruitment and binding of RGS2 to GPCRs to inhibit receptor and G protein-mediated signalling[162,163].

RGS2 is unique in the fact that it has relatively low binding affinity for Gαi/o proteins and therefore selectively attenuates Gαq/11-mediated signals to a greater extent than all other RGS proteins within this family[155]. Interestingly, we and others have demonstrated the ability of RGS2 to inhibit cAMP production facilitated by Gαs activation through a GAP-independent manner[150,164]. This is likely through its ability to bind and interact with Gαs and adenylyl cyclases[149–151], providing evidence of the ability of RGS2 to attenuate Gαs-mediated effects and to have functions apart from its GAP activity.

Gαq/11 is associated with several types of GPCRs, many of which are known to be important in the regulation of cardiomyocyte structure and function[127,136]. These include GPCRs such as endothelin-1, angiotensin II, M3 muscarinic, and α_1-adrenergic receptors[127], of which many show enhanced activity and result in pathological phenotypes such as hypertrophy of cardiomyocytes and increased susceptibility to atrial arrhythmia in mice lacking RGS2[165–167]. Additionally, *in vitro* studies show that the loss of endogenous RGS2 can exacerbate hypertrophic Gαq/11-mediated signalling[168]. Gαq/11 signalling has also been shown to activate particular protein kinases, such as members of the mitogen-activated protein kinase (MAPK) family and protein kinase B (Akt/PKB)[169], which are known to be induced by various stress signals ranging from inflammatory cytokines, osmotic stress, and heat shock[170]. These kinases are involved in cell stress

response and depending on the particular kinase activated, it may mediate cell survival, such as Akt/PKB[171], or regulate apoptotic pathways, such as JNK[172]. The activation of some MAP kinases are also linked to cellular hypertrophy, such as extracellular signal regulated kinase 1 and 2 (ERK1/2), JNK, and p38[127]. RGS proteins are known to govern the effects of GPCRs on MAPK signalling pathways such as ERK1/2[154,173,174], JNK[175], and p38[167,169,176]. In fact, i*n vitro* studies conducted in our lab show that overexpression of RGS2 can attenuate agonist-induced cellular hypertrophy mediated by both Gαq/11[167] and Gαs[166] signalling and also attenuate ERK1/2 and Akt activation which may contribute to its antihypertrophic effects[166,167]. Paradoxically, a study showed that the upregulation of RGS2 by ischemia lead to greater cell death in astrocytes and these effects were abolished upon the use of a p38 MAPK inhibitor[177]. This suggests a possible synergistic or feedback mechanism between MAP kinase activation and RGS2 expression. The relative contribution of various pathways of the MAPK system on stress response and how these are affected by RGS2 is not well understood. Since many of these stimuli also induce ER stress and ER-initiated apoptosis, RGS2 may be an adaptive protein in stress response. These collective findings suggest protective roles of RGS2 in cell stress, however, the molecular mechanisms regulating these responses is not well known and therefore this was investigated in the studies contained in this thesis.

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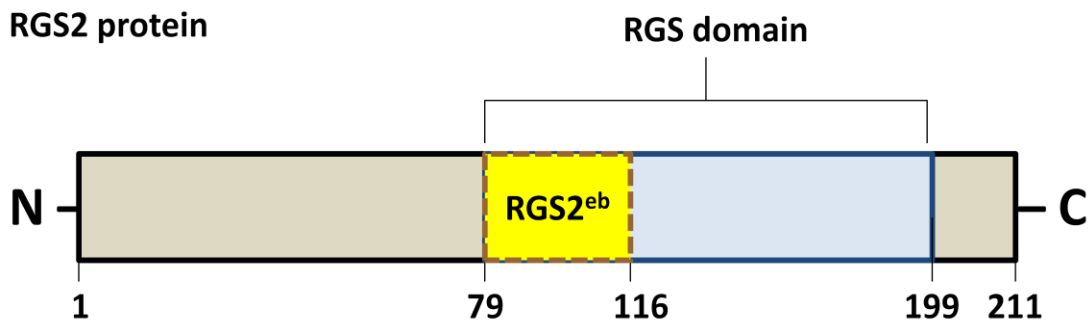


B

Figure 1.5. Schematic of the domains governing functions of RGS2. (**A**) Diagram of the full-length 211 amino acids RGS2 protein. Amino acid residues 79 to 199 house the functional GTPase accelerating domain conserved in all RGS proteins. Within this 120 amino acids RGS domain, the short 37 amino acids domain (residues 79 to 116) is determined to be the region mediating the binding of RGS2 to eIF2Bε to inhibit protein synthesis. (**B**) Comparison of the sequence homology between the established eIF2Bε-interacting domain of eIF2 (eIF2β) and the RGS2 eIF2Bε-binding domain (Nguyen *et al.*, 2009).

1.7.2 Distribution and regulation of RGS2 expression

RGS2 is ubiquitously expressed in all tissues throughout the body, with the mRNA detected in organs such as the heart, brain, lungs, and kidneys, as well as in cell types including pre-adipocytes, vascular smooth muscle cells, osteoblasts, immune cells, and chondrocytes at moderate to high amounts[178–181]. Stress stimuli can activate GPCRs, and agonist-induced Gαq/11 and Gαs signalling is shown to selectively upregulate RGS2 in cardiomyocytes but not other R4/B RGS proteins[166,167], demonstrating the functional importance of RGS2 in providing a feedback mechanism to regulate G protein signalling[182]. Furthermore, the expression of RGS2 is upregulated by several forms of stress including heat shock[159,183], bacterial infection[184], DNA damage[185], oxidative stress[186], and ischemia[177]. This suggests that RGS2 may be an important component in cellular stress response, however the specific outcomes mediated by RGS2 are not well known and were assessed in the studies presented in this thesis.

1.7.3 Physiological roles of RGS2

Dysregulation of G protein activity can lead to aberrant signalling and lead to stress within tissues that may ultimately lead to disease. RGS2-knockout mice have greater susceptibility to the development of atrial arrhythmias[165], are hypertensive, and show greater cardiac hypertrophy in response to pressure overload[168,187]. RGS2 also plays a role in hypertension[161,188,189] and anxiety[190] in humans. RGS2 mRNA expression levels are shown to be significantly lower in some groups of hypertensive patients, attributing to decreased modulation of G protein signals known to be involved in regulating vascular tone[189]. Several RGS2 gene polymorphisms have been identified within patients with panic disorders and thus RGS2 may play a role in the development of axiety[190]. In addition, RGS2-deficient mice exhibit a lean phenotype, where they have greatly reduced fat stores and do not develop age-related obesity as seen in wild-type counterparts[191].

RGS2 has also been shown to regulate the differentiation of various cell types including adipocytes[192], chondrocytes[193], myeloid cells[194], and cell types within the pituitary[195]. Other RGS2 loss-of-function phenotypes include abnormal renal solute handling[196], decreased T-cell proliferation and antiviral immunity[197]. As a result of the broad tissue

distribution of RGS2 throughout the body, changes in RGS2 expression or activity would be expected to have profound physiological effects.

1.7.4 Translational control by RGS2: Role of RGS2 in cellular stress response

Recent work on the functional molecular biology of RGS2 has shown its ability to affect translational machinery, suggesting novel functions of RGS2 distinct from its known roles as a negative modulator of G protein signalling. We previously discovered a 37 amino acid binding domain (herein termed RGS2^{eb}) found at residue 79 to 116 within the conserved RGS domain that can bind to the epsilon subunit of eIF2B (eIF2Bε) and inhibit translation (Fig. 1.5A)[160]. Binding of RGS2^{eb} to eIF2Bε interferes with the eIF2-eIF2B GTP exchange cycle, preventing the formation of eIF2-GTP-Met-tRNA$_i$ required in the initiation of mRNA translation, and leads to global reduction of protein synthesis (Fig. 1.6A).

As discussed previously, a similar interaction occurs with eIF2β and eIF2Bε, preventing the GDP-GTP exchange on eIF2, thereby preventing translation at initiation. This is heightened under states of stress as a result of the phosphorylation of eIF2α to regulate protein synthesis. RGS2^{eb} and the established eIF2Bε-interacting domain of eIF2β show 35% sequence similarity (Fig. 1.5B), while corresponding sequence comparisons with other RGS and RGS-like proteins did not show the same degree of similarity[160,198]. Furthermore, this activity of RGS2 to inhibit translation is independent of its effects on G proteins. We have shown that a point mutation in the critical contact point between RGS2 and Gα subunits by the replacement of asparagine residue 149 to an alanine or the removal of a substantial portion of the RGS2 domain within the carboxy terminus of the protein, was not able to increase agonist-induced GTP hydrolysis but was still able to prevent *de novo* protein synthesis[160]. It is therefore hypothesized that RGS2 may compete with eIF2 for the binding of eIF2B, and may be an important target in the modulation of stress-mediated translational control mechanisms and downstream effector pathways affected by the cellular state of translation. Furthermore, infections using adenoviruses to overexpress both full length RGS2 and RGS2^{eb} have been shown to inhibit *de novo* protein synthesis in multiple cell types[160] and to block agonist-induced cellular

hypertrophy[134,159]. Decreasing protein synthesis reduces protein misfolding while conserving cellular resources[24,29,122], suggesting a protective role by RGS2 in cell stress[159,160]. This would be beneficial to prevent diseases associated with protein stress, such as pathological hypertrophy, and may augment the effects of stress-activated kinases[159,199]. Additionally, RGS2 may provide an alternative way in regulating translation independent of eIF2α phosphorylation during stress (Fig. 1.6B). However, the full benefit of this is questionable as inhibition of translation can drive pathways leading to cell death, such as that mediated by the ATF4-CHOP pathway. How these effects by RGS2 modulate components of the UPR and apoptosis was therefore assessed in this thesis.

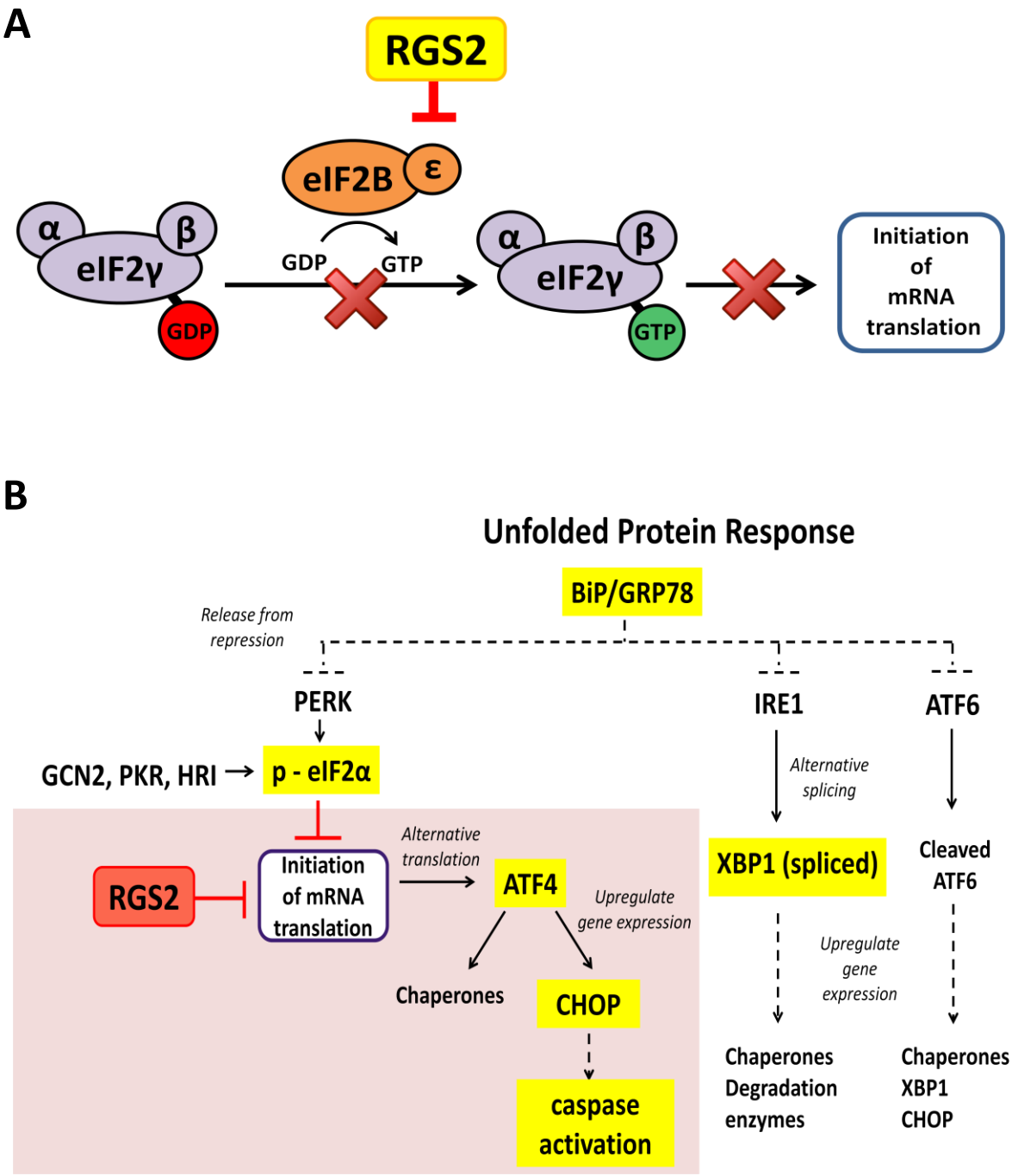


Figure 1.6. Mechanism of translational control by RGS2. (**A**) Binding of RGS2 to the ε-subunit of eIF2B prevents the necessary GDP-GTP exchange of eIF2, thereby preventing initiation of mRNA translation and the reduction of global protein synthesis. (**B**) Schematic of pathways involved in the unfolded protein response and the putative role that RGS2 may have in modulating the expression of endpoints associated in response to cell stress (highlighted in pink).

1.8 Rationale

RGS2 is upregulated by many of the same forms of stress that trigger eIF2 phosphorylation to reduce protein synthesis, suggesting that it may be an important modulator in stress response pathways and drive physiological outcomes. Apart from the GAP functions of RGS2 in regulating GPCR and G protein mediated-signalling, RGS2 can interfere with the translational machinery to inhibit initiation, and this has been mapped to a short 37 amino acid eIF2Bε-binding domain (RGS2^{eb}). Inhibition of protein synthesis is a hallmark response mediated by the UPR in order to alleviate stress. The benefit of this, however, is diminished by the fact that inhibition of initiation can result in the preferential upregulation of pro-apoptotic pathways. Furthermore, the expression of several proteins involved in such pathways are controlled by alternative translation mechanisms, triggered by eIF2α phosphorylation, and it is unclear whether the inhibitory effects on protein synthesis of RGS2 may provide a parallel pathway and drive similar outcomes during stress. Therefore, to better understand how the translational control abilities of RGS2 may contribute to the cellular stress response, we investigated its roles in relevant pathways, as outlined by the specific objectives below.

1.8.1 Hypothesis and Predictions

We hypothesized that RGS2 contributes to the cell stress response through its translational control abilities. We predicted that RGS2 would promote alternative translation and thereby affect the expression of factors involved in the unfolded protein response.

1.8.2 Objectives

It is well established that the phosphorylation of eIF2α by various stress-activated kinases inhibits initiation of mRNA translation and promotes expression of particular stress proteins through alternative translation mechanisms[34,64,117,200,201]. The inhibitory effects of RGS2 on protein synthesis, more specifically through its eIF2Bε-binding domain (RGS2^{eb}), perhaps provide a complementary pathway to prolong reduced translation during stress once dephosphorylation of eIF2α begins[202]. Whether this effect by RGS2

can promote alternative translation in a similar manner mediated by the phosphorylation of eIF2α is not known leading to my first research objective:

1. To determine the effect of RGS2 and RGS2^{eb} on alternative translation mechanisms.

This was directly investigated using cell based assays assessing the effect of RGS2 on the expression of stress-response proteins controlled by alternative translation mechanisms, such as ATF4. We expected that there would be enhanced expression of such protein if the inhibitory effects of RGS2 on translation do indeed promote alternative translation and thus may also affect cell stress response.

Key features of the cellular stress response include the regulation of the expression and/or the activity of stress-adaptive factors, or the induction of apoptosis should recovery be unsuccessful[29,117,203,204]. Whether the inhibitory effects of RGS2 on translation influence either of these facets of the stress response and the underlying mechanisms by which this occurs is not well understood, leading to the two other objectives of my research assessed in this thesis:

2. To determine the effects of RGS2 expression on the translational and transcriptional profiles of endpoints of the UPR during cell stress.

3. To determine the effects of RGS2 on stress-induced apoptosis.

Chapter 2

2 Materials and Methods

2.1 Cell culture

NIH-3T3, a well-established murine fibroblast cell line, were used to assess the effects of RGS2 expression on cellular stress and apoptosis. In addition to previously published studies looking at apoptosis in this cell line[205], preliminary studies in our lab have shown that NIH-3T3 fibroblasts overexpressing RGS2 resulted in the activation of caspase 3 after experimentally-induced stress. Other commonly used cell lines such as HEK-293 (human embryonic kidney cells) were also tested but did not appear to have any effect (unpublished data). Therefore, NIH-3T3 was chosen as the cellular model to assess our research questions on the effects of RGS2 expression in stress response pathways. NIH-3T3 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco Life Technologies) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco Life Technologies) at 37°C with 5% CO_2.

2.2 Adenoviruses

Recombinant adenoviruses, viral propagation, titre and multiplicity of infection determination

Replication-defective adenoviruses encoding GFP (Ad-GFP), full-length His_6-tagged human RGS2 (Ad-RGS2), and the His_6-tagged 37 amino acid eIF2Bε binding domain of RGS2 (Ad-RGS2^{eb}) were generated in our lab as previously described[134,160]. Adenoviruses were propagated in E1-producing HEK-293 cells. Briefly, cells were plated in 145 mm tissue culture plates in DMEM supplemented with 10% FBS, 24 hours prior to infection. Plates were checked and verified to be 80-90% confluent on the day of infection and 5 ml crude adenoviral stock was added to the cells. Cells were kept at 37°C, 5% CO_2, and infection proceeded until 80-90% of the cells had become spherical and detached from the plate. Adenoviruses were harvested from the cells via three freeze-thaw cycles. Cell lysates were centrifuged at 3000 rpm for 15 minutes at room

temperature to pellet cell debris. Supernatant containing viral particles were transferred to cryovials in 1 ml aliquots and stored at -80°C. Titring of the adenoviral constructs was carried out following the procedures described in Franceschi and Ge (2008)[206]. Levels of expression of adenoviral encoded proteins were also monitored through fluorescence microscopy for GFP. Cell lysates were collected and immunoblotted with rabbit anti-6X His tag ChIP grade antibody (1:1000, Abcam ab9108), chicken anti-RGS2 antibody (1:1000, Sigma-Aldrich GW22245F), or mouse anti-GFP (1:1000, Clontech 632381) to assess viral infection and levels of protein expression, as appropriate. See Appendix A, Fig. A1 for representative blots assessing the multiplicity of infection (MOI) of the viruses in 3T3 fibroblasts. Expression of polyhistidine-tagged RGS2^{eb} in cells via infection using the generated recombinant adenoviral vectors was verified in previous studies via immunofluorescent staining and dot blot analysis of whole cell lysates[134].

2.3 Reagents and drugs

Adenovirus-infected cells were subjected to stress via treatment with tunicamycin (TM), thapsigargin (TH), or apoptosis-inducing agents such as staurosporine (ST). Dimethyl sulfoxide (DMSO, 0.1% v/v) vehicle controls were run in parallel. Tunicamycin (Sigma-Aldrich T7765) was diluted to the indicated experimental concentrations from a 10 mg/m1 stock solution in DMSO. Staurosporine (TOCRIS Bioscience 1285) was diluted to the indicated concentrations from a 2 mM stock solution in DMSO. Thapsigargin (TOCRIS Bioscience 1138) was diluted to the indicated concentrations from a 5 mM stock solution in DMSO. Effective concentrations and length of time of drug treatments used in dose-response and time-course assays are indicated in figure legends and were selected as reported[42,107,207–214]. We confirmed that these stressors activated endpoints associated with stress response and/or apoptosis (see Appendix B, Fig. B4). Water-soluble forskolin (7β-deacetyl-7β-(γ-*N*-methylpiperazino)-butyryl, dihydrochloride foskolin, Calbiochem, La Jolla, CA) was used to induce endogenous RGS2 expression[182] (see Appendix A, Fig. A3).

2.4 Adenoviral infection and protein isolation

Cells were seeded in 12 well plates and grown to 60-70% confluency (approximate cell density of 5.8×10^5 cells/ml) on the day of infection. Cells were infected for 48 hours with Ad-RGS2, Ad-RGS2^{eb}, Ad-GFP (as an infection control), or left uninfected (NI), under 4 hours of serum deprivation, after which medium was removed and replaced with complete cell culture medium. Infection with the adenoviruses occurred for 48 hours, after which cells were treated with a chemical stressor at indicated concentrations and durations or subjected to a vehicle control. Cell lysates were prepared by washing with ice-cold 1X phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and scraping into 200 µl of ice-cold lysis buffer (250 mM NaCl, 50 mM Tris pH 8, 5 mM EDTA, 0.5% NP-40 (IGEPAL), phenylmethylsulfonyl fluoride protease inhibitor tablet (Roche), 20 mM $Na_4P_2O_7$, 10 mM NaF, and 20 mM Na_3VO_4). Cells were incubated in lysis buffer with rocking for 30 minutes at 4°C. Cell lysates were homogenized by vigorous pipetting through a 1.5 mm pipette tip followed by three freeze-thaw cycles with liquid nitrogen. Cell pellets were sedimented by centrifugation at 11 000 × *g* for 15 minutes at 4°C. Supernatants were collected and protein concentrations were determined using Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Scientific) and protein (bovine serum albumin) standard curve calculations.

2.5 Immunoblotting

Protein samples were prepared using 5X Laemmli loading (sample) buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.02% bromophenol blue) and balanced with 1X sample buffer for equal protein concentration. Unused protein samples were frozen immediately and stored at -20°C. Protein samples were heated to 99°C for 5 minutes prior to loading and gel electrophoresis. Equal amounts of protein (5 or 10 µg per lane) were separated by 10-12% SDS-PAGE and wet transferred onto nitrocellulose membranes (Whatman Protran). Membranes were incubated in blocking buffer (Tris-buffered saline, 0.1% Tween-20, 5% skim milk) and rocked for 1 hour at room temperature before overnight incubation with rocking at 4°C with respective antibodies to assess targeted endpoint proteins associated in ER stress and cell death

pathways: anti-BiP/GRP78 (1:1000, Pierce PA5-17423), anti-phospho-eIF2α (1:1000, Cell Signaling 9721), anti-CREB-2/ATF4 (1:5000, Santa Cruz sc-200X), anti-CHOP (1:1000, Cell Signaling 5554), anti-cleaved caspase 3 (1:1000, Cell Signaling 9964). Protein lysates extracted from full-body ATF4 knockout mouse embryos (E16.5) were run in parallel to determine ATF4-specific protein band, indicated by black arrowheads on representative immunoblots. ATF4 knockout mouse embryos were generously provided by Dr. Sean Cregan (Robarts Research Institute, London, ON). Anti-RGS2 (1:1000, Sigma-Aldrich GW22245F) was used to assess for endogenous and heterologously expressed levels of RGS2 under various stress conditions, anti-GFP (1:1000, Clontech 632381) and anti-6X His tag ChIP grade antibody (1:1000, Abcam ab9108) were used to probe for GFP and 6xHis-tagged RGS2, respectively, to assess adenoviral infection efficiency and expression. Purified His_6-tagged RGS2 protein samples (50 ng) were loaded into SDS PAGE gels as a positive control for expression of RGS2. Membranes were then incubated for 1 hour at room temperature with appropriate horseradish peroxidase-conjugated secondary antibodies: anti-rabbit IgG (1:3000, Pierce 31463), anti-mouse IgG (1:3000, Pierce 31437), or anti-chicken IgY (1:3000, Pierce SA1-72012). Immunoblots were visualized with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) and digitally imaged using Bio-Rad VersaDoc camera and Quantity One program (Bio-Rad, model GS-700). Immunoblots were stripped using Restore Western blot stripping buffer (Thermo Scientific) and re-probed to assess total protein species of ER stress and apoptotic endpoints such as anti-eIF2α (1:1000, Cell Signaling 9722), and anti-caspase 3 (1:1000, Cell Signaling 9665). Anti-β-tubulin (1:1000, Pierce PA5-16863) or anti-GAPDH (1:1000, Pierce PA1-988) were used to assess protein loading and were stable across experimental conditions.

2.6 Densitometry

Relative protein expression levels from immunoblots were quantified and analyzed by densitometry (Quantity One, Bio-Rad). Relative densitometric signal of target protein bands were determined with subtraction of background signal of immunoblots. For assessment of changes in caspase 3 activation, a densitometric ratio of cleaved caspase 3 to uncleaved caspase 3 was taken. For phosphorylated proteins, densitometric ratios of

phosphorylated to total species were taken. Data are presented as means ± SEM where the level of statistical significance was set at $\alpha = 0.05$. Statistical differences were further evaluated by post-hoc tests indicated, where p-values of <0.05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism® 5.01.

2.7 RNA isolation, reverse transcription (RT-PCR), and quantitative polymerase chain reaction (qPCR)

Cells were seeded in 24 well plates and grown to 60-70% confluency (approximate cell density of 5.8×10^5 cells/ml) on the day of infection. Cells were infected for 48 hours with Ad-RGS2, Ad-RGS2^{eb}, Ad-GFP (as an infection control), or left uninfected (NI), under 4 hours of serum starved conditions then replaced with complete cell culture medium. After 48 hours of infection, cells were treated with chemical stressors or vehicle control at the indicated concentrations and durations. Total RNA was then extracted from cells using TRIzol reagent (Invitrogen) following the manufacturer's protocol. RNA purity and concentrations were quantified through spectrophotometry (NanoDrop Lite, Thermo Scientific). RNA samples with an absorbance ratio ($A_{260\ nm}/A_{280\ nm}$) of 1.8-2.2 were determined to be pure for use in downstream PCR applications. RNA samples (2 μg) were reverse transcribed (RT-PCR) to generate first strand cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems) on a T100 Thermal Cycler (BioRad). Primer sets directed against target genes of interest were designed using the National Centre for Biotechnology Information Nucleotide sequences database (www.ncbi.nlm.nih.gov/nuccore) and Invitrogen's OligoPerfect Designer primer designing tool (www.thermofisher.com/oligoperfect/). Primers were custom manufactured by and purchased from Sigma-Aldrich Custom DNA Oligos (Table 2.1). ATF4 primers and sequences were generously provided by Dr. Sean Cregan (Robarts Research Institute, London, ON). Quantitative analysis of mRNA expression levels of endpoints of the cellular stress response: ATF4, CHOP, BiP/GRP78, and spliced XBP1 (XBP1s) were determined through qPCR carried out in 384 well plates using fluorescent nucleic acid dye SensiFAST SYBR Green No-ROX kit (Bioline) based assays, following manufacturer's protocol. Reactions were carried out on CFX384 Real Time PCR

Detection System and analyzed using CFX Manager 3.0 program (BioRad). The cycle threshold was set so that exponential increases in amplification were approximately level between all samples at the linear phase of the amplification curves. Relative mRNA levels of respective target genes were quantified using standard curves generated from five-fold serial dilutions of pooled cDNA samples, then normalizing all values to the geometric means of two reference genes (GAPDH and β2 microglobulin) measured in parallel. Reference genes were stable across experimental conditions to allow comparative assessments on the relative change in the expression of targeted genes of interest under indicated experimental conditions. Real time data are reported as mean ± SEM where the levels of statistical significance were set at $\alpha = 0.05$. Statistical differences were further evaluated by post-hoc tests indicated, where p-values of <0.05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism® 5.01.

Table 2.1. Primers (*Mus musculus*) used in qPCR reactions to assess for changes in gene expression of stress response targets.

Target	**Forward Primer**	**Reverse Primer**
ATF4/CREB-2	5'-TCTTGGACTAGAGGGGCAAA-3'	5'-GGGACAGATTGGATGTTGGA-3'
BiP/GRP78	5'-AGTTCTTCAATGGCAAGGAG-3'	5'-ACCAAGTGTAAGGGGACAAA-3'
CHOP/GADD153	5'-TACACCACCACACCTGAAAG-3'	5'-TTCTTCCTCTTCGTTTCCTG-3'
XBP1s	5'-GACACTGTTGCCTCTTCAGAT-3'	5'-ACATGGTCAAAACGAATGAGT-3'
RGS2	5'-TGACAAATATGCCAGGTCTCTA-3'	5'-CTGCACAGAGTGTGAGGTAAAT-3'
GAPDH	5'-GTTCCTACCCCCAATGTGT-3'	5'-GGAGTTGCTGTTGAAGTCG-3'
β2 microglobulin	5'-ACGCAGAAAGAAATAGCAATG-3'	5'-TGAGAAGTACAGAGGGTTTGG-3'

2.8 Quantitative fluorescence microscopy reporter assay

Sources of plasmids

To study the putative functions that RGS2 has within cellular stress signaling, we looked at the effect of RGS2 overexpression on the level of BiP ER stress response element (ERSE) promoter activity through quantitative fluorescence microscopy. The C-terminally FLAG-tagged human RGS2 plasmid (pcDNA3.1-hRGS2 WT-FLAG) was custom generated by and purchased from the Missouri S&T cDNA Resource Center (www.cdna.org). The BiP ERSE-tdTomato reporter construct[215,216] was generously provided by Dr. Patrick Lajoie (University of Western Ontario, London, ON). pcDNA3.1 empty vector controls were generously provided by Dr. Lina Dagnino (University of Western Ontario, London, ON).

Co-transfection and quantification of the BiP ERSE promoter activity

NIH-3T3 fibroblasts were seeded in 6 well plates and transfected with plasmids using Lipofectamine 2000 (Life Technologies) in reduced serum medium (Opti-MEM, Life Technologies) as per the manufacturer's instructions. Briefly, each well was co-transfected with 1 μg full-length RGS2 plasmid or 1 μg pcDNA3.1 empty vector backbone together with 1.25 μg BiP ERSE-tdTomato plasmid. After 24 hours of transfection, plates were imaged for initial baseline BiP ERSE promoter activity and monitored for transfection efficiency via quantitative fluorescence microscopy using a 10x objective and 547nm excitation, 581nm emission bandpass filter for tdTomato, prior to any pharmacological treatment (0 hour time point). Cells were then treated with 0.1% DMSO (vehicle control) or 5 μg/ml tunicamycin and imaged at 1.5, 3, 6, 12, and 24 hours post-treatment. Co-transfections and each treatment condition were carried out in triplicate. At each time point, live fluorescent images from three different fields per well were taken using an OlympusIX71 microinjection fluorescent microscope (Olympus Canada) and QCapture Pro camera and software (QImaging Canada). 8-bit greyscale images were taken at 10x magnification to capture the tdTomtato fluorescence of a population of cells at each visual field. Images were then analyzed to quantify total fluorescence using ImageJ (National Institutes of Health, Bethesda, MD) to assess for

differences in the level of ERSE promoter activity across treatment conditions. Thresholding function on ImageJ was used to quantify the total fluorescence from the images, measured by the sum of all the pixels that fall within a set threshold range of intensity graduations. The range to capture total intensity of the tdTomato fluorescence from background signal (tdTomato area/pixel^2) was set between 414 (lower threshold level) to 4095 (upper threshold level) which is within the linear range of signal and was used throughout our analyses. Areas were then multiplied by a conversion factor (51741.69 μm/pixel) to obtain values in tdTomato area/μm^2, followed by normalization to the total number of cells. Cell counts from the images were performed on ImageJ by setting cell circularity range between 0.20 – 0.90 to determine the total number of cells within each visual field. All fluorescence data are reported as means ± SEM in units of tdTomato area/cell of three independent experiments. All statistical analyses were performed using GraphPad Prism® 5.01, where the level of statistical significance was set at $\alpha = 0.05$. Statistical differences were further evaluated by post-hoc tests indicated in figure legends, where p-values of <0.05 were considered statistically significant.

2.9 Statistical Analysis

Grouped data are presented as mean ± SEM, where *n* represents the number of independent experiments. Differences between groups were determined using one-way ANOVA followed by Dunnett's post-hoc test. Statistical significance in experiments assessing the possible effects of either the infection condition (*i.e.*, Ad-RGS2, Ad-RGS2^{eb}, Ad-GFP, or uninfected cells) or stress treatment was determined using two-way ANOVA, followed by Bonferroni post-hoc tests. Between-group differences in total protein levels were analyzed using linear regression by constraining the y-intercept to a shared value for all data sets and comparing differences in the slopes of the fitted data. A *p* value < 0.05 was considered statistically significant throughout. All statistical analyses were performed using GraphPad Prism® 5.01.

Chapter 3

3 Results

3.1 RGS2 enhances alternative translation mechanisms and modulates cellular stress response pathways

Protein synthesis is a multistep process that involves the concerted activity of many molecular components. From a mechanistic and energetic standpoint, the regulation of translation occurs right at initiation to avoid unnecessary expenditure of energy and resources and to allow rapid control of gene expression. The phosphorylation of the initiation factor, eIF2, is a well known mechanism that reduces global protein synthesis and integrates several stress signals of varying origins. A growing body of work also demonstrates the increase in cellular protein levels of specific factors under impaired translation which leads to different physiological outcomes in response to stress. The expression of many of these factors is regulated by alternative open reading frames found in their transcript and translated via alternative translational mechanisms described previously.

Recently, we have demonstrated that the ability of RGS2 to bind and impede the functions of eIF2B provides another mechanism in the regulation of translation initiation[159,160]. This may function in parallel to the eIF2α-phosphorylation pathway to prolong reduced translation conditions under stress. The benefits of this are unclear, and whether this may modulate the expression and/or the activity of key components involved in the CSR was investigated. To determine whether RGS2 expression affects protein translation, we infected 3T3 cells with recombinant adenovirus expressing RGS2. Here, we show that the expression of RGS2 consistently resulted in significantly lower total protein concentrations, and the effect increased with the multiplicity of virus infection (Fig. 3.1, linear regression analysis, $p = 0.0063$). Cell confluency (80-90%) was consistent across all experimental conditions after 48 hour period of infection, assessed by light microscopy. This reduced the likelihood of confounding effects to our results that may be attributed with decreased cell number. This confirms previous findings from our lab on the ability of RGS2 to inhibit protein synthesis.

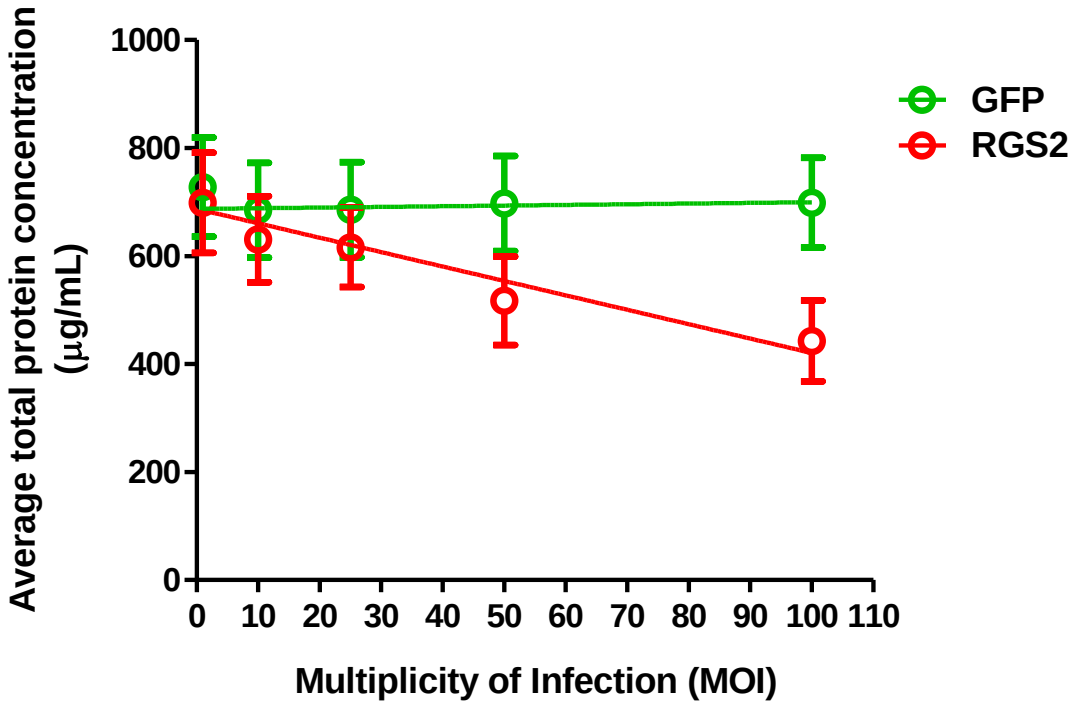


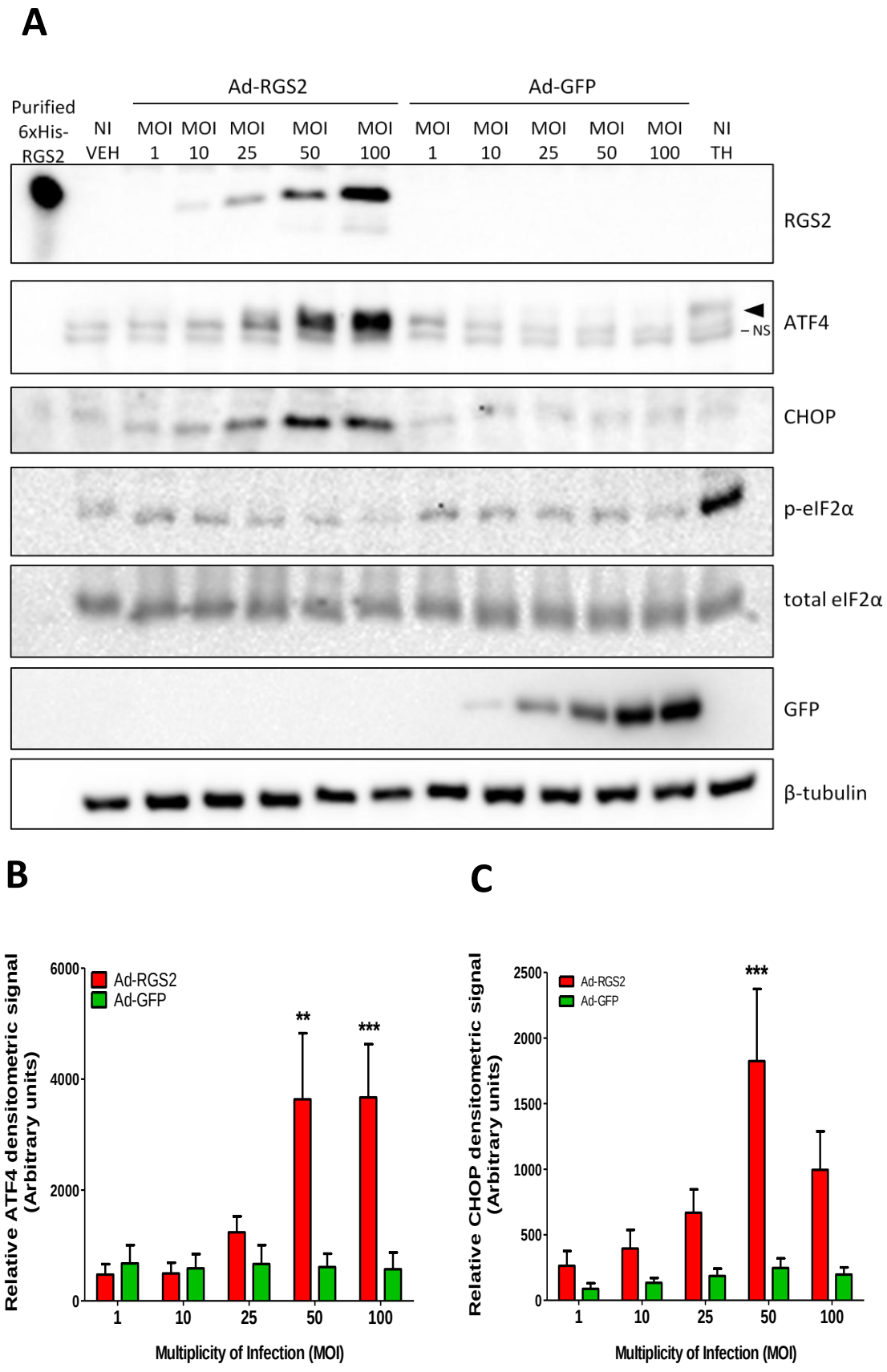
Figure 3.1. Expression of RGS2 results in reduced total cellular protein levels. 3T3 fibroblasts were infected with RGS2 or GFP-encoding adenovirus at the indicated multiplicities of infection (MOI). Following 48 hours of infection, cells were lysed and total protein concentrations of the collected lysates were quantified as described in Materials and Methods. Significantly lower total protein concentrations were observed from lysates of RGS2 expressing cells and the effect occurred in a concentration-dependent manner with increasing RGS2 (linear regression, $p = 0.0063$). Slopes of the fitted linear regression analysis for RGS2 (*y-int* = 687.40 ± 37.02, slope = -2.67 ± 0.87) and GFP (*y-int* = 687.40 ± 37.02, slope = 0.12 ± 0.87) were statistically different. Data presented are mean ± SEM, n = 8.

3.1.1 RGS2 and RGS2^{eb} increase ATF4 and CHOP protein levels

The expression of ATF4 is tightly regulated by the cellular state of translation and the efficiency of the translational machinery. During conditions of stress where the levels of p-eIF2α are increased, the availability of eIF2-GTP-Met-tRNA$_i$ ternary complex is reduced and scanning ribosomes do not acquire an initiator methionine in time to start translation. This delay allows ribosomes to bypass the two inhibitory upstream open reading frames (uORF1 and uORF2) of ATF4 and initiate translation at the ATF4 ORF instead to produce functional ATF4[34,70].

The ability of RGS2 to inhibit translation initiation has been specifically mapped to a stretch of 37 amino acid residues that is capable of binding to the epsilon subunit of eIF2B (RGS2^{eb})[160]. This binding was found to interfere with the eIF2-eIF2B GTPase cycle, which presumably accounts for its ability to inhibit the initiation of mRNA translation[160], comparable to the inhibitory effect associated with the phosphorylation of eIF2α. We therefore hypothesized that the translational control abilities of RGS2 may affect endpoints associated in stress response, regulated by alternative translational mechanisms, such as ATF4. We found that ATF4 protein levels increased with increasing expression of RGS2 (Fig. 3.2A). This effect was not a result of the viral infection *per se* as no ATF4 was detected in corresponding GFP expressing cells. RGS2 expression resulted in significant cellular levels of ATF4 (two-way ANOVA, $p = 0.0004$), and such levels were observed at multiplicities of infection of 50 and 100 (Fig. 3.2B, Bonferroni post-hoc tests, $^{**}p < 0.01$ and $^{***}p < 0.0001$, respectively). Additionally, the expression of CHOP significantly increased in RGS2 expressing cells (Fig. 3.2C, two-way ANOVA, $p < 0.0001$), similar to the expression pattern observed with ATF4 expression pattern. Interestingly, CHOP protein levels dropped at the highest level of infection. We speculate that this may be as a result of a saturation effect by even greater amounts of ATF4, as studies report that ATF4 becomes a transcriptional repressor at high concentrations[217]. CHOP is known to be upregulated in the stress response at the translational level[64,73,200,218,219] and as well its mRNA transcription is induced by transcription factors including ATF2[72,220,221], ATF4[39,72,73,222], ATF6[223,224], and XBP1s[45], so multiple regulatory components may be involved in modulating the level of CHOP expression.

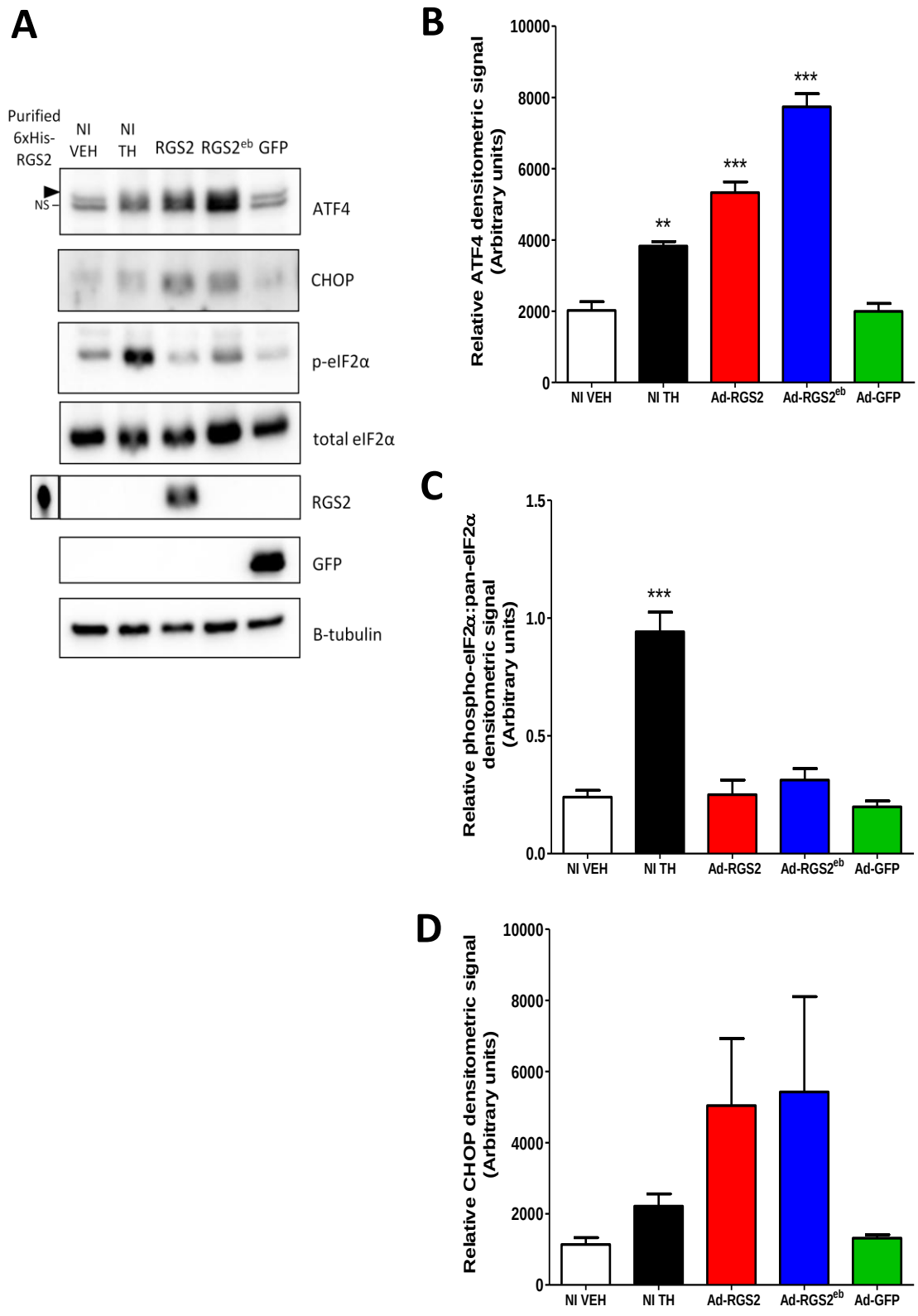
Figure 3.2. Dose dependent increase in ATF4 and CHOP protein levels with RGS2 expression. 3T3 fibroblasts were infected with adenoviruses encoding GFP (viral infection control) or full-length RGS2 at the indicated range of multiplicity of infection (MOI) for 48 hours. Non-infected (NI) fibroblasts were then treated with 2 μM thapsigargin (TH) for 2 hours as a positive control for the induction of eIF2α phosphorylation and ATF4, or were treated with 0.1% DMSO (vehicle control). ATF4-specific protein band is indicated by an arrowhead, NS = non-specific band. After incubation in sample buffer, proteins were separated by SDS-PAGE on two separate gels run in parallel. After protein transfer, membranes were cut horizontally. The top portion of the first membrane (approximately 40-300 kDa) was blotted with anti-ATF4, followed by stripping and reprobing with anti-β-tubulin (not shown). The bottom portion (up to 40 kDa) was blotted with anti-CHOP. The bottom portion was then stripped and reprobed with anti-RGS2, followed by a second strip and reprobe with anti-GFP to assess RGS2 and GFP expression, respectively. The top portion of the second membrane was blotted with anti-β-tubulin (shown) and the bottom portion was blotted with anti-p-eIF2α, followed by stripping and reprobing with anti-pan-eIFα, to assess the relative proportion of phosphorylated eIF2α. The same procedures were repeated for each independent experiment. (**A**) RGS2 expression significantly increased ATF4 protein levels in a dose dependent manner while no comparable increase was observed with GFP expressing cells, and this was independent of eIF2α phosphorylation. Levels of CHOP also significantly increased with RGS2 expression, correspondingly to the ATF4 expression pattern. Immunoblots shown are representative of seven independent experiments (thapsigargin controls were included in three of these). Corresponding densitometric data are summarized in bar graphs as mean ± SEM for ATF4 (**B**) and CHOP (**C**) protein levels. Statistical analysis was performed using two-way ANOVA followed by Bonferroni post-hoc test. **, Significant difference ($p < 0.01$). ***, Significant difference ($p < 0.0001$). For all targets assessed, immunoblotting for β-tubulin was used as the control for equal protein loading, here shown with one representative blot to demonstrate equal sample loading in our experiments (others not shown).


A
Ad-RGS2
Ad-GFP
Purified 6xHis-RGS2
NI VEH
MOI 1
MOI 10
MOI 25
MOI 50
MOI 100
MOI 1
MOI 10
MOI 25
MOI 50
MOI 100
NI TH
RGS2
ATF4
– NS
CHOP
p-eIF2α
total eIF2α
GFP
β-tubulin
B
Relative ATF4 densitometric signal (Arbitrary units)
Ad-RGS2
Ad-GFP
**
***
Multiplicity of Infection (MOI)
C
Relative CHOP densitometric signal (Arbitrary units)
Ad-RGS2
Ad-GFP
***
Multiplicity of Infection (MOI)

3.1.2 RGS2 and RGS2^{eb} induce ATF4 expression without eIF2α phosphorylation

The eIF2α-ATF4 axis is activated upon stress within cells and leads to changes in transcription and translation of downstream effectors. These effectors may function to prevent further damage, recover from the insult, or alternatively activate programmed cell death[3,39,64]. Interestingly, we show a novel way to increase cellular ATF4 levels that is independent of eIF2α phosphorylation. Here we show that the expression of either full-length RGS2 or the eIF2Bε-binding domain of RGS2 (RGS2^{eb}) is sufficient to significantly increase ATF4 expression without any detectable change in the level of p-eIF2α (Fig. 3.3). Translational control by RGS2 may therefore provide an alternative mechanism in the induction of stress response factors, parallel to pathways mediated by p-eIF2α. This may also consequently lead to changes in cellular adaptability to stress. Consistent with this idea, increased ATF4 levels with RGS2 and RGS2^{eb} expression correlated with comparable increases in its downstream target CHOP (Fig. 3.3A, D), suggesting that RGS2 modulates the ATF4-CHOP stress-mediated pathways and their effects. The implications from this are significant as to our knowledge, this is the first report of an alternative mechanism to upregulate ATF4 from the known eIF2α-ATF4 pathway and extends the known repertoire of mechanisms within the cellular stress response. This has important implications with respect to the molecular mechanisms involved in regulating the activity of cells during times of stress and understanding the cross-talk between stress response and apoptotic pathways. Additionally, this also suggests potential pharmacological targets important in modulating stress-related pathologies associated in the regulation of ATF4 expression.

Figure 3.3. RGS2 and RGS2^{eb} upregulates ATF4 protein levels independent of eIF2α phosphorylation. 3T3 fibroblasts were infected with adenoviruses encoding GFP (viral infection control), full-length RGS2, or the RGS2 eIF2Bε-binding domain (RGS2^{eb}) at a multiplicity of infection of 50 for 48 hours. Non-infected (NI) cells were then treated with 2 μM thapsigargin (TH) for 2 hours as a positive control for the induction of ATF4 and eIF2α phosphorylation, or treated with 0.1% DMSO (vehicle control). ATF4-specific protein band is indicated by an arrowhead, NS = non-specific band. Two gels were run in parallel as described for Figure 3.2, and the same subsequent procedures were performed to obtain the immunoblots shown. (**A**) RGS2 and RGS2^{eb} expression resulted in significant increase in cellular ATF4 levels (one-way ANOVA, $p < 0.0001$), while no comparable increase in levels of phosphorylated eIF2α was observed. CHOP protein levels increased in a similar pattern. Immunoblots shown are representative of three independent experiments, the densitometric data of which are summarized in bar graphs as mean ± SEM for ATF4 (**B**), phosphorylated eIF2α (**C**), and CHOP (**D**) protein levels. After immunoblot for levels of phosphorylated eIF2α, membranes were stripped and reprobed for total eIF2α protein levels. The relative level of eIF2α phosphorylation was determined by taking the ratio of p-eIF2α to signal obtained with pan-eIF2α antibody. Control for equal protein loading was assessed via immunoblotting for β-tubulin. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post-hoc test on each condition versus non-infected vehicle control condition. **, Significant difference ($p < 0.01$). ***, Significant difference ($p < 0.0001$).


A
Purified 6xHis-RGS2
NI VEH
NI TH
RGS2
RGS2eb
GFP
NS
ATF4
CHOP
p-eIF2α
total eIF2α
RGS2
GFP
B-tubulin
B
Relative ATF4 densitometric signal (Arbitrary units)
10000
8000
6000
4000
2000
0
**
***
***
NI VEH
NI TH
Ad-RGS2
Ad-RGS2eb
Ad-GFP
C
Relative phospho-eIF2α:pan-eIF2α densitometric signal (Arbitrary units)
1.5
1.0
0.5
0.0
***
NI VEH
NI TH
Ad-RGS2
Ad-RGS2eb
Ad-GFP
D
Relative CHOP densitometric signal (Arbitrary units)
10000
8000
6000
4000
2000
0
NI VEH
NI TH
Ad-RGS2
Ad-RGS2eb
Ad-GFP

3.1.3 RGS2 and RGS2^{eb} upregulates ATF4 and CHOP translationally but not transcriptionally

Our observations that RGS2 and RGS2^{eb} increase cellular protein levels of ATF4 and CHOP led us to examine whether such effects by RGS2 were strictly translational or due to increased gene expression, resulting in greater transcript levels available for translation. As shown in Figure 3.4, expression with RGS2 or RGS2^{eb} did not affect the relative mRNA levels of ATF4 compared to the levels observed in the positive control conditions via treatment with chemical stressors such as thapsigargin or tunicamycin. A similar ATF4 gene expression pattern was obtained using a different ATF4 primer set (Appendix D, Fig. D1). This suggests that the upregulation of ATF4 mediated by the expression of RGS2 and RGS2^{eb} observed in our immunoblot data (Fig. 3.3) was not due to increased transcript levels of ATF4. Rather, ATF4 is regulated via the ability of RGS2 to affect the translational machinery and inhibit initiation, as well as the intrinsic properties of ATF4 mRNA, governing its translation.

Surprisingly, CHOP mRNA levels did not increase as expected with the observed increases in ATF4 protein levels, which is a known transcriptional activator for CHOP gene expression. Similar to our assessment on ATF4 transcript levels, expression of RGS2 and RGS2^{eb} do not have an effect on CHOP transcription as no increase in CHOP transcript levels was observed (Fig. 3.5). Although CHOP gene expression can be induced by ATF4 and other stress-induced transcription factors, the CHOP gene also encodes multiple initiation sites, and as with ATF4, the translation of functional CHOP protein resulting from its proper open reading frame is enhanced by eIF2α phosphorylation[64,117,200,219]. The present results imply that RGS2 similarly is able to drive the expression of both ATF4 and CHOP via translational as opposed to transcriptional mechanisms.

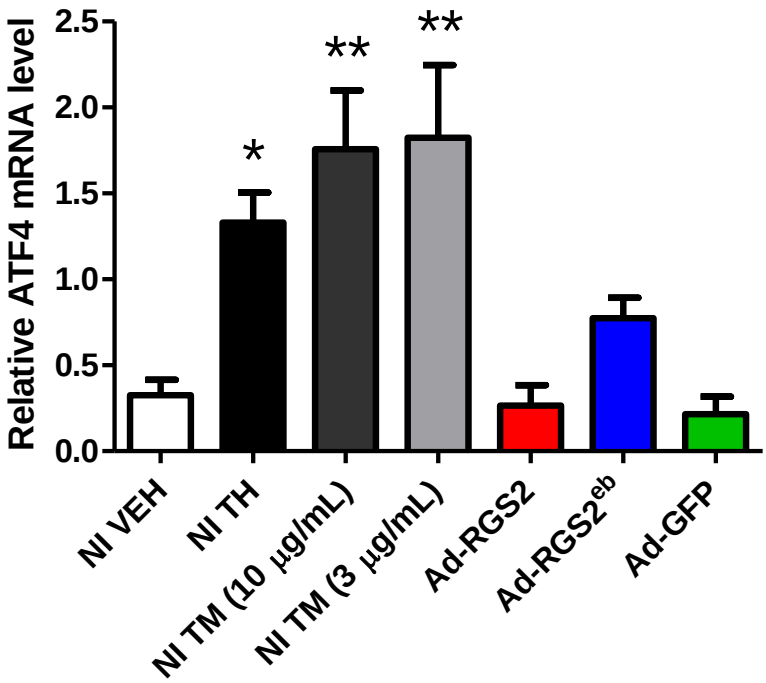


Figure 3.4. Effect of RGS2 and RGS2^{eb} on levels of ATF4 transcription. 3T3 fibroblasts were infected with adenoviruses encoding GFP (viral infection control), full-length RGS2, or the RGS2 eIF2Bε binding domain (RGS2^{eb}) at a multiplicity of infection of 50 for 48 hours. To confirm ATF4 upregulation, non-infected (NI) cells were treated with either 2 μM thapsigargin (TH) for 2 hours, 3 or 10 μg/ml tunicamycin (TM) for 6 hours, or 0.1% DMSO (vehicle control). All stressors increased ATF4 mRNA levels, where treatment with thapsigargin and tunicamycin resulted in a significant induction of ATF4 transcription. *, Significant difference ($p < 0.05$). **, Significant difference ($p < 0.01$). RGS2 and RGS2^{eb} expression did not result in an increase in ATF4 transcript levels. All mRNA levels are expressed as means normalized to the geometric mean of two stable reference genes (GAPDH and β2 microglobulin) ± SEM run in parallel through qPCR. Gene expression data presented are from three independent experiments, run in triplicate. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post-hoc test on each condition versus non-infected vehicle control condition.

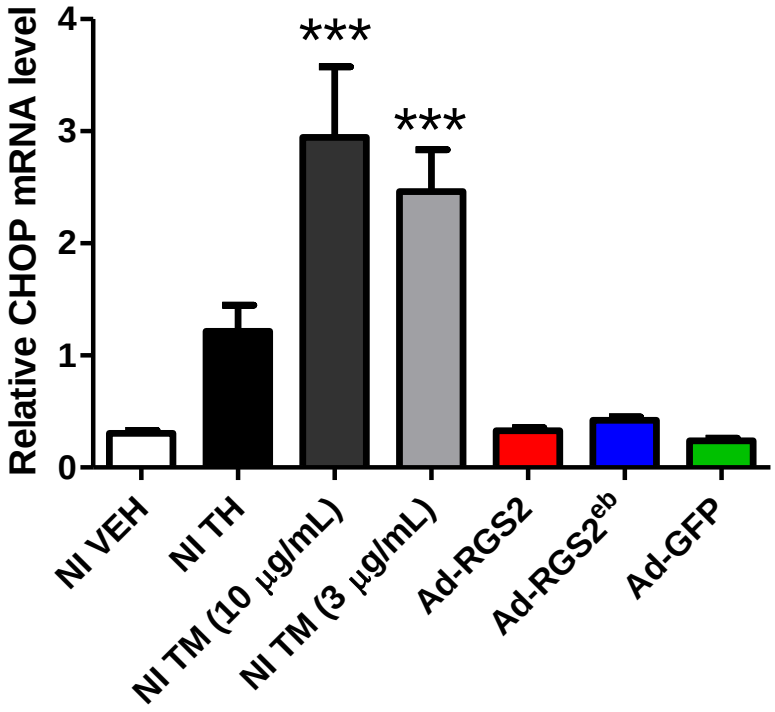
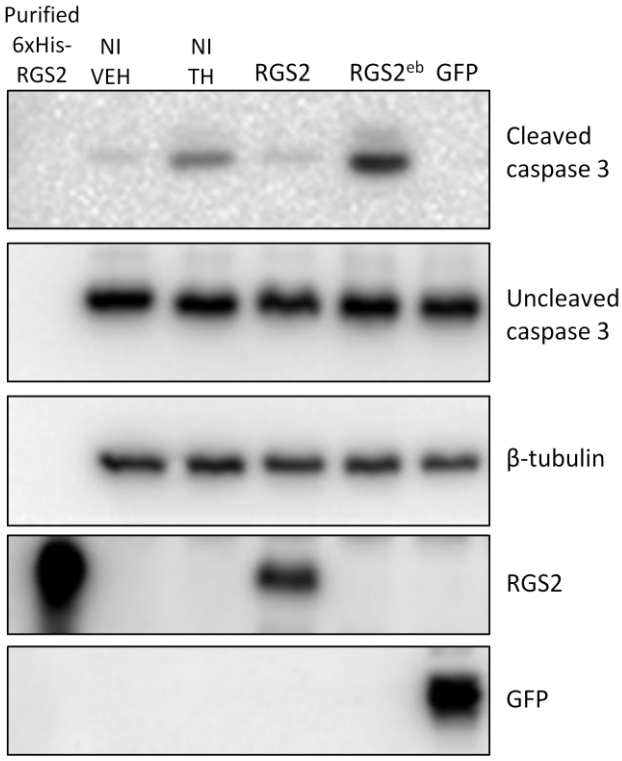


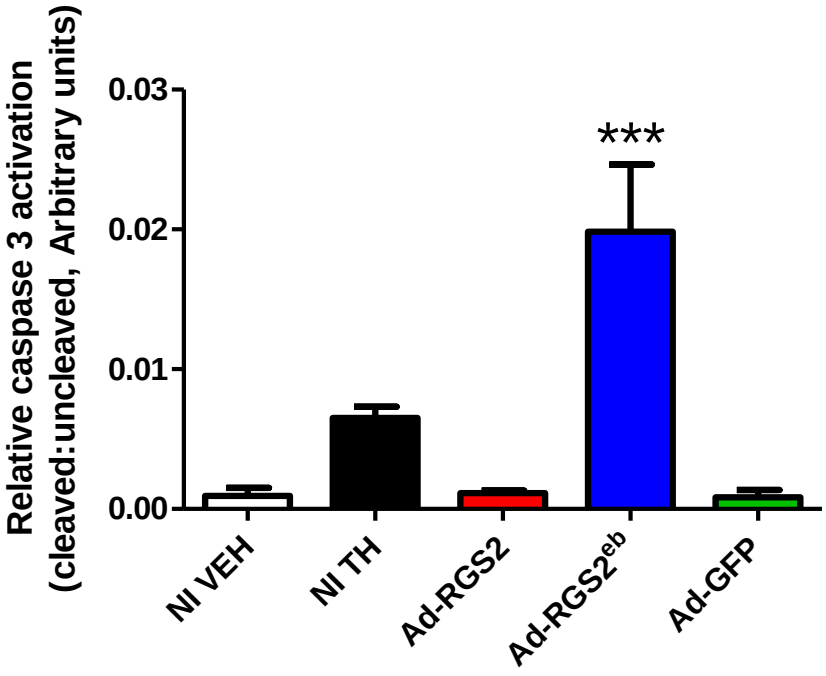
Figure 3.5. Effect of RGS2 and RGS2^{eb} on levels of CHOP transcription. 3T3 fibroblasts were infected with adenoviruses encoding GFP (viral infection control), full-length RGS2, or the RGS2 eIF2Bε binding domain (RGS2^{eb}) at a multiplicity of infection of 50 for 48 hours. To confirm CHOP upregulation, non-infected (NI) cells were treated with either 2 μM thapsigargin (TH) for 2 hours, 3 or 10 μg/ml tunicamycin (TM) for 6 hours, or 0.1% DMSO (vehicle control). Stressors increased CHOP mRNA levels, where treatment with tunicamycin resulted in a significant induction of CHOP transcription. ***, Significant difference ($p < 0.0001$). RGS2 and RGS2^{eb} expression did not result in an increase in CHOP transcript levels. All mRNA levels are expressed as means normalized to the geometric mean of two stable reference genes (GAPDH and β2 microglobulin) ± SEM run in parallel through qPCR. Gene expression data presented are from three independent experiments, run in triplicate. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post-hoc test on each condition versus non-infected vehicle control condition.

3.1.4 RGS2^{eb} induces caspase 3 activation

Since we found ATF4 and CHOP protein levels increased with RGS2 and RGS2^{eb} expression, we next assessed whether or not this may have downstream biological effects as the ATF4-CHOP pathway is known to promote apoptosis under irreparable stress. Apoptosis is a regulated and energy-dependent form of cell death that involves complex signalling pathways, one of the most well-known being the sequential activation of initiator caspases followed by effector caspases[87]. Caspase 3 is one of the main "executioner" caspases, the cleavage of which produces its active 17 kDa form that leads to cell destruction. Functions of activated caspase 3 include the cleavage of structural proteins, signalling molecules, other cytoplasmic and nuclear proteins, followed by the formation of apoptotic bodies which are then removed by macrophages[6,208,225–228]. Interestingly, our data show that only cells infected with RGS2^{eb} resulted in significant cleaved caspase 3 levels while cells infected with full-length RGS2 did not (Fig. 3.6, one-way ANOVA, $p = 0.0005$; Dunnett's post-hoc test, $p < 0.0001$). These results suggest possible functional domains found within full-length RGS2 that may be protective. While full-length RGS2 can reduce translation and drive increased levels of cellular ATF4 and CHOP, other protective effects might be in place to inhibit the activation of caspase 3.

Figure 3.6. Expression of RGS2^{eb} results in greater levels of cleaved caspase 3. 3T3 fibroblasts were infected with adenoviruses encoding GFP (viral infection control), full-length RGS2, or the RGS2 eIF2Bε binding domain (RGS2^{eb}) at a multiplicity of infection of 50 for 48 hours. Non-infected (NI) cells were then treated with 2 μM thapsigargin (TH) for 2 hours as a positive control for the induction of caspase 3 activation, or treated with 0.1% DMSO (vehicle control). RGS2^{eb} expression showed significant caspase 3 cleavage while no comparable level of cleaved caspase 3 was observed with full-length RGS2. Immunoblots shown are representative of three independent experiments, the densitometric data of which are summarized in the bar graph below as mean ± SEM by taking the ratio of cleaved caspase 3 to uncleaved caspase 3 levels. Control for equal protein loading was assessed via immunoblotting for β-tubulin. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post-hoc test on each condition versus non-infected vehicle control condition. ***, Significant difference ($p < 0.0001$).


Purified 6xHis-RGS2
NI VEH
NI TH
RGS2
RGS2eb
GFP
Cleaved caspase 3
Uncleaved caspase 3
β-tubulin
RGS2
GFP


Relative caspase 3 activation (cleaved:uncleaved, Arbitrary units)
0.03
0.02
0.01
0.00
***
NI VEH
NI TH
Ad-RGS2
Ad-RGS2eb
Ad-GFP

3.2 Modulation of the stress response by RGS2

While we showed that RGS2 expression resulted in decreased total cellular protein levels and increased protein levels of ATF4 and CHOP without the phosphorylation of eIF2, we next assessed whether such effects by RGS2 may influence the responses and physiological outcomes of cells under states of stress. Increased expression and/or activity of regulatory components involved in protein quality control, such as ER molecular chaperones (*e.g.*, BiP/GRP78, GRP94, calnexin), and processing enzymes (*e.g.*, protein disulfide isomerase), would be adaptive to maintain cellular integrity, functionality, and survival, until homeostasis is re-established or the stressful stimulus is removed[229]. When stresses exceed tolerable limits and cells become dysfunctional however, activation of factors leading to apoptosis, such as ATF4 and CHOP, may be favourable.

To investigate this, we infected 3T3 fibroblasts with adenoviruses at a multiplicity of viral infection of 10 that may more closely reflect moderate levels of RGS2 that may occur during stress (see Appendix A, Fig. A1 and A3) and still lead to a reduction in protein synthesis (refer to Fig. 3.1). Following infection, we used well-known chemical agents to induce cellular stress, such as tunicamycin, a natural inhibitor of N-linked protein glycosylation from *Streptomyces sp.* which leads to the disruption of post-translational modification of proteins in the ER[229], or thapsigargin, a compound that prevents calcium reuptake by inhibiting endoplasmic or sarcoplasmic reticulum calcium-ATPases, thereby inducing ER stress, and is also shown to induce autophagy in mammalian cells[107,108]. In our studies, we also used staurosporine, a general protein kinase C inhibitor, as a way to induce cellular stress, as this agent is known to be a strong inducer of apoptosis[211,230].

We have been able to verify that our selection of pharmacological treatments does induce stress within fibroblasts. Factors involved in the cellular stress response and the UPR such as ATF4, CHOP, BiP/GRP78, XBP1s, and the activation of caspase 3, indicative of apoptosis, were indeed upregulated with these treatments (see Appendix B, Fig. B4) and thus served as appropriate positive controls in our experiments. Furthermore, we hypothesized that the translation inhibition abilities of RGS2 may augment the cellular

stress response pathways and the UPR. To test this idea, we assessed the role of RGS2 on the expression of key components involved in such pathways under experimentally induced stress conditions.

3.2.1 RGS2 is transcriptionally upregulated by tunicamycin treatment

RGS2 is known to be upregulated by various forms of stress including heat shock[159,183], ischemia[177], oxidative stress[186], as well as agonist-induced Gαs- and Gαq/11-mediated signals associated in cellular hypertrophy[166,167,182]. Here, we observed that RGS2 gene expression is upregulated by tunicamycin in 3T3 fibroblasts (Fig. 3.7, two-way ANOVA, $p = 0.0124$). Additionally, this result is not from the infection using adenoviruses that encode for RGS2 as it was not detected by our primers (see Table 2.1) due to species differences of the cell type used (mouse) and the viral RGS2 construct (human). RGS2 mRNA levels increased in a concentration-dependent manner with tunicamycin stress and treatment at 10 μg/ml resulted in an approximately two-fold increase compared to vehicle control conditions.

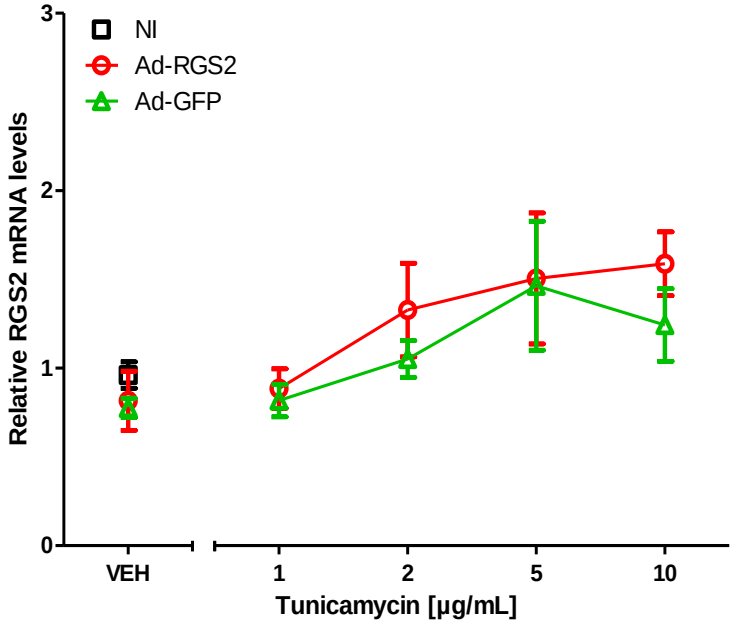


Figure 3.7. Dose-dependent increase in RGS2 gene expression with tunicamycin treatment. 3T3 fibroblasts were infected for 48 hours with adenoviruses encoding RGS2 or GFP at an MOI of 10, or were left uninfected (NI). RNA was then isolated 6 hours after treatment with 0.1% DMSO (vehicle control) or tunicamycin at the indicated concentrations. Treatment with increasing concentrations of tunicamycin significantly increased cellular mRNA levels of RGS2 ($p = 0.0124$) and no effect on RGS2 transcription was seen as a result of the infection with adenoviruses ($p = 0.2763$). Relative mRNA levels of RGS2 are expressed as means normalized to the geometric mean of two stable reference genes (GAPDH and β2 microglobulin) ± SEM run in parallel through qPCR. Data presented are from three independent experiments, run in triplicate. Statistical analysis was performed using two-way ANOVA, followed by Bonferroni post-hoc test.

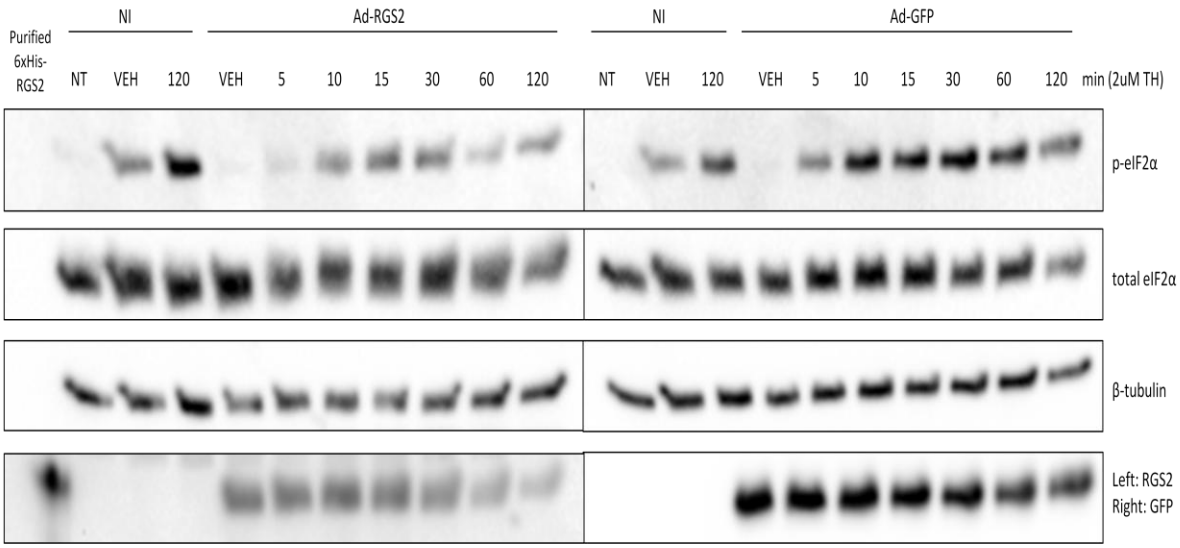
3.2.2 Effect of RGS2 expression on stress-induced phosphorylation of eIF2α

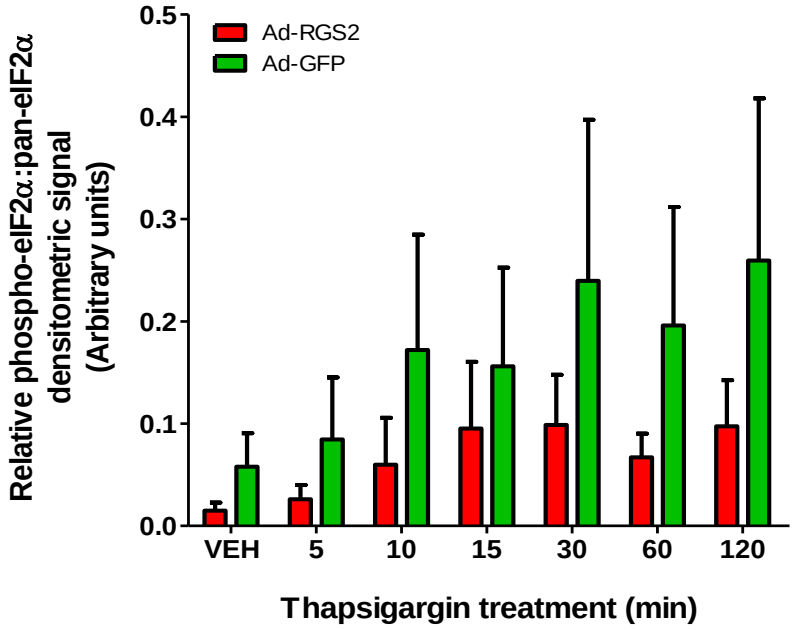
Phosphorylation of eIF2α is a hallmark of stress-induced inhibition of translation in order to decrease cellular protein load to conserve energy and resources. In mammals, four key kinases activated by different types of stress are known to converge and phosphorylate eIF2α[120,199]. These include GCN2, activated upon deficiencies in essential amino acids[69,119]; PERK, activated upon ER stress primarily as a result of dysregulation of protein synthesis[35,118,203]; PKR, activated during the presence of viral double-stranded RNA[118,120]; and HRI, activated during heme deficiency, oxidative stress, osmotic and heat shock[120,121]. Inhibition of *de novo* protein synthesis is considered a major defense mechanism against a wide variety of cellular stresses[3]. While global protein synthesis is reduced, particular key components involved in alleviating stressful conditions, such as the transcription factor, ATF4, are selectively translated through previously described alternative translational mechanisms. The eIF2α-ATF4 pathway is associated with several stress-response functions such as amino acid biosynthesis, ER-associated degradation, autophagy, and apoptosis[70]. Dysregulation of this pathway has been linked to numerous diseases such as cancer, metabolic disease, and neurodegenerative disorders[3,61,119,231].

To investigate whether the expression of RGS2 may modulate the activity of the eIF2α-ATF4 pathway and provide a parallel mechanism to regulate stress response via its ability to inhibit translation at initiation, we first looked at the levels of eIF2α phosphorylation in RGS2 expressing cells under stress. We have assessed the induction of phosphorylated eIF2α using both tunicamycin and thapsigargin, however, treatment with tunicamycin gave very limited phosphorylation of eIF2α (data not shown) while thapsigargin was a more robust inducer of ER stress[76] and induced the phosphorylation of eIF2α (Fig. 3.8). Interestingly, infection of cells with RGS2 adenovirus consistently resulted in significantly lower levels of phosphorylated eIF2α compared to GFP-infection controls (Fig. 3.8, two-way ANOVA, $p = 0.0323$). Induction of p-eIF2α peaked around 15 minutes of thapsigargin treatment in RGS2 expressing cells whereas levels of p-eIF2α were still detected up to 2 hours of thapsigargin treatment in corresponding GFP

expressing cells. This suggests that RGS2 may have been helping to alleviate ER stress. Furthermore, this data supports our current findings on the translational control of ATF4 and CHOP expression by RGS2 which appears to be independent of eIF2α phosphorylation.

Figure 3.8. RGS2 expression leads to lower levels of stress-induced eIF2α phosphorylation. 3T3 fibroblasts were infected with RGS2 or GFP-encoding adenoviruses (MOI = 10), or left uninfected (NI). Following 48 hours of infection, cells were treated with 2 μM of thapsigargin (TH) over the course of 2 hours and lysates were immunoblotted for levels of phosphorylated eIF2α. 0.1% DMSO was used as vehicle control. Under RGS2 infection conditions, significantly lower phosphorylated eIF2α levels were observed (two-way ANOVA, $p = 0.0323$), with peak amounts at 15 min of treatment with thapsigargin. GFP-infection controls showed sustained levels of phosphorylated eIF2α over the course of treatment. Membranes were stripped and reprobed for total eIF2α protein levels. Immunoblots shown are representative of four independent experiments, the densitometric data of which is summarized in the bar graph below as mean ± SEM, showing the relative level of eIF2α phosphorylation, determined by taking the ratio of p-eIF2α to signal obtained with pan-eIF2α antibody. Control for equal protein loading was assessed via immunoblotting for β-tubulin.


Purified 6xHis-RGS2
NI
Ad-RGS2
NI
Ad-GFP
NT VEH 120 VEH 5 10 15 30 60 120 NT VEH 120 VEH 5 10 15 30 60 120 min (2uM TH)
p-eIF2α
total eIF2α
β-tubulin
Left: RGS2
Right: GFP


Ad-RGS2
Ad-GFP
Relative phospho-eIF2α:pan-eIF2α densitometric signal (Arbitrary units)
0.5
0.4
0.3
0.2
0.1
0.0
VEH 5 10 15 30 60 120
Thapsigargin treatment (min)

3.2.3 Effects of RGS2 on the transcriptional regulation of stress-related targets of the UPR

To assess the role of RGS2 in the CSR, we looked at changes in the expression of endpoints of the UPR, such as BiP/GRP78 and XBP1s, in response to RGS2 expression under induced stress. Treatment with tunicamycin or thapsigargin generally led to increased protein levels of both of these targets compared to non-stressed states (Appendix B, Fig. B4). However, the low sensitivity of the available antibodies to consistently detect stress-induced changes of these endpoints led us to assess our questions using more sensitive, robust, and quantitative methods, such as changes in gene expression through real-time PCR. While our data on ATF4 and CHOP protein levels provided insights on translational effects with the expression of RGS2, we also examined whether RGS2 may regulate the activity of other pathways of the UPR at the transcriptional level.

3.2.3.1 Effect of RGS2 on BiP/GRP78 gene expression

BiP/GRP78 is an ER chaperone belonging to the heat shock protein 70 kDa (Hsp70) family of proteins and has quality control functions in the synthesis of secretory proteins by mediating proper protein folding. BiP is also known as a stress signal-regulating protein, keeping the activity of the three major transmembrane sensors that mediate ER stress response pathways (PERK/ATF4, IRE1/XBP1, and ATF6) in check[215,216,232–234].Treatment with tunicamycin significantly increased mRNA levels of BiP/GRP78 in a dose dependent manner (Fig. 3.9, two-way ANOVA, $p < 0.0001$). However, no significant effect was seen with RGS2 expression on the gene expression of BiP (two-way ANOVA, $p = 0.5191$). Assessment with Bonferroni post-hoc test showed that at the highest dose of tunicamycin, there was a significant difference ($*p < 0.05$) in BiP transcript levels in RGS2 expressing cells compared to GFP viral infection controls.

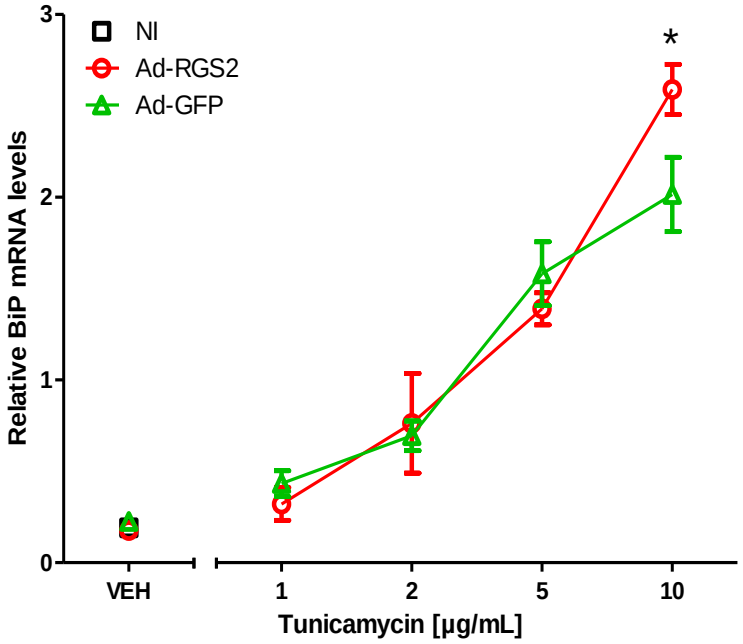


Figure 3.9. Dose-dependent increase of BiP gene expression with tunicamycin. 3T3 fibroblasts were infected for 48 hours with adenoviruses encoding for RGS2 or GFP at an MOI of 10, or were left uninfected (NI). RNA was then isolated 6 hours after treatment with 0.1% DMSO (vehicle control) or tunicamycin at the indicated concentrations. Treatment with increasing concentrations of tunicamycin significantly increased cellular mRNA levels of BiP. Relative BiP mRNA levels are expressed as means normalized to the geometric mean of two stable reference genes (GAPDH and β2 microglobulin) ± SEM run in parallel through qPCR. Gene expression data presented are from three independent experiments, run in triplicate. Statistical analysis was performed using two-way ANOVA followed by Bonferroni post-hoc test, where a significant difference in BiP transcript levels were observed in RGS2 expressing cells at the highest concentration of tunicamycin (*$p < 0.05$).

3.2.3.1.1 Effect of RGS2 on BiP ER stress response element promoter activity

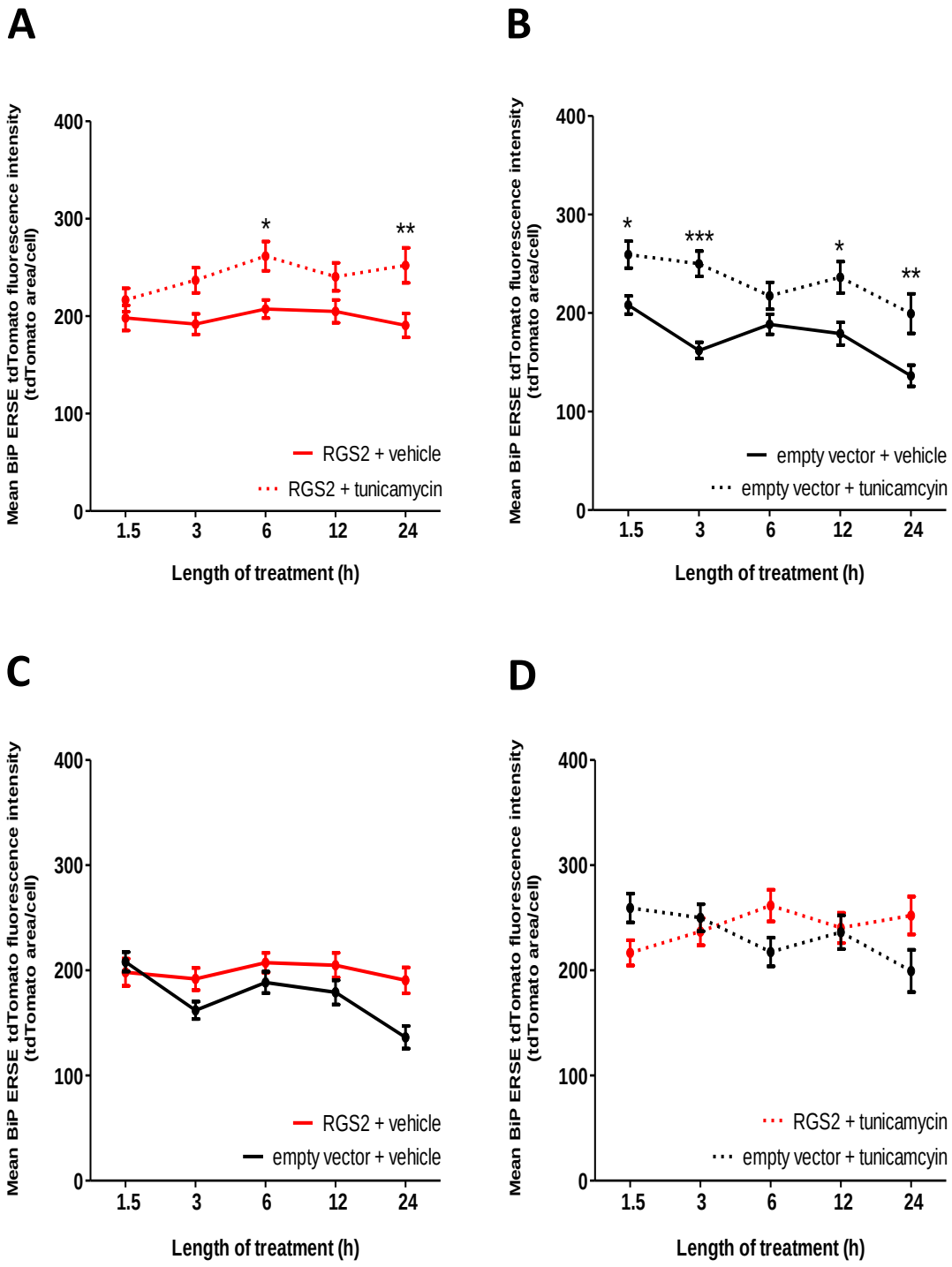
In response to the BiP/GRP78 real-time data obtained, we looked at the effects of RGS2 expression on the activity of promoters containing ER stress response elements (ERSE), such as that present in the BiP gene[215], as another method to evaluate whether RGS2 may affect gene expression of stress-adaptive proteins. Previous studies characterizing the promoter of *BiP/GRP78* showed it to be highly active under stress[215,235]. This promoter region contains several heat shock elements (HSEs) and an ERSE that is regulated by ATF6[65]. This suggests a positive feedback mechanism between BiP and ATF6 within the UPR to increase protein folding capacity. Disruption of protein glycosylation processes within the ER results in increased expression of BiP/GRP78, however, little is known about the regulatory elements involved in the transcriptional activation of BiP/GRP78[215].

RGS2 is upregulated during thermal stress[159,186] and recent studies show that this is mediated by the binding of heat shock transcription factor 1 (HSF1) to an HSE recently characterized in the *RGS2* promoter[183]. HSF1 functions principally as an activator of heat shock proteins, such as HSP70 proteins (of which BiP is a member), to protect against proteotoxic stress resulting from heat shock[236,237]. Whether there is competition for HSF1 binding during thermal stress is not known, but the fact that both RGS2 and BiP are upregulated by HSF1 in response to heat shock may lead to synergistic effects towards cell recovery. RGS2 functions to inhibit translation and whether this modulates the activity of parallel stress-response processes, such as in the expression of chaperones to increase protein folding capacity, is not well understood. Additionally, RGS2 has been shown to attenuate the transcription of target genes associated with aberrant protein synthesis that can lead to pathological hypertrophy[166,167]. Therefore, it is unclear whether RGS2 may affect expression through its ability to inhibit translation or perhaps by directly modulating the activity of ERSEs found in the promoters of stress proteins like BiP, of which we assessed the latter.

We co-transfected plasmids that encoded for FLAG-tagged RGS2 together with plasmids that encoded the BiP ERSE promoter region into 3T3 fibroblast cells. Relative changes in the activity of the promoter were quantified via fluorescence microscopy of the tdTomato

fluorescent reporter, tagged to BiP ERSE (see Appendix C, Fig. C1, for representative fluorescent images). Treatment with 5 μg/ml tunicamycin resulted in significantly greater tdTomato fluorescence compared to 0.1% DMSO vehicle-treated controls (Fig. 3.10A and B, two-way repeated measures ANOVA, $p < 0.001$). No temporal effects of tunicamycin treatment on BiP ERSE activity were apparent. Moreover, no significant effects were seen with RGS2 expression on BiP ERSE promoter activity (Fig. 3.10C and D, two-way repeated measures ANOVA, $p > 0.05$), which this corresponds to our real-time data where we showed no effect by RGS2 on BiP transcription under tunicamcyin-induced stress.

Figure 3.10. Dose- and time-dependent effects of tunicamycin and RGS2 expression on BiP ERSE promoter activity. Experiments were performed as described in materials and methods, where 3T3 fibroblasts were co-transfected with plasmids encoding for BiP ERSE-tdTomato together with full-length RGS2 (indicated with red lines) or pcDNA3.1 empty vector (indicated with black lines) for 24 hours. Cells were then treated with 0.1% DMSO (vehicle control, indicated with solid lines) or 5 μg/ml tunicamycin (indicated with dotted lines). Fluorescent images were taken at the indicated time points of treatment. Data presented is the relative fluorescence intensity averaged from three independent experiments. Treatment with 5 μg/ml tunicamycin significantly increased BiP ERSE promoter activity (**A** and **B**, two-way repeated measures ANOVA, $p < 0.001$). Such levels were observed at 6 hours and 24 hours of treatment in RGS2-transfected conditions (**A**, Bonferroni post-hoc test, $*p < 0.05$ and $**p < 0.01$) and at 1.5, 3, 12, and 24 hours after treatment in empty vector controls (**B**, Bonferroni post-hoc test, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$). In panels **C** and **D** the same data were replotted and reanalyzed to evaluate whether RGS2 influences BiP promoter activity, however, no difference was observed between RGS2 and empty vector expressing cells in either the absence (**C**) or presence (**D**) of tunicamycin (two-way repeated measures ANOVA, $p > 0.05$).


A
B
C
D
Mean BiP ERSE tdTomato fluorescence intensity (tdTomato area/cell)
Length of treatment (h)
1.5
3
6
12
24
0
100
200
300
400
*
**
***
RGS2 + vehicle
RGS2 + tunicamycin
empty vector + vehicle
empty vector + tunicamcyin

3.2.3.2 Effect of RGS2 on XBP1s transcript levels

The activity of IRE1 makes up one of the three major branches of the UPR that is activated upon the accumulation of misfolded proteins during ER stress. One of the most well studied functions of activated IRE1 is its endoribonuclease activity to result in the splicing of a 26-nucleotide intron within the XBP1 mRNA transcript. This causes a frame-shift during the translation of this spliced variant to produce a functional transcription factor (XBP1s)[11,40]. In mammals, XBP1s activates the expression of targets associated in cell differentiation, lipid synthesis, ER biogenesis, protein folding and degradation[11,43], and is therefore an important factor in the regulation of cellular responses during stress. Similar to our assessment on the role of RGS2 on BiP gene expression under stressed conditions, treatment with tunicamycin significantly increased XBP1s transcript levels in a dose dependent manner (Fig. 3.11, two-way ANOVA, $p < 0.0001$). However, no significant effect was seen with RGS2 expression on the level of XBP1s transcription (two-way ANOVA, $p = 0.2674$).

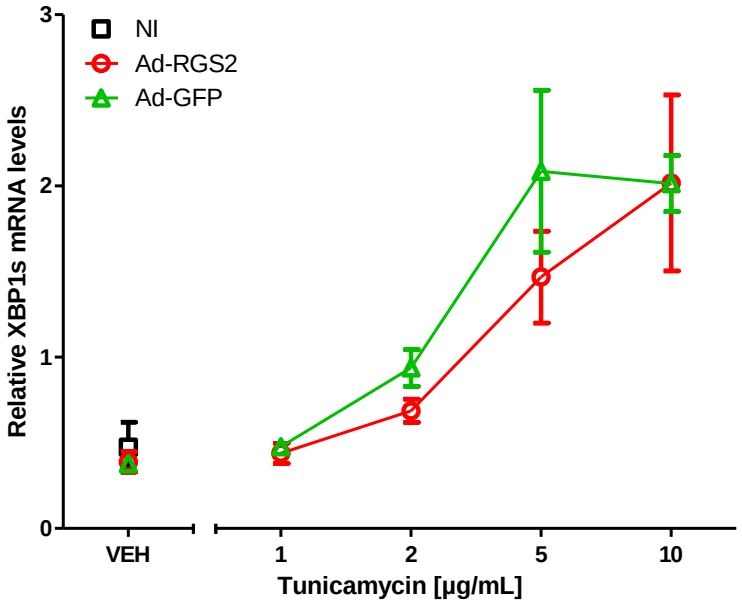


Figure 3.11. Dose-dependent increase of XBP1s transcript levels with tunicamycin. 3T3 fibroblasts were infected for 48 hours with adenoviruses encoding for RGS2 or GFP at an MOI of 10, or were left uninfected (NI). RNA was then isolated 6 hours after treatment with 0.1% DMSO (vehicle control) or tunicamycin at the indicated concentrations. Treatment with increasing concentrations of tunicamycin significantly increased cellular mRNA levels of XBP1s. Relative XBP1s mRNA levels are expressed as means normalized to the geometric mean of two stable reference genes (GAPDH and β2 microglobulin) ± SEM run in parallel through qPCR. Gene expression data presented are from three independent experiments, run in triplicate. Statistical analysis was performed using two-way ANOVA followed by Bonferroni post-hoc test, where no significant difference was seen with the expression of RGS2.

3.2.3.3 Effect of RGS2 on CHOP gene expression

CHOP is a major transcription factor that functions predominately to promote cell death by initiating and enhancing the activity of apoptotic pathways[58,72,238]. Downstream of the ATF4-CHOP mediated apoptotic pathway during the UPR, CHOP upregulates a number of effector proteins such as Bax and Bak that functionally carry out the destruction of cells, meanwhile decreasing the expression of anti-apoptotic factors, such as those belonging to the Bcl-2 family of proteins[55,72,80,239]. CHOP gene expression significantly increased in a concentration-dependent manner with tunicamycin induced stress (Fig. 3.12, two-way ANOVA, p <0.0001). Similar to BiP and XBP1s however, no significant differences in CHOP mRNA levels were seen with RGS2 expression (two-way ANOVA, p = 0.9571), suggesting no additive effect by RGS2 on stress-induced upregulation of CHOP. This further supports our findings presented in section 3.1.3 where although we showed that RGS2 and RGS2^{eb} expression on its own led to increased protein levels of CHOP in cells, the effect by RGS2 appeared to be translational in nature as no effect was seen on the transcription of CHOP.

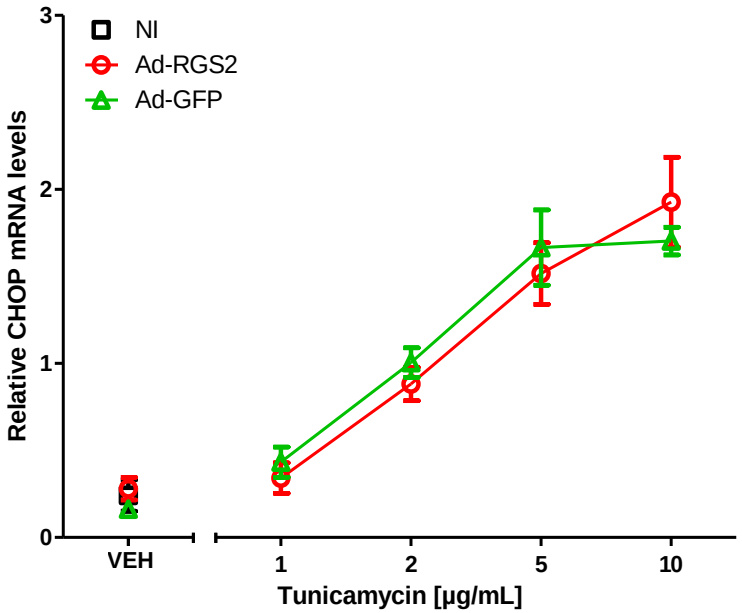


Figure 3.12. Dose-dependent increase of CHOP transcript levels with tunicamycin. 3T3 fibroblasts were infected for 48 hours with adenoviruses encoding for RGS2 or GFP at an MOI of 10, or were left uninfected (NI). RNA was then isolated 6 hours after treatment with 0.1% DMSO (vehicle control) or tunicamycin at the indicated concentrations. Treatment with increasing concentrations of tunicamycin significantly increased cellular mRNA levels of CHOP. Relative CHOP mRNA levels are expressed as means normalized to the geometric mean of two stable reference genes (GAPDH and β2 microglobulin) ± SEM run in parallel through qPCR. Gene expression data presented are from three independent experiments, carried out in triplicate. Statistical analysis was performed using two-way ANOVA followed by Bonferroni post-hoc test, where no significant difference was seen with the expression of RGS2.

3.2.4 Expression of ATF4 and CHOP in response to cell stress

ATF4 is a transcriptional activator of stress response targets involved in metabolism, nutrient uptake and amino acid biosynthesis, control of redox status, apoptosis and autophagy[222,240,241]. It does so by binding to CCAAT/enhancer binding protein-activating transcription factor (C/EBP-ATF) response element sequences in such target genes to increase their expression[3,63]. Since RGS2 can prevent translation at initiation, similar to the effect of p-eIF2α by inhibiting the formation of complete ribosomal complexes, we hypothesized that RGS2 might affect the expression of ATF4 and thereby modulate its known anti-apoptotic and pro-apoptotic effects during cellular stress. In section 3.1.3, we demonstrated that both full-length RGS2 and its eIF2Bε-binding domain, RGS2^{eb}, were sufficient to substantially increase ATF4 protein levels in cells. Here, we assessed whether this effect would be enhanced during stressed states at moderate levels of RGS2 expression. ATF4 protein levels increased in a concentration-dependent manner with tunicamycin, as shown in Figure 3.13 (A and B). Similarly, protein levels of CHOP increased in a concentration-dependent manner with tunicamycin treatment as well (Fig. 3.13 A and C). Interestingly, no significant differences were seen with RGS2 expression however. This protein expression profile for CHOP parallels the corresponding gene expression data obtained in section 3.2.3.3, where CHOP mRNA levels increased in a dose dependent manner with tunicamcyin treatment but no difference in this effect was observed between RGS2- and GFP-expressing cells.

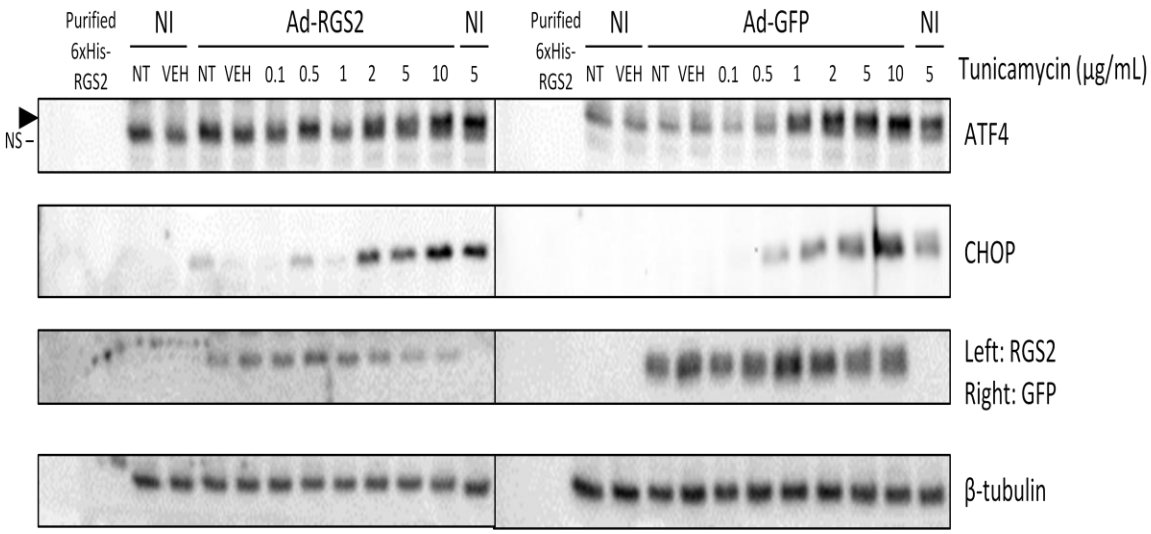
3.2.5 Effect of RGS2 on stress-induced apoptosis

During times of stress, it is expected that the primary responses of cells would be to increase cellular functions toward recovery and survival. However, if such responses are unsuccessful in mitigating the stressful conditions, the accumulation of dysfunctional cells and processes may be detrimental to the organism. Cells would therefore engage in a series of metabolic changes and activate caspase cascades, leading to apoptosis[57]. As described previously, caspase 3 is one of the effector caspases that contributes to the molecular and biochemical changes within cells leading to programmed cell death. Staurosporine is reported to be a strong inducer of apoptosis in various cell lines[208,209,213,242,243] and was found to induce caspase 3 cleavage in our experiments (see

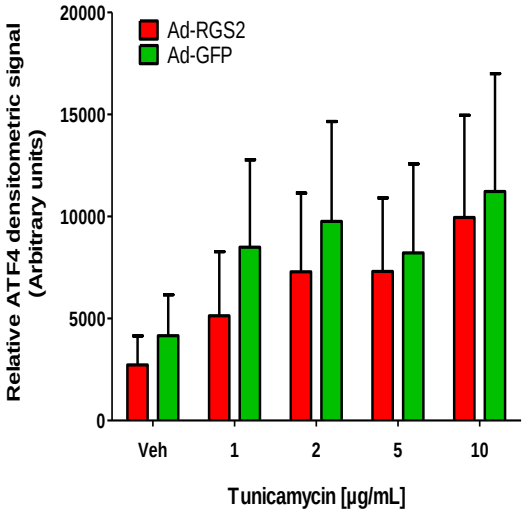
Appendix B, Fig. B4 F), and thus was used to investigate possible roles of RGS2 in stress-related apoptosis. Morphological changes characteristic of apoptosis were seen at all concentrations of staurosporine treatment, including cell shrinking, blebbing, formation of apoptotic bodies, and cytoplasmic fragmentation (see Appendix B, Fig. B2 and B3), although these were not quantified. Viral infection on its own with either of the adenoviruses at an MOI of 10 did not appear to evoke cell death (Fig. 3.14). Caspase 3 activation significantly increased in a dose-dependent manner with staurosporine treatment (Fig. 3.14, two-way ANOVA, $p = 0.0001$). Significant differences with RGS2 expression were only observed at the highest dose of staurosporine, where greater caspase 3 activation was observed in RGS2 expressing cells than GFP expressing cells (Bonferroni post-hoc test, $*p < 0.05$).

Figure 3.13. Effects of RGS2 on stress-induced ATF4-CHOP pathways. 3T3 fibroblasts were infected for 48 hours with adenovirus encoding for RGS2 or GFP (infection control) at an MOI of 10, or left uninfected (NI). Cells were then treated for 6 hours with 0.1% DMSO (vehicle control), tunicamycin at the indicated concentrations, or not treated (NT). (**A**) Immunoblots show a dose-dependent increase in ATF4 and CHOP protein levels with tunicamycin treatment. ATF4-specific protein band is indicated by an arrowhead, NS = non-specific band. Immunoblot data shown are representative of three independent experiments where changes in ATF4 and CHOP protein levels were quantified via densitometry, as summarized in the corresponding bar graphs below, presented as mean ± SEM (**B** and **C**). Control for equal protein loading was assessed via immunoblotting for β-tubulin.

A


Purified 6xHis-RGS2
NI
Ad-RGS2
NI
Purified 6xHis-RGS2
NI
Ad-GFP
NI
NT VEH NT VEH 0.1 0.5 1 2 5 10 5
NT VEH NT VEH 0.1 0.5 1 2 5 10 5
Tunicamycin (µg/mL)
NS
ATF4
CHOP
Left: RGS2
Right: GFP
β-tubulin

B


Relative ATF4 densitometric signal (Arbitrary units)
20000
15000
10000
5000
0
Ad-RGS2
Ad-GFP
Veh
1
2
5
10
Tunicamycin [µg/mL]

C

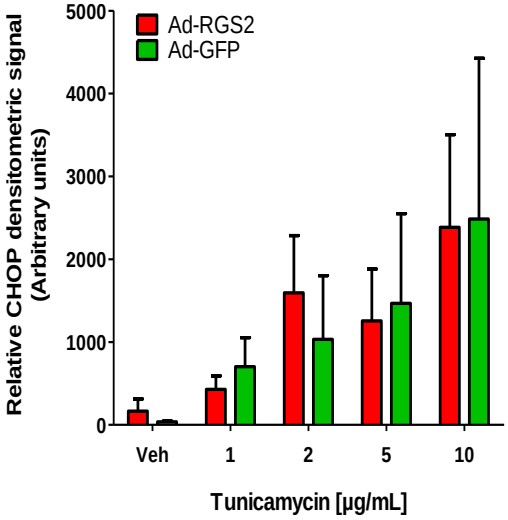
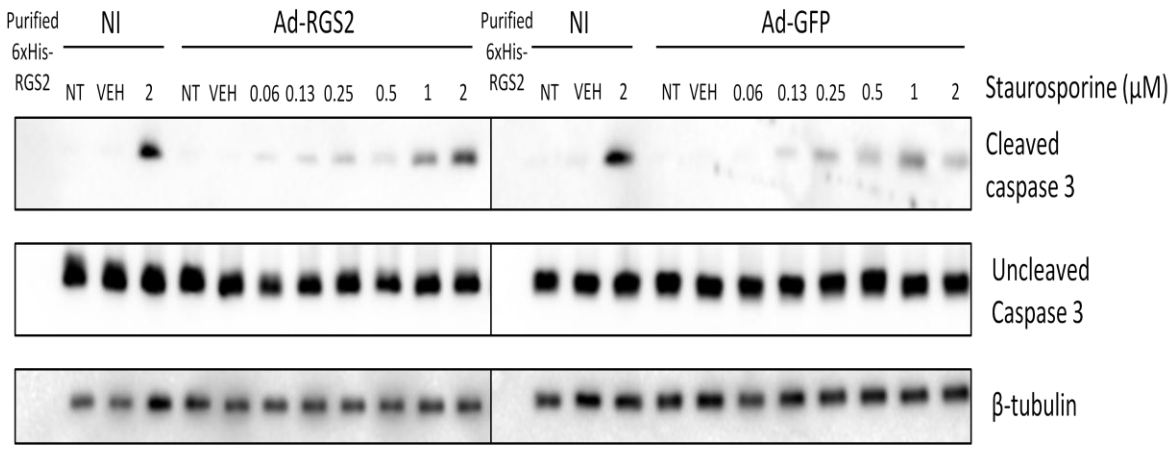
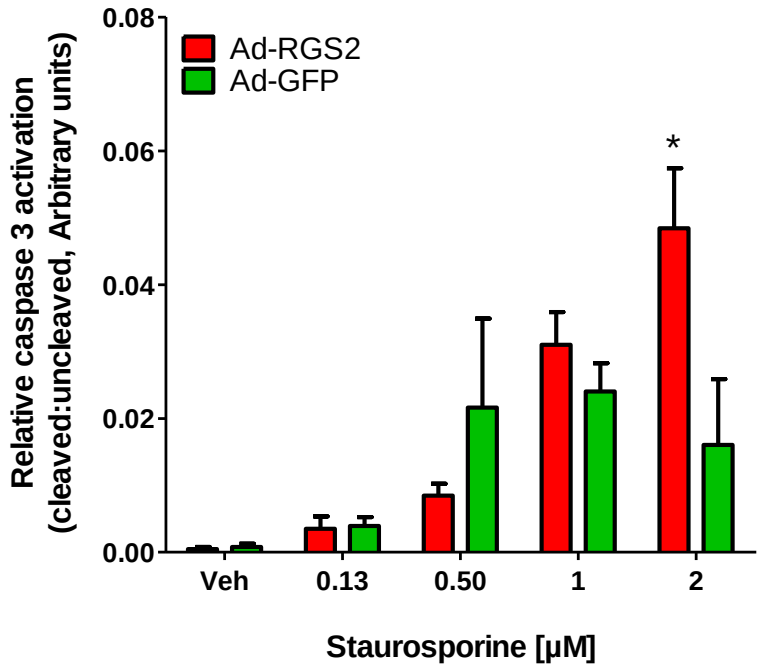

Relative CHOP densitometric signal (Arbitrary units)
5000
4000
3000
2000
1000
0
Ad-RGS2
Ad-GFP
Veh
1
2
5
10
Tunicamycin [µg/mL]

Figure 3.14. Effects of RGS2 on stress-induced apoptosis. 3T3 fibroblasts were infected for 48 hours with adenovirus encoding for RGS2 or GFP (infection control) at an MOI of 10, or left uninfected (NI). To assess the role of RGS2 expression on apoptosis, cells were treated with staurosporine at the indicated concentrations for 4 hours. Treatment with staurosporine increased the levels of cleaved caspase 3 in a dose-dependent manner. Membranes were then stripped and reprobed to assess total uncleaved caspase 3 protein levels. Relative caspase 3 activation was then taken as a ratio of cleaved caspase 3 signal to uncleaved caspase 3 signal. Immunoblot data shown are representative of three independent experiments and quantified via densitometry, as summarized in the corresponding bar graph below, presented as mean ± SEM. Control for equal protein loading was assessed via immunoblotting for β-tubulin.


Purified 6xHis-RGS2
NI
Ad-RGS2
Purified 6xHis-RGS2
NI
Ad-GFP
NT VEH 2 NT VEH 0.06 0.13 0.25 0.5 1 2 NT VEH 2 NT VEH 0.06 0.13 0.25 0.5 1 2
Staurosporine (μM)
Cleaved caspase 3
Uncleaved Caspase 3
β-tubulin


Relative caspase 3 activation (cleaved:uncleaved, Arbitrary units)
0.08
0.06
0.04
0.02
0.00
Ad-RGS2
Ad-GFP
*
Veh
0.13
0.50
1
2
Staurosporine [μM]

Chapter 4

4 Discussion

We have previously shown that RGS2 is able to inhibit *de novo* protein synthesis as a result of its interaction with eIF2B and interference with eIF2-eIF2B GTP-exchange activity[160]. This limits the amount of activated eIF2-GTP that is a necessary component within the translational machinery for initiation to occur[160]. This effect is similar to those triggered by stresses that promote the phosphorylation of eIF2α, and in fact, many of the same stressors that trigger eIF2α phosphorylation also upregulate RGS2[186,244]. The inhibition of initiation leads to a reduction of global protein synthesis, allowing the conservation of energy and resources, but it also upregulates factors preferentially produced through alternative translation mechanisms, such as ATF4[61]. Whether or not the translational control abilities of RGS2 may contribute to alternative translation mechanisms and modulate stress response is not well understood. The goals of the studies presented in this thesis were to delineate other possible mechanisms in the regulation of stress response pathways, including apoptosis, and how they may be modulated by RGS2. We provide evidence demonstrating that RGS2 can modulate pathways of the UPR, in particular the eIF2α-ATF4 axis, and this adds to the current knowledge of the molecular mechanisms and components involved in the cellular stress response.

4.1 Translational control by RGS2 increases the expression of stress response factors: A novel mechanism independent of eIF2α phosphorylation

Mechanisms of the cellular stress response are highly conserved in all cells and, for the most part, the end result of exposure to stress depends on the damage sustained (*e.g.*, if protein or DNA was damaged or if the stress was as a result of an imbalance in organic molecules and ions *etc.*) and less on the specific stimuli that first evoked the damage[2]. For example, thermal stress (*i.e.*, heat shock), oxidative stress (*i.e.*, reactive oxygen species), or known chemical compounds that can disrupt protein synthesis at the ER (*e.g.*, tunicamcyin or thapsigargin), all can lead to the misfolding of proteins and result in cellular dysfunction. Consistent with this idea, several key kinases activated by such

stresses have been found to converge and phosphorylate eIF2 to inhibit initiation, suggesting that this is a pivotal target and molecular pathway taken to alleviate stress[29,239].

A variety of mechanisms exist that lead to the reduction of protein synthesis, most of which predominantly involve regulation at initiation. The rate of initiation is limited by the activities of eIF2 and eIF4F. eIF4F is a complex of eIF4 initiation factors composed of eIF4A, E, and G which are involved in the recruitment of an mRNA to a ribosome for translation to occur[245]. In particular, eIF4E is responsible for the recognition of the 5'cap structure of mRNAs facilitating their recruitment to a ribosome[245]. A family of eIF4E-binding proteins blocks such functions of eIF4E, thereby regulating cap-dependent translation[246]. Moreover, the rate-limiting step in initiation is the GTP exchange cycle that occurs between eIF2 and eIF2B in order to generate activated eIF2 that is required to bring the initiator methionine to start the process of protein synthesis[114,247]. The most well-known mechanism to impede this involves the phosphorylation of eIF2α, which increases its binding affinity and sequesters the activity and availability of eIF2B[61,115,116]. While this reduces protein synthesis in general, some stress response proteins are selectively upregulated via alternative translation. Previously, our lab has shown that RGS2 can bind to eIF2B and inhibit protein synthesis in various cell types[160]. This provides a similar regulatory effect on protein translation. However, whether this effect by RGS2 modulates the expression of proteins produced through alternative translation mechanisms during stress was not known and was one of the aims addressed in this research.

Thapsigargin induces ER stress by depleting ER calcium levels which further leads to the loss of activity of calcium-dependent ER chaperones, such as calnexin, resulting in the accumulation of unfolded proteins[16]. Interestingly, we showed that expression of RGS2 under thapsigargin-induced stress led to lower levels of phosphorylated eIF2α. A possible explanation to this may be as a result of reduced protein synthesis by RGS2. The data presented in this thesis showed that the expression of RGS2 does indeed decrease total protein levels, implying an effect by RGS2 on translation, and complements previous studies from our lab which showed that RGS2 can interfere with the initiation machinery

and prevent *de novo* protein synthesis[160]. This would reduce protein load and the amount of unfolded proteins, thereby alleviating ER stress. Reversion of pathways of the UPR would be expected, such as the re-association of BiP to PERK, thereby silencing its kinase activity, which would explain the decreased levels of phosphorylated eIF2α. As well, total eIF2 appears to have been increased in RGS2-expressing cells (data not shown, $p = 0.0271$), which could also have affected the ratio of phosphorylated to total eIF2 in these cells. This increase in eIF2 may perhaps act as a compensatory mechanism as a result of reduced protein synthesis by RGS2 to balance basal translational needs of the cell to maintain functionality.

The increased translation of ATF4 is generally attributed to the decrease in eIF2 activation as a result of the phosphorylation of eIF2α. While previous studies from our lab identified RGS2 as another means to reduce levels of activated eIF2, it was not known whether this would increase ATF4. The current studies directly tested this and demonstrated that RGS2 does substantially induce ATF4 under non-stressed states. In fact, this effect could be achieved with just the 37 amino acids eIF2Bε-binding domain of RGS2 ($RGS2^{eb}$). Furthermore, no measurable increase in level of phosphorylated eIF2α could be detected in either of these cases. This suggests that it is the inhibition of eIF2 and not its phosphorylation that contributes to the molecular changes associated with reduced translation.

To summarize, two major findings arise as a result of these studies. Firstly, RGS2 expression can modulate the translation of genes normally only expressed under times of stress-induced inhibition of protein synthesis, and this is most likely attributed to the ability of RGS2 to inhibit translation and drive alternative translation mechanisms. Secondly, the upregulation of stress-induced effectors mediated by alternative translation mechanisms is a result of decreased eIF2 activity and not necessarily its phosphorylation. These findings outline a novel way to modulate pathways of stress response by RGS2, in addition to the known molecular repertoire mediated by phosphorylated eIF2a.

4.2 Effects of RGS2 on the enhanced expression of stress response factors occurs through translational means and not through transcription

Our data presents an interesting finding in regards to how the expression of stress proteins is regulated. We showed that both RGS2 and RGS2^{eb} substantially increased cellular protein levels of ATF4 and CHOP without an increase in transcription of either of these two stress response factors.

The mRNA molecules encoding stress-activated proteins are in some cases found to contain multiple start sites (triplet AUG sequences), leading to alternative open reading frames that give rise to different protein isoforms of a target gene, depending on where the ribosomal machinery initiates translation. These protein isoforms may have very different physical properties, such as in protein biochemistry and structure, which may influence its stability, activity, and/or functionality, and affect cellular physiology. As mentioned previously, the structural features of the mRNA transcripts themselves, the "scanning" mechanism of ribosomes to translate mRNA, and the availability of components required for the formation of competent initiation complexes (*i.e.*, eIF2-GTP-Met-tRNAi) can affect where ribosomes initiate translation and lead to the production of such protein isoforms[34,126]. During times of stress, the relative scarcity of activated eIF2-GTP leads to a delay in initiation, resulting in the tendency of scanning ribosomes to bypass upstream AUG sequences and promote initiation at downstream AUG sequences[34]. ATF4 is one such stress protein whose expression is regulated by such mechanisms, controlled by two open reading frames located upstream of the main opening reading frame that encodes for functional ATF4 in its transcript[63]. Here we provide direct evidence that this can also be achieved by RGS2 and more specifically through its inhibitory effects on translation by RGS2^{eb}. RGS2^{eb} interacts with eIF2B which impedes the GTP-exchange activity between eIF2B and eIF2[160] thereby reducing the amount of activated eIF2. ATF4 translation would therefore be expected to increase, which was observed in these present studies. Furthermore, as reported in literature, the presence of cellular ATF4 is predominantly regulated by its translation[62,63,70] and post-translational modifications affecting its stability[62], and less on its transcription. ATF4

mRNA is ubiquitously found and in relatively abundant amounts[62,248]. This possibly explains why no increase in ATF4 transcript levels was observed with RGS2 or RGS2^{eb} and also strongly points to its regulation more so by translational means. Less is known regarding the transcription of ATF4, although some studies report that transcriptional control of ATF4 can be stress-dependent[249,250]. The lack of a transcriptional effect by RGS2 is not due to its absence from the nucleus or inefficiencies in translocation to the nucleus. Although RGS2 does not have a nuclear import signal, nuclear accumulation of RGS2 does occur and it enters the nucleus by passive diffusion[251]. Many RGS proteins localize to the nucleus and in some cases have been shown to affect transcription[252,253], although this has not been seen with RGS2.

ATF4 is a transcriptional activator of CHOP, thus we expected that greater CHOP transcription would occur as a result of the increase in levels of ATF4 with RGS2 expression. Interestingly, cells infected with RGS2 or RGS2^{eb} showed increased CHOP protein expression whereas CHOP transcription was essentially unaltered. CHOP mRNA consists of an upstream open reading frame that encodes a short 31 amino acids protein that strongly represses basal translation of functional CHOP by inhibiting translation reinitiation at the downstream CHOP open reading frame during quiescent conditions[64,218]. Therefore, similar to ATF4, CHOP protein expression is enhanced under times of reduced translation via alternative translation mechanisms which would explain our findings on the increased CHOP protein levels by RGS2. Additionally, the transcription of CHOP is known to be regulated by other stress-activated transcription factors, such as ATF6 and XBP1[72,223]. In fact, it has been reported that the presence of multiple transcription factors in the pathways of the UPR is required to maximally induce the transcription of CHOP[223,224]. A study by Okada *et al.* (2002) showed that the expression of ATF6 only induced a 2-fold increase in CHOP gene expression and overexpression of ATF4 alone was not sufficient for the induction of CHOP mRNA while the induction of both pathways resulted in more than a 20-fold induction of CHOP mRNA[224]. It is possible that CHOP gene expression may have been transiently upregulated by RGS2-promoted ATF4 translational upregulation but returned to baseline during the relatively long infection period, as opposed to the acute treatment times with

thapsigargin and tunicamycin. This provides a possible reason for the lack of an increase in CHOP transcription even though RGS2 significantly increased ATF4 protein levels.

The disparate outcomes in protein and gene expression of both ATF4 and CHOP as a result of the expression of RGS2, plus the translational control abilities of RGS2, strongly suggests that RGS2 can promote alternative translation as a part of its role in the stress response.

4.3 Effects of RGS2 on the expression of stress-activated endpoints of the UPR

For the most part, moderate expression of RGS2 did not appear to either increase or decrease the expression of components of the UPR under experimentally induced stress. This is surprising considering the effects we observed on ATF4 and CHOP protein expression as a result of the expression of RGS2 or RGS2^{eb} on its own, as well as the lowered levels of phosphorylated eIF2α with RGS2 expression. These included endpoints associated with cell recovery, such as the XBP1 splice variant (XBP1s), which is a reportedly active and stable transcription factor involved in the transcriptional activation of genes encoding for chaperones and degradation enzymes[203]. While levels of the spliced transcript of XBP1 significantly increased with tunicamycin induced stress, there was no effect with RGS2, suggesting that RGS2 may not affect the IRE1-XBP1s branch of the UPR. However, although RGS2 did not appear to affect XBP1s expression, RGS2 may influence stress-activated IRE1 pathways through other means. For example, we have not tested whether the expression of RGS2 may affect other stress-response processes mediated by IRE1 such as the activation of mRNA degradation (*i.e.*, RIDD)[32,47] or apoptotic pathways[11,13], or perhaps on the activation of IRE1 itself through assessing its phosphorylation.

BiP is a chaperone that is essential for the proper folding of proteins in the ER. Inhibition of translation by RGS2 would decrease protein load to prevent further accumulation of unfolded or misfolded proteins, and thus alleviates or reduces the effects of stress. This would reduce the need for the expression of BiP and conserve energy and resources. Therefore, it was predicted that BiP expression levels would be lower in conditions with

RGS2; however this was not the case. Treatment with tunicamycin significantly increased BiP transcript levels and RGS2 expression did not appear to have an effect on this. In fact, at the highest concentration of tunicamycin, greater BiP transcript levels were seen with RGS2 expression. The reasons for this are unclear but points to the fact that BiP appears to be regulated transcriptionally under times of cell stress. Also, this provides further evidence that RGS2 does not appear to have functions in transcription, consistent with the ATF4 and CHOP gene expression data presented. This is further supported through our assessment on BiP ERSE promoter activity where we saw no effect by RGS2 on tunicamycin-induced BiP promoter activity. This suggests that other factors may be recognizing and binding to the BiP ERSE, such as ATF6[50,65], that is driving its activity during stress but not RGS2.

4.4 Effects of RGS2 on stress-induced apoptosis

A recent study by Endale *et al.* (2010) showed that the upregulation of RGS2 by ischemic stress resulted in greater astrocyte cell death[177]. Since RGS2 can prevent translation at initiation, similar to the effects by eIF2α phosphorylation, we hypothesized that RGS2 might modulate the expression of factors of the CSR, such as ATF4, and thereby modulate its downstream anti-apoptotic or pro-apoptotic effects under states of stress. We found that ATF4 protein levels increased in a concentration-dependent manner with tunicamycin induced stress. Similarly, protein levels of CHOP increased in a concentration-dependent manner with tunicamycin treatment as well and these increases were comparable to levels found in neurons treated with tunicamycin[223]. However, RGS2 expressing cells did not induce any greater levels of ATF4 or CHOP proteins as that seen with the GFP viral infection controls under stress. Also, cells infected with adenoviruses encoding for the expression of RGS2 and treated with staurosporine only showed greater caspase activity than GFP-infection controls at the highest concentration of the stress inducer. Therefore, whether RGS2 may be pro-apoptotic during stress remains elusive.

RGS2 can perhaps affect different but overlapping pathways of the cell stress response other than the UPR. These include the mitogen-activated protein kinase (MAPK) pathways, heat shock response, or oxidative stress response[1,2], and the multiplicity of its

effects may possibly explain the minimal effects by RGS2 on endpoints of the CSR under experimentally-induced stress seen here. Previous studies conducted in our lab showed intriguing results whereby RGS2 expression appeared to alter the activation of various MAP kinases. MAPKs make up one of the largest family of protein kinases involved in the transduction and amplification of cellular signals, including those initiated by GPCRs, and regulate major cellular processes such as cell proliferation, differentiation, and death[254–256]. Additionally, MAPK signalling pathways are shown to be activated in response to ER stress and form part of the UPR[255,257]. Our lab has previously shown that RGS2 can inhibit agonist-induced Gαq/11- and Gαs-mediated hypertrophy in cardiomyocytes and this may be attributed to the observed decreases in activation of the MAP kinase ERK1/2 or other stress-activated kinases such as Akt[166,167]. This makes sense as ERK 1/2 and Akt are both highly involved in cell growth and proliferation functions[254–257], so a decrease in the activity of such MAPKs would be expected to prevent hypertrophy. Those studies also showed an increase in the levels of activated JNK and p38 with RGS2 overexpression[166,167], and these MAP kinases are linked to pro-apoptotic functions[254,255,257–259], possibly explaining the pro-apoptotic effects of RGS2. Indeed, the activation of p38 appears to be an important component in RGS2-induced astrocyte cell death under ischemic stress[177]. Endale *et al.* (2010) showed that the inhibition of p38 activation by a selective inhibitor prevented RGS2 upregulation and resulted in greater astrocyte survival during ischemia[177]. These studies indicate a relationship between p38 MAPK activation and RGS2 to affect physiological outcomes, however, this may be cell-type specific as thapsigargin-treated RGS2-expressing 3T3 fibroblasts did not affect p38 activation (data not shown, $p > 0.05$), and this may also be attributed to the timing of detection of its activation. Thus, the cross-talk between RGS2 and the activity of the MAP kinase pathways may very well influence the molecular pathways in cellular stress response but this remains equivocal based on our results and may involve other factors.

The protein data presented here suggests that RGS2 may be pro-apoptotic. Upstream of caspase 3, increases in ATF4 and CHOP were observed with RGS2 and RGS2^{eb} expression. Additionally, other pathways of the cell stress response and the UPR, such as those regulated by IRE1, are known to mediate apoptosis as well[13] and they may have

contributed to the effects we observed, although we did not specifically test them in this current body of work. Moreover, expression of RGS2^{eb} resulted in significant increases in caspase 3 cleavage. Under *in vitro* models of stress, greater levels of cleaved caspase 3 were observed in RGS2 expressing cells compared to GFP viral infection controls, albeit at the highest concentration of the stress treatment with staurosporine, further suggesting a pro-apoptotic effect by RGS2. Depending on the context and physiological state of the cell, cell death may be preferable when the stress cannot be resolved to remove damaged and dysfunctional cells before detrimental effects occur.

4.5 Role of RGS2 in the stress response

The relative benefits of RGS2 expression during stress are unclear and based on the findings presented here, there may potentially be multiple roles for RGS2 in the stress response, some of which may be distinct from its translational control ability. Decreased protein synthesis is beneficial during times of stress to conserve energy and resources but this also leads to the preferential upregulation of proteins such as ATF4 and CHOP, which are known to promote apoptosis. Outside of the pro-apoptotic effects of ATF4 and CHOP, both also have important physiological and protective functions in cell stress responses. ATF4 upregulates the expression of NADH-cytochrome B5 reductases and asparagine synthetases to combat oxidative stress or increase amino acid transport during times of amino acid deficiency, respectively[62]. CHOP regulates the expression of a variety of genes involved in cell differentiation, proliferation and immune functions[72]. Therefore, the expression of these two factors forms an important component in stress response. We showed that the expression of RGS2 and RGS2^{eb} alone can substantially increase the levels of both of these factors and therefore may modulate physiological outcomes in cell stress responses. One may speculate that the structural and biochemical differences between the full-length RGS2 and the much shorter RGS2^{eb} may lead to differences in their cellular localization (*i.e.*, RGS2^{eb} may be more strongly and rapidly recruited to ribosomal complexes to affect initiation factors, resulting in greater induction of ATF4 and CHOP) and/or potency. Indeed, Heximer *et al.* (2001) showed that truncation mutants of RGS2 exhibit different subcellular localization, so this is highly probable with RGS2^{eb} as well and it might lead to different physiological outcomes[251].

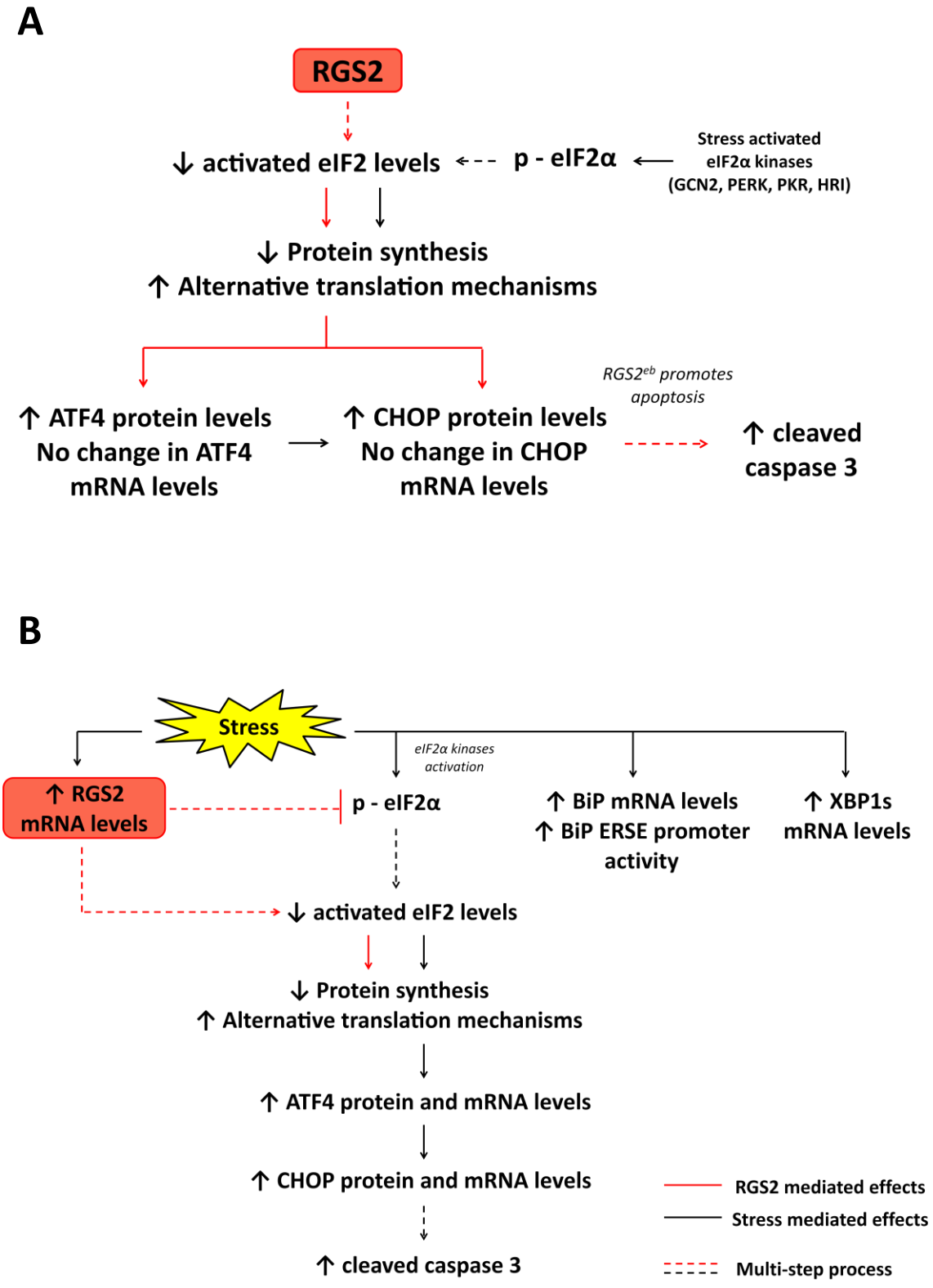
Additionally, the expression of RGS2^{eb} increased the levels of cleaved caspase 3, implying pro-apoptotic effects. Whether this is linked to the increase in ATF4 and CHOP expression is uncertain as such increases in caspase 3 activation were not seen with the expression of full-length RGS2. This suggests that RGS2 has different functional domains and these are required to "fine-tune" its effects in stress response. On one hand, the translational control ability mediated by its RGS2^{eb} domain ensures the reduction in protein synthesis and drives the upregulation of stress response factors such as ATF4 and CHOP for their protective effects during stress. On the other hand, other domains within the full-length RGS2 may exist to ensure the repression of ATF4-CHOP mediated apoptotic effects.

We and others have identified different domains of RGS2 that mediate other important cellular processes. Canonical functions of RGS2 include its activities to modulate GPCR and G protein-mediated signals, either through its GTPase-accelerating activity on Gα subunits mapped to the conserved 120 amino acids RGS domain[142], or indirectly via affecting G protein-mediated effectors[149–151,164,260]. The physiological importance of this is shown where overexpressed RGS2 can block agonist-induced Gαq/11- and Gαs-mediated cellular hypertrophy[166,167], while a loss of endogenous RGS2 can exacerbate cellular hypertrophy[168]. Our lab has also shown that inhibition of heat shock-induced RGS2 upregulation via siRNA knockdown diminishes the repression of *de novo* protein synthesis, implicating that RGS2 expression as a cellular mechanism in regulating translation in response to stress[159]. Furthermore, several studies have shown the importance in the regulation of G protein-mediated signals and protein synthesis and the cross-talk between these two cellular processes in regulating cardiac physiology[127,261,262]. RGS2 has been shown to be important in these processes, where the eIF2B-interacting domain of RGS2 is shown to be protective against GPCR agonist-induced hypertrophy in cardiomyocytes[134], and RGS2-null mice experience greater cardiac morbidity and mortality with induced pressure-overload on the heart[187]. Other functional domains identified in RGS2 include a stretch of amino acids from residues 1–82, situated outside of the RGS domain, that interacts with TRPV6 ionotropic receptors and disrupt ionic currents[158], while another 20 amino acids domain, also located outside of the RGS domain, appears to have functions in enhancing microtubule polymerization affecting

cellular structure[157]. A study by Dusonchet *et al.* (2014) also showed RGS2 to be an interacting partner of leucine-rich repeat kinase 2 (LRRK2), likely via its GAP domain. A growing body of work show that mutations in the LRRK2 gene have emerged to be a genetic determinant in Parkinson's disease, and the same group of researchers showed that RGS2 appears to be protective against mutant LRRK2-induced neurite shortening by regulating the GTPase and kinase activities of LRRK2[263]. Several important functional domains exists in full-length RGS2 that would not be present in RGS2^{eb} which may explain some of the molecular differences seen in these present studies between RGS2 and RGS2^{eb}. While it is uncertain whether these domains or ones yet to be identified in RGS2 may function to modulate pathways of the UPR and be a repressor of caspase 3, it is clear from these current results that the RGS2 eIF2Bε-binding domain appears to drive an opposite and pro-apoptotic effect.

Recently, studies have characterized four initiator methionines in the mRNA of RGS2 at residues 1, 5, 16, and 33 that give rise to different protein isoforms of RGS2[264]. Structurally, all four protein products still contain the conserved RGS domain and RGS2^{eb}. Functionally, all four RGS2 isoforms are still able to carry out its activities in attenuating Gαq/11-mediated signals, however isoforms arising from translation at residues 16 and 33 result in RGS2 products having impaired adenylyl cyclase activity[264]. The presence of alternative translation start sites suggests the possibility of RGS2 being differentially regulated during times of stress, similar to that of other stress proteins, further suggesting roles of RGS2 in stress response. Currently, it is not known whether particular initiation start sites are preferentially used during stress and, if so, whether the resulting products have altered activity in its other functional domains, *i.e.*, RGS2^{eb}, thereby affecting its translational control abilities. Ribosomal footprint profiling, a technique which can provide a global snapshot of only the mRNAs that are actively being translated, *i.e.*, bound by ribosomes, at a particular moment[265], may be valuable to determine which initiation start sites of RGS2 are being translated under different conditions, such as during stressed states. Collectively, the results presented here demonstrate the potential for multiple roles of RGS2 in the stress response (Fig. 4.1).

Figure 4.1. Summary of findings. Currently, our data shows the potential for multiple roles of RGS2 in the stress repsonse. (**A**) We have demonstrated that RGS2, apparently via its eIF2B-binding domain ($RGS2^{eb}$), can promote alternative translation in a manner analogous to that mediated by the phosphorylation of eIF2α. Furthermore, the present results imply that RGS2 and $RGS2^{eb}$ drive the expression of both ATF4 and CHOP via translational as opposed to transcriptional mechanisms, and that $RGS2^{eb}$ is pro-apoptotic. (**B**) As expected, stress induced the phosphorylation of eIF2α, increased the expression of factors invovled in cell stress response such as ATF4, CHOP, BiP, XBP1s, and increased caspase 3 cleavage. Additionally, treatment with tunicamycin resulted in an increase in RGS2 mRNA levels. In contrast to our original speculation, moderate expression of RGS2 did not appear to either increase or decrease the expression of any of these factors under experimentally induced stress, although lower levels of phosphorylated eIF2α were observed in RGS2 expressing cells.


A
RGS2
↓ activated eIF2 levels
p - eIF2α
Stress activated eIF2α kinases (GCN2, PERK, PKR, HRI)
↓ Protein synthesis
↑ Alternative translation mechanisms
↑ ATF4 protein levels
No change in ATF4 mRNA levels
↑ CHOP protein levels
No change in CHOP mRNA levels
RGS2eb promotes apoptosis
↑ cleaved caspase 3
B
Stress
eIF2α kinases activation
↑ RGS2 mRNA levels
p - eIF2α
↑ BiP mRNA levels
↑ BiP ERSE promoter activity
↑ XBP1s mRNA levels
↓ activated eIF2 levels
↓ Protein synthesis
↑ Alternative translation mechanisms
↑ ATF4 protein and mRNA levels
↑ CHOP protein and mRNA levels
↑ cleaved caspase 3
RGS2 mediated effects
Stress mediated effects
Multi-step process

4.6 Conclusion and Future Perspectives

In summary, our data strongly implies that RGS2 is an important component of the stress response. We show that RGS2 is a key component in driving alternative translation-dependent mechanisms that activate the ATF4-CHOP pathway associated with the CSR. More specifically, $RGS2^{eb}$ on its own was sufficient to significantly upregulate cellular ATF4 protein levels, without the presence of p-eIF2α. Previously, the activation of ATF4 was predominantly known to be regulated by decreased eIF2 activation as a result of stress-induced phosphorylation of eIF2α. We are the first to show that the upregulation of ATF4 and its downstream effects can occur without the phosphorylation of eIF2α and we present a novel mechanism mediated in part by translational functions of RGS2. We provide evidence that alternative translation mechanisms triggered by eIF2α phosphorylation are due to decreased eIF2 activity and not phosphorylation *per se*, and thus extend the known repertoire of mechanisms involved in the CSR. $RGS2^{eb}$-infected fibroblasts also increased levels of ATF4, CHOP, and cleaved caspase 3, suggesting that this may be the domain in RGS2 that promotes apoptosis under stressed conditions and may be an important target to mitigate apoptosis-mediated diseases and pathologies.

Investigating the physiological implications of RGS2-mediated activation of the ATF4-CHOP pathway would be of interest to assess whether or not the effects seen here with RGS2 and $RGS2^{eb}$ expression do lead to greater cell death. Differences in cell viability between RGS2-positive and RGS2-negative cells under stress may be indicative of the relative protective or detrimental effects correlated with the expression of RGS2 as it currently appears that RGS2 may have multiple roles in the stress response that may be cell type- or stress-dependent.

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Appendices

Appendix A: Verification of adenoviral titres, multiplicity of infection, and viral infection efficiency

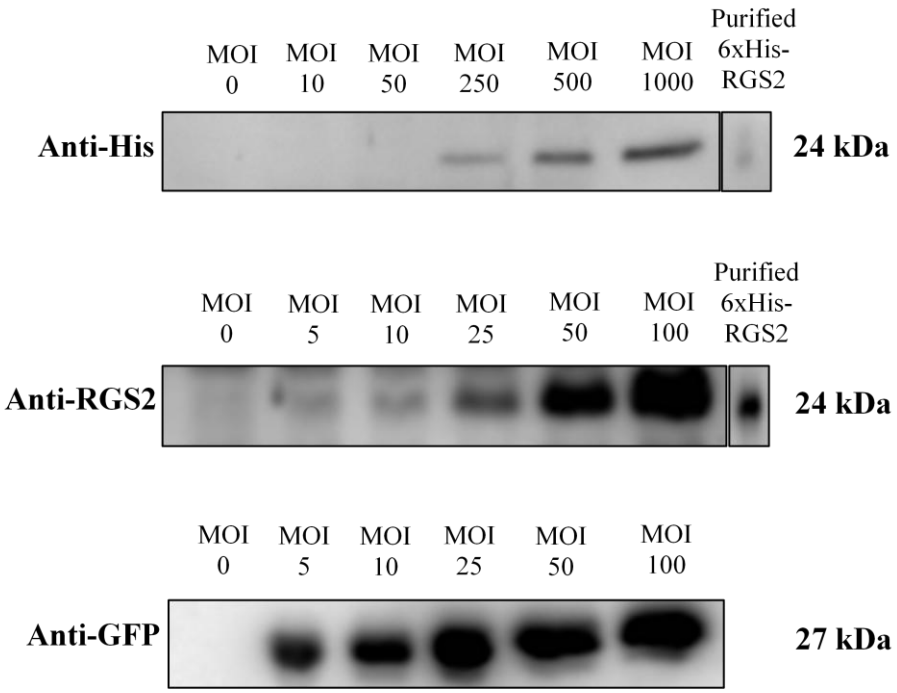


Figure A1. Verification of adenoviral titres and protein expression via immunoblotting. 3T3 fibroblasts were infected for 48 hours at the indicated multiplicity of infection (MOI) with adenoviruses encoding full-length His_6-tagged human RGS2 or GFP. Purified His_6-tagged RGS2 protein samples were run in parallel in SDS-PAGE gels as positive control for RGS2 expression.

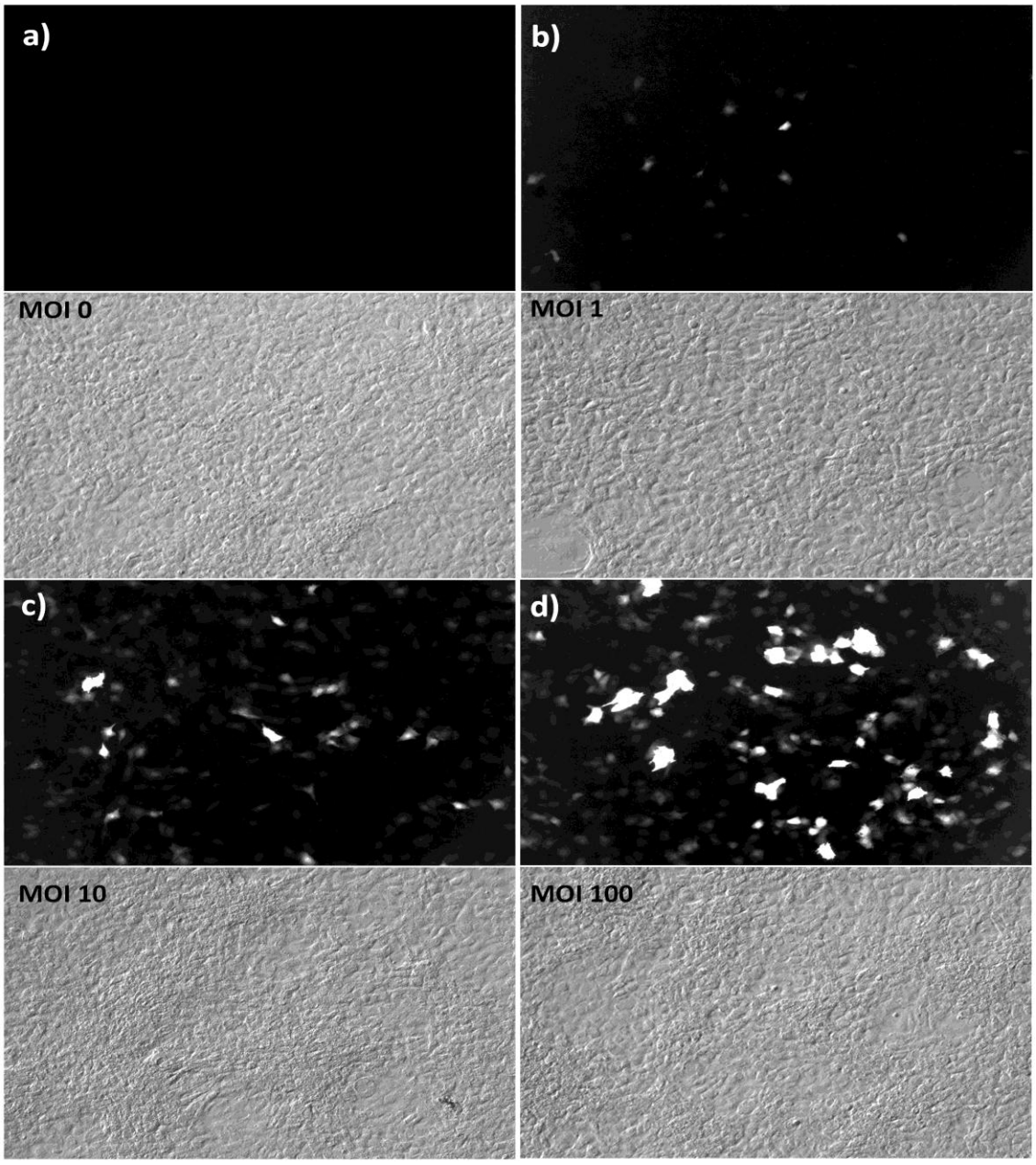


Figure A2. Expression of adenoviral encoded proteins. 3T3 fibroblasts were infected with increasing amounts of adenoviruses encoding for the expression of GFP, starting at an MOI of 0 (no virus) up to 100. Levels of expression of adenoviral encoded proteins were assessed via fluorescent microscopy for GFP expressing cells. Representative images on the level of GFP expression are shown with respective light microscopy images below to show equal cell density after 48 hours of infection with the adenoviruses (**a-d**).

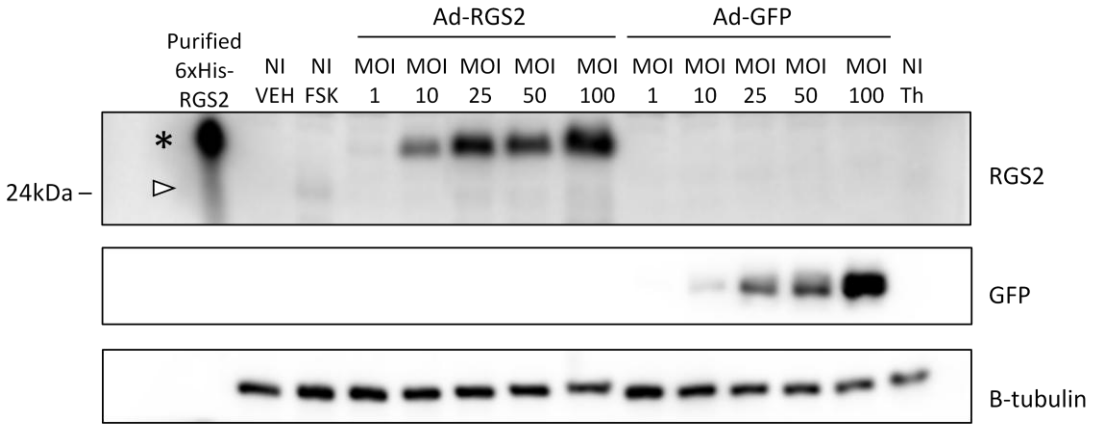


Figure A3. Assessment of endogenous levels of RGS2 with forskolin treatment. 3T3 fibroblasts were infected with adenoviruses encoding GFP (viral infection control) or full-length RGS2 at the indicated range of multiplicity of infection (MOI). A sample of uninfected fibroblasts (NI) was treated with 100uM water-soluble forskolin (FSK) or was treated with 0.1% DMSO (vehicle control) for 3h to assess physiological levels of endogenous RGS2 during upregulation. 6xHis-tagged RGS2 protein band from the viral infections is indicated by an asterisk (*), endogenous RGS2 protein band is indicated with a white arrowhead. An MOI of 10 was selected in our studies to study the effects of moderate levels of RGS2 expression on pathways of the CSR under experimentally induced stress.

Appendix B: Verification of drug treatments to induce cell stress and the expression of stress-activated targets

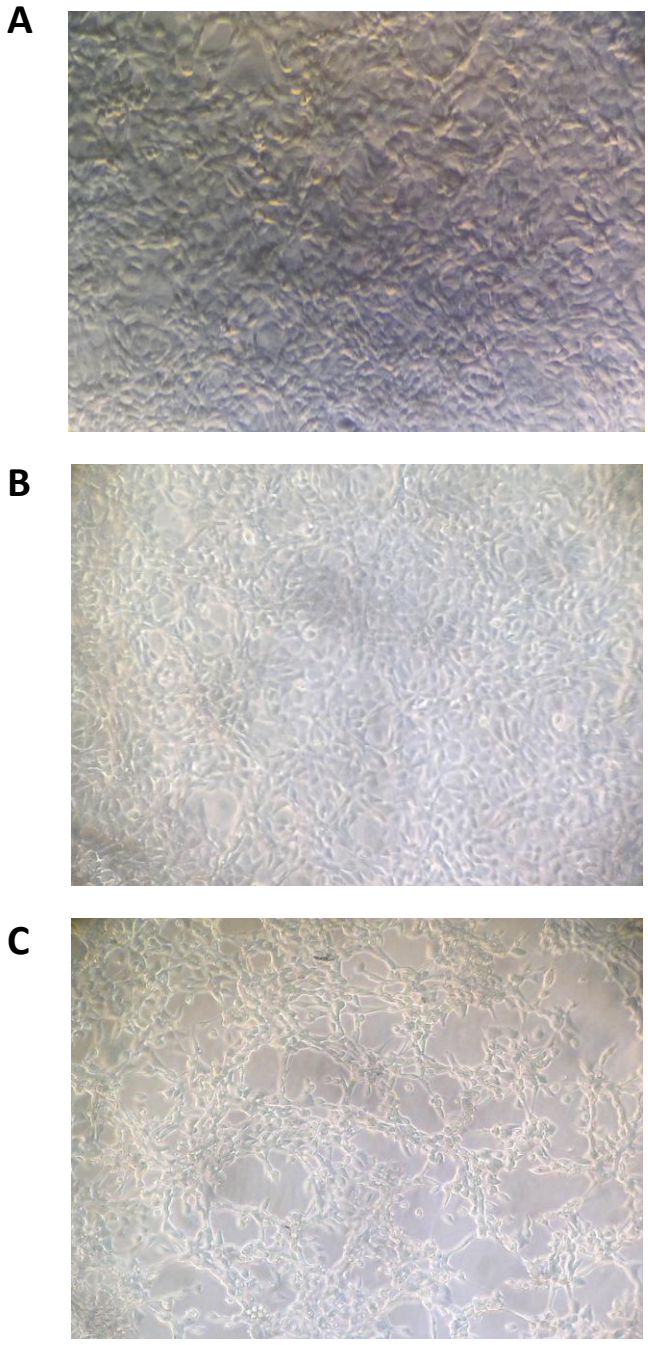


Figure B1. Morphological changes in 3T3 fibroblasts after treatment with tunicamycin. (**A**) Untreated 3T3 cells grown to approximately 80-90% confluency after infection with adenoviruses. (**B**) 0.1% DMSO (vehicle control) treated cells grown to the same confluency post-infection. (**C**) Tunicamycin (5 μg/ml) treated cells grown to the same confluency post-infection.

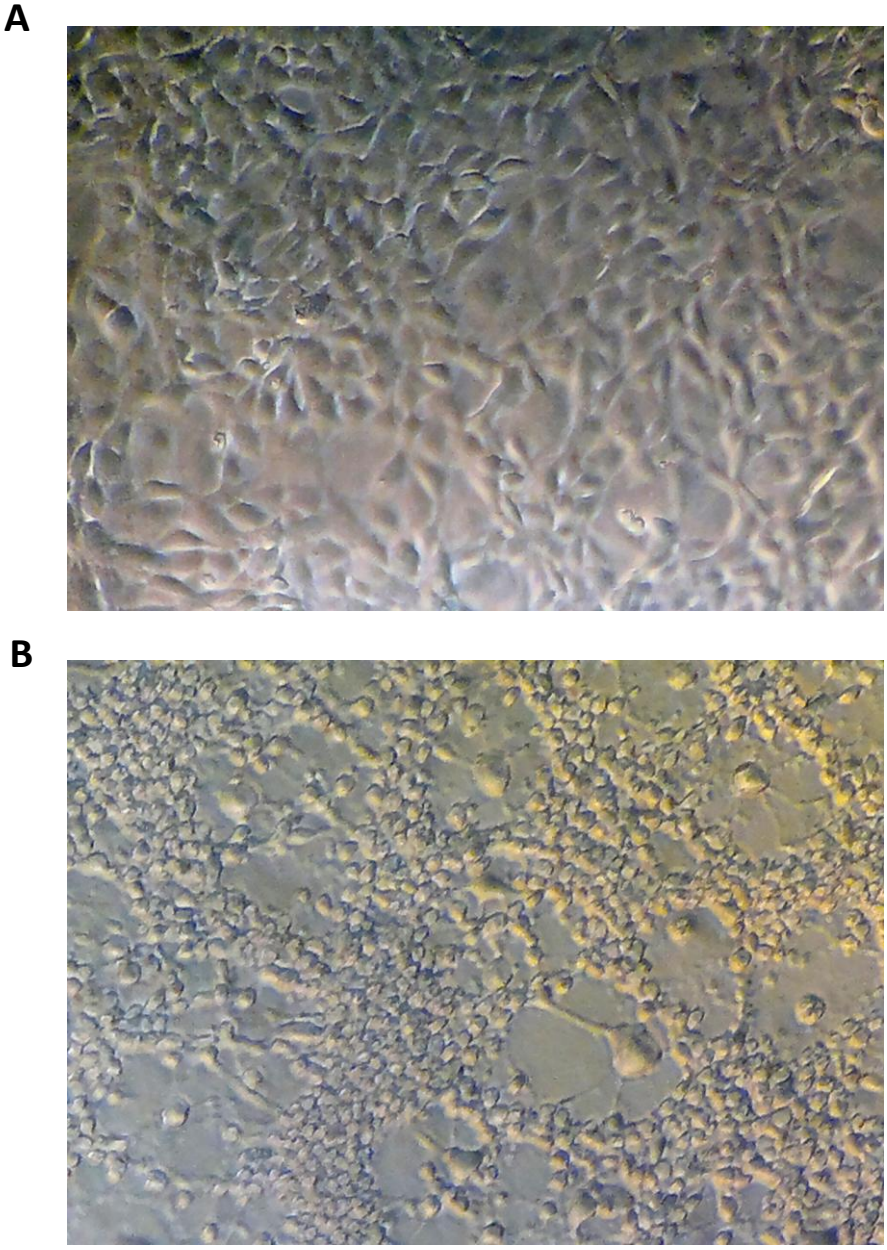


Figure B2. Morphological changes in 3T3 fibroblasts after treatment with staurosporine. (**A**) Untreated 3T3 cells grown to approximately 80-90% confluency after infection with adenoviruses. (**B**) Staurosporine (2 μM) treated cells grown to the same confluency post-infection. Cell blebbing and shrinkage was evident as early as 4 hours of treatment.

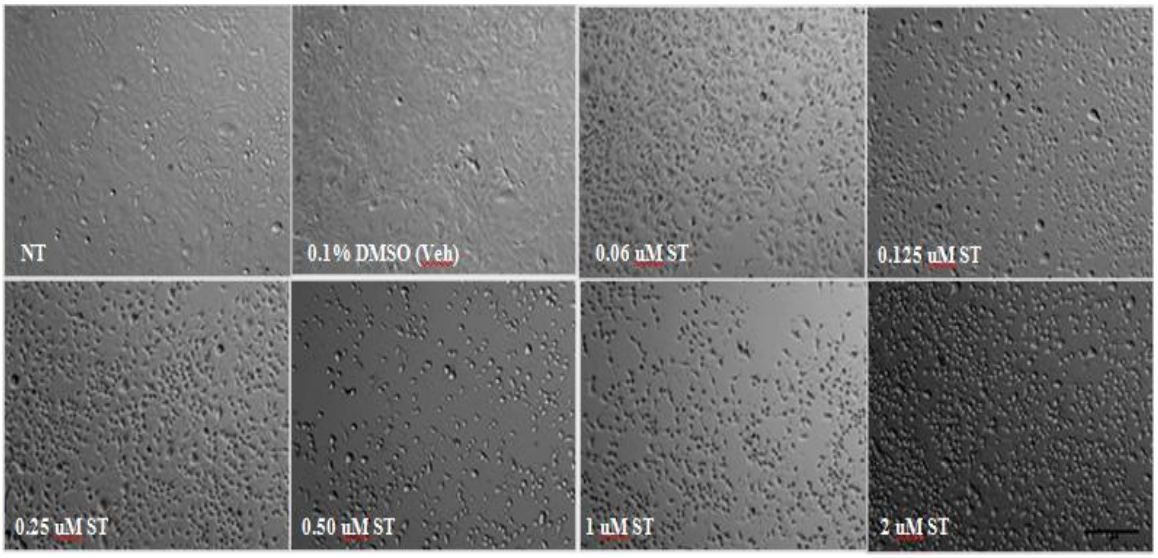


Figure B3. Dose-dependent effects of staurosporine (ST) on the morphology of 3T3 fibroblasts. Cytotoxic effects such as cellular blebbing and shrinkage associated with cell death was evident with staurosporine treated cells compared to non-treated (NT) or 0.1% DMSO (Veh) treated controls. Images were obtained 4 hours post-treatment with staurosporine at the indicated concentrations.

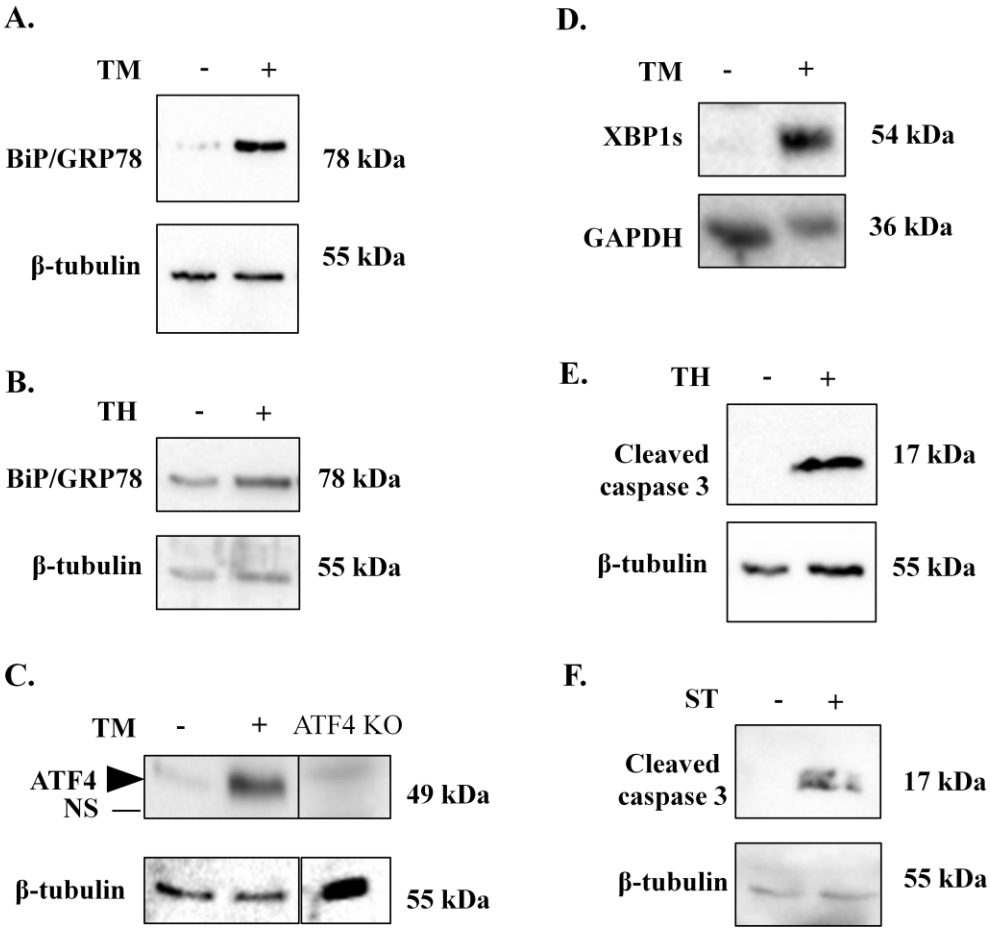


Figure B4. Immunoblots showing the effects of tunicamycin (TM), thapsigargin (TH), or staurosporine (ST) treatment on the expression of stress-activated proteins in 3T3 fibroblasts. Cells were treated with the indicated drug (+) or 0.1% DMSO vehicle control (-).Treatment with TM (5 μg/ml) or TH (1 μM) lasted for 24 hours, treatment with ST (2 μM) lasted for 6 hours, cells were then lysed and total protein from lysates were collected and immunoblotted for the indicated targets (n = 2-3). BiP/GRP78 was upregulated by TM and TH treatment (**A, B**). TM also induced ATF4 expression as highlighted by the arrowhead, NS = non-specific protein band. Protein lysates extracted from a full-body ATF4 knockout mouse embryo (E16.5) was used as a negative control (**C**). Greater levels of the spliced XBP1 variant (XBP1s) were observed in cells treated with TM (**D**). Treatment with TH and ST was shown to activate caspase 3 (**E, F**). β-tubulin or GAPDH was used as a protein loading control.

Appendix C: Effects of RGS2 on BiP ER stress response element promoter activity

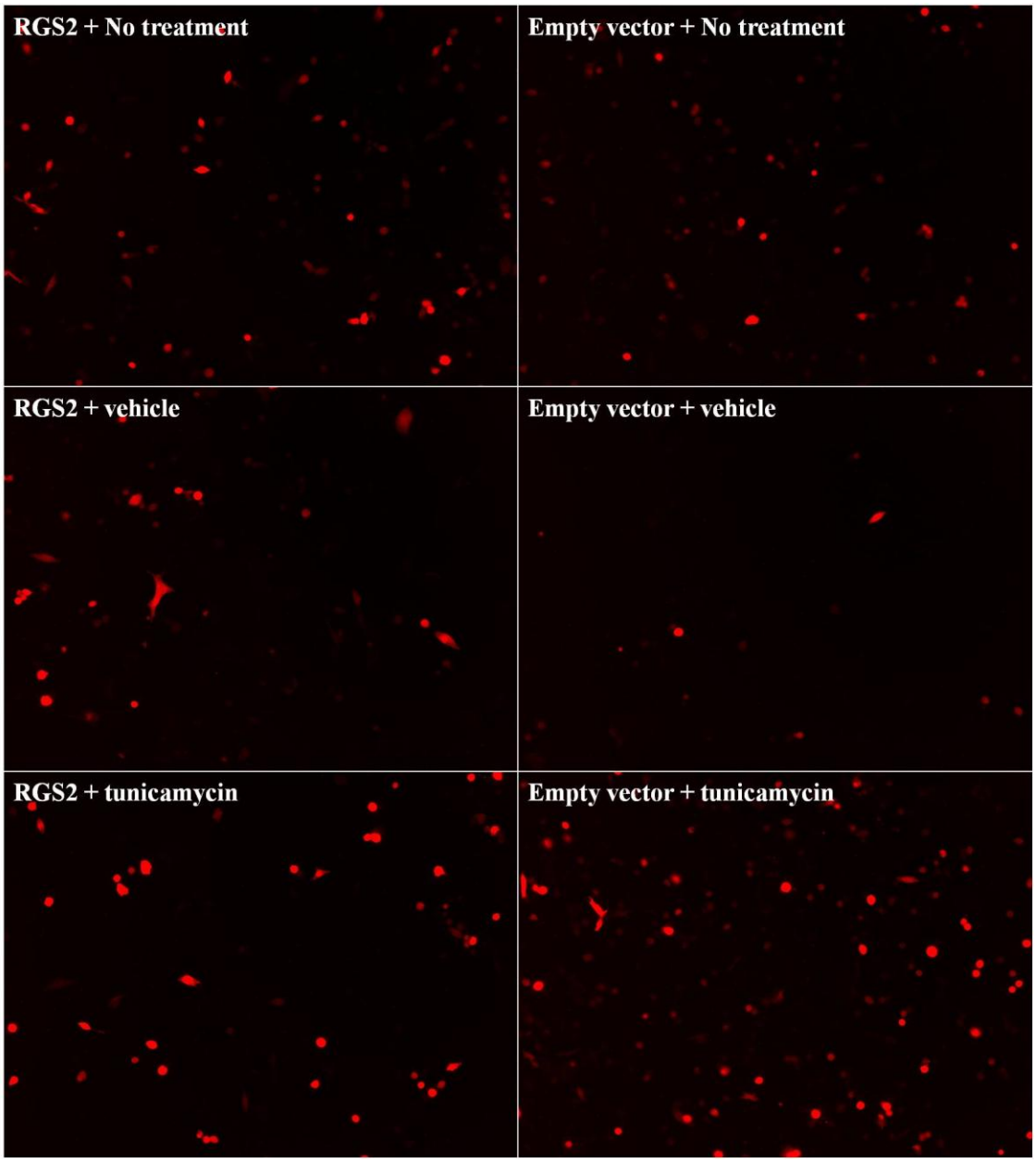
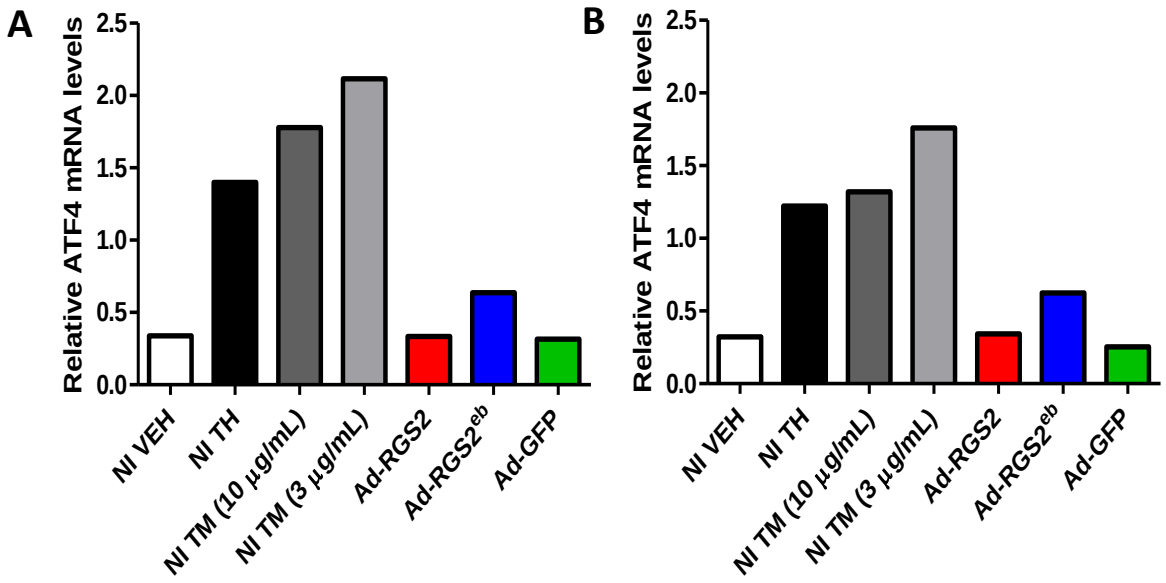


Figure C1. Representative fluorescent images showing the effects of tunicamycin and RGS2 expression on BiP ERSE-tdTomato promoter activity. 3T3 fibroblasts were co-transfected with plasmids encoding for the BiP ERSE promoter tagged with tdTomato together with full-length RGS2 or pcDNA3.1 empty vector (transfection control) for 24 hours. Cells were then treated with 0.1% DMSO (vehicle control) or 5 μg/ml tunicamycin and fluorescent images were taken over a course of 24 hours to assess for differences in the level of BiP ERSE promoter activity. Image analysis to quantify total fluorescence was performed using ImageJ as described in Materials and Methods. Images are representative of three independent experiments.

Appendix D: Effects of RGS2 and RGS2^{eb} expression on ATF4 transcription



Forward Primer

5'-TCTTGGACTAGAGGGGCAAA-3'

Reverse Primer

5'-GGGACAGATTGGATGTTGGA-3'

Forward Primer

5'-GATCTTTTTGCCCCTCTAGTC-3'

Reverse Primer

5'-TCAACTTCACTGCCTAGCTCT-3'

Figure D1. Effect of RGS2 and RGS2^{eb} expression on ATF4 mRNA levels. 3T3 fibroblasts were infected with adenoviruses encoding GFP (viral infection control), full-length RGS2, or the RGS2 eIF2Bε binding domain (RGS2^{eb}) at a multiplicity of infection of 50 for 48 hours. To confirm ATF4 upregulation, non-infected (NI) cells were treated with either 2 μM thapsigargin (TH) for 2 hours, 3 or 10 μg/ml tunicamycin (TM) for 6 hours, or 0.1% DMSO (vehicle control). All stressors increased ATF4 mRNA levels while ATF4 transcript levels did not show a significant change with RGS2 and RGS2^{eb} expression. Here we show that similar ATF4 gene expression profile was obtained using two different ATF4 primer sets (**A** and **B**), of which the primer sequences are listed below. All mRNA levels are expressed as means normalized to the geometric mean of two stable reference genes (GAPDH and β2 microglobulin) run in triplicate in parallel through qPCR (n = 1).

Curriculum Vitae

Name: Chang-Hui (Jenny) Wang

Post-secondary Education and Degrees:
University of Toronto
Mississauga, Ontario, Canada
2009-2013 H.B.Sc.

The University of Western Ontario
London, Ontario, Canada
2013-2015 M.Sc.

Honours and Awards:
Canadian Institute of Health Research Graduate Scholarship (CIHR-CGS-M)
2014-2015

Ontario Graduate Scholarship (OGS)
2014-2015
Declined in favour of CIHR-CGS-M

Related Work Experience
Teaching Assistant
Phys3130 (Physiology Laboratory)
The University of Western Ontario
2013-2015

Conferences Attended (poster presenter):
2014 – London Health Research Day (London, ON)
2015 – London Health Research Day (London, ON)

Publications:

Paluzzi J-PV, Bhatt G, **Wang C-HJ**, Zandawala M, Lange AB, Orchard I. Identification, functional characterization, and pharmacological profile of a serotonin type-2b receptor in the medically important insect, *Rhodnius prolixus*. *Frontiers in Neuroscience*. 2015;9:175. doi:10.3389/fnins.2015.00175.