Cellular Signalling 59 (2019) 163–170

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

RGS2 promotes the translation of stress-associated proteins ATF4 and CHOP via its eIF2B-inhibitory domain

Chang-Hui (Jenny) Wang[a], Peter Chidiac[a,b,*]

[a] Department of Physiology and Pharmacology, Schulich School of Medicine and Dentistry, University of Western Ontario, London, Ontario N6A 5C1, Canada
[b] Department of Biology, Faculty of Science, University of Western Ontario, London, Ontario N6A 5B7, Canada

ARTICLE INFO

Keywords:
Regulator of G protein signaling 2 (RGS2)
Cell stress response
Translation
Eukaryotic initiation factor 2 (eIF2)
ATF4
CHOP

ABSTRACT

Regulator of G protein signaling 2 (RGS2) is upregulated by multiple forms of stress and can augment translational attenuation associated with the phosphorylation of the initiation factor eIF2, a hallmark of several stress-induced coping mechanisms. Under stress-induced translational inhibition, key factors, such as ATF4, are selectively expressed via alternative translation mechanisms. These factors are known to regulate molecular switches that control cell fate by regulating pro-survival and pro-apoptotic signals. The molecular mechanisms that balance these opposing responses to stresses are unclear. The present results suggest that RGS2 may be an important regulatory component in the cellular stress response 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 demonstrate that the expression of either full length RGS2 or its eIF2B-interacting domain (RGS2^{eb}) significantly increases levels of ATF4 and CHOP, both of which are linked to stress-induced apoptosis. Furthermore, we show that these effects are translationally regulated and independent of eIF2 phosphorylation. The present results thus point to a novel function of RGS2 in the stress response directly related to its ability to reduce global protein synthesis.

1. Introduction

Cells are exposed to a constantly changing environment which may include stressful stimuli such as nutrient deprivation, temperature fluctuations, hypoxia, oxidative damage, exposure to toxins or ultraviolet radiation, mechanical damage, and viral or bacterial infections, any of which can affect important cellular processes and lead to dysfunction. Appropriate responses to stress therefore must be in place to prevent or ameliorate aberrant processes. The cellular or integrated stress response (ISR) is a highly conserved mechanism coordinating gene expression and protein translation to serve as an adaptive response to alleviate the stressful state [1,2]. A key component of the ISR is a decrease in protein synthesis, typically achieved via the phosphorylation of the heterotrimeric translation initiation factor, eIF2, as a means to conserve energy and resources under states of stress. Phosphorylation at the conserved serine residue of the α-subunit of eIF2 (eIF2α) converts it to a competitive inhibitor of the guanine nucleotide exchange factor, eIF2B, impeding the initiation step in translation and resulting in reduced protein synthesis [3–5]. During translational attenuation, preferential increases in the expression of components of the stress proteome are observed through alternative translation mechanisms associated with reduced initiation, such as the use of internal ribosome entry sites (IRESs) or the bypass of inhibitory upstream open reading frames (uORFs) [6–8]. Notably, activating transcription factor 4 (ATF4) is translationally upregulated under states of decreased protein synthesis. Translation of ATF4 mRNA is limited by inhibitory uORFs and is conserved across many species, where these regions of the ATF4 transcript under normal conditions inhibit initiation at the start codon of the main ATF4 ORF [9–12]. Under states of stress leading to the phosphorylation of eIF2α, decreased abundance of activated eIF2 results in the delay of the formation of competent ribosomal complexes, bypassing ATF4 uORFs, and instead facilitates ribosome initiation at the main ORF to produce functional ATF4 [10,13]. ATF4 further regulates the transcription of downstream genes that can promote both cell recovery and cell death in response to stress [14–19].

Studies from our lab have identified Regulator of G protein signaling 2 (RGS2) as a component in the repertoire of proteins involved in translational control in addition to its canonical functions in regulating G protein-mediated signaling [20]. Specifically, we discovered a short 37 amino acid residue domain within the conserved RGS domain (herein termed RGS2^{eb}) that mediates the binding of RGS2 to the ε-subunit of eIF2B, blocking its ability to activate eIF2 for translation

* Corresponding author at: Department of Physiology and Pharmacology, University of Western Ontario, 1151 Richmond Street, London N6A 5C1, Canada.
E-mail address: peter.chidiac@schulich.uwo.ca (P. Chidiac).

https://doi.org/10.1016/j.cellsig.2019.02.007
Received 22 August 2018; Received in revised form 15 February 2019; Accepted 26 February 2019
Available online 01 March 2019
0898-6568/ © 2019 Published by Elsevier Inc.

initiation and thus resulting in the inhibition of protein synthesis [20]. Notably, RGS2 is upregulated by many of the same forms of stress that trigger eIF2 phosphorylation [21–25], suggesting that it may be an important modulator in the stress response. We have shown that the stress-induced upregulation of RGS2 serves to arrest *de novo* protein synthesis via a mechanism that is independent of eIF2α phosphorylation [26]. However, it is unclear how this inhibitory effect of RGS2 on the initiation of translation might impact other facets of the stress response, for example the synthesis of stress proteins via alternative translation mechanisms. Here we hypothesize that the effects of RGS2 in the ISR may complement and fine-tune changes triggered by eIF2α phosphorylation.

Inhibition of protein synthesis is a hallmark response to alleviate stress, however, the benefit of this is diminished by the fact that inhibition of initiation can drive the preferential upregulation of pro-apoptotic proteins, and indeed one study has shown RGS2 to promote stress-induced apoptosis [25]. In this study, we show evidence that RGS2 expression can upregulate the expression of ATF4 and CHOP, all in a manner that is independent of eIF2α phosphorylation *per se*. Furthermore, we show that the effects of RGS2 on the enhanced expression of stress response factors are translationally regulated rather than through transcription. Our current study extends the known mechanisms involved in stress response mediated in part by RGS2.

2. Materials and methods

2.1. Cell culture

NIH-3T3 fibroblasts were used to assess the effects of RGS2 expression on cellular stress. Cells were maintained in Dulbecco's Modified Eagle Medium (Gibco Life Technologies) supplemented with 10% (v/v) fetal bovine serum (Gibco Life Technologies) and incubated at 37 °C with 5% CO_2.

2.2. Reagents

Cells were subjected to stress via treatment with tunicamycin (TM) or thapsigargin (TH) as positive controls in the expression of stress markers. 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. Thapsigargin (TOCRIS Bioscience 1138) was diluted to the indicated concentrations from a 5 mM stock solution in DMSO.

2.3. Recombinant adenoviruses

Replication-defective adenoviruses encoding Green Fluorescent Protein (Ad-GFP), full-length His_6-tagged human RGS2 (Ad-RGS2), and the 37 amino acid eIF2Bε binding domain of RGS2 (Ad-RGS2^{eb}) were generated in our lab as previously described [20,27]. Titring of the adenoviral constructs was carried out following the procedures described in Franceschi and Ge [28]. Infection efficiency in 3T3 fibroblasts was monitored through fluorescence microscopy for GFP. Additionally, sample cell lysates were immunoblotted with rabbit anti-6× 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. Expression of polyhistidine-tagged RGS2^{eb} in cells via infection was verified in previous studies via immunofluorescent staining and dot blot analysis of whole cell lysates [27,29].

2.4. Adenoviral infection of 3T3 fibroblasts

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 h with Ad-RGS2, Ad-RGS2^{eb}, Ad-GFP (as an infection control), or left uninfected (NI), under 4 h of serum deprivation, after which medium was removed and replaced with complete cell culture medium. 48 h post-infection, cells were treated with a chemical stressor at indicated concentrations and durations as positive control for the expression of stress markers or subjected to a vehicle control.

2.5. Protein isolation and immunoblotting

Cell lysates were prepared by washing with ice-cold 1× 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 min 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 min 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.

Protein samples were prepared using 5× 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 1× sample buffer for equal protein concentration. Protein samples were heated to 99 °C for 5 min 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 h at room temperature before overnight incubation with rocking at 4 °C with respective primary antibodies to assess targeted endpoint proteins associated with the stress response: anti-phospho-eIF2α (1:1000, Cell Signaling 9721), anti-CREB-2/ATF4 (1:5000, Santa Cruz sc-200X), and anti-CHOP (1:1000, Cell Signaling 5554). Anti-RGS2 (1:1000, Sigma-Aldrich GW22245F) and anti-GFP (1:1000, Clontech 632381) were used to assess adenoviral infection and expression of RGS2 and GFP, respectively. 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 h 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 stress markers such as anti-eIF2α (1:1000, Cell Signaling 9722). Anti-β-tubulin (1:1000, Pierce PA5-16863) was used to verify that protein loading was consistent 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 phosphorylated proteins, densitometric ratios of phosphorylated to total species were taken. Data are presented as means ± SEM.

2.7. RNA isolation and quantitative RT-PCR

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

Table 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′
CHOP/GADD153	5′-TACACCACCACACCTGAAAG-3′	5′-TTCTTCCTCTTCGTTTCCTG-3′
GAPDH	5′-GTTCCTACCCCCAATGTGT-3′	5′-GGAGTTGCTGTTGAAGTCG-3′
β2 microglobulin	5′-ACGCAGAAAGAAATAGCAATG-3′	5′-TGAGAAGTACAGAGGGTTTGG-3′

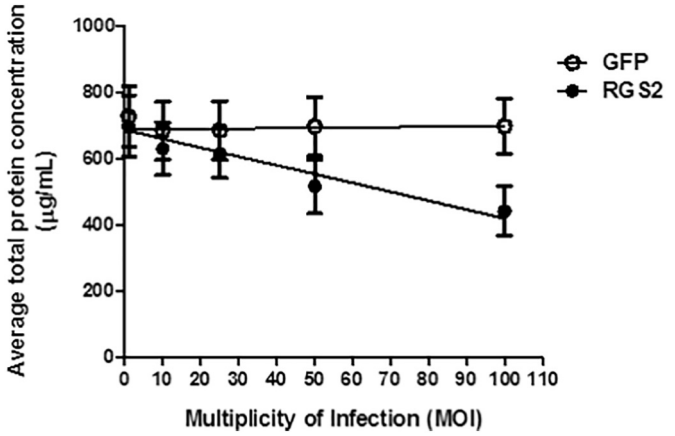

Fig. 1. Expression of RGS2 results in reduced total cellular protein levels. Significantly lower total protein concentrations were observed from lysates of RGS2-infected cells and the effect occurred in a concentration-dependent manner with increasing RGS2. Slopes of the fitted linear regression analysis for RGS2 (−2.67 ± 0.87) and GFP (0.12 ± 0.87) were statistically different ($p = 0.0063$). Data presented are mean ± SEM, $n = 8$.

infection. Cells were infected for 48 h with Ad-RGS2, Ad-RGS2^{eb}, Ad-GFP (as an infection control), or left uninfected (NI), under 4 h of serum starved conditions then replaced with complete cell culture medium. After 48 h 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 sufficiently 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 Center 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 1). Quantitative analyses of mRNA expression levels of ATF4 and CHOP 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.

2.8. 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. The level of statistical significance was set at $\alpha = 0.05$, where a p value < 0.05 was considered statistically significant throughout. All statistical analyses were performed using GraphPad Prism® 5.01.

3. Results

3.1. Expression of RGS2 reduces total cellular protein levels

Previously, our lab has shown that RGS2 can bind to eIF2B at its ε-subunit and inhibit protein synthesis [20]. Here, we show that the expression of RGS2 consistently results in significantly lower total cellular protein concentrations, an effect which increases with the multiplicity of virus infection (Fig. 1, linear regression analysis, $p = 0.0063$). Cell confluency (80–90%) was consistent across all experimental conditions after a 48 h period of infection, assessed by light microscopy, and thus the loss of total protein does not appear to reflect decreased cell numbers. The proportional decrease observed here is consistent with reported changes in protein synthesis in response to either overexpression or loss of RGS2 [20,26,27,30], although possible minor effects of RGS2 expression on cell number cannot be strictly ruled out in the present study. Overall, the ability of RGS2 to inhibit protein synthesis suggests a role in mediating the cell stress response through the regulation of protein translation, wherein such inhibition may augment the effects of eIF2 phosphorylation by stress-activated kinases.

3.2. RGS2 promotes increases in ATF4 and CHOP protein levels

The expression level of various stress-induced proteins is tightly regulated by the efficiency of the translational machinery. We therefore hypothesized that the translational control abilities of RGS2 may affect expression of key factors of the stress response regulated by alternative translational mechanisms, such as ATF4. Indeed, we found that ATF4 protein levels increased with increasing expression of RGS2 (Fig. 2A). This effect was specific to RGS2 overexpression and no increase in ATF4 was detected in control cells infected with an adenovirus encoding GFP. RGS2 overexpression resulted in significantly increased cellular levels of ATF4 (two-way ANOVA, $p < 0.0001$), and such levels were observed at multiplicities of infection of 50 and 100 (Fig. 2B, Bonferroni post-hoc tests, $^{***}p < 0.001$). Additionally, the expression of CHOP significantly increased in RGS2 expressing cells (Fig. 2C, two-way ANOVA, $p < 0.0001$), similar to the pattern observed with ATF4 expression.

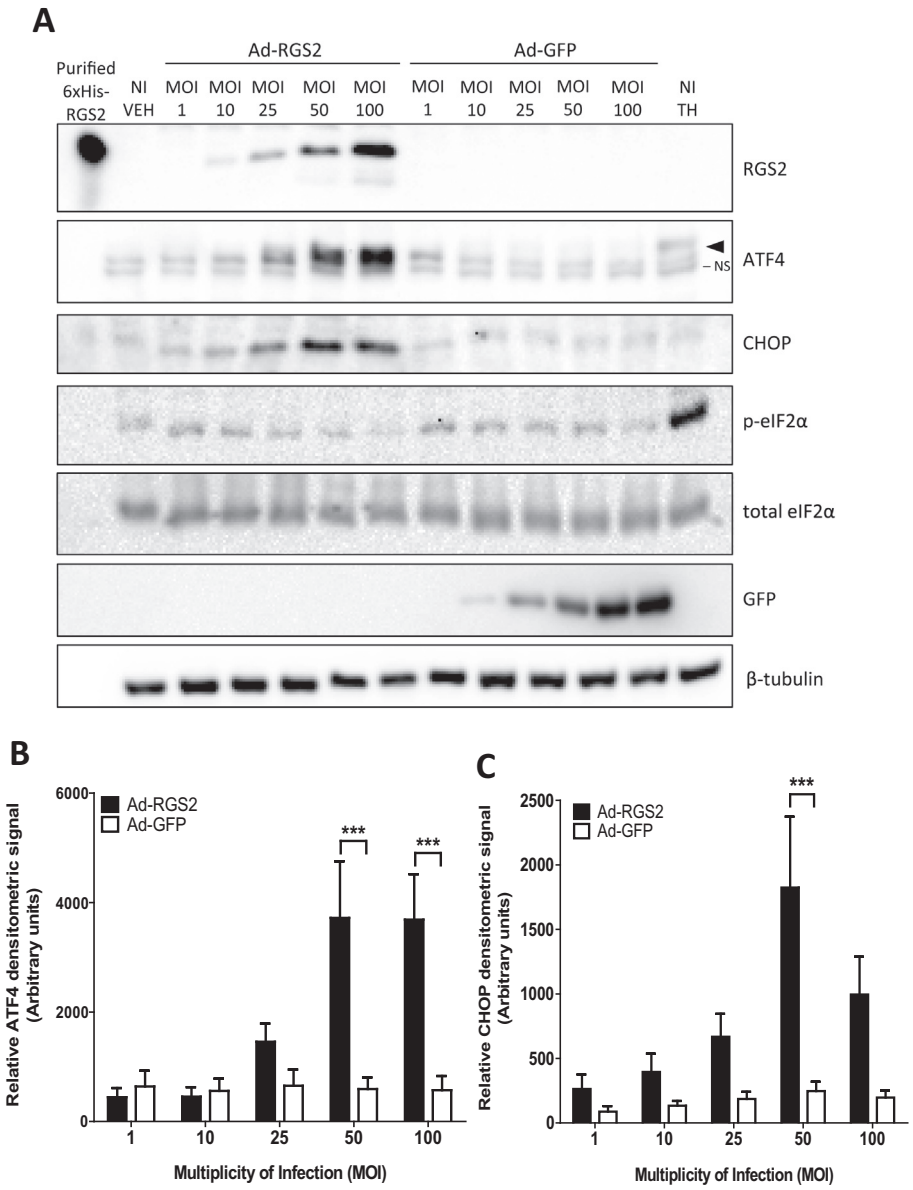

Fig. 2. Dose dependent increase in ATF4 and CHOP protein levels with RGS2 expression. NIH-3T3 fibroblasts were infected with adenoviruses encoding GFP (viral infection control) or full-length RGS2 at the indicated range of multiplicity of infection (MOI) for 48 h. Non-infected (NI) fibroblasts were then treated with 2 μM thapsigargin (TH) for 2 h 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. (A) RGS2 expression significantly increased ATF4 protein levels in an MOI-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, comparable to the ATF4 expression pattern. Corresponding densitometric data are summarized in bar graphs as mean ± SEM for (B) ATF4, $n = 8$ and (C) CHOP, $n = 7$ protein levels. Statistical analysis was performed using two-way ANOVA followed by Bonferroni post-hoc test. ***, Significant difference ($p < 0.001$).

3.3. 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. Interestingly, the present results point to a novel mechanism to increase cellular ATF4 levels that is independent of eIF2α phosphorylation. In contrast thapsigargin, a robust inducer of stress, strongly increased eIF2α phosphorylation in these experiments. To distinguish whether the increase in stress-induced proteins associated with RGS2 stems from its inhibitory effects on protein synthesis or from its inhibitory effects on G protein-mediated signaling, we compared the effects of RGS2 to those of its isolated eIF2B-binding domain (RGS2^{eb}), which exhibits no appreciable effect on signaling [20,27]. The data presented in Fig. 3 show that the expression of either full-length RGS2 or RGS2^{eb} is sufficient to significantly increase ATF4 expression without any detectable change in the level of p-eIF2α. Translational control by RGS2 may therefore provide an alternative or supplementary pathway to induce the expression of stress response factors, in addition to their induction via 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. 3A, D), suggesting that RGS2 may modulate the ATF4-CHOP pathway and their effects. The implications from this are significant as we report an alternative mechanism to upregulate ATF4 apart from the eIF2α-ATF4 pathway which extends the repertoire of mechanisms involved in the cellular stress response.

3.4. ATF4 and CHOP mRNA levels are unchanged in RGS2-overexpressing cells

The observed ability of RGS2 and RGS2^{eb} to increase cellular protein levels of ATF4 and CHOP could reflect changes at the transcriptional level, the translational level, or both, and thus we examined mRNA levels of these genes by qPCR. As shown in Fig. 4, expression of RGS2 or RGS2^{eb} did not affect the relative mRNA levels of ATF4 in contrast to the levels observed in the positive control conditions via treatment with chemical stressors such as thapsigargin or tunicamycin. This suggests that the upregulation of ATF4 mediated by the expression of RGS2 and RGS2^{eb} observed in our immunoblot data was not due to increased transcript levels of ATF4. Rather, ATF4 would appear to be regulated via the ability of RGS2 to affect the translational machinery

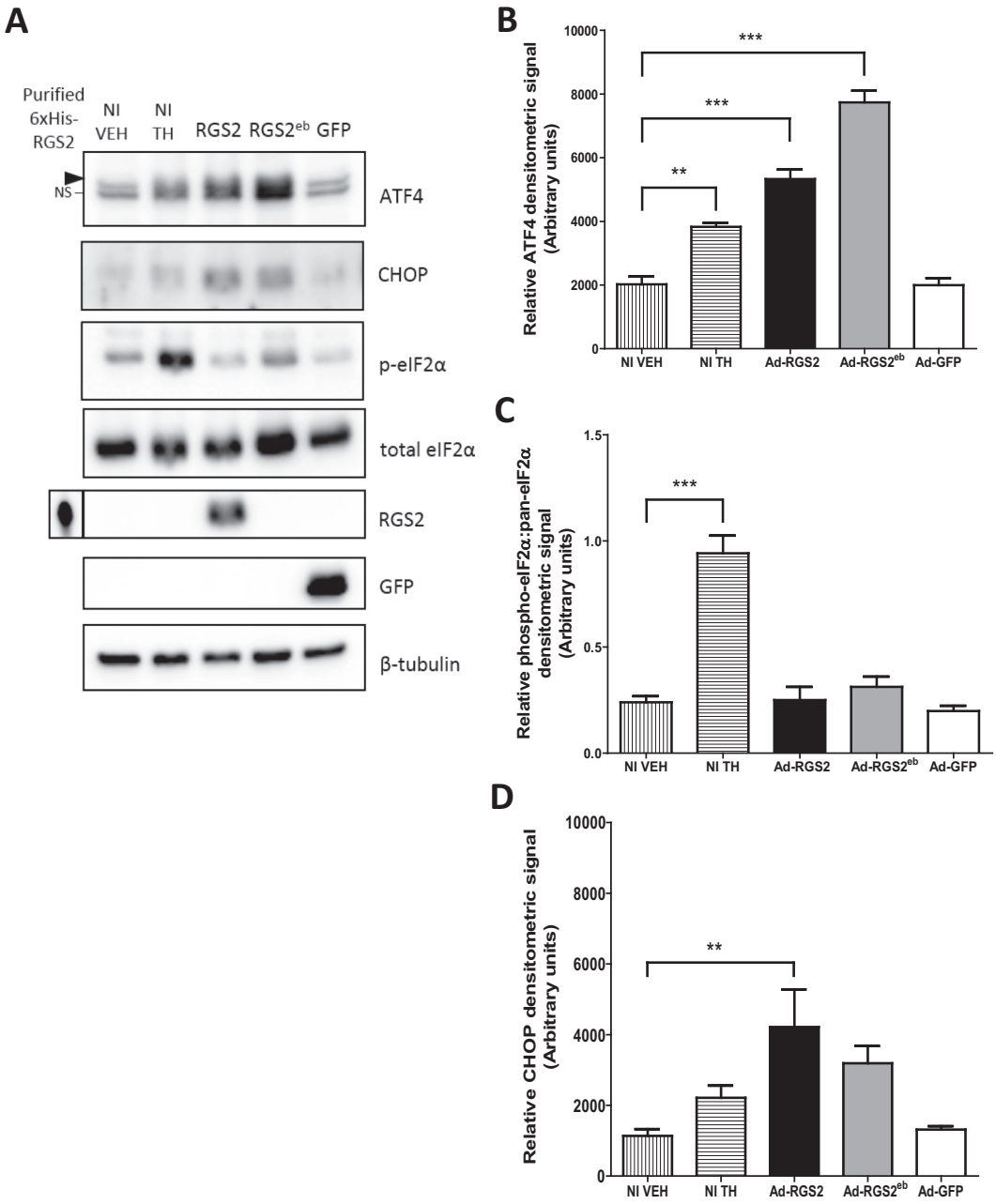

Fig. 3. RGS2 and RGS2^{eb} upregulate ATF4 protein levels independent of eIF2α phosphorylation. NIH-3T3 fibroblasts were infected with adenoviruses encoding GFP (viral infection control), full-length RGS2, or the RGS2 eIF2Bε-binding domain (RGS2^{eb}) at a multiplicity of infection of 50 for 48 h. Non-infected (NI) cells grown in parallel were then treated with 2 μM thapsigargin (TH) for 2 h 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. (A) RGS2 and RGS2^{eb} expression resulted in significant increases in cellular ATF4 levels (one-way ANOVA, $p < 0.0001$), while no comparable increase in levels of phosphorylated eIF2α was observed. A significant increase in CHOP protein level was observed with RGS2 expression (one-way ANOVA, $p = 0.0132$). Densitometric data are summarized in bar graphs as mean ± SEM for (B) ATF4, (C) phosphorylated eIF2α, and (D) CHOP relative protein levels, $n = 3$. 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 ($p < 0.001$).

and delay initiation, resulting in increased ATF4 protein levels.

CHOP mRNA levels did not measurably increase with either RGS2 or RGS2^{eb}, (Fig. 5). This result was somewhat surprising given the concurrent increases in ATF4 protein levels, which is a known transcriptional activator of CHOP gene expression. Although CHOP gene expression can be induced by ATF4 and other stress-induced transcription factors, the CHOP gene also encodes multiple translation initiation sites, and as with ATF4, the translation of functional CHOP protein resulting from its proper open reading frame is enhanced by eIF2α phosphorylation [11,31–34]. The present results do not support a role for RGS2 in the transcriptional upregulation of either ATF4 or CHOP and thus it would appear that their protein levels are likely increased via the effects of RGS2 and RGS2^{eb} on translation.

4. Discussion

We have previously shown that RGS2 is able to inhibit *de novo* protein synthesis as a result of its interaction with eIF2B [20] and that the induced expression of RGS2 contributes to reduced mRNA translation during the cellular stress response [26]. The results of the current

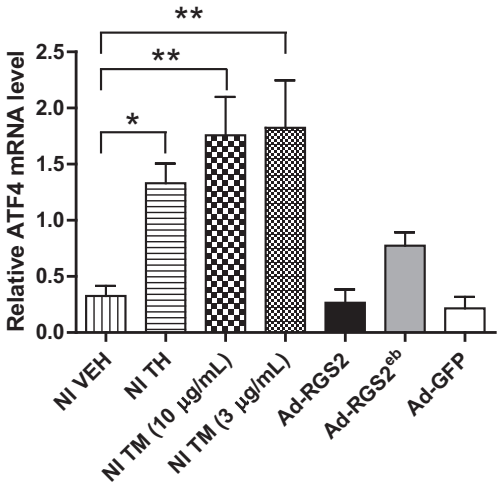

Fig. 4. Effect of RGS2 and RGS2^{eb} on levels of ATF4 transcription. NIH-3T3 fibroblasts were infected with adenoviruses encoding GFP (viral infection control), full-length RGS2, or the RGS2 eIF2Bε binding domain (RGS2^{eb}) at a multiplicity of infection of 50 for 48 h. To confirm ATF4 upregulation, non-infected (NI) cells were treated with either 2 μM thapsigargin (TH) for 2 h, 3 or 10 μg/ml tunicamycin (TM) for 6 h, 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. Data presented are $n = 3$, 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.

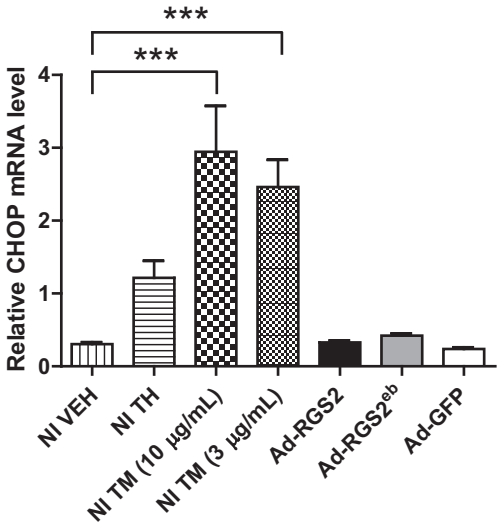

Fig. 5. Effect of RGS2 and RGS2^{eb} on levels of CHOP transcription. NIH-3T3 fibroblasts were infected with adenoviruses encoding GFP (viral infection control), full-length RGS2, or the RGS2 eIF2Bε binding domain (RGS2^{eb}) at a multiplicity of infection of 50 for 48 h. To confirm CHOP upregulation, non-infected (NI) cells were treated with either 2 μM thapsigargin (TH) for 2 h, 3 or 10 μg/ml tunicamycin (TM) for 6 h, or 0.1% DMSO (vehicle control). Treatment with tunicamycin increased CHOP mRNA levels. ***, Significant difference ($p < 0.001$). RGS2 and RGS2^{eb} expression did not result in an increase in CHOP transcript levels. Data presented are $n = 3$, 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.

study further show that via its eIF2B-interacting domain, RGS2 can modulate pathways associated with the stress response. Specifically, we present evidence that RGS2^{eb} can promote the translation of ATF4 and CHOP, both of which mediate cellular stress [19,35,36].

Translation is principally regulated at the initiation stage to allow rapid, spatial control of gene expression [13] and it is well established that protein synthesis is slowed as a result of the phosphorylation of eIF2α by stress-activated kinases [4,5,33]. This promotes the translation of stress response proteins encoded by mRNA molecules with one or more upstream open reading frames (through ribosome scanning) or containing internal ribosome entry sites [6–8,37]. The present findings with RGS2 imply that slowing the rate of mRNA translation via a mechanism independent of eIF2α phosphorylation can similarly foster the production of stress-induced proteins through ribosome scanning.

Our current findings show that RGS2 increases ATF4 and CHOP protein levels while having no measurable effect on eIF2α phosphorylation. Recent studies have shown that other proteins that impact eIF2 function, including Obg-like ATPase 1 (OLA1) [38], eIF5, and eIF5 mimic protein (5MP) [39], may also promote ATF4 translation, however RGS2 appears unique in being a stress-induced protein that does so in a manner independent of eIF2 phosphorylation. Both RGS2 and RGS2^{eb} substantially increased cellular protein levels of ATF4 and CHOP with no apparent change in the transcription of either. RGS2 is not known to affect protein degradation, but formally we cannot rule out such effects on ATF4 or CHOP based on the present results. Still, given that ATF4 and CHOP are encoded by mRNAs that contain upstream open reading frames that attenuate translation of functional protein under normal conditions [9–13,31–34,40], whereas the translation of both proteins is enhanced via alternative mechanisms under conditions of reduced global protein synthesis, the most reasonable interpretation is that RGS2 increases these proteins via its effects on translation. Additionally, the transcription of CHOP is known to be regulated by ATF4 and other stress-activated transcription factors, such as ATF6 and XBP1 [19,41]. It is possible that CHOP gene expression may have been transiently upregulated by RGS2-promoted ATF4 translational upregulation in the present study but returned to baseline during the relatively long infection period, as opposed to the acute treatment times with thapsigargin and tunicamycin [42]. This could explain the lack of an observable increase in CHOP transcription notwithstanding that RGS2 significantly increased ATF4 protein levels.

Some elements of the ISR can contribute to both pro-survival and pro-apoptotic processes, and the function of RGS2 may be similarly context dependent. A previous report showed that RGS2 may be pro-apoptotic in astrocytes under ischemic stress [25]. Intriguingly, a recent study showed that miRNA-22, which targets RGS2 and several other proteins, is diminished in Huntington's and Alzheimer's disease brains, and that miRNA-22 overexpression resulted in reduced caspase activation and apoptosis and promoted survival in primary neuronal cultures [43]. In contrast to the foregoing studies pointing to pro-apoptotic functions of RGS2, Dong et al. (2017) showed that pancreatic β-cells from RGS2-knockdown mice exhibited excessive glucose and exendin-4-induced insulin secretion and had greater stress-induced β-cell death while overexpression of RGS2 mitigated those effects [44]. Over-secretion of insulin can engender β-cell exhaustion and lead to β-cell death that could presumably be limited by RGS2. Whether the effects of RGS2 on pancreatic islet cell mass and function occur via its ability to regulate G protein signaling or translation is uncertain, however, the results from the study show that RGS2 appears to be anti-apoptotic. The determination between cell survival and cell death depends on the duration and severity of the stress, with severe and prolonged stress signals triggering the expression of pro-apoptotic factors farther down the cell-death pathway that are generally less stable and rapidly degraded [42]. Gq- and Gs-mediated signals in many systems have been found to promote apoptosis [45], and the ability of RGS2 to inhibit such signaling [20,27,46,47] might tip the balance away from apoptosis depending on the cellular context. RGS2 may potentially impede or shorten the duration of such signals and thereby prevent G-protein mediated apoptosis. Indeed, RGS2 overexpression reduces the potency of isoproterenol to stimulate cellular cAMP levels [48], which is shown

to be needed to promote apoptosis in myocytes via sustained beta-adrenergic receptor signaling [45,49].

Temporally, the contribution of RGS2 to the stress response may occur subsequently to the rapid effects of stress-activated kinases since concurrent stress-induced RGS2 mRNA transcription [21–25] would then need to be followed by RGS2 protein synthesis. Indeed, the effects of RGS2 and stress-activated kinases may overlap, as the rate of heat shock-induced RGS2 mRNA expression is accelerated in MEF cells lacking the conserved eIF2α phosphorylation site targeted by these kinases [26]. The ability of RGS2 to inhibit translation may reduce the need for eIF2α phosphorylation and raises the possibility of RGS2 to supplant or complement multiple aspects of the integrated stress response. We thus examined whether RGS2 might influence the response of cells to agents that induce stress. Using an intermediate level of infection of RGS2 (MOI = 10), we were not able to detect any clear decrease in either eIF2α phosphorylation in response to thapsigargin (Supplementary materials, Fig. S1) or IRE1 activation in response to tunicamycin (measured via RT-PCR for the amount of spliced XBP1 transcripts), an established hallmark of ER stress [50–53] (Supplementary materials, Fig. S2). Attempts to detect RGS2-dependent increases in ATF4 and CHOP protein levels above those triggered by simultaneous administration of tunicamycin were also unsuccessful (data not shown). While the present results fail to show any appreciable direct effects of RGS2 on pathways of the ISR, further studies may reveal RGS2 effects on other stress-dependent biochemical changes and extend on the repertoire of stress response mechanisms.

Overall, RGS2 may contribute to cell survival during stress by promoting the conservation of energy and resources but conversely the apparent translational upregulation of ATF4 and CHOP in the present study implies an ability to trigger apoptotic signaling [25,53–57]. The present findings overall support and further define a role for RGS2 in the cellular stress response, consistent with its upregulation by stressful stimuli. RGS2 is a ubiquitously expressed [58] multifunctional protein [59,60] that has been implicated in multiple physiological and pathophysiological processes, however it is not always clear which of its biochemical properties underlie which of its biological effects. As well, RGS2 is considered to be an immediate early gene [61] and its transcription is induced by a wide array of stimuli including stress, G protein-mediated signaling, and developmental changes [20–25,46–48]. In summary, our findings imply that RGS2 is able to drive alternative translation-dependent mechanisms that activate the ATF4-CHOP pathway associated with the ISR. More specifically, $RGS2^{eb}$ on its own was sufficient to significantly upregulate cellular ATF4 and CHOP protein levels. Previously, the activation of ATF4 was predominantly known to be triggered by decreased eIF2 activation as a result of stress-induced phosphorylation of eIF2α. Here we show that the upregulation of ATF4 can occur without the phosphorylation of eIF2α and we present a novel mechanism mediated in part by translational functions of RGS2.

Acknowledgements

This work was supported by operating grants from the Canadian Institutes of Health Research. ATF4 primers and sequences were generously provided by Dr. Sean Cregan from the Robarts Research Institute, University of Western Ontario, London, ON. We thank Dr. Patrick Lajoie for his advice and helpful comments on the manuscript and Dr. Alexey Pereverzev for technical advice.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2019.02.007.

References

[1] S. Fulda, A.M. Gorman, O. Hori, A. Samali, Cellular stress responses: cell survival and cell death, Int. J. Cell Biol. (Feb 21, 2010), https://doi.org/10.1155/2010/214074.
[2] D. Kültz, Molecular and evolutionary basis of the cellular stress response, Annu. Rev. Physiol. 67 (2005) 225–257.
[3] F. Gebauer, M.W. Hentze, Molecular mechanisms of translational control, Nat. Rev. Mol. Cell Biol. 5 (2004) 827–835.
[4] M.S. Sheikh, A.J. Fornace, Regulation of translation initiation following stress, Oncogene. 18 (1999) 6121–6128.
[5] A. Sudhakar, A. Ramachandran, S. Ghosh, S.E. Hasnain, R.J. Kaufman, K.V. Ramaiah, Phosphorylation of serine 51 in initiation factor 2 alpha (eIF2 alpha) promotes complex formation between eIF2 alpha(P) and eIF2B and causes inhibition in the guanine nucleotide exchange activity of eIF2B, Biochemistry 39 (2000) 12929–12938.
[6] M.E. Filbin, J.S. Kieft, Toward a structural understanding of IRES RNA function, Curr. Opin. Struct. Biol. 19 (2009) 267–276.
[7] M. Kozak, Structural features in eukaryotic mRNAs that modulate the initiation of translation, J. Biol. Chem. 266 (1991) 19867–19870.
[8] M. Holcik, N. Sonenberg, Translational control in stress and apoptosis, Nat. Rev. Mol. Cell Biol. 6 (2005) 318–327.
[9] K. Ameri, A.L. Harris, Activating transcription factor 4, Int. J. Biochem. Cell Biol. 40 (2008) 14–21.
[10] K.M. Vattem, R.C. Wek, Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 11269–11274.
[11] P.D. Lu, H.P. Harding, D. Ron, Translation reinitiation at alternative open reading frames regulates gene expression in an integrated stress response, J. Cell Biol. 167 (2004) 27–33.
[12] H.P. Harding, I. Novoa, Y. Zhang, H. Zeng, R. Wek, M. Schapira, D. Ron, Regulated translation initiation controls stress-induced gene expression in mammalian cells, Mol. Cell 6 (2000) 1099–1108.
[13] R.J. Jackson, C.U. Hellen, T.V. Pestova, The mechanism of eukaryotic translation initiation and principles of its regulation, Nat. Rev. Mol. Cell Biol. 11 (2010) 113–127.
[14] C. Chaveroux, V. Carraro, L. Canaple, J. Averous, A.C. Maurin, C. Jousse, Y. Muranishi, L. Parry, F. Mesclon, E. Gatti, J. Mallet, P. Ravassard, P. Pierre, P. Fafournoux, A. Bruhat, In vivo imaging of the spatiotemporal activity of the eIF2 -ATF4 signaling pathway: insights into stress and related disorders, Sci. Signal. 8 (2015) rs5.
[15] J.L. Armstrong, R. Flockhart, G.J. Veal, P.E. Lovat, C.P. Redfern, Regulation of endoplasmic reticulum stress-induced cell death by ATF4 in neuroectodermal tumor cells, J. Biol. Chem. 285 (2010) 6091–6100.
[16] P.S. Lange, J.C. Chavez, J.T. Pinto, G. Coppola, C.W. Sun, T.M. Townes, D.H. Geschwind, R.R. Ratan, ATF4 is an oxidative stress-inducible, prodeath transcription factor in neurons in vitro and in vivo, J. Exp. Med. 205 (2008) 1227–1242.
[17] C. Deval, C. Chaveroux, A.C. Maurin, Y. Cherasse, L. Parry, V. Carraro, D. Milenkovic, M. Ferrara, A. Bruhat, C. Jousse, P. Fafournoux, Amino acid limitation regulates the expression of genes involved in several specific biological processes through GCN2-dependent and GCN2-independent pathways, FEBS J. 276 (2009) 707–718.
[18] M. Schröder, R.J. Kaufman, The mammalian unfolded protein response, Annu. Rev. Biochem. 74 (2005) 739–789.
[19] S. Oyadomari, M. Mori, Roles of CHOP/GADD153 in endoplasmic reticulum stress, Cell Death Differ. 11 (2004) 381–389.
[20] C.H. Nguyen, H. Ming, P. Zhao, L. Hugendubler, R. Gros, S.R. Kimball, P. Chidiac, Translational control by RGS2, J. Cell Biol. 186 (2009) 755–765.
[21] A. Ota, M. Sawai, H. Sakurai, Stress-induced transcription of regulator of G protein signaling 2 (RGS2) by heat shock transcription factor HSF1, Biochimie. 95 (2013) 1432–1436.
[22] R.L. McCaffrey, P. Fawcett, M. O'Riordan, K.D. Lee, E.A. Havell, P.O. Brown, D.A. Portnoy, A specific gene expression program triggered by Gram-positive bacteria in the cytosol, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 11386–11391.
[23] L. Song, R.S. Jope, Cellular stress increases RGS2 mRNA and decreases RGS4 mRNA levels in SH-SY5Y cells, Neurosci. Lett. 402 (2006) 205–209.
[24] J.W. Zmijewski, L. Song, L. Harkins, C.S. Cobbs, R.S. Jope, Oxidative stress and heat shock stimulate RGS2 expression in 1321N1 astrocytoma cells, Arch. Biochem. Biophys. 392 (2001) 192–196.
[25] M. Endale, S.D. Kim, W.M. Lee, S. Kim, K. Suk, J.Y. Cho, H.J. Park, Y. Wagley, S. Kim, J.W. Oh, M.H. Rhee, Ischemia induces regulator of G protein signaling 2 (RGS2) protein upregulation and enhances apoptosis in astrocytes, Am. J. Physiol. Cell Physiol. 298 (2010) C611–C623.
[26] C.H. Nguyen, P. Zhao, A.J. Sobiesiak, P. Chidiac, RGS2 is a component of the cellular stress response, Biochem. Biophys. Res. Commun. 426 (2012) 129–134.
[27] P. Chidiac, A.J. Sobiesiak, K.N. Lee, R. Gros, C.H. Nguyen, The eIF2B-interacting domain of RGS2 protects against GPCR agonist-induced hypertrophy in neonatal rat cardiomyocytes, Cell. Signal. 26 (2014) 1226–1234.
[28] R.T. Franceschi, C. Ge, Gene delivery by adenoviruses, Methods Mol. Biol. 455 (2008) 137–147.
[29] K.N. Lee, X. Lu, C. Nguyen, Q.P. Feng, P. Chidiac, Cardiomyocyte specific overexpression of a 37 amino acid domain of regulator of G protein signalling 2 inhibits cardiac hypertrophy and improves function in response to pressure over load in mice, J. Mol. Cell. Cardiol. 108 (2017) 194–202.
[30] W. Zhang, T. Anger, J. Su, J. Hao, X. Xu, M. Zhu, A. Gach, L. Cui, R. Liao, U. Mende, Selective loss of fine tuning of Gq/11 signalling by RGS2 protein exacerbates cardiomyocyte hypertrophy, J. Biol. Chem. 281 (2006) 5811–5820.
[31] R.C. Wek, H.Y. Jiang, T.G. Anthony, Coping with stress: eIF2 kinases and translational control, Biochem. Soc. Trans. 34 (2006) 7–11.

[32] G.D. Pavitt, D. Ron, New insights into translational regulation in the endoplasmic reticulum unfolded protein response, Cold Spring Harb. Perspect. Biol. (Jun 1, 2012), https://doi.org/10.1101/cshperspect.a012278.
[33] L.R. Palam, T.D. Baird, R.C. Wek, Phosphorylation of eIF2 facilitates ribosomal bypass of an inhibitory upstream ORF to enhance CHOP translation, J. Biol. Chem. 286 (2011) 10939–10949.
[34] Y. Ma, J.W. Brewer, J.A. Diehl, L.M. Hendershot, Two distinct stress signaling pathways converge upon the CHOP promoter during the mammalian unfolded protein response, J. Mol. Biol. 318 (2002) 1351–1365.
[35] H.P. Harding, Y. Zhang, H. Zeng, I. Novoa, P.D. Lu, M. Calfon, N. Sadri, C. Yun, B. Popko, R. Paules, D.F. Stojdl, J. Bell, T. Hettmann, J.M. Leiden, D. Ron, An integrated stress response regulates amino acid metabolism and resistance to oxidative stress, Mol. Cell 11 (3) (2003) 619–633. Available at http://www.ncbi.nlm.nih.gov/pubmed/12667446.
[36] S.P. Persengiev, M.R. Green, The role of ATF/CREB family members in cell growth, survival and apoptosis, Apoptosis 8 (3) (2003) 225–228. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12766482.
[37] K.A. Spriggs, M. Stoneley, M. Bushell, A.E. Willis, Re-programming of translation following cell stress allows IRES-mediated translation to predominate, Biol. Cell. 100 (2008) 27–38.
[38] H. Chen, R. Song, G. Wang, Z. Ding, C. Yang, J. Zhang, Z. Zeng, V. Rubio, L. Wang, N. Zu, A.M. Weiskoff, L.J. Minze, P.V.S. Jeyabal, O.C. Mansour, L. Bai, W.C. Merrick, S. Zheng, Z.Z. Shi, OLA1 regulates protein synthesis and integrated stress response by inhibiting eIF2 ternary complex formation, Sci. Rep. 5 (2015) 13241.
[39] C. Kozel, B. Thompson, S. Hustak, C. Moore, A. Nakashima, C.R. Singh, M. Reid, C. Cox, E. Papadopoulos, R.E. Luna, A. Anderson, H. Tagami, H. Hiraishi, E.A. Slone, K. Yoshino, M. Asano, S. Gillaspie, J. Nietfeld, J.P. Perchellet, S. Rothenburg, H. Masai, G. Wagner, A. Beeser, U. Kikkawa, S.D. Fleming, K. Asano, Overexpression of eIF5 or its protein mimic 5MP perturbs eIF2 function and induces ATF4 translation through delayed re-initiation, Nucl. Acids Res. (2016), https://doi.org/10.1093/nar/gkw559.
[40] C. Jousse, A. Bruhat, V. Carraro, F. Urano, M. Ferrara, D. Ron, P. Fafournoux, Inhibition of CHOP translation by a peptide encoded by an open reading frame localized in the CHOP 5′UTR, Nucleic Acids Res. 29 (2001) 4341–4351.
[41] G. Chen, Z. Fan, X. Wang, C. Ma, K.A. Bower, X. Shi, Z.J. Ke, J. Luo, Brain-derived neurotrophic factor suppresses tunicamycin-induced upregulation of CHOP in neurons, J. Neurosci. Res. 85 (2007) 1674–1684.
[42] D.T. Rutkowski, S.M. Arnold, C.N. Miller, J. Wu, J. Li, K.M. Gunnison, K. Mori, A.A. Sadighi Akha, D. Raden, R.J. Kaufman, Adaptation to ER stress is mediated by differential stabilities of pro-survival and pro-apoptotic mRNAs and proteins, PLoS Biol. 4 (2006) e374.
[43] A. Jovicic, J.F. Zaldivar Jolissaint, R. Moser, F. Silva Santos Mde, R. Luthi-Carter, MicroRNA-22 (miR-22) overexpression is neuroprotective via general anti-apoptotic effects and may also target specific Huntington's disease-related mechanisms, PLoS One. 8 (2013) e54222.
[44] H. Dong, Y. Zhang, J. Wang, D.S. Kim, H. Wu, B. Sjogren, W. Gao, L. Luttrell, H. Wang, Regulator of G protein signaling 2 is a key regulator of pancreatic β-cell mass and function, Cell Death Dis. 8 (5) (2017) e2821.
[45] V. Yanamadala, H. Negoro, B.M. Denker, Heterotrimeric G proteins and apoptosis: intersecting signaling pathways leading to context dependent phenotypes, Curr. Mol. Med. 9 (2009) 527.
[46] A.A. Roy, C. Nunn, H. Ming, M.X. Zou, J. Penninger, L.A. Kirshenbaum, S.J. Dixon, P. Chidiac, Up-regulation of endogenous RGS2 mediates cross-desensitization between Gs and Gq signaling in osteoblasts, J. Biol. Chem. 281 (2006) 32684–32693.
[47] M.X. Zou, A.A. Roy, Q. Zhao, L.A. Kirshenbaum, M. Karmazyn, P. Chidiac, RGS2 is upregulated by and attenuates the hypertrophic effect of alpha1-adrenergic activation in cultured ventricular myocytes, Cell. Signal. 18 (2006) 1655–1663.
[48] C. Nunn, M.X. Zou, A.J. Sobiesiak, A.A. Roy, L.A. Kirshenbaum, P. Chidiac, RGS2 inhibits beta-adrenergic receptor-induced cardiomyocyte hypertrophy, Cell. Signal. 22 (2010) 1231–1239.
[49] W.Z. Zhu, M. Zheng, W.J. Koch, R.J. Lefkowitz, B.K. Kobilka, R.P. Xiao, Dual modulation of cell survival and cell death by β_2-adrengergic signaling in adult mouse cardiac myocytes, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 1607–1612.
[50] H. Yoshida, ER stress and diseases, FEBS J. 274 (2007) 630–658.
[51] D. Ron, P. Walter, Signal integration in the endoplasmic reticulum unfolded protein response, Nat. Rev. Mol. Cell. Biol. 8 (2007) 519–529.
[52] J.B. DuRose, A.B. Tam, M. Niwa, Intrinsic capacities of molecular sensors of the unfolded protein response to sense alternate forms of endoplasmic reticulum stress, Mol. Biol. Cell 17 (2006) 3095–3107.
[53] B.F. Teske, M.E. Fusakio, D. Zhou, J. Shan, J.N. McClintick, M.S. Kilberg, R.C. Wek, CHOP induces activating transcription factor 5 (ATF5) to trigger apoptosis in response to pertubations in protein homeostasis, Mol. Biol. Cell 24 (2013) 2477–2490.
[54] M. Matsumoto, M. Minami, K. Takeda, Y. Sakao, S. Akira, Ectopic expression of CHOP (GADD153) induces apoptosis in M1 myeloblastic leukemia cells, FEBS Lett. 395 (1996) 143–147.
[55] E.V. Maytin, M. Ubeda, J.C. Lin, J.F. Habener, Stress-inducible transcription factor CHOP/gadd153 induces apoptosis in mammalian cells via p38 kinase-dependent and –independent mechanisms, Exp. Cell Res. 267 (2001) 193–204.
[56] S. Oyadomari, K. Takeda, M. Takiguchi, T. Gotoh, M. Matsumoto, I. Wada, S. Akira, E. Araki, M. Mori, Nitric oxide-induced apoptosis in pancreatic beta cells is mediated by the endoplasmic reticulum stress pathway, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 10845–10850.
[57] T. Gotoh, S. Oyadomari, K. Mori, M. Mori, Nitric oxide-induced apoptosis in RAW264.7 macrophages is mediated by endoplasmic reticulum stress pathway involving ATF6 and CHOP, J. Biol. Chem. 277 (2002) 12343–12350.
[58] C. Larminie, P. Murdock, J.P. Walhin, M. Duckworth, K.J. Blumer, M.A. Scheideler, M. Garnier, Selective expression of regulators of G-protein signaling (RGS) in the human central nervous system, Brain Res. Mol. Brain Res. 122 (2004) 24–34.
[59] M. Abramow-Newerly, A.A. Roy, C. Nunn, P. Chidiac, RGS proteins have a signalling complex: interactions between RGS proteins and GPCRs, effectors, and auxiliary proteins, Cell. Signal. 18 (2006) 579–591.
[60] S. Gu, C. Cifelli, S. Wang, S.P. Heximer, RGS proteins: identifying new GAPs in the understanding of blood pressure regulation and cardiovascular function, Clin. Sci. 116 (2009) 391–399.
[61] T. Ingi, A.M. Krumins, P. Chidiac, G.M. Brothers, S. Chung, B.E. Snow, C.A. Barnes, A.A. Lanahan, D.P. Siderovski, E.M. Ross, A.G. Gilman, P.F. Worley, Dynamic regulation of RGS2 suggests a novel mechanism in G-protein signaling and neuronal plasticity, J. Neurosci. 18 (1998) 7178–7188.