THE MECHANISMS REGULATING THE TRANSCRIPTION FACTOR ATF5 AND ITS FUNCTION IN THE INTEGRATED STRESS RESPONSE

Donghui Zhou

Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the Department of Biochemistry and Molecular Biology
Indiana University

November 2010

Accepted by the Faculty of Indiana University, in partial
fulfillment of the requirements for the degree of Doctor of Philosophy.

_____

Ronald Wek, Ph.D., Chair

_____

Robert Harris, Ph.D.

Doctoral Committee

_____

Lawrence Quilliam, Ph.D.

September 20, 2010

_____

Nuria Morral, Ph.D.

DEDICATION

This thesis is first dedicated to my teachers who have inspired me and taught me how to appreciate the learning process. Included in this group are my parents: Xin Zhou and Shulan Yang, Dr. Muzhen Fan, Dr. Nuria Morral, Dr. Lawrence Quilliam, Dr. Robert Harris and Dr. Ronald Wek.

I am also lucky to have friends who have inspired me to think for myself and reach beyond what I first thought impossible. These include my brother and sister: Dongjie and Wenzhao, Jingliang Yan, Lin Lin and Sixin Jiang.

I would like to thank my parents, Xin Zhou and Shulan Yang, and my mother- and father-in-law, Xihong Mo and Bin Zhong, for making the life of my wife and kids at home infinitely easier.

Finally, this thesis is dedicated to my daughters, Jiaming and Jiayi, and my wife, Minghua, who give me great joy and hope. It is my hope that these studies and the future research that builds upon them will positively impact their lives.

ACKNOWLEDGEMENTS

I first thank the other graduate students of the Wek lab, Brian Teske, Souvik Dey, Kirk Staschke, Reddy Palam and Thomas Baird, for their advice and encouragement. I have learned a lot from their work and discussions. I want to express gratitude to Sheree Wek for her selfless support. I also owe Li Jiang, Helen Jiang and Jana Narasimhan, a big thank you for their persistence and attention to experimental detail.

The members of my advisory committee, Dr. Nuria Morral, Dr. Lawrence Quilliam, Dr. Robert Harris provided me with scientific guidance during the course of my research. I also owe a tremendous amount of thanks to my mentor, Ronald Wek. His enthusiasm for research is contagious and I do not know of any laboratories where the scientific training is better. This work was supported by grants from the National Institutes of Health.

ABSTRACT

Donghui Zhou

THE MECHANISMS REGULATING THE TRANSCRIPTION FACTOR ATF5 AND ITS FUNCTION IN THE INTEGRATED STRESS RESPONSE

Phosphorylation of eukaryotic initiation factor 2 (eIF2) is an important mechanism regulating global and gene-specific translation during different environmental stresses. Repressed global translation by eIF2 phosphorylation allows for cells to conserve resources and elicit a program of gene expression to alleviate stress-induced injury. Central to this gene expression program is eIF2 phosphorylation induction of preferential translation of *ATF4*. ATF4 is a transcriptional activator of genes involved in stress remediation, a pathway referred to as the Integrated Stress Response (ISR). We investigated whether there are additional transcription factors whose translational expression is regulated by eIF2 kinases. We found that the expression of the transcriptional regulator ATF5 is enhanced in response to many different stresses, including endoplasmic reticulum stress, arsenite exposure, and proteasome inhibition, by a mechanism requiring eIF2 phosphorylation. *ATF5* is regulated by translational control as illustrated by the preferential association of *ATF5* mRNA with large polyribosomes in response to stress. *ATF5* translational control involves two upstream open reading frames (uORFs) located in the 5′-leader of the *ATF5* mRNA, a feature shared with *ATF4*. Mutational analyses of the 5′-leader of *ATF5* mRNA fused to a luciferase reporter suggests that the 5′-proximal uORF1 is positive-acting, allowing scanning ribosomes to reinitiate translation of a downstream ORF. During non-stressed conditions, when eIF2 phosphorylation is low, ribosomes reinitiate translation at the next ORF, the inhibitory

uORF2. Phosphorylation of eIF2 during stress delays translation reinitiation, allowing scanning ribosomes to bypass uORF2, and instead translate the ATF5 coding region. In addition to translational control, ATF5 mRNA and protein levels are significantly reduced in mouse embryo fibroblasts deleted for *ATF4*, *or* its target gene, the transcriptional factor C*HOP*. This suggests that ISR transcriptional mechanisms also contribute to *ATF5* expression. To address the function of ATF5 in the ISR, we employed a shRNA knock-down strategy and our analysis suggests that ATF5 promotes apoptosis under stress conditions via caspase-dependent mechanisms. Given the well-characterized role of CHOP in the promotion of apoptosis, this study suggests that there is an ATF4-CHOP-ATF5 signaling axis in the ISR that can determine cell survival during different environmental stresses.

Ronald Wek, Ph.D., Chair

TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS

ATF	activating transcription factor
ATF4	activating transcription factor 4
ATF5	activating transcription factor 5
ATF6	activating transcription factor 6
bZIP	basic zipper
CHOP	C/EBP homologous protein
C-terminus	carboxy terminus
DsRBM	double-stranded RNA-binding motif
DTT	dithiothreitol
ERSE	ER stress response element
EBER	Epstein-Barr Virus Small RNA
eIF	eukaryotic initiation factor
eIF2	eukaryotic initiation factor-2
eIF2B	eukaryotic initiation factor B
ER	endoplasmic reticulum
GAAC	general amino acid control
GADD34	Growth arrest and DNA damage-inducible protein 34
GCN	general control nonderepressible
GCN2	general control nonderepressible 2
GEF	guanine nucleotide exchange factor
HisRS	histidyl-tRNA synthetase
HIV-1	human immunodeficiency virus type 1

HRI	heme-regulated inhibitor
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ISR	integrated stress response
Met-tRNAi	Met initiator methionyl-tRNA
Min	minute(s)
mRNA	messenger RNA
mTOR	mammalian target-of-rapamycin
NaF	sodium fluoride
PCR	polymerase chain reaction
PEK	pancreatic eIF2 kinase
PERK	PKR-like ER kinase
PKR	double-stranded RNA-activated kinase
PMSF	phenylmethylsulfonyl fluoride
PP1	Protein phosphatase 1
PP1c	catalytic subunit of protein phosphatase 1
qRT	quantitative reverse transcription
RT	reverse transcriptase
S.E.	standard error
SS	signal sequence
TC	ternary complex
TF	transcription factor
TOR	target-of-rapamycin
TM	transmembrane

uORF	upstream open reading frame
UPR	unfolded protein response
UTR	untranslated region
WRS	Wolcott-Rallison Syndrome

INTRODUCTION

1. Cellular stress responses: a gateway to life or death

Environmental stresses, such as accumulation of misfolded protein in the endoplasmic reticulum (ER stress), nutrient deprivation, UV irradiation, and oxidative damage can trigger a variety of physiological and pathological responses. One example of this stress response pathway involves phosphorylation of eukaryotic initiation factor-2 (eIF2). eIF2 phosphorylation is a well-characterized translational control mechanism, which is induced by a family of protein kinases that each respond to a unique set of stress conditions (Fig. 1) (1). This translation control process, which is described in detail below, can mitigate cellular damage and determine the threshold between cell survival and apoptosis.

The eIF2 kinase stress response has three main parts. The first is the upstream stress signal that activates the eIF2 kinase response pathway. For example, heme deficiency in erythroid cells results in activation of the eIF2 kinase, Heme-regulated inhibitor (HRI). Unique stress signals also activate the other members of the eIF2 kinase family, including endoplasmic reticulum (ER) stress (PKR-like ER kinase, PERK), double stranded RNA produced during viral infection (Double-stranded RNA activated protein kinase, PKR), and nutrient deprivation (general control nonderepressible 2, GCN2) (Fig. 1) (2). The second part of the eIF2 kinase response is the system adaption to the underlying stress, which involves reconfiguration of gene expression. For example, ER stress elicits the unfolded protein response (UPR), involving induction of genes that facilitate the folding and transport of secretory proteins, ER-associated protein degradation (ERAD), and selected metabolic processes. As detailed further below,

phosphorylation of eIF2 reduces global translation coincident with preferential translation of ATF4, a transcriptional activator that can participate in the UPR during ER stress. The eIF2 kinase PERK and ATF4 function in conjunction with other UPR sensory proteins, including ATF6 (3, 4), a transcriptional activator that can bind ER stress response elements (ERSEs) in the promoters of UPR-responsive genes, and IRE1, which is an ER transmembrane protein kinase and endonuclease that facilitates cytoplasmic splicing of *XBP1* mRNA (5, 6). *XBP1* also encodes a transcriptional activator of the UPR (7, 8). The combination of gene expression directed by eIF2 phosphorylation and these UPR-specific regulators allows for the transcriptome to be tailored for the specific stress condition.

The final part of the eIF2 kinase stress pathway involves resolution of the stress damage and cell survival, or alternatively apoptosis. PERK promotes cell viability in response to ER stress, and loss of PERK induces cell death in pancreatic β-cells (9), indicating that this eIF2 kinase contributes to survival during ER stress. The PERK/ATF4 pathway can also induce the expression of CHOP, a transcription factor that can elicit apoptosis (10-13). This reflects the dual functions of the eIF2 kinase pathway in the stress context. Initially, this stress response pathway triggers adaption to restore the homeostasis. However, if the extent or duration of the stress is heightened, the eIF2 kinase response can instead switch to the progression of cell death.

2. eIF2 phosphorylation: a key regulator of protein synthesis in response to stress

2A. eIF2 is essential for the initiation of translation

eIF2 is composed of three subunits α, β, and γ, which forms a ternary complex (TC) with GTP and initiator Met-tRNAi^{Met} (14). The primary role of eIF2 in translation

initiation is to escort the initiator Met-tRNAi^{Met} to the translation machinery. The eIF2 TC assembles with 40s ribosomal subunits, and participates in the ribosomal recognition of the AUG start codon (15, 16). This process proceeds with hydrolysis of eIF2-GTP to eIF2-GDP and Pi. AUG recognition allows the release of Pi and eIF2-GDP (17). Joining of 60s ribosomal subunit yields a translation-competent 80s ribosome with the start codon and associated initiator tRNA in the P site. To facilitate the subsequent rounds of translation initiation, the GDP bound form of eIF2 is subsequently recycled to eIF2-GTP, a process facilitated by a guanine nucleotide exchange factor (GEF), eIF2B (Fig. 2).

2B. The recycling of eIF2 by eIF2B is a highly regulated step in protein synthesis

eIF2B is heteropentameric complex that is composed of five subunits α, β, γ, δ and ε (18-20). eIF2B γ and ε share sequence homology and form a binary catalytic subcomplex that catalyzes the regeneration of eIF2-GTP. The α, β and δ subunits form the regulatory part that can facilitate inhibition of eIF2B GEF activity in response to stress conditions. During translation initiation, eIF2 is released from the ribosome in the GDP bound form. Since eIF2-GTP is required to deliver Met-tRNAi^{Met} to 40S subunits, eIF2-GDP must be converted to eIF2-GTP. As eIF2 has a higher affinity for GDP, eIF2B is required to catalyze guanine nucleotide exchange. Because eIF2B promotes the release of GDP from eIF2, modulation of the GEF activity of eIF2B is a key regulatory step for translation.

The process of guanine nucleotide exchange by eIF2B is inhibited by the phosphorylation of the α subunit of eIF2 on serine 51(1). Phosphorylated eIF2α is thought to bind to the regulatory complex of eIF2B (α, β and δ subunits), leading to the

inhibition of the catalytic portion of the GEF (γ and ε subunits) (Fig. 2) (21). Because eIF2 is present at a much higher cellular concentration than eIF2B, only a portion of eIF2 is required to be phosphorylated to significantly block the guanine nucleotide exchange activity of eIF2B. The resulting reduction of eIF2-GTP lowers general translation (15), thus allowing cells to conserve enough resources and providing additional time to reconfigure gene expression designed to alleviate the damage elicited as a consequence of the underlying stress.

2C. Dephosphorylation of eIF2α and translational recovery

Since sustained repression of protein synthesis by eIF2 phosphorylation can have negative consequences, cells have developed a strategy to feedback control this translational control response. Growth Arrest and DNA Damage-inducible 34 (GADD34) is involved in this feedback process. GADD34 is a regulatory subunit for the type 1 protein phosphatase 1 catalytic subunit (PP1c) and is transcriptionally induced by ATF4 during the eIF2 kinase response. As a consequence, enhanced levels of GADD34 bind to PP1c, facilitating its recognition and dephosphorylation of eIF2α (22, 23). This feedback mechanism would allow for a resumption of translation once the stress-related genes have been induced by ATF4. Interestingly, viruses also utilize a similar strategy to overcome the cellular response that down-regulates global translation and inhibits virus replication and spread in the host. $\gamma_1$34.5, a virulence factor of herpes simplex virus has sequence homology to GADD34, and recruits PP1c to preclude phosphorylation of eIF2α triggered by PKR during viral infection (24). Mutations that disrupt the interaction between $\gamma_1$34.5 and PP1c inhibit both eIF2 dephosphorylation and viral replication. These results are

consistent with the function of GADD34 in the recovery of the shutoff of protein synthesis. Therefore, activation of GADD34 and the attendant dephosphorylation of eIF2α serve to provide a means for cells to attenuate the stress response once the stress damage has been alleviated.

2D. eIF2 kinases regulate translation during different stress conditions

A family of eIF2 kinases have been characterized in mammalian cells. As diagrammed in Fig. 3, these protein kinases each contain a conserved protein kinase domain, along with a unique regulatory region that allows for specific recognition and activation by different stresses. PERK (PEK/EIF2AK3) is an ER-resident transmembrane protein kinase, with its cytosolic portion containing the protein kinase domain, and the ER luminal part containing the regulatory elements for PERK. The regulatory elements facilitate dimerization and associate with the repressing protein, Glucose-regulated protein 78 (GRP78/BiP), a major ER chaperone whose expression is induced by UPR during ER stress (25-27).

GRP78/Bip has an ATPase domain in its N-terminus, and a peptide binding domain in its C-terminus. GRP78 binds to the hydrophobic patches of nascent polypeptides in ER with its peptide-binding domain and uses the energy from the hydrolysis of ATP to promote proper polypeptide folding and to prevent aggregation (28-30). GRP78 is also suggested to function as a regulator of the UPR by binding to ER stress sensors, such as PERK. In non-stressed cells, GRP78 associates with the luminal portion of PERK and blocks the dimerization of this eIF2 kinase; however, the overwhelming load of misfolded protein in ER stress is proposed to titrate GRP78 away

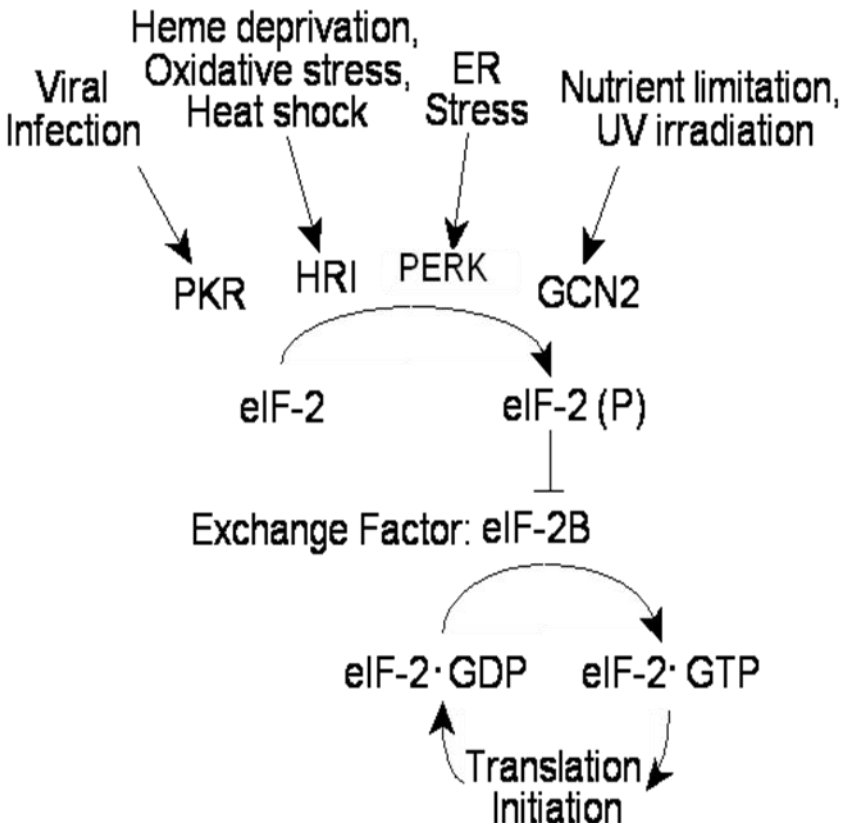


Figure 1. Protein kinases, PKR, HRI, PERK and GCN2 each respond to distinct stress conditions and phosphorylate the α subunit of eIF2 at serine-51.

Phosphorylation of eIF2α inhibits the function of the guanine nucleotide exchange factor, eIF2B, which is required for the exchange of eIF2-GDP to eIF2-GTP. The resulting reduction in eIF2-GTP levels block translation initiation, leading to a lowered global protein synthesis.

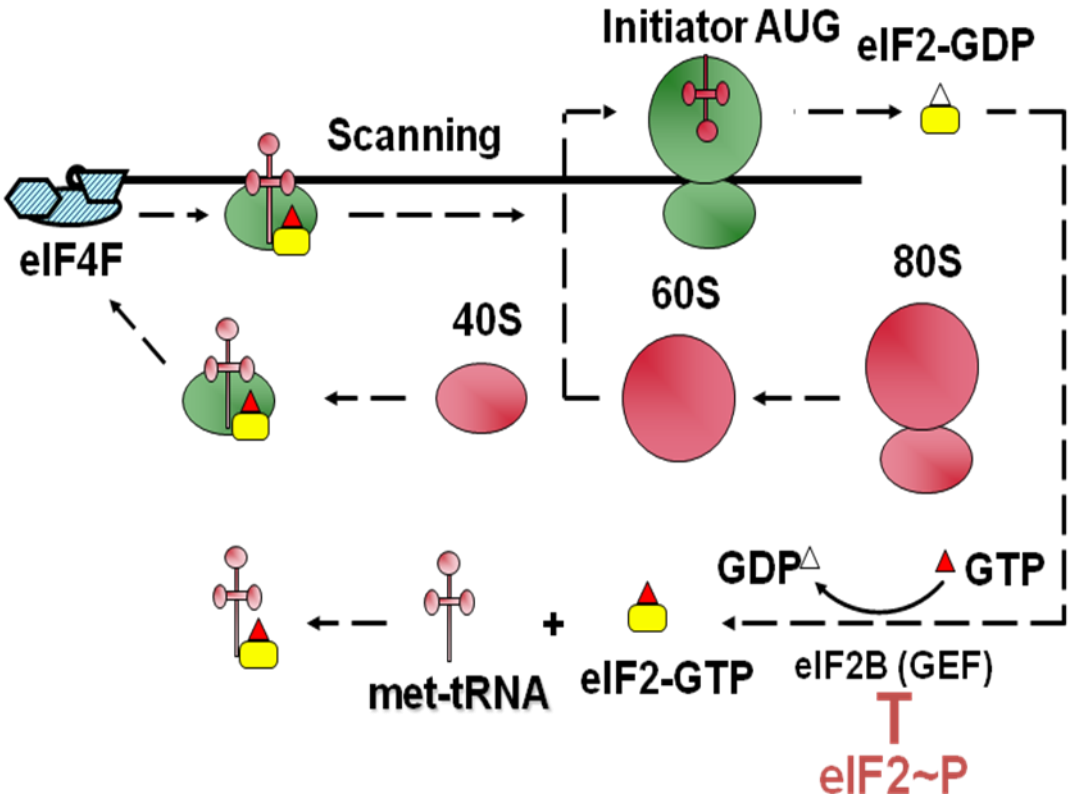


Figure 2. eIF2 associates with initiator Met-tRNA$_i^{Met}$ and GTP, and participates in the ribosomal selection of the start codon. The eIF2-GTP combines with initiator Met-tRNAi^{Met} and via additional translation initiation factors associates with the small 40S ribosome, resulting in a 43S complex. This ribosomal complex then combines with the 5'-cap structure of mRNAs consisting of the 7'methyl guanosine cap of the mRNA and associated cap-binding protein, eIF4F. The 40S ribosome and associated eIF2 TC then scans processingly 5'- to 3'- along the mRNA until an AUG initiation codon is recognized. The initiation codon bound to initiator tRNA are situated in the P site, and then the 60S ribosome joins to form the competent 80S ribosome, allowing for the elongation phase of protein synthesis to follow. Prior to this joining of the ribosomal subunits, eIF2 which has been hydrolyzed to eIF2-GDP and Pi are released, completing the cycle. A family of protein kinases phosphorylates the α subunit of eIF2 at serine-51 in

response to different environmental stresses. Phosphorylation of eIF2α converts this translation factor from a substrate to an inhibitor of eIF2B. The resulting reduction in eIF2-GTP levels lowers general translation, allowing cells sufficient time to correct the stress damage, and selectively enhance gene-specific translation that is important for stress remediation.

from PERK, facilitating PERK dimerization, which leads to auto-phosphorylation and activation of PERK. Reduced translation during ER stress is accompanied by the UPR that enhances the expression of genes involved in assembly and processing of secreted proteins. For example, PERK phosphorylation of eIF2α induces ATF4 translation by a mechanism of delayed ribosomal reinitiation (see Figure 4). ATF4 functions in conjunction with ATF6 and XBP1 to direct the UPR genes. Enhanced ATF4 translation is suggested to improve β cell survival in mice (31, 32), and *PERK* deletions lead to Wolcott Rallison Syndrome in humans, which features loss of insulin-secreting β cells (33). Since PERK is abundantly expressed in the secretory cells, and overload of insulin over time causes chronic ER stress that is proposed to lead to β cell loss, these findings suggest that PERK has an important role in proliferation and viability of secretory cells, especially pancreatic β cells.

GCN2 (EIF2AK4) functions to regulate translation from yeast to mammals, Phosphorylation of eIF2α increases the translation of ATF4 in mammals, and GCN4 in yeast *Saccharomyces cerevisiae* in response to deprivation for amino acids (34, 35). GCN2 contains a partial kinase domain, a protein kinase domain, a histidyl-tRNA synthetase-related region (HisRS), and a C-terminal region required for ribosome binding and dimerization (Fig. 3). The HisRS-related domain monitors the availability of amino acids, while the C-terminus facilitates GCN2 dimerization and ribosome association (36). The C-terminus of GCN2 has also been suggested to play an auto-inhibitory role by

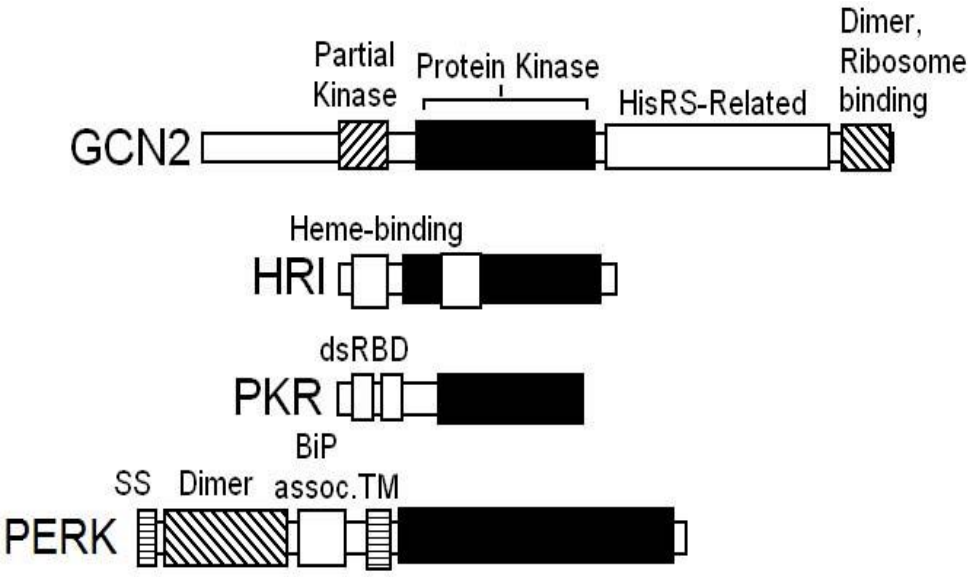


Figure 3. eIF2 kinases have different regulatory elements that facilitate recognition of unique stress conditions. Diagram of GCN2, HRI, PKR and PERK (PEK). Each eIF2 kinase has a conserved protein kinase domain represented by a black box, flanked by a divergent regulatory domain that participate in the recognition of diverse stress conditions. GCN2 contains a HisRS-related domain that monitors amino acid availability by binding to uncharged tRNAs that accumulate during nutrient deprvation, and a C-terminal region that provides for GCN2 ribosome association and GCN2 dimerization. HRI has two heme-binding domains that mediate HRI repression when heme is readily available in erythroid cells. The two dsRNA-binding domains (dsRBD) of PKR are involved in activation of the eIF2 kinase by dsRNA produced during viral infections. The PEK regulatory elements include a signal sequence (SS) important for its entry into the ER, an ER transmembrane (TM) region, and an ER lumenal region that regulates PEK dimerization and association with ER chaperones, such as GRP78/BiP (37, 38). The resulting phosphorylation of eIF2α during ER stress reduces protein synthesis, lowering the influx of nascent polypeptides into the stressed ER.

binding to the protein kinase domain (39). Amino acid starvation leads to accumulation of uncharged tRNAs, which bind to the HisRS-related domain of GCN2, eliciting a conformational change that is proposed to release the association between the kinase domain and C-terminus domain, thus enhancing the eIF2 kinase activity(40). GCN2 also functions to control translation upon treatment with UV irradiation or with exposure to drugs that inhibit the proteasome (41-43).

There is also cross-talk between GCN2 and other stress response pathways. The target of rapamycin (TOR) is a serine/threonine protein kinase and a sensor of cellular nutritional status in yeast and mammalian cells. Rapamycin, an inhibitor of TOR, induces eIF2 phosphorylation by GCN2 in yeast (44, 45). Decreased phosphorylation of 4E-BP and S6K1, two regulators of translation initiation controlled by mTOR, is blocked after leucine starvation in the liver of *GCN2* knockout mice (46). These findings indicate that GCN2 is integrated with the mTOR pathway to control protein synthesis.

In addition to preferential translation of ATF4, GCN2 phosphorylation of eIF2α can lower the synthesis of IκBα in response to UV irradiation, which is an inhibitory protein of NF-κB (47). NF-κB plays a key role in immune responses, the control of cellular proliferation, and apoptosis (48-50). The lowered synthesis of IκBα, coupled with its rapid turnover, releases the inhibitor from NF-κB, which then is transported into the nucleus. After nuclear translocation, NF-κB binds at DNA elements in the promoters of its target genes, including those involved in mitigation of stress damage and regulation of apoptosis. Loss of GCN2 or the RelA/p65 subunit of NF-κB enhances activation of Caspases 3 and 8, thus increasing apoptosis in response to UV irradiation (47). These

findings support the idea that GCN2 regulation of NF-κB is important for signaling apoptosis.

As noted above, GCN2 recognizes stresses other than nutritional deprivation, namely UV irradiation and proteasome inhibition (41-43). In response to UV irradiation, GCN2 enhancement of NF-κB activity is suggested to have a pro-survival function, whereas GCN2 activation by proteasome inhibition can facilitate a pro-apoptotic pathway. Therefore, while different stress arrangements induce GCN2 phosphorylation of eIF2α, this stress response can play different roles in cell survival. These findings suggest that GCN2 functions in conjunction with additional stress response pathways to induce a program of gene expression to modulate the stress damage.

HRI (EIF2AK1) is a heme-binding protein expressed predominantly in erythroid cells (51, 52). HRI contains two heme-binding sites, one in the N-terminus, and a second located in an insert region in the middle of the protein kinase domain (Fig. 3). Heme binding inhibits HRI kinase activity, and in response to heme deficiency, heme dissociates from the heme-binding site, leading to activation of HRI (53). The resulting phosphorylation of eIF2α down-regulates globin synthesis, the predominant synthesized polypeptide in reticulocytes. In this way, HRI serves to balance the globin synthesis and heme availability. Hemoglobin is composed of α-globin, β-globin, and heme strictly at the ratio of 2:2:4; and imbalance of this ratio is harmful. Iron is the main component of heme. In iron deficiency and low level of heme, the main adaptive response of wild-type mice is to prevent the globin synthesis through HRI phosphorylation of eIF2α, this is characterized by the red blood cell hypochromia and microcytosis; but in HRI deficient mice, globin devoid of heme aggregates improperly, characterized by hyperchromia,

compensatory erythroid hyperplasia and accelerated apoptosis (52, 54). Together, these findings illustrate that HRI not only maintains the balance between globin synthesis and heme availability, but also is required for erythroid survival upon iron deficiency.

PKR (EIF2AK2) is ubiquitously expressed in all cells at low abundance. As an integral part of anti-viral infection system, PKR is transcriptionally induced by interferon, and this eIF2 kinase is activated on binding to double-stranded RNA (dsRNA) created during viral replication (55, 56). The anti-viral effect is achieved by blocking protein synthesis, both cellular and viral, as a result of induced PKR phosphorylation of eIF2α. In the N-terminal portion of PKR are two dsRNA-binding domains (dsRBDs), while the protein kinase domain is located at the C-terminus. During virus invasion, binding of double-strand RNA to the dsRBDs brings two PKR molecules in close proximity to form dimers, and induces PKR autophosphorylation and activation, thereby inhibiting cell growth and viral replication.

Interestingly, viruses have developed several strategies to counteract the PKR mechanism. As discussed above, Herpes simplex virus $\gamma_1$34.5 is homologous to GADD34, and recruits PP1c to preclude phosphorylation of eIF2α triggered by virus infection (24). EBERs (Epstein-Barr Virus Small RNA) are noncoding RNAs expressed by Epstein-Barr virus that binds to PKR. EBERs are thought to have a similar affinity for PKR as dsRNA, which activate PKR; thus EBERs can compete for the dsRBDs of PKR and prevent PKR dimerization and activation (57). The human immunodeficiency virus type 1 evades the human immune system. One strategy is through its transcription regulatory protein, TAT, which is thought to act as the substrate homologue for PKR, competing with eIF2 for PKR phosphorylation (58). The block of PKR phosphorylation

of eIF2α would allow the virus protein synthesis to proceed. Finally, PKR is also reported to be involved in p53-mediated tumor suppression. As a target gene of p53, PKR is suggested to have an important effect in tumor-suppressor function of p53 (59).

3. Target genes regulated by eIF2α phosphorylation

3A. Phosphorylation of eIF2α induces translation of *ATF4* mRNA

Together with reduced protein synthesis, phosphorylation of eIF2α also increases the preferential translation of specific mRNAs. An important example of such preferential translation is ATF4 in mammals, and GCN4, a transcriptional activator in the yeast *Saccharomyces cerevisiae*. ATF4 is a basic zipper (bZIP) transcription activator that is important for directing the expression of genes involved in metabolism, the redox status of cells, and apoptosis. Decreased protein synthesis conserves energy and provides sufficient time for ATF4, and other stress-responsive transcription factors, to reconfigure gene expression that would block or ameliorate damage elicited by the underlying stress. Enhanced ATF4 expression during stress-induced eIF2 phosphorylation occurs primarily by translational control, as illustrated by increased association of *ATF4* mRNA with polysomes (60). Central to *ATF4* translational control is the 5'-leader of the *ATF4* mRNA, which encodes two upstream open reading frames (uORFs) that have opposing functions. *ATF4* translation begins with the 40S ribosomal subunit bound to eIF2/GTP /Met-tRNA$_i^{Met}$ scanning from the 5′-end of the ATF4 mRNA and initiating translation at the positive-acting uORF1. Following uORF1 translation, ribosomes are thought to retain association with *ATF4* mRNA and reinitiate translation at a downstream coding region. (61-63). In non-stressed cells, when eIF2 phosphorylation is low and there is abundant

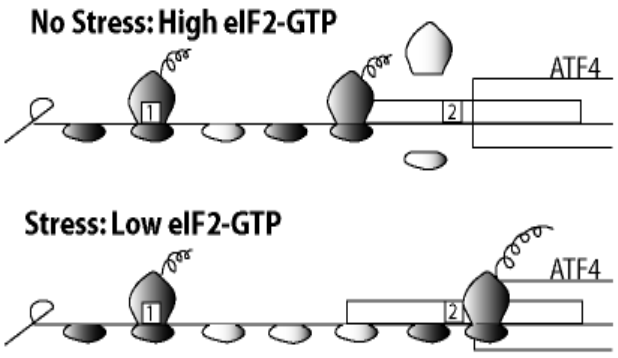


Figure 4. ATF4 translational control by its leader sequences. Illustration of the model for ATF4 translational control. In the 5'-leader of the ATF4 mRNA are two uORFs that function differently in the regulation of *ATF4* translation. The regulatory mechanism begins with translation of uORF1, which allows for retention of ribosomes and reinitiation at a downstream ORF. In nonstressed conditions, eIF2-GTP is available at high levels, and scanning ribosomes rapidly reinitiate translation at the next available ORF, uORF2. After translation of the inhibitory uORF2, ribosomes dissociate from the *ATF4* mRNA; thus there is low expression of ATF4. Cellular stress and the ensuing eIF2 phosphorylation lower the levels of eIF2-GTP, which delays translation reinitiation and allows for scanning ribosomes to bypass the uORF2 initiation codon. With the bypass of the uORF2 initiation codon, the scanning ribosomes have additional time to reacquire eIF2/GTP/Met-tRNA$_i^{Met}$ and begin translation at the *ATF4* coding region.

eIF2-GTP, ribosomes scanning downstream from uORF1 readily reinitiate translation at the next available ORF, the inhibitory uORF2. Following translation of uORF2, ribosomes are suggested to dissociate from the *ATF4* transcript, leading to lowered translation of the *ATF4* coding region. During stress conditions, elevated phosphorylation of eIF2α reduces eIF2-GTP levels, thus increasing the time required for scanning ribosomes to become competent to reinitiate translation. Following translation of uORF1, delayed reinitiation would allow for a portion of the ribosomes to bypass the uORF2 initiation codon, and instead translate the *ATF4* coding region (Fig. 4). Elevated expression of *ATF4* would lead to enhanced binding of ATF4 to the promoters of the target genes and increased transcription.

3B. uORFs regulate *GCN4* mRNA translation

The central feature of the delayed ribosome reinitiation controlling translation of *ATF4* mRNA in response to eIF2 phosphorylation is shared with the bZIP transcriptional regulator GCN4 in yeast *Saccharomyces cerevisiae* (34). GCN4 is the "master regulator" in the general amino acid control (GAAC) pathway (64). In the GAAC, amino acids starvation induces by GCN2, the only eIF2 kinase in this yeast. As sensor of amino acid depletion, activation of GCN2 requires the accumulation of uncharged tRNAs, which bind to the HisRS-related domain of GCN2 (Fig. 5). GCN2 phosphorylation of eIF2α reduces its activity, and the resulting lowered global translation allows cells to conserve resources and provides time to reconfigure the transcriptome to alleviate nutrient stress. The *GCN4* transcript has four uORFs in its 5'-leader sequence, each contributing to translational control. The uORF1 serves as a positive-acting element, allowing ribosomes

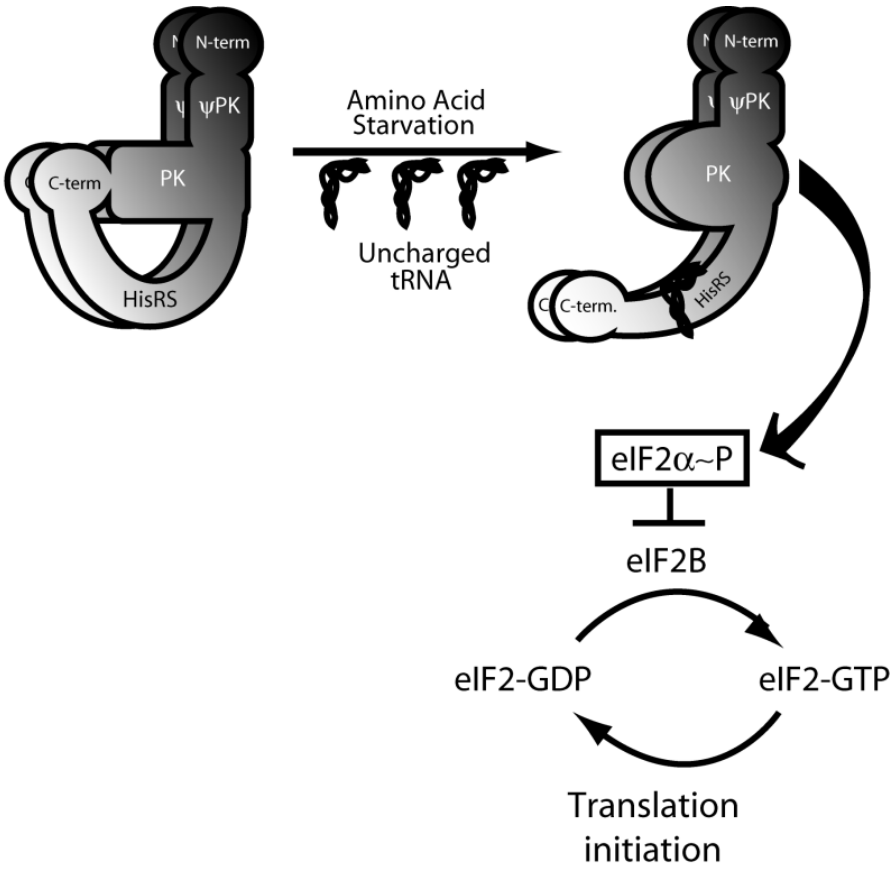


Figure 5. GCN2 protein kinase activity is enhanced by uncharged tRNA that accumulates during amino acid starvation. Upon nutrient depletion, accumulating uncharged tRNAs are proposed to bind to the HisRS-related domain of GCN2, eliciting a conformational change that results in GCN2 autophosphorylation and activation of GCN2 kinase activity. GCN2 phosphorylation of eIF2α converts the translation initiation factor into a potent inhibitor of the guanine nucleotide exchange factor eIF2B, which reduces translation initiation.

to reinitiate at a downstream uORF. uORFs 2, 3, and 4 function as inhibitory elements, and translation of one of these inhibitory uORFs is thought to facilitate dissociation of ribosomes from the mRNA, and thus lower translation of the GCN4 coding region (65). Upon amino acid starvation, with elevated phosphorylation of eIF2α and low eIF2-GTP levels, ribosomes would translate the 5'-proximal- uORF1. Following translation of the positive-acting uORF1, ribosomes would resume scanning, but there is delayed translation reintiation. With this delay, the ribosomes would scan through the inhibitory uORFs 2, 3, and 4. During the interval between uORF4 and the *GCN4* coding region, the scanning ribosomes would reacquire the eIF2 ternary complex and translate the *GCN4* coding region (Fig. 6) (66-68). Increased GCN4 protein levels would then enhance the transcription of the hundreds of genes subject to the yeast GAAC.

3C. Role of ATF4 in response to diverse cellular stresses

Induction of ATF4 mediates the integrated stress response (ISR) initiated by phosphorylation of eIF2α, which can protect cells against metabolic consequences of ER stress. ATF4 can form homodimers or heterodimers with other bZIP transcription factors, and elevated ATF4 synthesis directly contributes to increased binding of this transcription activator to the promoters of targeted genes. Activation of ATF4 induces many ISR target genes, including GADD34, which as described above facilitates feedback dephosphorylation of eIF2α. Microarray studies utilizing *PERK*$^{-/-}$ and *ATF4*$^{-/-}$ mouse embryo fibroblast (MEF) cells reported that of the genes requiring PERK activation and eIF2 phosphorylation for their induction in response to ER stress, about

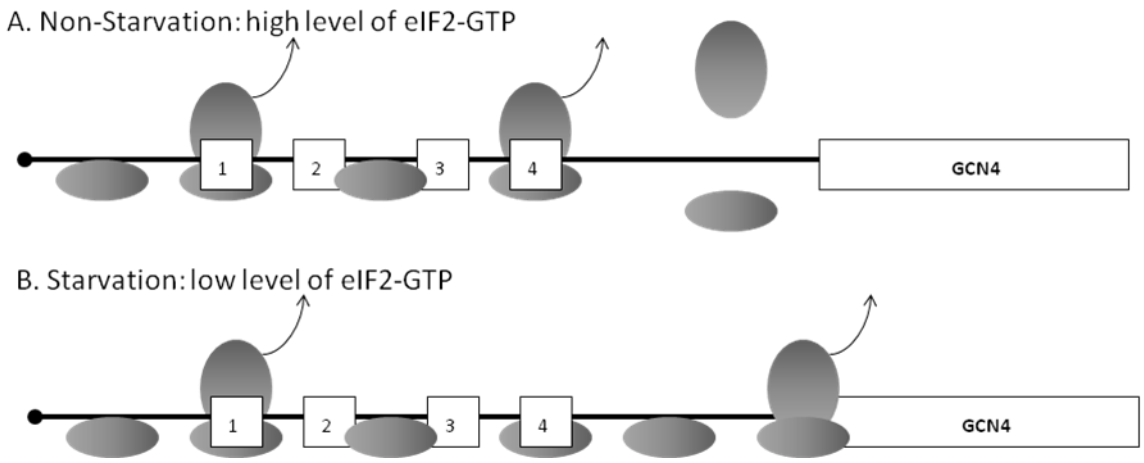


Figure 6. Model for *GCN4* translation in amino acid starvation. In nonstressed cells, ribosomes completing translation of the 5'-proximal uORF1 reinitiate at a downstream uORF, uORFs 2, 3, and 4, which function as inhibitory elements. Translation of one of these inhibitory uORFs facilitates dissociation of ribosomes from the mRNA, and thereby lowers translation of the *GCN4* coding region. During amino acid starvation, uncharged tRNA activates GCN2 phosophorylation of eIF2. The resulting low levels of eIF2-GTP allows for the 40S ribosomes to scan through the inhibitory uORFs 2, 3, and 4 located in the 5'-leader of the *GCN4* mRNA. In the interval between ORF4 and the *GCN4* coding sequences, scanning ribosomes associate with eIF2-GTP and initiate translation at the *GCN4* coding sequences. Increased levels of GCN4 then enhances the transcription of genes subject to the GAAC.

half required ATF4 function (69). These results suggest that there may be additional transcription factors that are important for directing the eIF2 kinase pathway and are subject to translational control.

4. Integration of ATF5 into the eIF2 kinase stress response

4A. General properties of ATF5

As noted above, four different eIF2 kinases have been identified in mammals, each participating in a complex network of stress-related gene expression. In addition to ATF4, other targets of preferential translation are thought to facilitate regulation of gene expression by eIF2 kinases. One candidate is ATF5, a bZIP transcriptional regulator that is encoded by an mRNA that contains two uORFs with analogous proximity to that described for the *ATF4* transcript (Fig. 7).

ATF5 belongs to the activating transcription factor ATF/cAMP response-element binding protein (CREB) family. When the original ATF5 cDNA clone was isolated and characterized, it was named differently as ATFx (70). ATF5 was first determined to be a binding partner of G-CSF gene promoter element 1-binding protein (GPE1-BP) and was classified as a member of the ATF4 subgroup due to its 55% sequence identity with the ATF4 protein. As an ATF4 subfamily member, ATF5 contains a C-terminal leucine zipper that directs homophilic dimerization (71). Additionally, ATF5 has a central proline-rich domain for DNA transactivation (72).

The human ATF5 gene is 5.2-kb in length, and is placed in chromosome 19 at the cytogenetic band: 19q13.3 at 50,431,974-50,437,192 bp (Chormosome accession: NC_000019.9, RefSeq Accession: NM 012068). ATF5 has two mRNA isoforms (*ATF5α*

and *ATF5β*) that encode the same protein, with different 5'-leader sequences (72). *ATF5α* is predominant during development and in the adult with the highest levels present in liver, while *ATF5β* mRNA is secondary, with expression restricted to development (71); however, due to the unavailability of a satisfactory antibody, ATF5 protein expression has not been detected reliably. As described further in the Results section, our laboratory produced ATF5 antibody and we have been able to reliably detect ATF5 protein by immunoblot analysis.

Examination of the 5'-leader sequence of *ATF5α* mRNA revealed two uORFs that are conserved among many different vertebrates, including human, mouse, rat, cow, and frogs (Fig. 7). The 5′-proximal uORF1 encodes a polypeptide that is only three amino acid residues in length, Met-Ala-Leu, that is conserved among the different *ATF5* orthologs. The downstream uORF2 encodes a polypeptide ranging from 59 residues in length in human and cow, to 53 residues in the frog *ATF5* mRNA. In each example, the uORF2 overlaps, out of frame, with the *ATF5* coding region. Based on the similarity of 5'-leader structure and protein level between *ATF4* and *ATF5*, *ATF5* is a potential target for uORF-mediated translational control. Although our understanding of the biological function of ATF5 is limited and fragmentary, ATF5 has recently emerged as a key player in cell differentiation, cell survival and apoptosis (73-81). It is likely that the characteristics of ATF5 function are cell-type-dependent and context-dependent, and this transcription factor is a central subject of this thesis.

4B. Functional role of ATF5 in nervous system

The main focus of published research on ATF5 concerns its functions in neuroprogenitor cells. *ATF5* transcript was first detected in olfactory epithelium and vomeronasal organ of the nasal cavity during mouse embryonic development, suggesting its role in olfactory sensory neuron differentiation (72). Comparisons between PC12 pheochromocytoma cells before and after a 9 days of NGF exposure suggested that ATF5 is involved in neuronal differentiation (77). NGF promotes PC12 differentiation rather than proliferation. Before NGF exposure, *ATF5* transcripts are highly expressed in the cells, compared to a 25-fold decrease in *ATF5* mRNA levels after NGF treatment. Constitutive expression of *ATF5* was reported to block NGF-induced neuron differentiation, and knock-down of *ATF5* using RNAi enhanced NGF-promoted neurite outgrowth (77). These findings led to the idea that ATF5 serves as a negative regulator of neuronal differentiation. This function for ATF5 is further supported by its presence in the ventricular zone of the E12, E14, and E17 rat telencephalon; such an expression pattern is not detected on the surface of the developing cortex, where the neuronal marker, Tubulin bIII (TUJ1) is expressed. ATF5 is also suggested to block the differentiation of the neuroprogenitor cells to astrocytes (82) and oligodendrocytes (83). Based on these findings, appropriate expression of ATF5 is proposed to be critical for neural differentiation.

4C. ATF5 and cell survival

Apart from its role in neuroprogenitor cells, ATF5 is also suggested to have a role in cell survival. Repression of ATF5 mRNA has been reported to correlate with the

induction of apoptosis in response to growth factor deprivation in multiple cell lines and primary cells (84). Ectopic expression of ATF5 suppresses apoptosis induced by cytokine deprivation in an IL-3 dependent cell line. Further supporting the role of ATF5 in cell survival, high expression of ATF5 was detected in glioblastoma cells, and interference with ATF5 function resulted into glioma cell death (82). High abundance of ATF5 was also found in other neoplasms, leading to speculation that ATF5 provided an advantage in cell proliferation and survival (74). Finally, a role of ATF5 in cell survival is also suggested by its ability to upregulate the level of MCL1, an antiapoptotic B cell leukemia-2 (BCL2) family member, and to block the p53-dependent apoptosis induced by ionizing irradiation (78, 85). Aside from these target genes, early growth response factor 1 (*Egr-1*), a transcription factor that promotes cell survival, is also upregulated by ATF5 (79).

By contrast, Wei and his colleagues suggested that ATF5 facilitated cisplatin-induced apoptosis. Although the precise mechanisms for the ATF5-mediated apoptosis are not clear, it is suggested that ATF5 can up-regulate transcription of cyclin D3 during cisplatin treatment (86). Furthermore, it was suggested that ATF5 protein degradation through the E3 ligase, Cdc34, mediates is blocked by cisplatin (87). These findings suggest that ATF5 may have diverse functions that can regulate cell survival in response to stress. Whether ATF5 plays a pro-survival or pro-apoptotic functions may depend on the precise stress arrangement and the cell types.

4D. ATF5 and its target genes

Limited information is known about the downstream gene targets for ATF5. Several reports suggested that ATF5 binds to CRE elements and represses their activation by CREB (88). However, overexpression of ATF5 was reported to increase the levels of *HSP27* transcripts in H9c2 cells partly via a CRE site and loss of ATF5 function inhibits *HSP27* expression. These data suggest that ATF5 function in CRE-containing promoters may be dependent on cellular context. Overexpression of ATF5 is also reported to induce *CYP2B6, Cyclin D3* and *MCL1*, although the exact mechanisms are not clear (80, 85, 86). Considering the sequence similarity between ATF4 and ATF5 proteins, as well as the possible association of ATF5 with the eIF2 kinase network, it is reasonable to consider the role of ATF5 in the cellular stress context in conjunction with other signaling pathways. Deciphering the regulatory mechanisms of ATF5 expression is central for understanding the role of ATF5 in survival and differentiation.

5. Role of eIF2 phosphorylation in disease

Translational control by eIF2 phosphorylation is associated with several medically related stress conditions, including anemia, viral immunity, stroke, cancer, neurological dysfunctions, and diabetes. One of the best illustrated examples is a rare autosomal recessive disease, Wolcott-Rallison Syndrome (WRS) that results from mutations in the *PERK (PEK/EIF2AK3)* gene (33, 89, 90). WRS patients present with neonatal insulin-dependent diabetes, but do not display auto-antibodies diagnostic of type I diabetes. WRS patients can also have epiphyseal dysplasia, osteoporosis and growth retardation. Frequently, afflicted patients suffer from multisystemic pathologies,

including hepatic and renal complications, cardiovascular disease and mental retardation. Interestingly, mice deleted for *PERK* present with the characteristic diseases described in WRS patients (91, 92). This includes dysfunction in both endocrine and exocrine tissues in the pancreases, and deficiency in osteoblast differentiation and maturation, with *PERK*-deficient mice being severely osteopenic. The trafficking and secretion of collagen I is compromised and collagen I is abnormally retained in the ER. Loss of PERK in tumor cells also results in impaired regeneration of intracellular antioxidants and accumulation of oxidative DNA damage induced by reactive oxygen species (ROS) (93). This has led to the proposal that PERK plays an important role in the progression of solid tumors (93).

There is also a connection between dysfunction of the other eIF2 kinases and disease. A "knock-in" mouse with an alanine substituted for the phosphorylation site, serine-51, in eIF2α (A/A) dies within 12 to 48 hours of birth due to apparent metabolic conflicts, including severe hypoglycemia (94). *GCN2*$^{-/-}$ mice fed on a leucine deprived diet show a marked loss of skeletal muscle mass compared to their wild-type littermates (95), with about 40% of the *GCN2*$^{-/-}$ mice expiring within three days of the nutrient stress (96). *GCN2*-deficient mice also have been reported to develop hepatic steatosis and exhibit reduced lipid mobilization when fed a leucine-deprived diet (97). HRI disruption exacerbates the microcytic and hypochromic consequences during iron-deficient anemia (51), and *PKR*-deficient mice display a differential virus sensitivity, with for example *PKR*$^{-/-}$ mice being more permissive for vesicular stomatitis virus infection than its wild-type counterpart (98-100). The active form of PKR is overexpressed in the brain of

patients with Alzheimer's disease, suggesting that this eIF2 kinase participates in the neurodegeneration process (101).

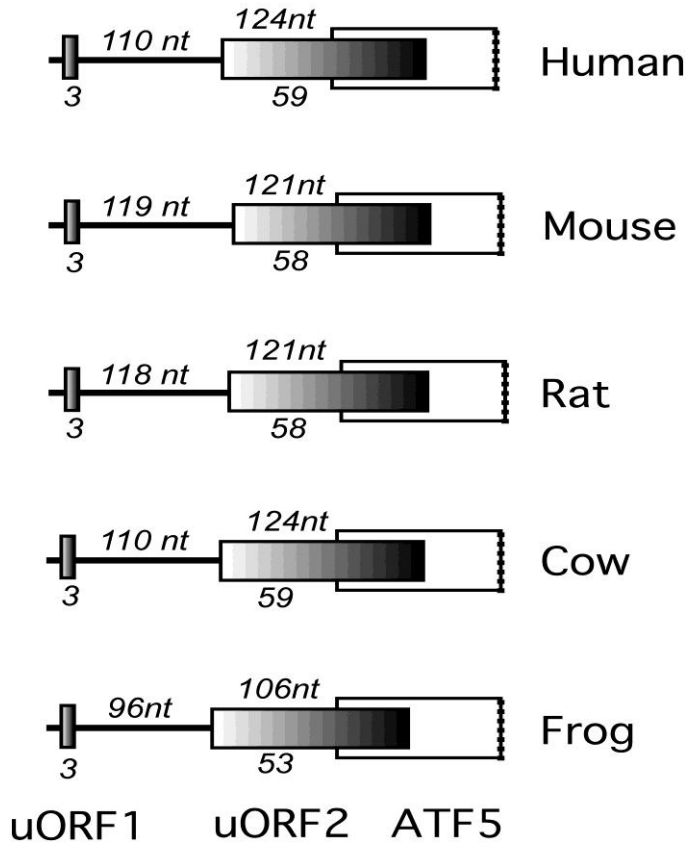


Figure 7. Two uORFs are present in the 5′-leader of the *ATF5* mRNA from different vertebrates. Representative cDNAs encoding ATF5 orthologs, including human (GenBank™ accession number AB073613), mouse (AF375475), rat (BC061786), cow (DV917530), and frog, Xenopus tropicalis (BC076876), indicate that each mRNAs contain a similar 5′-leader configuration with two uORFs. In each of the panels, the dark-colored boxes represent the two uORFs, and the white box overlapping uORF2 represents the 5′-portion of the *ATF5* coding region. The number of nucleotides between uORF1 and uORF2, and between the first residue of uORF2 and the *ATF5* coding region, is indicated on the top of each panel. The numbers indicated below each panel highlight the number of amino acid residues encoded by each uORF. The uORF1 in each of the *ATF5* transcripts encodes a three residue polypeptide, Met-Ala-Leu.

MATERIALS AND METHODS

1. Expression of Recombinant ATF5 and ATF4 and Antibody Production

A cDNA encoding human ATF5 was inserted between the BamHI and XhoI restriction sites of plasmid pET28, yielding p834 that encodes an N-terminal polyhistidine-tagged version of full-length *ATF5* expressed from an inducible T7 promoter. This plasmid was introduced into *Escherichia coli* (*E. coli*) strain BL21(DE3) (F^- ompTrB^- containing lysogen DE3), and bacterial cells were grown at 37 °C with shaking in Luria-Bertani medium supplemented with 100 µg/ml ampicillin until an A_{600} of between 0.4 and 0.6. One mM IPTG was added to cultures to enhance *ATF5* expression from the T7 promoter, and after further incubation at 37 °C for 6 h, cells were collected by centrifugation. The cell pellet was suspended in Buffer A solution (20 mM Tris [pH 7.9], 500 mM NaCl, and 10% glycerol) containing 10 mM imidazole and lysed using a French press. Proteins in the soluble and insoluble portions of the lysates were separated by SDS-PAGE and visualized by staining with Coomassie R-250. The majority of recombinant ATF5 protein was found to be in the insoluble fraction. The soluble lysate portion was applied to nickel-nitrilotriacetic acid-agarose (Qiagen) equilibrated with Buffer A and incubated at 4 °C. The agarose was washed with Buffer A solution containing 50 mM imidazole, and the ATF5 protein was eluted with buffer A solution containing 200 mM imidazole. The purified recombinant ATF5 protein was M*r* ~ 35,000 and was specifically recognized by antibody (Santa Cruz) recognizing the polyhistidine tag.

Cloning and expression of human *ATF4* in *E. coli* was performed in the similar strategy. Amplified products were cleaned using QIAquick Gel Extraction Kit

(QIAGEN), digested with BamHI and XhoI, and ligated with appropriately digested pET28. Recombinant plasmids were transformed into competent *E. coli* DH5α. Individual clones were isolated from transformants, and sequenced to confirm nucleotide identity. The resulting plasmid p842 encodes ATF4 with an N-terminal polyhistidine-tag. Then, freshly transformed cells of *E. coli* strain BL21(DE3) harboring p842, were grown in 1 liter of LB supplemented with 100 μg/ml ampicillin until an A_{600} of between 0.4 and 0.6. To induce protein expression, freshly prepared IPTG was added to the culture to a final concentration of 0.5mM and growth was continued for a further 6 hours at 37 °C. Cells were harvested by centrifugation and stored at −20 °C until further processing. The cell pellets were then washed and re-suspended in Buffer A solution containing 10 mM imidazole and lysed using a French press. Proteins in the soluble and insoluble portions of the lysates were separated by SDS-PAGE and visualized by staining with Coomassie R-250. The soluble lysate portion was applied to nickel-nitrilotriacetic acid-agarose (Qiagen) equilibrated with Buffer A and incubated at 4 °C. The agarose was then packed into a disposable column. The column was eluted with 10 column volumes of Buffer A solution containing 50 mM imidazole to remove contaminating proteins, and the ATF4 protein was eluted with 3-4 ml buffer A solution containing 200 mM imidazole. The purified recombinant ATF4 protein was Mr ~ 55,000 and was specifically detected by anti-His antibody.

To prepare ATF5 and ATF4-specific antibodies, the recombinant proteins ATF4 or ATF5 were separated by SDS-PAGE respectively, and the ATF5 and ATF4 protein were sliced from the polyacrylamide gel and injected into rabbits. The antisera of ATF5 or ATF4 that were generated were affinity-purified using recombinant protein. The

purification involved the transfer of the recombinant protein onto nitrocellulose by electrophoretic transfer, staining of the protein by Ponceau S, and slicing the band corresponding to the recombinant proteins from the filter. The antisera were incubated with the filter slices overnight at 4 °C. The antibodies were then eluted from the filter band with 0.1M glycine-HCl [pH 2.7], neutralized with 1/10 volume of 2 M Tris (pH 8.0). As described further below in the immunoblot analyses, the ATF5- and ATF4-specific antibody preparations recognized purified recombinant ATF5 and ATF4 protein, respectively, and the specific transcription factor in mouse and human cell lines that was induced by different stress conditions.

2. Cell Culture and Stress Conditions

Mouse embryo fibroblast (MEF) cells that were derived from *S/S* (wild type eIF2α) and *A/A* (mutant eIF2α-Ser51A) mice were previously described (94, 102). *ATF4*$^{-/-}$ and *CHOP*$^{-/-}$ MEF cells, and their wild-type counterparts, were reported previously (103). MEF cells knocked down for *ATF5* expression by shRNA, as further described below. MEF cells were cultured in Dulbecco's modified Eagle's medium supplemented with 1 mM non-essential amino acids, 100 units/ml penicillin, 10% fetal bovine serum, and 100 μg/ml streptomycin. ER stress was elicited in MEF cells by the addition of either 0.1 μM or 1 μM thapsigargin to the medium, as indicated, followed by incubation for up to 6 h. Alternatively, 20 μM arsenite or 1 μM of the proteasome inhibitor, MG132, was added to the culture medium, and the MEF cells were cultured for up to 24 h, as indicated.

3. shRNA Lentivirus Knock-Down of *ATF5*

To establish MEF cell lines with stable knockdown of *ATF5*. pLKO.1-puro Control plasmid and five pLKO.1 plasmids (Sigma Mission shRNA) containing *ATF5* lentiviral shRNAs TRCN0000075553 (CCGGCCCTCCATTCCACTTTCCTATCTC GAGATAGGAAAGTGGAATGGAGGGTTTTTG), TRCN0000075554: (CCGGCC TGCTAATTGAGGTGTATAACTCGAGTTATACACCTCAATTAGCAGGTTTTTG), TRCN0000075555 (CCGGAGTACGTGAAGGACCTGCTAACTCGAGTTAGCAGG TCCTTCACGTACTTTTTTG, TRCN0000075556 (CCGGGCGGGAGATCCAGTACG TGAACTCGAGTTCACGTACTGGATCTCCCGCTTTTTG) and TRCN0000075557 (CCGGTGACGGCTTCTCTGATTGGATCTCGAGATCCAATCAGAGAAGCCGTCA TTTTTG), targeting the *ATF5* coding sequence or 3'-UTR were separately transduced into 293T cells along with Lentiviral Packaging Mix, consisting of an envelope and packaging vector (Sigma) to produce lentivirus packed with shRNA cassettes using the standard procedure. Viruses were harvested from the media on day 3 by centrifugation at 3000 × g for 15 min at 4 °C.

MEF cells (at 1×10^4 $well^{-1}$) were seeded in 96-well plates with 120 μL fresh medium for overnight. Puromycin was added at concentration of 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3, 5, 7, 10, 15 and 20 μg/ml. Cells viability was examined every two days. The medium containing puromycin was replaced every 3 days. The minimum concentration of puromycin that causes complete MEF cell death was 3μg/ml.

MEF cells were then incubated with viral supernatant in the presence of 10 μg/ml of polybrene. After 1 day of incubation, the medium was changed to DMEM. Puromycin was added 2 days after transduction at 3 μg/ml to select for the transduced cells.

Transduced cells were cultured until all the non-transduced cells were dead in 2 days. Puromycin-resistant colonies were isolated and expanded. Each clone was assayed for knockdown of *ATF5*.

4. Preparation of Protein Lysates and Immunoblot Analyses

MEF cells cultured in stressed or non-stressed conditions were washed two times with chilled phosphate-buffered solution, and lysed in a solution containing 50 mM Tris-HCl (pH 7.9), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 100 mM NaF, 17.5 mM β-glycerolphosphate, 10% glycerol supplemented with protease inhibitors (100 μM of phenylmethylsulfonyl fluoride, 0.15 μM aprotinin, 1 μM leupeptin, and 1 μM of pepstatin) and sonication for 30 seconds. Cell lysates were clarified by centrifugation to remove the pellet, and protein content was determined by the Bio-Rad protein quantitation kit for detergent lysis following the manufacturer's instructions. Equal amounts of each protein sample were separated by SDS-PAGE, and proteins were then transferred to nitrocellulose filters. Polypeptide markers of known molecular weights (Bio-Rad) were included to determine the size of proteins identified in the immunoblot analysis. Transferred filters were then incubated in TBS-T solution containing 20 mM Tris-HCl (pH 7.9), 150 mM NaCl, and 0.2% Tween 20 supplemented with 4% nonfat milk, followed by incubation with TBS-T solution with antibody that specifically recognized the indicated proteins. ATF5 and ATF4 polyclonal antibodies were prepared against the recombinant human protein, as described above. Cleavaged-Caspase-9 (#9509) and Cleavaged-Caspase-3 (#9664) were from Cell Signaling Technology. CHOP (sc-7351) antibody was obtained from Santa Cruz Biotechnology, and β-actin

monoclonal antibody (A5441) was purchased from Sigma. Polyclonal antibody that specifically recognized phosphorylated eIF2α at Ser-51 was purchased from BioSource (44-728G). Monoclonal antibody that recognizes either phosphorylated or nonphosphorylated forms of eIF2α was provided by Dr. Scot Kimball (Pennsylvania State University, College of Medicine, Hershey, PA). Filters were then washed three times in TBS-T, and the protein-antibody complexes were visualized using horseradish peroxidase-labeled secondary antibody and chemiluminescent substrate. Autoradiograms shown in the figures are representative of three independent experiments.

5. RNA Isolation and Analyses

Northern analyses were carried out as previously described (98-100). Total cellular RNA was isolated from *S/S, A/A, CHOP*$^{-/-}$, and *ATF4*$^{-/-}$ MEF cells treated with 1 μM thapsigargin, 20 μM sodium arsenite, or no stress, for the indicated number of hours using the TRIzol reagent (Invitrogen) following the manufacturer's instructions. 10 μg of total RNA from each sample preparation was separated by electrophoresis using a 1.2% agarose gel and visualized by using ethidium bromide staining and UV light. RNA was transferred onto nylon filters and hybridized to ^{32}P-labeled-DNA probes specific for the indicated genes. Filters were washed using high stringency conditions and visualized by autoradiography. Levels of ATF5-luciferase mRNA expressed in *S/S* and *A/A* MEF cells transfected with the ATF5-Luc fusion constructs were treated with 0.1 μm thapsigargin for 6 h, or no stress. A ^{32}P-labeled probe complementary to the luciferase reporter gene was used in a Northern blot analysis to measure ATF5-Luc transcripts.

Quantitative RT-PCR was carried out by two-step protocol (Roche). Total cellular RNA was isolated from *S/S, CHOP*$^{-/-}$, and *ATF5* KD MEF cells treated with 1 μM MG132, 20 μM sodium arsenite, or no stress, for the indicated number of hours using the TRIzol reagent following the manufacturer's instructions. The samples were then treated for 0.5 hr at 37 °C with DNase I using a DNase kit to ensure complete removal of the DNA (Promega), followed by the addition of DNase inactivation reagent. In the RT step, cDNA was synthesized using reverse transcription kit (Roche), and then the PCR step was carried out with gene specific probes (Applied Biosystems) for detection of target genes.

6. Plasmid Constructions and Luciferase Assays

For ATF5 luciferase reporter, PCR was used to generate a HindIII-PagI fragment DNA encoding the full-length *ATF5* mRNA leader and *ATF5* initiation codon, which was inserted between HindIII and NcoI restriction sites in a derivative of plasmid pGL3. The resulting plasmid contains the 5′-portion of the *ATF5* coding sequence fused to the luciferase reporter gene downstream of a minimal TK promoter. The ATG initiation codons in each of the uORFs in the *ATF5* mRNA were mutated individually or in combination to AGG using the site-directed mutagenesis kit (Stratagene), following the manufacturer's instructions. All mutations were sequenced to ensure that there were only the desired changes. Plasmid transfections were performed using the *S/S* and *A/A* MEF cells grown to 40% confluency and the FuGENE 6 transfection reagent (Roche Applied Science). Co-transfections were carried out in triplicate using wild type or mutant versions of the *ATF5-Luc* fusion plasmids and a *Renilla* luciferase plasmid serving as an

internal control (Promega, Madison, WI). 24 h after transfection, MEF cells were treated with 0.1 μM thapsigargin for 6 h, or with no ER stress. Dual luciferase assays were carried out as described by the Promega instruction manual. Values are a measure of a ratio of firefly *versus Renilla* luciferase units (relative light units) and represent the mean values of three independent transfections. Results are presented as means ± S.E. that were derived from three independent experiments. The Student's *t* test was used to determine the statistical significance.

7. Transcriptional Start Site of *ATF5* Transcripts

The cDNAs corresponding to the 5′-ends of the ATF5-Luc transcripts expressed in S/S MEF cells treated with 0.1 μM thapsigargin, or no stress, were amplified using a RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE kit, Ambion) following the manufacturer's instructions. Alternatively, there was amplification of cDNAs corresponding to the 5′-ends of ATF5 mRNA prepared from human HepG2 hepatoma cells treated with this ER stress condition, or no stress. Briefly, 10 μg of total RNA was treated with calf intestinal phosphatase, resulting in the removal of free 5′-phosphates from RNAs other than mRNAs containing intact 5′-cap structures. The RNA preparations were then treated with tobacco acid pyrophosphatase to remove the cap structure, leaving a 5′-monophosphate that was ligated using T4 RNA ligase to a 45-base RNA adapter oligonucleotide that was supplied in the kit. A random-primed reverse transcription (RT) reaction and nested PCR were then carried out to amplify the 5′-end of endogenous ATF5 transcripts, as well as transfected thymidine kinase-minimal promoter driven ATF5-Luc mRNAs. The primers corresponding to the 5′-RACE adapter sequence

were provided by the manufacturer. The sequences of the two nested antisense primers specific to endogenous ATF5 mRNA were the outer primer 5′-TTCCCATAGTCTA CGAGCCATCCC-3′ and inner primer 5′-ACATGGCTGTAGCACAGGTGCT-3′. The outer primer used for amplifying the 5′-ends of ATF5-Luc transcript was 5′-CCATCTT CCAGCGGATAGAA-3′, which was combined with the same inner primer described earlier. A portion of the amplified DNA products were analyzed by agarose gel electrophoresis, and the DNA was visualized by ethidium bromide staining and UV irradiation. The major DNA band was excised from the gel and sequenced. The transcriptional start site was determined as the first nucleotide residue that was 3′- to the adapter sequence that was ligated to 5′- of the cDNA.

8. Polysome Analysis of ATF5 Translational Control

S/S cells were cultured in Dulbecco's modified Eagle's medium, as highlighted above, in the presence of 1.0 μM thapsigargin, or to no stress, for 6 h. 10 μg/ml cycloheximide was added to the medium prior to collection and analysis. Cells were washed in cold phosphate-buffered-saline solution supplemented with10 μg/ml cycloheximide, and then lysed with ice-cold lysis buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 100 mm NaCl, 0.4% Nonidet P-40, and 10 μg/ml cycloheximide. The extracts were passed through a 23-gauge needle for proper lysis of cells, incubated for 10 min on ice, and insoluble material was collected by microcentrifugation at 10,000 rpm for 10 min at 4 °C. The resulting supernatant was then applied onto a 15-45% sucrose gradient containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 100 mM NaCl, and 10 μg/ml cycloheximide, and subjected to centrifugation for 2 hours at 40,000 rpm in a

Beckman SW-41Ti rotor. Following centrifugation, the gradients were fractionated, and the absorbance of cytosolic RNA at 254 nm was recorded by an in-line UV monitor. Total RNA was isolated from a portion of each fraction using TRIzol reagent as described by the manufacturer's instructions (Invitrogen). The mRNAs from fractions collected were amplified using RT-PCR kit (Invitrogen) and the following primers: ATF4 forward (5′-TCACGAAATCCAGCAGCAGTG-3′), ATF4 reverse (5′-CAAGCCAT CATCCATAGCCG-3′), ATF5 forward (5′-CTACCCCTCCATTCCACTTTCC-3′), ATF5 reverse (5′-TTCTTGACTGGCTTCTCACTTGTG-3′), β-actin forward (5′-TTCTTTGCAGCTCCTTCGTTGCCG-3′), and β-actin reverse (5′-TGGATGG CTACGTACATGGCTGGG-3′). For RT-PCR analysis of cytosolic mRNA levels, equal volumes were reverse transcribed using oligo(dT), then 50 ng of cDNA from each fraction was amplified with Bullseye R-Taq (MIDSCI). PCR was carried out for 25, 27, 30, 33, and 35 cycles to determine the linear range of amplification. In this study, 25 cycles were used for PCR for ATF4, and 27 cycles for ATF5 and β-actin. Densitometry was performed using the software provided with Quantity One imaging system (Bio-Rad).

9. Cellular survival assays

Wild type, *CHOP*$^{-/-}$ and two *ATF5* knockdown MEF cell lines were used for a CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega G4001). This colormetric assay is based on the living cell conversion of a tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] of the Dye solution into formazan product. This assay was performed in a 96 well plate format and read on a 96

well plate reader at a wavelength of 570 nm. 5,000 cells were plated in 50 µl DMEM supplemented with 10% FBS, penicillin (100 international units/ml) and streptomycin (100 µg/ml). After overnight incubation when cells adhered to the bottom of the wells, a final concentration of 1 µM MG132 or media control was added to each well. Cells were incubated in the presence of the MG132 for 1, 3, 6, 12, or 18 hours, or no treatment. At the indicated times, the media containing MG132 was removed by gentle aspiration with a 0.5-10 µl pipet tip, and cells were then washed with warm media and subject, recovery to 24 hours from the time of the treatment with MG132. For example, the cells treated with MG312 for 1 hour, were washed and then cultured in the absence of this stress agent for 23 hours. After 24 hours, 15 µl of the dye solution was added to a final volume of 115 µl, and then incubated with the cells for 4 hours followed by the addition of 100 µl solubilization solution, as described by the manufacturer's protocol. The absorbance of the solution was then measured at 570 nm using a (Spectra Max 340 96-well plate reader from Molecular Devices.

RESULTS

1. Phosphorylation of eIF2α is required for increased ATF5 protein levels in response to diverse stress conditions

The *ATF5* mRNA has two uORFs that are conserved among many different vertebrates, including human, mouse, rat, cow, and frogs (Fig. 7). The 5′-proximal uORF1 encodes a polypeptide that is only three amino acid residues in length, Met-Ala-Leu, that is conserved among the different ATF5 orthologs. The downstream uORF2 encodes a polypeptide ranging from 59 residues in length in human and cow, to 53 residues in the frog *ATF5* mRNA. In each example, the uORF2 overlaps, out of frame, with the *ATF5* coding region (Fig. 7). Given the importance of uORFs in *ATF4* translational control in response to eIF2α phosphorylation, we addressed whether the levels of ATF5 protein were increased in response to environmental stresses by a mechanism requiring eIF2α phosphorylation. Wild-type MEF cells, designated *S/S*, and a mutant version containing alanine substituted for the serine-51 phosphorylation site in eIF2α, termed *A/A*, were exposed to three different stress conditions known to activate eIF2α phosphorylation and its downstream target, ATF4. The first stress arrangement involved treatment of these MEF cells with thapsigargin, a well-characterized ER stress agent that specifically activates the eIF2 kinase PERK (62, 104). The second was oxidative stress that was elicited by arsenite exposure. Arsenite appears to activate multiple eIF2 kinases, because deletion of any one eIF2 kinase gene in MEF cells does not block phosphorylation of eIF2α. The third stress involved treatment with MG132, a potent inhibitor of proteasome function that preferentially activates GCN2 phosphorylation of eIF2α in MEF cells (43). Each of these three stress conditions

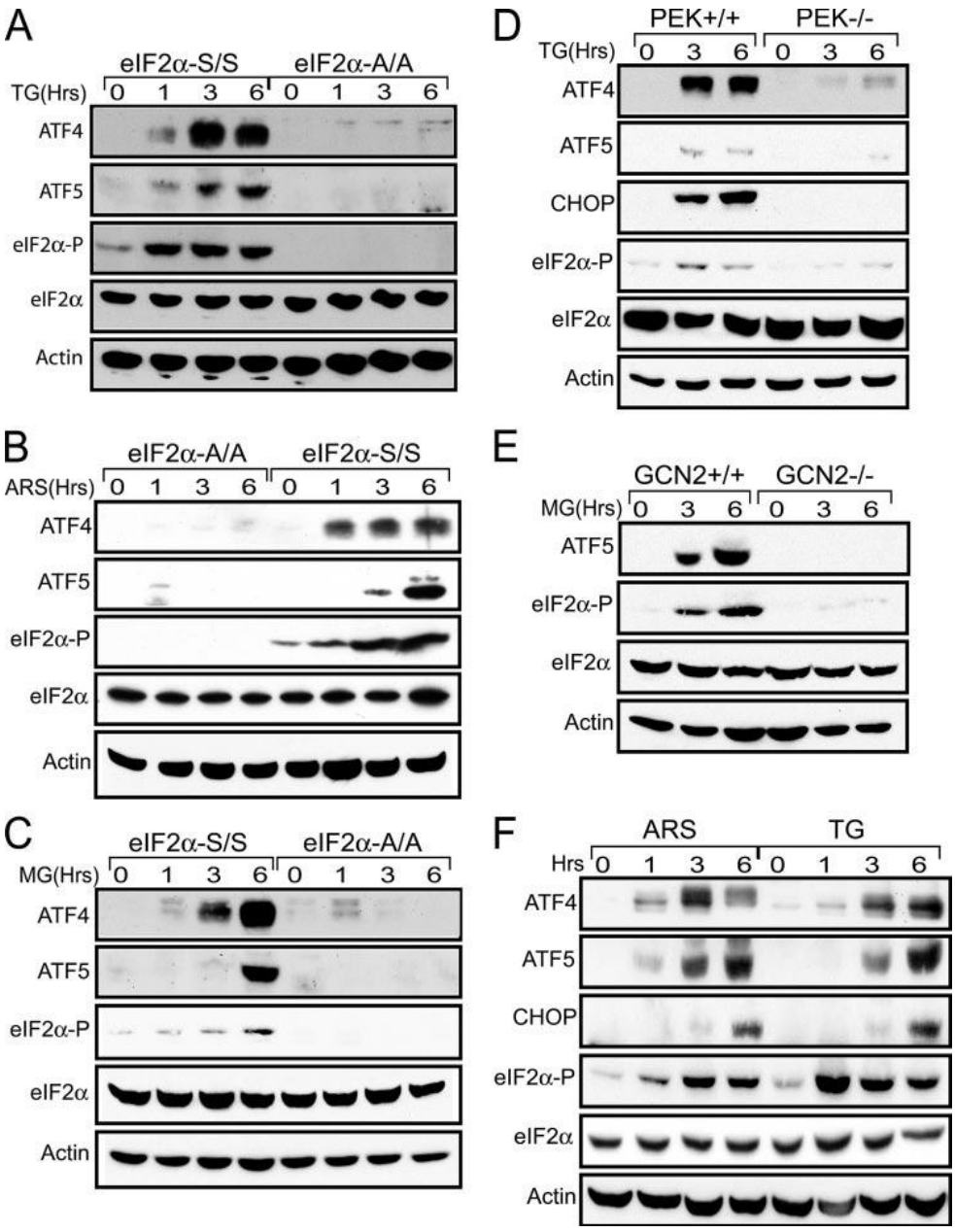


Figure 8. Phosphorylation of eIF2α is required for increased levels of ATF5 protein in response to diverse stress conditions. Wild-type *S/S* MEF cells, and *A/A* cells containing an alanine residue substituted for the phosphorylated serine-51 in eIF2α, were treated with 1 μM thapsigargin (*A*, *TG*), 20 μM arsenite (*B*, *ARS*), or 1μM MG132 (*C*, MG) for 1, 3, or 6 hours, or to no stress (0 hour), as indicated. Alternatively, *PEK*$^{-/-}$

(PERK$^{-/-}$) and *GCN2$^{-/-}$* MEF cells, and their wild-type counterparts, were treated with 1μM thapsigargin (*D, TG*), or 1μM MG132 (*E, MG*) for 3 or 6 hours, or to no stress (0 hour), as listed. Protein lysates were prepared from the cultured cells, and the levels of ATF5, ATF4, CHOP, phosphorylated eIF2α, total levels of eIF2α, and β-actin were measured by immunoblot analysis using antibody specific to each protein. In *F*, a similar immunoblot analysis was carried out using lysates prepared from human HepG2 hepatoma cells treated with either arsenite (*ARS*) or thapsigargin for up to 6 hours, or to no stress (0 hour). Each *panel* is representative of three independent experiments.

activates the eIF2 kinase pathway in *S/S* cells, with enhanced eIF2α phosphorylation and increased ATF4 protein levels within 1-3 hours of treatment (Fig. 8, *A*-*C*). As expected, in the *A/A* cells there was no measurable phosphorylation of eIF2α and minimal ATF4 protein. Importantly, there were increased ATF5 protein levels in *S/S* cells in response to each of the stress treatments. Upon thapsigargin exposure, ATF5 expression was induced after 1 hour, with high levels of this bZIP transcriptional activator within 3 hours of the onset of ER stress (Fig. 8*A*). Arsenite and MG132 treatments also showed robust increases in ATF5 expression, although high levels of ATF5 protein were detected only after 6 hours of the stress treatments (Fig. 8, *B* and *C*). No ATF5 protein was detected in the *A/A* MEF cells, which are devoid of eIF2α phosphorylation, in response to each of the three stress conditions.

These central observations were extended to other MEF cells and to human HepG2 hepatoma cells that have been well studied for regulation of the eIF2 kinase/ATF4 pathway (103, 105, 106). $PEK^{-/-}$ MEF cells, and its wild-type counterpart, were exposed to thapsigargin for up to 6 h. ATF5 expression was increased in the $PEK^{+/+}$ cells after 3 hours of ER stress, coincident with elevated eIF2α phosphorylation and increased levels of ATF4, and its target gene *CHOP* (Fig. 8*D*). By contrast in the *PEK* deficient cells, there was lowered ATF5 expression, along with reduced eIF2α phosphorylation and downstream targets ATF4 and CHOP. In response to MG132, loss of *GCN2* blocked both eIF2α phosphorylation and induced ATF5 expression, supporting the essential role of the eIF2 kinase pathway for increased ATF5 levels in response to proteasome inhibition (Fig. 8*E*). Finally, human HepG2 hepatoma cells have been reported to display a robust eIF2 kinase stress response during different stress

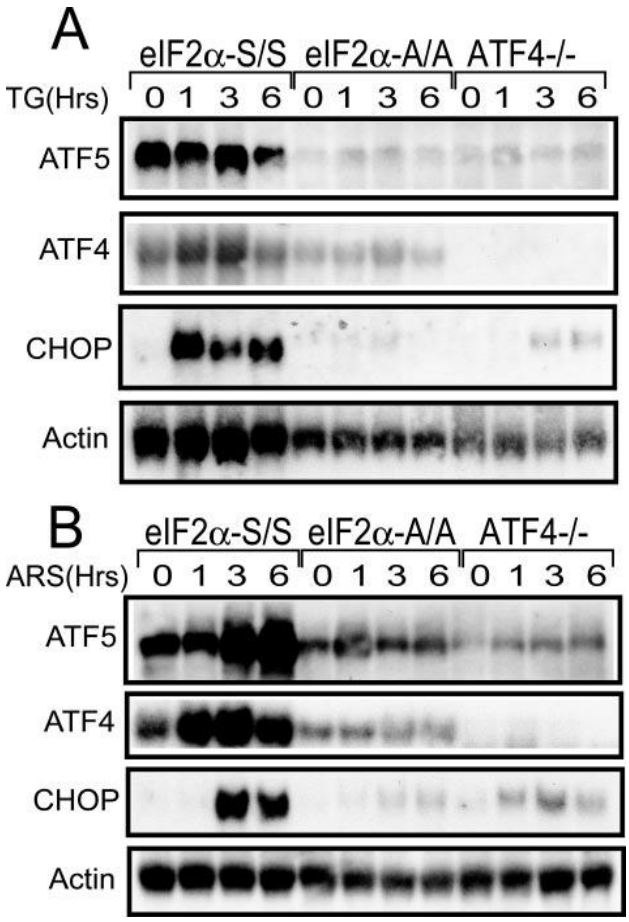


Figure 9. Deletion of eIF2α phosphorylation, or its target gene ATF4, reduces the levels of *ATF5* mRNA. Wild-type, *A/A* or *ATF4*$^{-/-}$ MEF cells were treated with 1 μM thapsigargin (*A, TG*), or 20 μM arsenite (*B, ARS*), for 1, 3, or 6 hours, or to no stress (0 hour), as indicated. RNA was prepared from the cultured cells, and the levels of mRNAs encoding ATF5, ATF4, CHOP, and β-actin were measured by Northern analyses using radiolabeled probes specific to each gene. The panels are representative of three independent experiments.

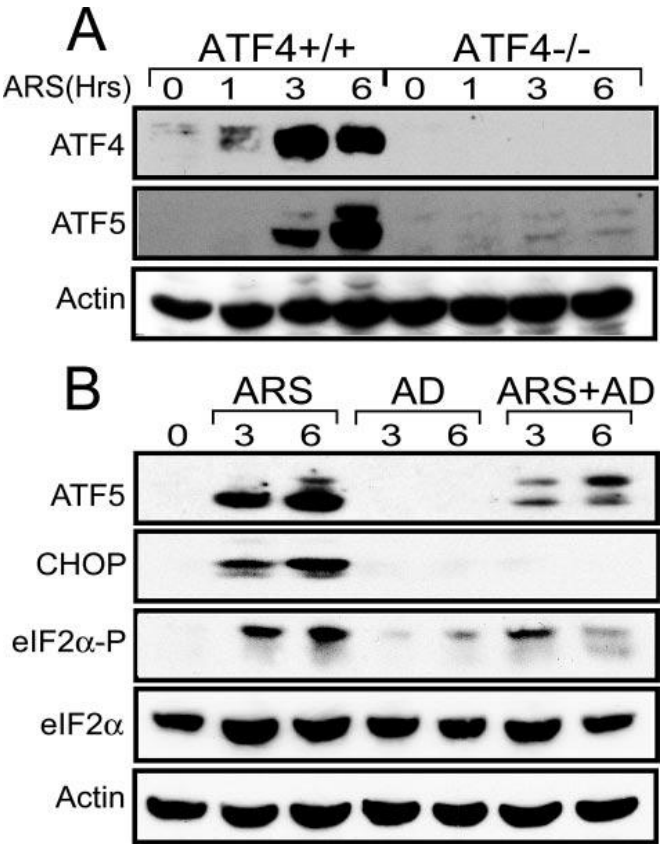


Figure 10. Increased *ATF5* expression involves transcriptional and post-transcriptional regulation in response to arsenite stress. A, *ATF4*$^{+/+}$ and *ATF4*$^{-/-}$ MEF cells were treated with 20 μM arsenite (ARS) for 1, 3, or 6 hours, or to no cellular stress (0 hour). Protein lysates were prepared from the cultured cells, and the levels of ATF4, ATF5, and β-actin were measured by immunoblot analysis. B, wild-type MEF cells were treated with 20 μM arsenite (ARS) for 3 or 6 hours, or to no cellular stress (0 hour). Alternatively, the MEF cells were treated with 10 μM actinomycin D (AD) alone, or in combination, with arsenite (ARS+AD), and immunoblot analyses were carried out to measure the levels of ATF5, CHOP, phosphorylated eIF2α (eIF2α-P), total eIF2α, and β-actin.

conditions (105, 106). We treated the HepG2 cells with thapsigargin or arsenite for up to 6 hours and found that ATF5 levels were increased in a time frame similar to that of ATF4 in response to both stress conditions (Fig. 8*F*). Levels of the ATF4 target, CHOP, were increased later, after about 6 hours of each stress. These results indicate that ATF5 expression is induced by a diverse range of environmental stresses by a mechanism requiring eIF2α phosphorylation.

2. Phosphorylation of eIF2α and ATF4 are required for high Levels of ATF5 mRNA

The eIF2 kinase pathway can increase both translational and transcriptional expression in response to cellular stress. To determine whether *ATF5* mRNA levels change in response to stress, we carried out Northern analyses using RNA prepared from *S/S* and *A/A* MEF cells treated with thapsigargin or arsenite. There were increases in *ATF4* mRNA levels in *S/S* cells treated with thapsigargin or arsenite treatment for up to 6 h (Fig. 9, *A* and *B*). No *ATF4* mRNA was detected in *ATF4*$^{-/-}$ MEF cells that were treated with either stress agent, confirming the identity of the transcripts in the Northern analyses. In these studies, *ATF4* mRNA levels were reduced, albeit measurable, in the *A/A* cells treated with thapsigargin or arsenite (Fig. 9, *A* and *B*). The lowered levels of *ATF4* transcripts in the MEF cells devoid of eIF2α phosphorylation could result from decreased transcription or increased decay of the *ATF4* mRNA that was inefficiently translated. These experiments suggest that regulation of *ATF4* expression during stress can involve both the well characterized translational control and changes in mRNA levels. ATF4 binds to the *CHOP* promoter, increasing *CHOP* transcription in response to different cellular stresses (105, 107, 108). Consistent with this premise, our Northern

analysis showed that *CHOP* mRNA levels are significantly increased in response to treatment with either thapsigargin or arsenite (Fig. 9). Minimal *CHOP* expression was detected in the stressed *ATF4*$^{-/-}$ cells, or the *A/A* cells, which expressed low levels of ATF4 protein. The levels of *ATF5* mRNA were virtually unchanged in *S/S* MEF cells in response to thapsigargin treatment. By comparison, there was an increase in the amount of *ATF5* mRNA following 3 hours of arsenite stress, although *ATF5* transcripts were readily measurable in non-stressed condition. Interestingly, there was a significant decrease in the amount of *ATF5* mRNA in the *ATF4*$^{-/-}$ and *A/A* cells, including lowered transcript levels in the basal conditions, and no detectable increase upon stress treatment. These results suggest that the eIF2 kinase pathway, specifically the transcriptional activator ATF4, is responsible for elevating *ATF5* mRNA levels in both the basal and stressed conditions. As will be highlighted further in the Discussion section, some eIF2 kinase target genes are regulated by both translational and transcriptional control mechanisms. ATF5 is a candidate for such an arrangement given the important role of ATF4 in increased *ATF5* mRNA levels, and the fact that ATF5 protein is measurable in the *S/S* MEF cells only following environmental stress, despite the availability of *ATF5* mRNA.

3. Expression of ATF5 is regulated by post-transcriptional control mechanisms

We next wished to address the importance of post-transcriptional regulation in the induction of ATF5 expression in response to stress. Comparison of ATF5 protein levels

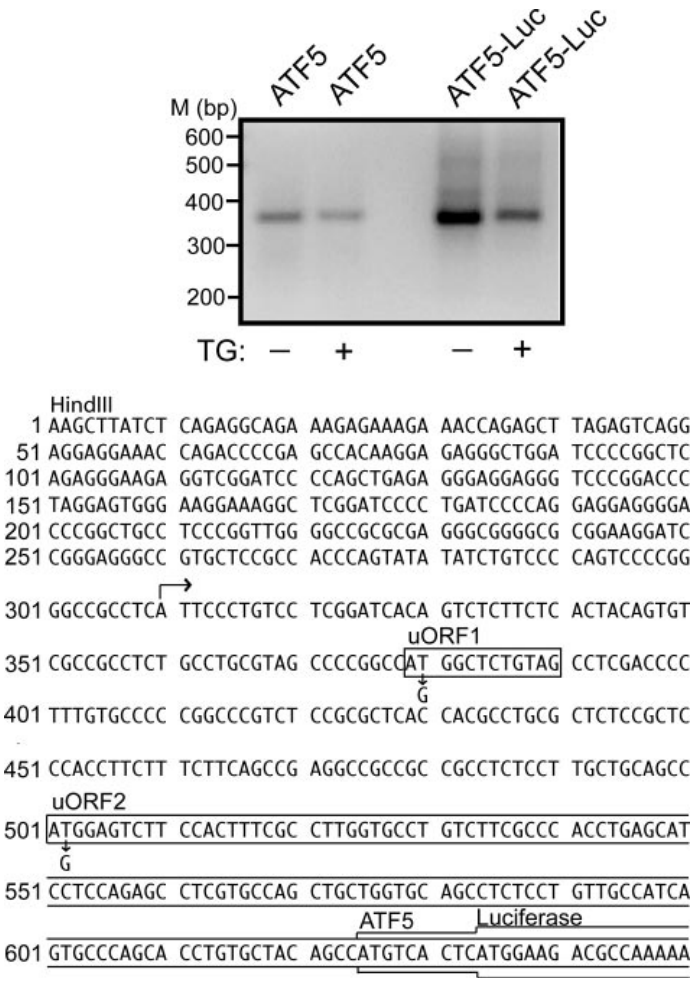


Figure 11. Sequence of the 5'-leader of *ATF5* mRNA fused to the luciferase reporter gene. Top panel, DNA was derived by 5'-RACE using RNA prepared from *S/S* MEF cells expressing the ATF5-luciferase (*ATF5-Luc*) reporter that were treated with 0.1µM thapsigargin (*TG*) or no stress. As a control, 5'-RACE was also carried out using RNA preparations from HepG2 treated with the ER stress agent, or no stress condition. ATF5 indicates 5'-RACE products prepared from the endogenous *ATF5* mRNA, and ATF5-Luc indicates products derived from the ATF5-reporter. DNA size markers listed to the left are indicated in base pairs. Bottom panel, the sequence of the 5'-leader of the *ATF5* mRNA, with boxes indicating the uORF1 and uORF2 sequences upstream of the *ATF5*-

Luc coding region. Note the uORF2 sequence overlaps, out of frame, with the ATF5-Luc reporter sequence. A HindIII restriction site was engineered into the ATF5 DNA. The major transcription start site of the ATF5 gene, as determined by sequencing of 5'-RACE products, is indicated by an arrow. The initiation codons in uORF1 and uORF2 were substituted to AGG, as indicated below the sequences.

between *ATF4*$^{+/+}$ and *ATF4*$^{-/-}$ MEF cells subjected to arsenite stress confirmed that induced *ATF5* expression required ATF4 (Fig. 10*A*). Although ATF5 protein was fully increased following 3 h of treatment of arsenite exposure, there was minimal ATF5 protein in the stressed *ATF4*$^{-/-}$ cells. This requirement for *ATF4* was comparable to that found for *CHOP*, which has been shown to require ATF4 transcriptional activation in response to several different stress conditions, including arsenite treatment (Fig. 9, *A* and *B*)(60, 69, 108, 109)

To begin to delineate the role of transcriptional regulation from post-transcription modes of control, we pretreated the wild-type MEF cells with actinomycin D, a known inhibitor of transcription, prior to arsenite stress. ATF5 protein levels were increased in response to the combined actinomycin D and arsenite treatment, supporting the idea that post-transcriptional mechanisms significantly contribute to induced ATF5 expression in response to environmental stress (Fig. 10B). It is noted that ATF5 levels were reduced in the combined actinomycin D and arsenite treatment compared with arsenite alone, suggesting that transcriptional mechanisms are a contributor to *ATF5* expression. Interestingly, an additional higher molecular weight version of the ATF5 protein was readily detected in the combined treatment preparation, suggesting that ATF5 may be subject to post-translational modification(s), such as protein phosphorylation (Fig. 10*B*). This higher molecular weight form of ATF5 was also found in cells treated with arsenite alone, although not as prevalent as when cells were first pretreated with actinomycin D. Finally, there was minimal ATF5 protein in the MEF cells exposed to only actinomycin D, indicating that this treatment regimen alone did not lead to enhanced ATF5 expression (Fig. 10*B*).

4. uORF1 and uORF2 differentially regulate translation of *ATF5* mRNA

To address the role of translational control in *ATF5* expression in response to stress, we constructed a luciferase reporter system that included the *ATF5* mRNA leader sequence and initiation codon that were inserted upstream of a luciferase reporter gene (Fig. 11). Given that this region of the *ATF5* mRNA is well conserved among mammals, we elected to use the human *ATF5* version for this reporter construct. The ATF5-luciferase fusion was expressed downstream of a minimal thymidine kinase promoter in the *S/S* and *A/A* MEF cells. The major transcriptional start site of the *ATF5-Luc* reporter gene was analyzed by 5′-RACE and DNA sequencing and found to be identical to that of the endogenous *ATF5* expressed in HepG2 cells (Fig. 11). The 5′-leader sequence is 315 nucleotides in length and includes the two uORFs preceding the ATF5 coding region. This leader configuration was unchanged in response to ER stress, with the same major ATF5 transcription initiation site in the ATF5-luciferase reporter or endogenous transcript. ATF5-luciferase was measured in the *S/S* and *A/A* MEF cells treated with thapsigargin for 6 h, or no stress. There was a 5-fold increase in ATF5-luciferase activity in the *S/S* MEF cells in response to thapsigargin treatment (Fig. 12). By comparison, the activity of ATF5-luciferase in the *A/A* cells was minimal, even during ER stress. Measurements of the ATF5-luciferase mRNA revealed minimal differences in transcript levels in the transfected *S/S* and *A/A* cells (Fig. 13). These results support the idea that *ATF5* expression is subject to translational control by a mechanism involving the 5′-leader of the *ATF5* mRNA and eIF2α phosphorylation.

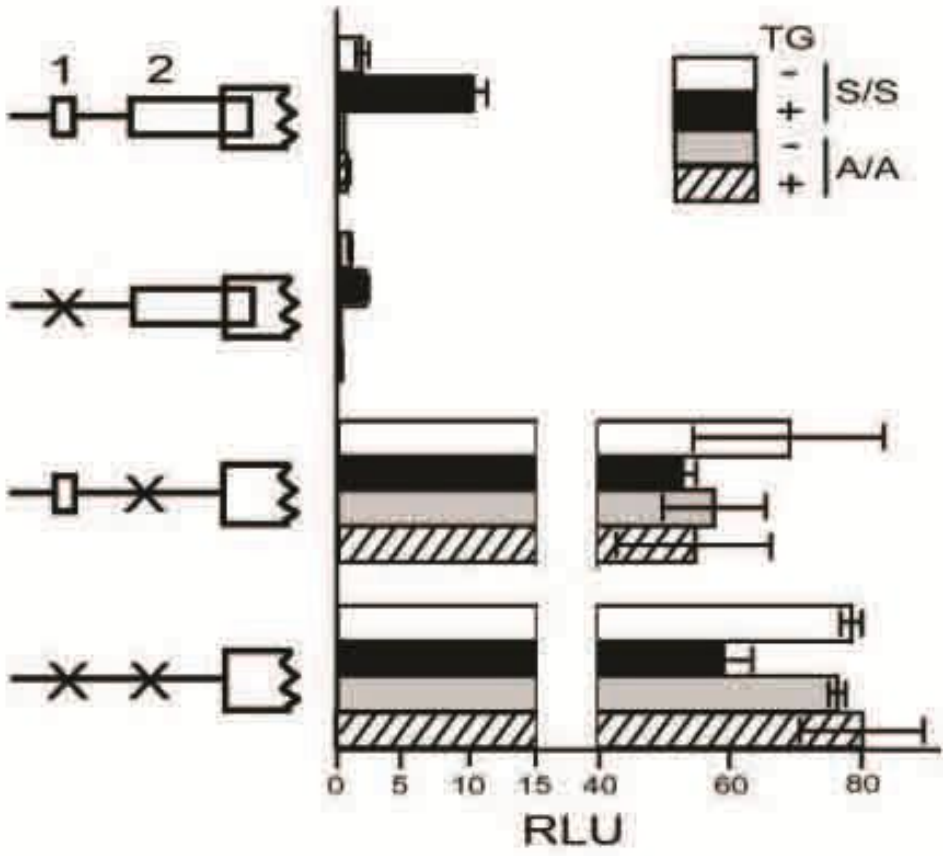


Figure 12. uORF1 functions as activator and uORF2 as an inhibitor in the mechanism regulating the *ATF5* translation. The wild-type and different mutant versions of the 5'-leader sequences of the *ATF5* mRNA fused to luciferase are shown to the left of each luciferase measurement. The box represents the wild-type versions of uORF 1 and uORF2, and an "X" indicates a mutation in the initiation of the uORF, rendering it non-functional. Wild-type *S/S* and mutant *A/A* MEF cells were co-transfected with the listed ATF5-Luc plasmid and a control Renilla luciferase plasmid. The transfected cells were treated with 0.1 μM thapsigargin (TG), or no stress agent, as indicated in the figure legend. For clarity, the histograms are represented in two different scales. Numbers presented are means ± S.E. derived from three independent experiments.

To delineate the roles of uORF1 and uORF2 in the mechanisms regulating ATF5 translation, the start codons for each uORF were mutated to AGG, rendering them non-functional for translation initiation. The ATF5-luciferase reporter containing the uORF1 and uORF2 mutations individually, or in combination, were then introduced into the *S/S* and *A/A* MEF cells and assayed for expression in the presence or absence of ER stress. The uORF1 mutation resulted in only low levels of ATF5-luciferase activity in the *S/S* or *A/A* cells, independent of thapsigargin treatment (Fig. 12). This result defines the 5′-proximal uORF1 as a positive-acting element in ATF5 translational control. By comparison, the uORF2 mutation led to an over 35-fold increase in ATF5-luciferase activity in both *S/S* and *A/A* cells, as compared with the repressed version of ATF5-luciferase reporter containing both uORF1 and uORF2 intact (Fig. 12). It is noteworthy that this elevated ATF5-luciferase activity was constitutive, occurring during either stressed or non-stressed conditions. Constitutively high levels of ATF5-luciferase were also seen when the uORF1 and uORF2 mutations were combined into the ATF5-luciferase reporter (Fig. 12). Northern analyses of the different mutant versions of ATF5-luciferase mRNA in the *S/S* and *A/A* cells indicated that changes in transcript levels were not a significant contributor to the differences in ATF5-luciferase expression (Fig. 13). These results indicate that uORF2 functions as an inhibitory element in ATF5 translational control. Only after uORF2 is removed is uORF1 dispensable for ATF5 expression (Fig. 12). We conclude that uORF1 and uORF2 have opposing functions in the regulation of ATF5 translation, with uORF1 enabling ribosomes to overcome the inhibitory affects of uORF2.

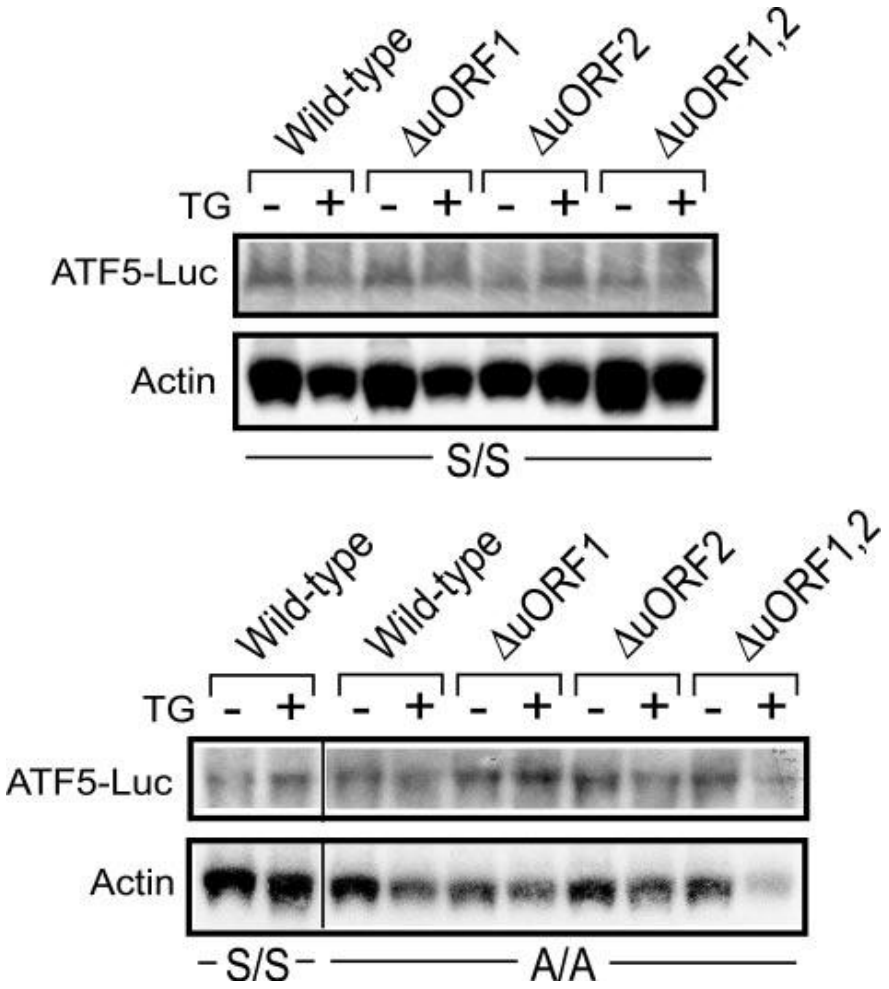


Figure 13. The levels of wild-type and mutant versions of the *ATF5-Luc* reporter mRNA are similar in the MEF cells. Total RNA was prepared from *S/S* and *A/A* MEF cells transfected with the wild-type or mutant versions of the ATF5-luciferase (*ATF5-Luc*) reporter plasmids, and Northern blots were carried out to measure the mRNA levels for the ATF5-Luc reporter and β-actin. The MEF cells were treated with 0.1 μM thapsigargin (+) or no stress agent (-), as indicated. Wild-type indicates that both uORF1 and uORF2 were present in the 5'-leader of the reporter mRNA. ΔuORF1 and ΔuORF2 indicates that the reporter transcript contains a mutation in the initiation codon of the specified uORF, rendering this reading frame nonfunctional for translational control. The combined ΔuORF1 and ΔuORF2 highlights that there were mutations in initiation codons

of both reading frames. The *top panel* illustrates the Northern analysis of RNA prepared from transfected *S/S* cells. The *bottom panel* was derived from *A/A* cells, and for comparison purposes RNA from S/S cells expressing the wild-type reporter from *S/S* cells was included in this *panel*. In this *lower panel*, the *A/A* and *S/S* Northern lanes were derived from the same Northern blot experiment.

5. *ATF5* mRNA is preferentially translated in response to stress

The ATF5-luciferase reporter assays indicated that *ATF5* mRNA is preferentially translated in response to stress. To directly address this idea, we measured the efficiency of mRNA association with translating ribosomes by polysome profile analyses. In this technique, sucrose gradient centrifugation is used to separate free ribosomal subunits and monosomes from polyribosomes (110-112). Transcripts that are efficiently translated are bound to multiple ribosomes, or large polysomes, whereas those mRNAs that are weakly translated localize to monosomes or disomes. In the non-stressed *S/S* cells, *β-actin* mRNA was bound to large polyribosomes (Fig. 14, fractions 12-14). By comparison *ATF4* mRNA, which is poorly translated in the absence of stress, was associated with fewer ribosomes (Fig. 14, fractions 7 *and* 8).

Upon ER stress, total protein synthesis is reduced due to PEK phosphorylation of eIF2α. The resulting lowered eIF2-GTP levels leads to reduced polysomes and accumulation of free ribosomal subunits in the polysome profile (Fig. 14, top panels). This stress arrangement led to a shift in the *ATF4* mRNA to the larger polysome fractions (Fig. 14, fractions 7-11). By comparison, there was some lowering in the number of ribosomes associated with *actin* mRNA. Our analysis of *ATF5* mRNA in the polysome profiles revealed a pattern that paralleled that described for ATF4. In the non-stressed condition, when protein synthesis is plentiful, the *ATF5* mRNA was associated with fewer ribosomes, as compared with the large polysomes associated with *actin* mRNA. In response to ER stress, *ATF5* mRNA was readily detected in the larger polysome fractions, consistent with the idea that *ATF5* mRNA was bound to multiple ribosomes and was more efficiently translated. These results further support the idea that

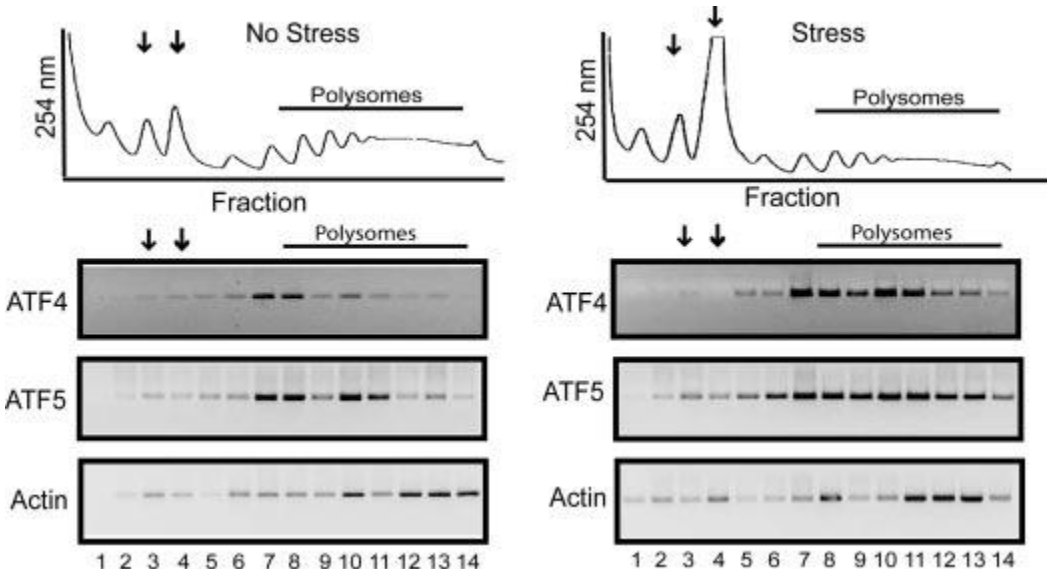


Figure 14. Cellular stress triggers enhanced *ATF5* mRNA association with polysomes. Wild-type *S/S* MEF cells were treated with 1.0 µM thapsigargin (Stress) or no stress condition (No Stress) for 6 hours, and cell lysates were preparedand subjected to sucrose gradient centrifugation. Gradients were then fractionated, and RNA was monitored by absorbance at 254 nm. Arrows indicate peaks corresponding 40 S and 80 S ribosomal fractions,and polysomes are highlighted. Levels of *ATF5, ATF4,* and *β-actin* mRNA were measured by RT-PCR in each fraction. PCR products corresponding to the indicated transcripts were separated by electrophoresis, stained with ethidium bromide for visualization using UV light, and imaged electronically.

translational control is an important underlying reason for increased *ATF5* expression during environmental stress.

6. CHOP is required for full induction of ATF5 protein levels in response to diverse stresses

Phosphorylation of eIF2α promotes translation initiation of ATF4, leading to transcription of the ATF4 downstream target CHOP. To determine whether CHOP is required for induced ATF5 expression in response to stress, *CHOP*$^{-/-}$ MEF cells and their wild-type counterparts were exposed to thapsigargin, arsenite, or MG132 for up to 6 hours (Fig. 15). As expected, there were increased ATF5 protein levels in wild-type cells in response to each of these stress treatments, consistent with the increased levels of ATF4 and CHOP. However, there was lowered ATF5 protein expression in *CHOP*$^{-/-}$ cells, along with the absence of CHOP expression in response to each of these stress conditions (Fig. 15). This requirement of CHOP for induced ATF5 protein in response to these different stress conditions suggests that CHOP is upstream of ATF5 in the ISR. Next we measured *ATF5, CHOP* and *ATF4* mRNA levels in wild-type and *CHOP*$^{-/-}$ cells subjected to arsenite exposure (Fig. 16, *A, B* and *C*). While *ATF4* mRNA was induced upon stress, ATF5 transcript levels remained low in the *CHOP*$^{-/-}$ cells. These findings suggest that CHOP is a direct or indirect activator of *ATF5* transcription in the ISR.

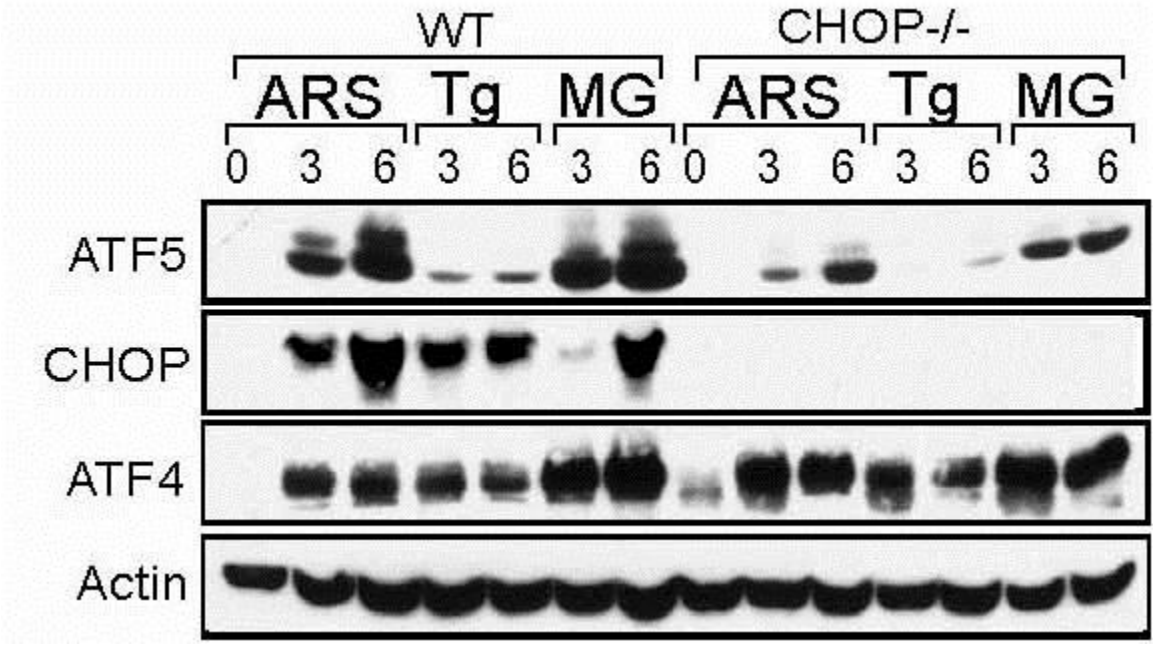
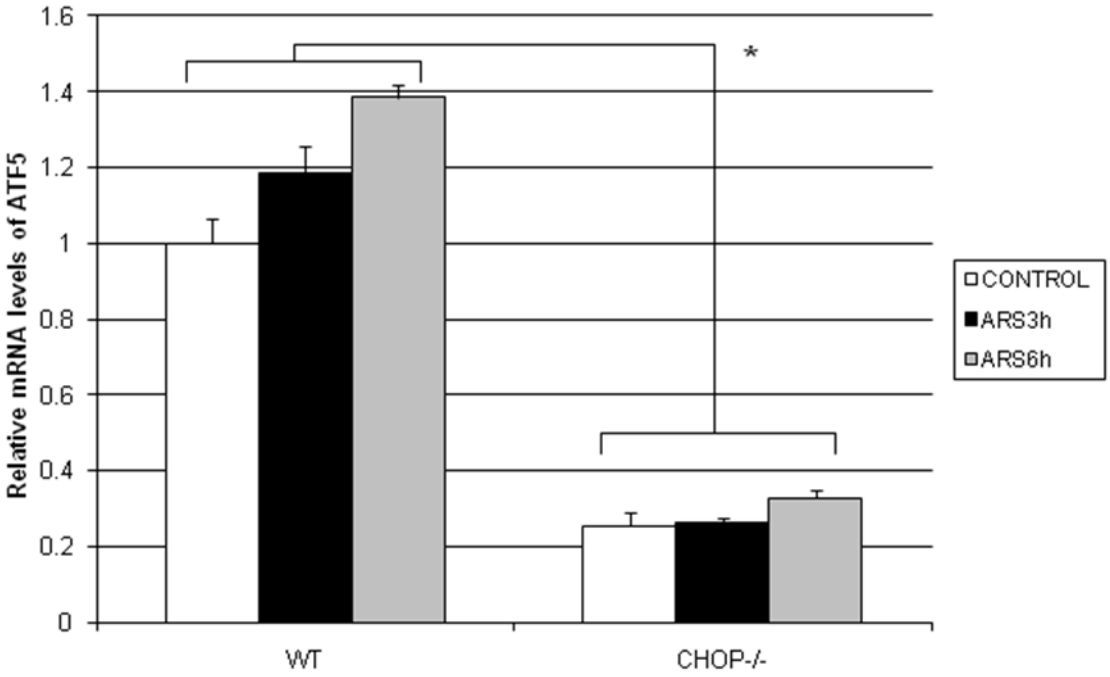
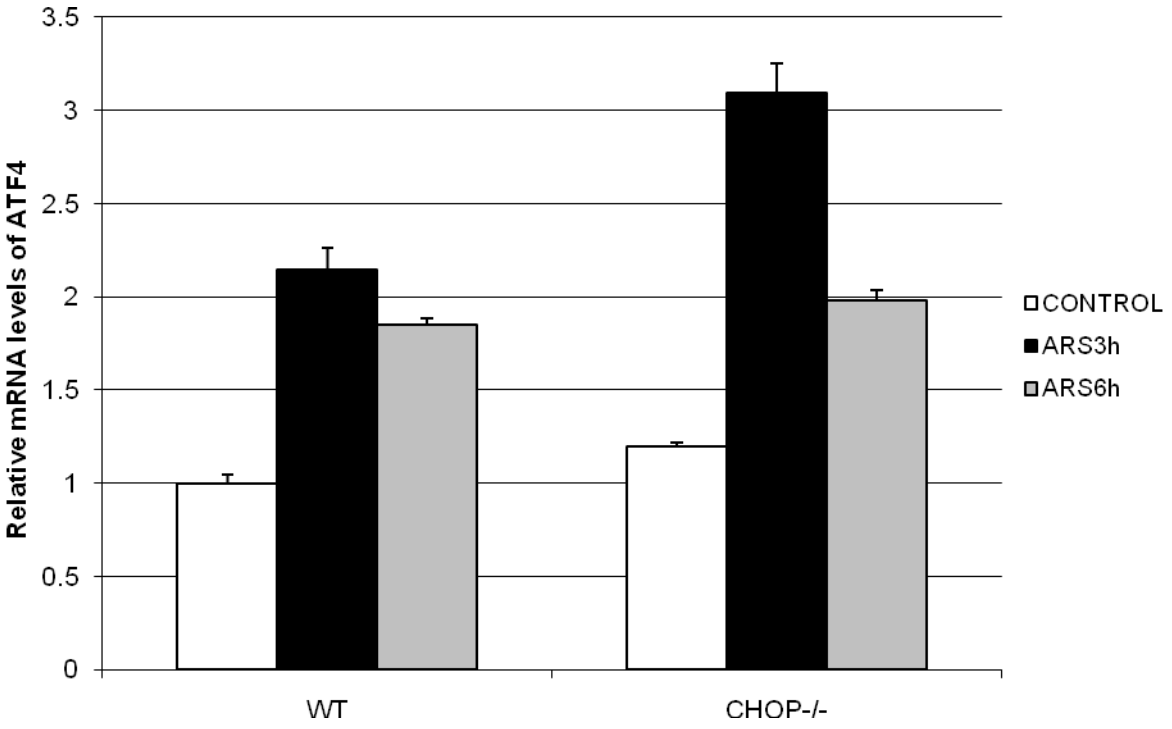


Figure 15. Expression of ATF5 in wild-type and *CHOP*$^{-/-}$ MEF cells. (A) *CHOP*$^{+/+}$ and *CHOP*$^{-/-}$ were treated with 1 μM thapsigargin (TG), 20 μM arsenite (ARS), or 1 μM MG132 (MG) for 3 or 6 hours, or to no stress (0 h), as indicated. Protein lysates were prepared from the cultured cells, and the levels of ATF5, ATF4, CHOP and β-actin were measured by immunoblot analysis using antibody specific to each protein.

A


1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Relative mRNA levels of ATF5
*
WT
CHOP-/-
CONTROL
ARS3h
ARS6h

B


3.5
3
2.5
2
1.5
1
0.5
0
Relative mRNA levels of ATF4
WT
CHOP-/-
CONTROL
ARS3h
ARS6h

C

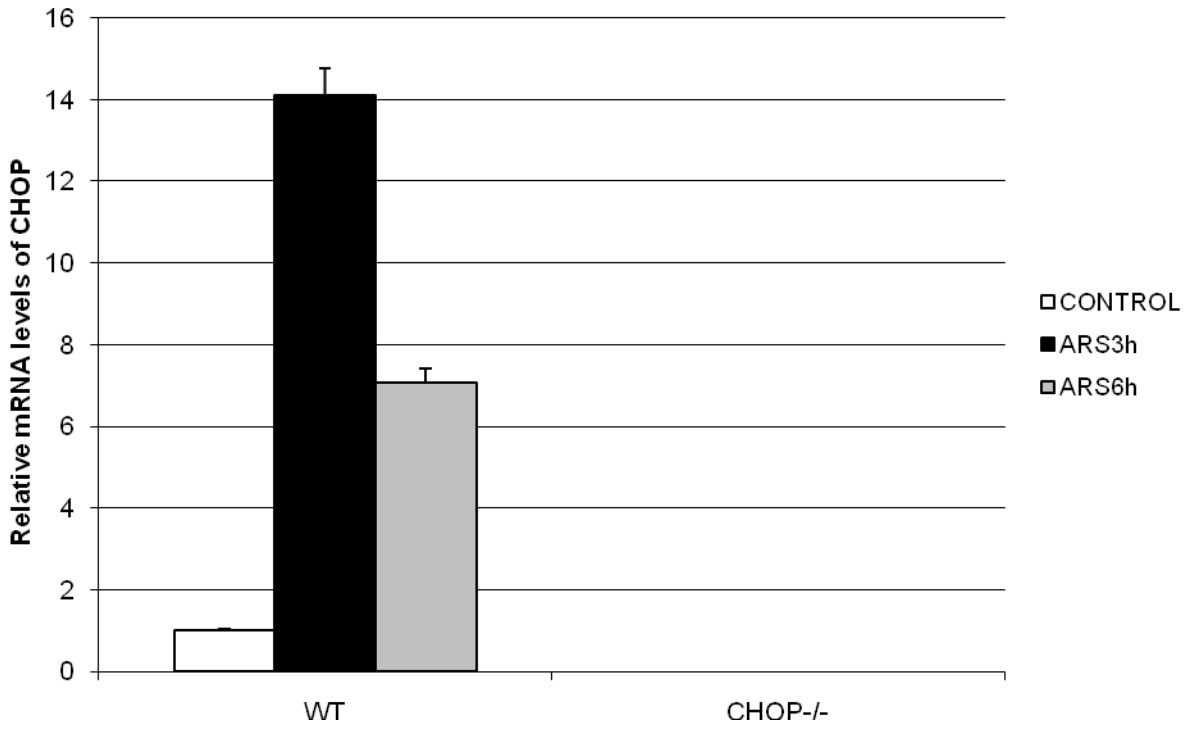


Figure 16. CHOP is required for increased ATF5 mRNA in response to arsenite stress. Wild type and *CHOP*$^{-/-}$ MEF cells were treated with 20 μM arsenite (ARS) for 3 or 6 hours, or to no stress (0 hours). Total RNA was isolated from samples and the levels of *ATF5*, *ATF4* and *CHOP* mRNA normalized to 18S rRNA were measured by qRT-PCR. Values were plotted compared to the no treatment control, and the panel is presented as averages ± SD of three indepenedent experiments, with each measurement performed in triplicate (* $P<0.05$).

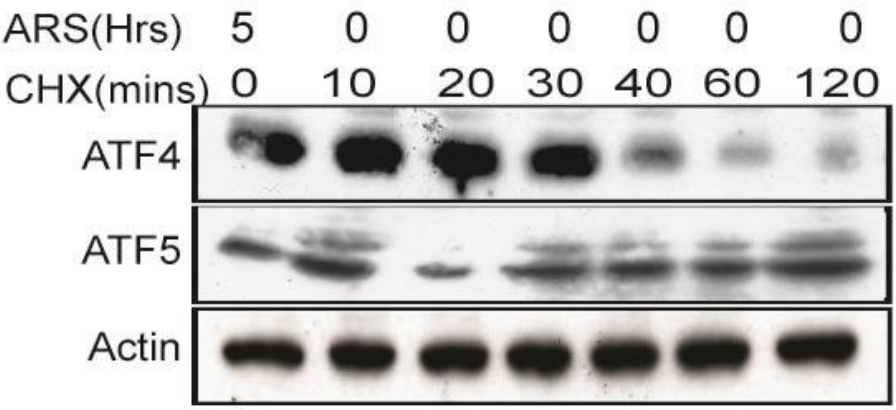
7. Assessment of ATF4 and ATF5 protein turnover

ATF4 has been shown to be an unstable protein with a half-life of less than 30 minutes (113). Considering the similarity between ATF4 and ATF5, posttranslational regulation by protein degradation can be a crucial mechanism regulating ATF5 expression. Therefore, to compare the half-lives of ATF4 and ATF5, after 5 hour of exposure to arsenite, we monitored the decay of ATF4 and ATF5 proteins following inhibition of new protein synthesis. ATF5 had a longer half-life (more than 2 h). By contrast, ATF4 decayed rapidly (half-life about 30min) (Fig. 17), consistent with the previously reported lability of ATF4.

8. Assess the function of ATF5 in cell survival

We next addressed the role of ATF5 in the cell survival, and the functional consequences of the absence of induced ATF5 expression. Lentivirus containing small hairpin ATF5 RNA (shRNA/ATF5) was introduced into the MEF cells. The levels of *ATF5* mRNA and protein in the knock-down cells were significantly reduced compared with wild-type cells and the cells transfected with nontarget vector control virus (Figs. 18 and 19). Proteasome inhibition triggered apoptosis has been shown to be facilitated by cleavage and activation of a cascade of caspase proteases (42). Cleavage of caspase 9 was measured by immunoblot and found to be significantly enhanced in the wild type MEF after 18 hours of the MG132 treatment. By comparison, cleavage of this caspase was significantly reduced in the two different cloned MEF cells knockdown for *ATF5* expression. Cleavage of PARP, another measure of apoptosis, was also detected to be at a lowered level in ATF5-deficient cells treated with MG312 compared with the wild type

A



B

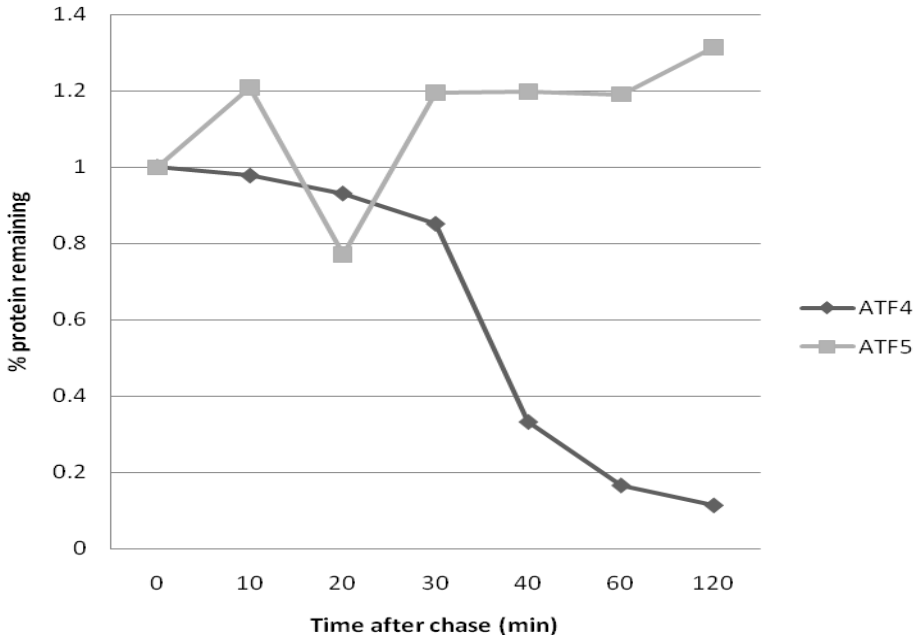


Figure 17. Measurements of ATF5 and ATF4 protein turnover during arsenite stress. (A) Wild type MEF cells were exposed to 20 μM arsenite (*ARS*) for 5 hours, and then 10 μg/ml cycloheximide (CHX) was then added, and the cultures were incubated for the indicated length of time. Immunoblot analyses for ATF4, ATF5 and β-actin were

carried out using whole-cell extracts and antibodies specific to the indicated proteins. (B) The intensity of the protein bands were quantified using ImageJ software and plotted as the percentage of protein remaining versus time following cycloheximide treatment.

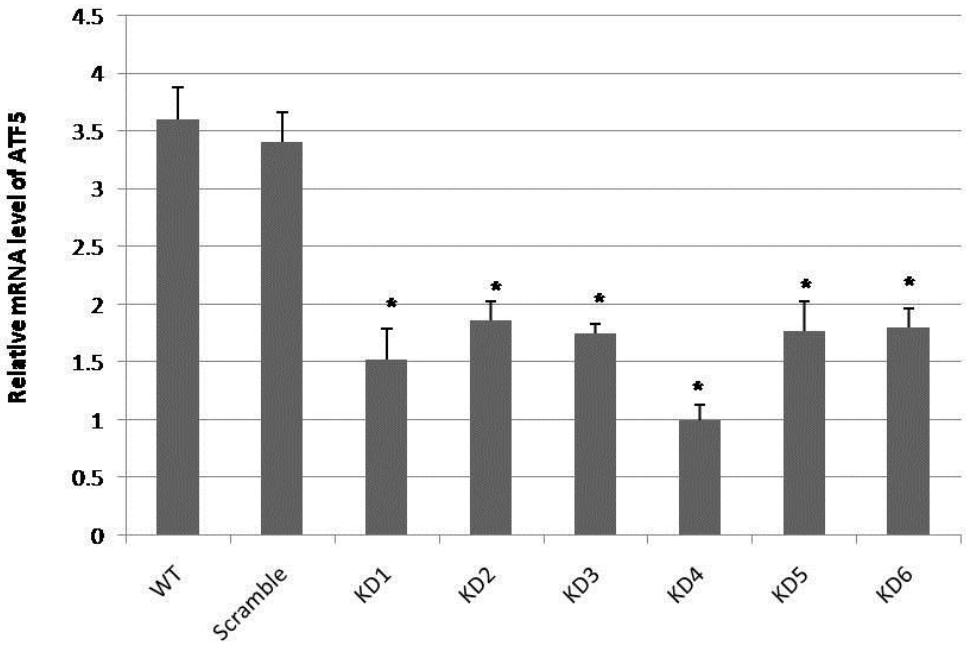


Figure 18. Levels of ATF5 mRNA in wild-type and *ATF5* knock-down MEF cells. Total RNA was isolated from MEF cells expressing scrambled shRNA or *ATF5*-specific shRNA (KD1--KD6). The levels of *ATF5* mRNA, normalized for 18S rRNA, were measured by qRT-PCR. Values from ATF5 knock-down cells were presented compared to the control, and the panel indicates the averages ± SD derived from three independent experiments (* $P<0.05$).

cells (Fig. 19). These data support a role for ATF5 for eliciting apoptotic pathways in response to stress conditions that block proteasome function.

To address whether ATF5 depletion alters cell survival in response to stress, we conducted the CellTiter 96® Non-Radioactive Cell Proliferation Assay that provides a rapid and convenient method to determine viable cell number in proliferation. Wild type, two *ATF5* knockdown cells (KD1 and KD2) and *CHOP*$^{-/-}$ MEF cells were subjected to proteasome inhibition for up to 18 h. We found that proteasome inhibition significantly reduced survival of wild type MEF cells as judged by the Cell Proliferation Assay. As a pro-apoptotic factor and positive control, CHOP expression has a negative consequence following MG132 treatment. Deletion of CHOP restored cell survival in response to proteasome inhibition (Fig. 20). Importantly, loss of ATF5 enhanced MEF cell survival during this stress treatment. These results indicate that ATF5, which is downstream of CHOP, enhances cell death in response to proteasome inhibition.

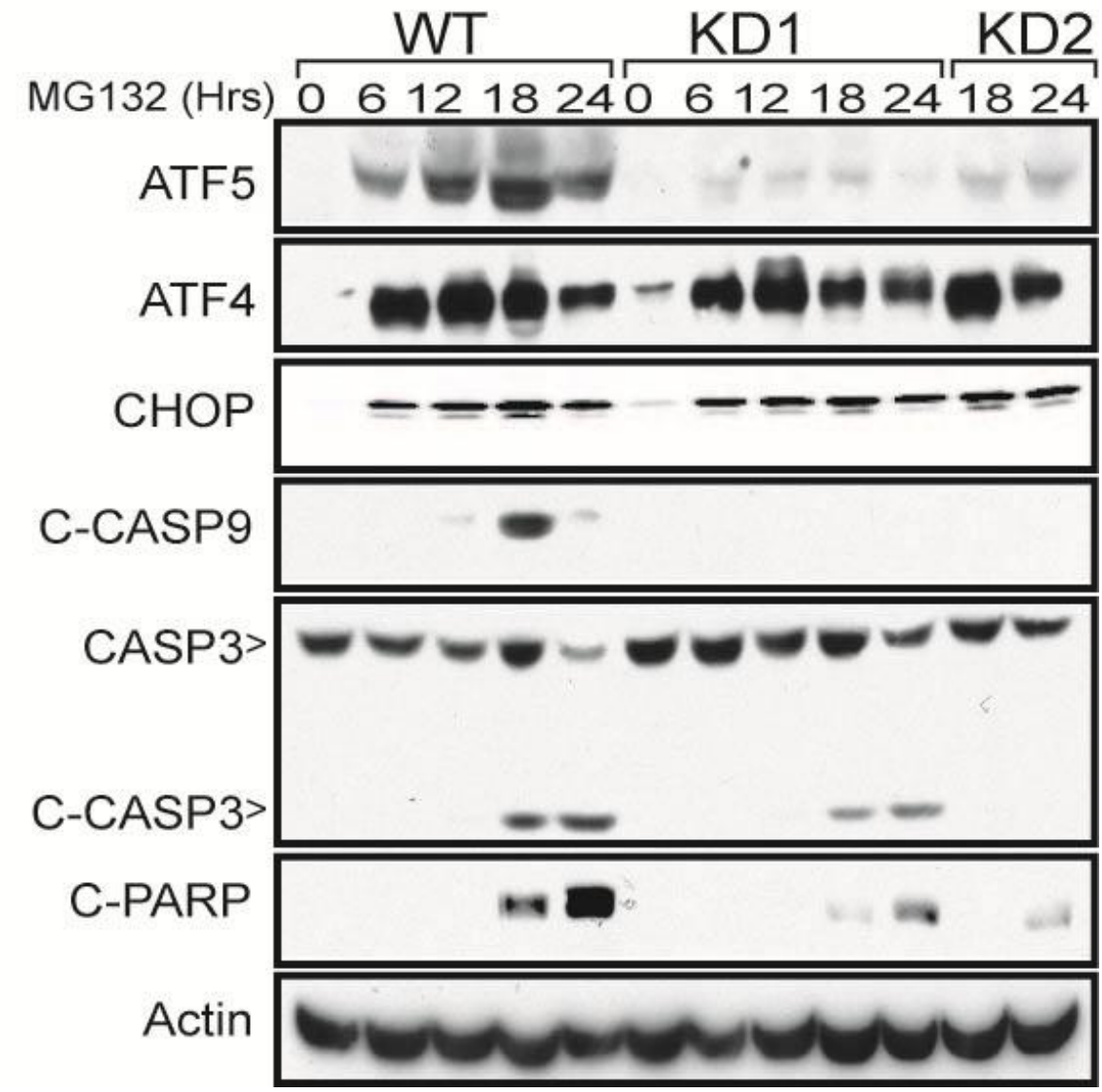


Figure 19. ATF5 facilitates cleavage and activation of caspase proteases. Wild-type MEF cells, and ATF5 knockdown cells were treated with 1μM MG132 for 6, 12, 18, or 24 hours, or to no stress (0 hours) as indicated. Protein lysates were prepared from the cultured cells, and the levels of ATF5, ATF4, CHOP, cleavage of caspase-3(C-CASP3), cleavage of caspase-9 (C-CASP9), PARP and β-actin were measured by immunoblot analysis using antibody specific to each protein.

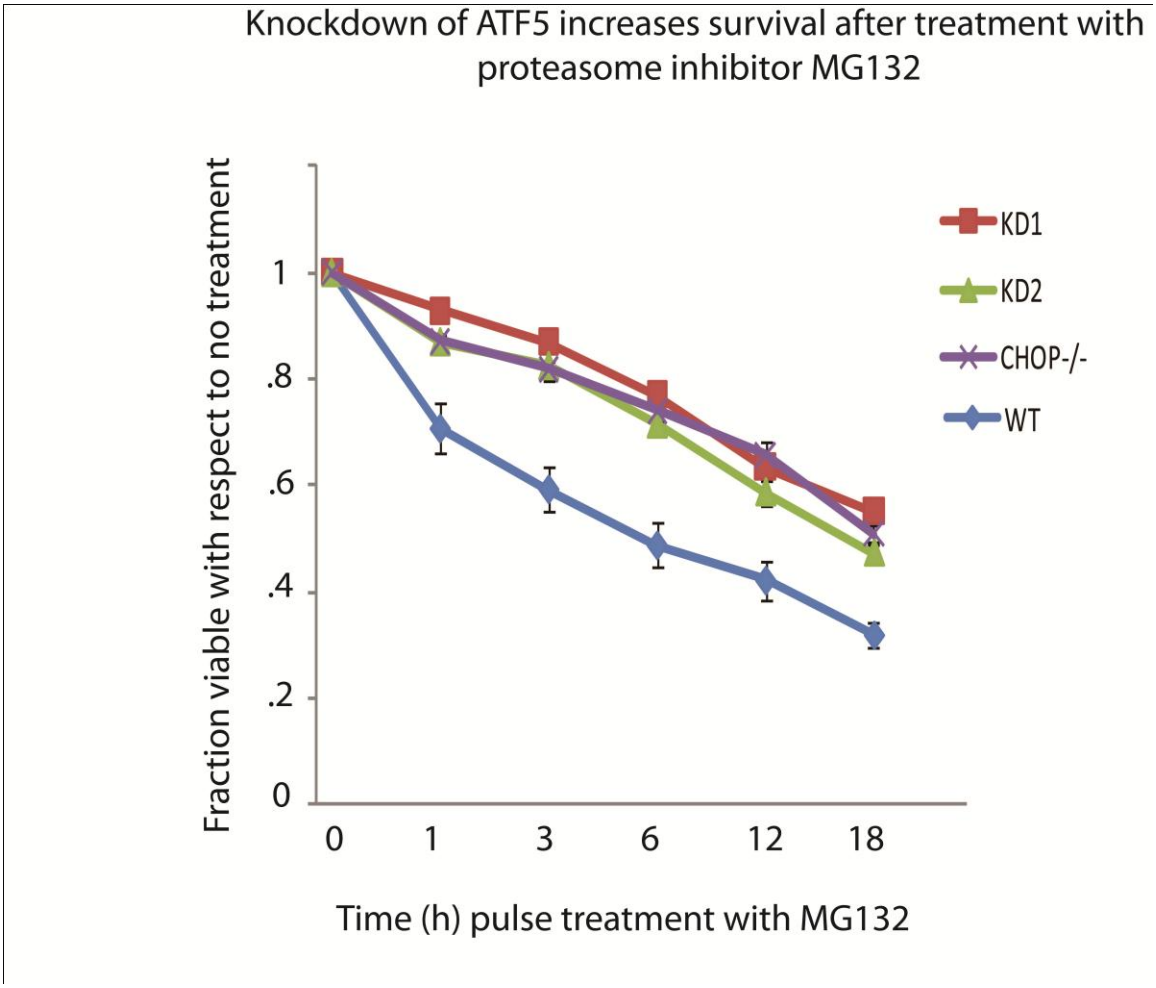


Figure 20. Knockdown of ATF5 increases survival after treatment with MG132.

WT, CHOP$^{-/-}$, ATF5 KD1 and ATF5 KD2 MEF cells (at 5×10^3 well^{-1}) were seeded in 96-well plates and allowed to adhere overnight. Cells were treated with 1µM MG132 for 1, 3, 12, 18 hours, or to no stress (0 hour), as indicated. The MG132 was then removed and then cells were incubated culture medium in the absence of the stress agent for a total of 24 hours from the onset on the stress induction. Optical density representing viable cells was then determined by the CellTiter 96® Non-Radioactive Cell Proliferation Assay. Results are calculated as percentage of viable cells compared with control cultures that were not treated with MG132 (mean ± SD, n = 3).

DISCUSSION

1. Phosphorylation of eIF2α is required for ATF5 expression

Phosphorylation of eIF2α induced in response to diverse environmental stress conditions elicits translational and transcriptional regulatory mechanisms to direct the expression of genes that alleviate cell damage, or alternatively induce apoptosis. eIF2α phosphorylation represses general protein synthesis coincident with preferential translation of select of stress-related genes. Translational control of the *ATF4* gene is the best studied example of selective translation by eIF2α phosphorylation in mammalian cells. In this study, we showed that eIF2α phosphorylation is also required for enhanced expression of the bZIP transcriptional regulator ATF5 in response to each of three different stress conditions: ER stress, arsenite exposure, and proteasome inhibition (Fig. 8). *ATF5* is subject to translational control. There was minimal expression of ATF5 protein in non-stressed conditions, despite the availability of *ATF5* mRNA (Figs. 8 and 9). only in response to stress conditions and enhanced eIF2α phosphorylation, was there a significant increase in ATF5 protein (Fig. 8). In fact, increased levels of ATF5 protein during stress occurred even in the presence of actinomycin D, indicating that transcription is not obligatory for the enhanced *ATF5* expression during stress (Fig. 10*B*). *ATF5* mRNA was preferentially associated with large polysomes in response to ER stress, directly supporting the idea that there is increased translation of *ATF5* mRNA. Analysis of an ATF5-luciferase reporter demonstrated that the 5′-leader of the *ATF5* mRNA directs *ATF5* translational control. The underlying mechanism involves a positive-acting uORF1 that allows reinitiating ribosomes to bypass an inhibitory uORF2 in stressed conditions (Figs. 11 and 12).

2. The mechanisms by which eIF2α phosphorylation enhances ATF5 expression

The key features of *ATF5* translational control share those described for *ATF4* (61). The regulatory mechanism begins with translation of uORF1, which allows for retention of ribosomes and reinitiation at a downstream ORF. In non-stressed conditions, eIF2-GTP is readily available, allowing for scanning ribosomes to rapidly reinitiate translation at the next ORF, uORF2. Following translation of the inhibitory uORF2, terminating ribosomes would be positioned downstream of the start codon of the *ATF5* coding region, and thus incapable of *ATF5* expression. Furthermore, upon translation of uORF2, terminating ribosomes may dissociate from the *ATF5* mRNA. Therefore, translation of the inhibitory uORF2 would lead to lowered synthesis of the ATF5 transcription regulator. This central feature of the model, in which uORF2 plays an inhibitory role in ATF5 translational control, is also suggested in a report by Watatani et al. (114). In response to stress, the reduced levels of eIF2-GTP that occur during eIF2α phosphorylation delay reinitiation, allowing for scanning ribosomes to bypass the uORF2 initiation codon. Ribosomes scanning the interval between the initiation codon of uORF2 and the *ATF5* coding region would reacquire eIF2/GTP/Met-tRNAi^{Met} and begin translation at the *ATF5* coding region. Elevated levels of ATF5 protein would then be available to regulate transcription of targeted genes.

A second mechanism contributing to *ATF5* expression involves accumulation of *ATF5* mRNA. Although in wild-type MEF cells there was minimal increase in *ATF5* transcript levels following up to 6 hours of ER stress, there was some elevation in *ATF5* mRNA levels during arsenite treatment. This increase in mRNA levels during this oxidative stress condition may contribute to the increased synthesis of ATF5 protein.

Interestingly, in *A/A* MEF cells, *ATF4*$^{-/-}$, and *CHOP*$^{-/-}$ cells there was a significant lowering in *ATF5* mRNA (Fig. 9, *A* and *B*, Fig. 16, *A*). This suggests that ATF4 and CHOP, downstream targets of eIF2α phosphorylation, contributes directly or indirectly to increased *ATF5* transcription. Importantly, the requirement of ATF4 or CHOP for accumulation of *ATF5* mRNA is visible even during non-stressed conditions, suggesting that there is a basal level of ATF4 or CHOP protein that directs elevated *ATF5* mRNA levels. *ATF4*$^{-/-}$ cells were reported to be sensitive to oxidative stress, requiring reducing agents in the medium (69). This observation suggests that ATF4 can have critical functions in cells even in the absence of treatment with stress agents. In this case, there may be physiological stresses that trigger modest, transient increases in ATF4 levels. These results suggest that translational control and ATF4 regulation of *ATF5* mRNA function together to enhance *ATF5* expression in response to different stress conditions. This suggests that there is a cascade of activated transcription factors, ATF4>CHOP>ATF5, that direct each downstream effector via transcriptional control. Therefore, a combination of transcriptional regulation and translational control is central for ATF5 function in the ISR.

In order to identify potential ATF4 binding sites in the *ATF5* promoter, we analyzed the 5-kb sequence of *ATF5* gene and flanking promoter sequences using a number of software programs that identify potential binding sites for transcription factors (rVISTA, ConSite, and TFSEARCH). This analysis was performed on the *ATF5* promoters from different species and we did not detect an identifiable ATF binding site. It has been reported that C/EBP/ATF site is located within the intron responsible for transcriptional regulation of the *SNAT2* gene (115). Considering the complexity of

regulatory elements, to maximize the identification of functional sites, we also scanned the sequences of the 5'-leader of the *ATF5* mRNA, introns, ATF5 coding region, 3'-UTR of the ATF5 mRNA, and 1-kb downstream of the ATF5 gene. There was no identifiable ATF site in these regions as well. This suggests that ATF4 functions indirectly to induce ATF5 mRNA in response to stress, through another transcription factor directly under its control, e.g. CHOP. We found a predicted CHOP_01 binding site in the ATF5 promoter (-2529-2541). These results suggest that translational control and direct CHOP regulation of *ATF5* mRNA expression can function together to enhance *ATF5* activity in response to different stress conditions.

These key features of combined translational and transcriptional mechanisms have also been described for GCN4 orthologs in fungi, such as *Candida albicans*, *Neurospora crassa*, and *Aspergillus nidulans* (35, 116-118). In each of these cases, increased synthesis of mRNA was suggested to be important for insuring maximal expression of these GCN4-related transcriptional activators. Transcriptional expression of these GCN4 orthologs is suggested to be coupled to translational control mechanisms involving uORFs and translation reinitiation that is delayed by eIF2α phosphorylation. However, it is noteworthy that, although a single "master regulator" directs the eIF2 kinase pathway in these fungi (64), this report shows that mammals have multiple transcriptional regulators subject to translational control in response to eIF2α phosphorylation.

3. ATF5 functions in the ISR pathway ATF4/CHOP/ATF5

Given that translational control has the most immediate effect on gene expression, this combination of transcriptional and translational regulation suggests that the translational induction of ATF5 is invoked first in stressed cells, while transcription regulation would facilitate further enhancement of ATF5 protein levels in the longer term. The adaptive function of the UPR during ER stress have been viewed as being directed by enhancing the preferential stabilities of mRNAs and proteins that facilitate adaptation versus those that lead to cell death (13). *ATF4* and *CHOP* are unstable at the mRNA and protein levels, and as a consequence the pro-apoptotic CHOP accumulates only during prolonged stress. By contrast, key stress adaptive proteins, such as chaperones, are more stable during stress insults (13). ATF5 protein appears to be stable (Fig. 17) and the half-life of ATF5 mRNA is 11 h (119). Thus elevated expression level of *ATF5* would extend significantly beyond the period of stress, resulting in a scenario where ATF5 remains up-regulated, while ATF4 and CHOP do not. This may be significant for determining the patterns of gene expression during stress conditions and the resulting cell survival.

4. A Possible Mechanism of Adaptation

Given the well-characterized role of *CHOP* in stress-induced apoptosis, it is reasonable to propose a significant pro-apoptotic function for ATF5 in MEF cells. MG132 can induce caspase-dependent apoptosis. We demonstrated that depletion of ATF5 in MEF cells conferred resistance to cell death induced by MG132. Such protection from apoptosis is associated with the reduced cleavage of caspase-9 and caspase-3, as well as PARP (Fig. 19). This was accompanied by increased cell survival as

measured by a 96® Non-Radioactive Cell Proliferation Assay in ATF5 knock-down cells (Fig. 20). Disruption of ATF5 was effective in diminishing key apoptotic markers and preventing cell death, thus indicating the importance of ATF5 in stress-induced MEF cell death. These data suggest that ATF5, as a downstream target of CHOP, is a key mediator of CHOP-induced apoptosis.

How might ATF5 mediate apoptosis in MEF cells? Our results suggest that the induction of ATF5 is an intermediate event in stress-induced apoptosis and that it is not downstream events of caspase activation. It was also reported that ATF5 represses CRE-dependent transcription (88), and CREB and its associated factors play a role in cell survival (120, 121). These findings indicate that repression of CRE transcription could be one of the mechanisms inducing apoptosis that is promoted by ATF5.

Other mechanisms of ATF5 actions are also possible. For example, ATF5 can also heterodimerize with ATF4 and C/EBP factors (122). Other gene promoters are likely to bind ATF5 containing heterodimers whose transcriptional products may induce apoptosis during stress. It is noteworthy that CHOP also has downstream targets linked to apoptosis, such as Dr5, GADD34 and Bcl2. These genes might also be the target genes of ATF5. GADD34 is CHOP-dependent, and directly activated by CHOP (123), and a conserved ATF binding site was also found in the GADD34 promoter (23). ATF4 was reported to directly bind to the GADD34 promoter in a ER-stress dependent manner (23). As an ATF4 subfamily member, ATF5 might also bind to the GADD34 promoter at the same ATF site. For example, interaction among ATF4, ATF5 and CHOP may collectively contribute to the regulation of GADD34.

CHOP also sensitizes cells to ER stress by down-regulating Bcl2 expression. Overexpression of Bcl2 protects cells from ER stress-induced cell death (124). However, no CHOP binding site has been located in the *Bcl2* promoter. An alternative explanation is that ATF5, as a downstream effecter of CHOP, regulates Bcl2 expression. ATF and CREB binding site were also located in Bcl2 promoter. Given the role of a repressive action on CRE sites, ATF5 expression may contribute to the downregulation of Bcl2, which can promote cell death.

5. Future directions

Prior studies on ATF5 have largely focused on *ATF5* mRNA expression, the role of ATF5 in neural differentiation, and the potential role of ATF5 in cancer progression (72, 77, 82, 83, 125). Our findings that ATF5 expression is induced by stress by mechanisms requiring eIF2α phosphorylation may be integral to each of these research topics. Concerning *ATF5* expression, two versions of *ATF5* mRNA, derived from alternative splicing, were reported to be expressed in humans and mice (72). ATF5α mRNA, the major form studied in this thesis, is expressed during early mouse development and in adult tissues, with highest levels in liver and certain neural tissues (72). Given our findings that ATF5 is subject to translational control, *ATF5* transcript levels are not necessarily an accurate measure of ATF5 protein and activity. A second version of *ATF5* mRNA, designated *ATF5β*, is present at much lower levels, and was reported to be restricted to early mouse development (72). Interestingly, *ATF5β* shares an identical *ATF5* coding region with *ATF5α*, differing only in the 5′-leader region of the mRNA. Three uORFs are present in the *ATF5β* transcript, each differing from that

described for the *ATF5α* version. Like the *ATF5α* transcript, the 3′-proximal uORF in *ATF5β* is longest, encoding a 63-amino acid residue polypeptide that overlaps out-of-frame with the *ATF5* coding region. It is inviting to speculate that this alternative 5′-leader configuration in the *ATF5β* mRNA may change important features of translational control as compared with the *ATF5α* transcript. For example, the 5′-leader of *ATF5β* mRNA may alter the efficiency of ribosome scanning and change the timing of translation reinitiation, thus altering the sensitivity of translational control to eIF2α phosphorylation.

Another area that needs to be considered is the function of homo- or hetero-dimerization of ATF5 in transcriptional regulation of the ISR. The structural similarity of ATF4 and ATF5 suggests dimerization between ATF5 and ATF4, or other b-ZIP transcription factors. Differential binding to bZIP partners may provide for unique binding elements in target promoters, enhancing the diversity of transcription during stress conditions.

In addition to its role in the activation of transcription, ATF5 was also reported to regulate proliferation and differentiation of neuroprogenitor cells. In PC12 cells, *ATF5* transcripts, which were highly expressed prior to NGF exposure, fell 25-fold following NGF treatment (77); however, our preliminary immunoblot data demonstrated that the transient induction of ATF5 in PC12 cells in response to proteasome inhibition, support the idea that expression of ATF5 is also inducible in these neural cells, rather than being constitutively expressed. Further experiments to address the regulation mechanism of ATF5 in neuroprogenitor cells versus differentiated neurons may shed light on the role of ATF5 in neural development.

6. Summary

We are only beginning to understand the biological functions of ATF5. Recent studies suggest that ATF5 is highly expressed in neural progenitor cells, and lowered *ATF5* mRNA expression is a prerequisite for differentiation into neurons and glia (77, 82, 83, 125). Illustrating this point, overexpression of ATF5 in rat PC12 cells repressed neurite outgrowth in response to NGF, while expression of a dominant-negative version of ATF5 accelerated neuritogenesis (77). In keeping with the idea that ATF5 represses differentiation of neural progenitor cells and facilitates proliferation, it was observed that ATF5 is expressed in a number of different human glioblastomas (82). In fact, the presence of ATF5 mRNA has been suggested to be a good prognostic marker in histopathologic examinations (126). The finding that ATF5 expression is integrated into the eIF2 kinase response suggests that this pathway may be a factor in neural differentiation and cancer, and that stress-induced ATF5 may alter these processes. Understanding the complex regulation of ATF5 leading to cell death or survival may allow the identification of novel molecular targets and facilitate the design of therapeutic strategies to specifically target tumor and neurodegenerative diseases.

REFERENCES

1. Wek RC. 1994. eIF-2 kinases: regulators of general and gene-specific translation initiation. *Trends in Biochemical Sciences* 19: 491-6

2. Wek RC, Jiang HY, Anthony TG. 2006. Coping with stress: eIF2 kinases and translational control. *Biochemical Society Transactions* 34: 7-11

3. Rutkowski DT, Kafuman RJ. 2004. A trip to the ER: coping with stress. *Trends in Cell Biology* 14: 20-8

4. Shen J, Chen X, Hendershot L, Prywes R. 2002. ER stress regulation of ATF6 localization by dissociation of BiP/GRP78 binding and unmasking of Golgi localization signals. *Dev Cell* 3: 99-111

5. Harding HP, Calfon M, Urano F, Novoa I, Ron D. 2002. Transcriptional and translational control in the mammalian unfolded protein response. *Ann Rev Cell Dev Biol* 18: 575-99

6. Kaufman RJ, Scheuner D, Schroder M, Shen X, Lee K, Lin CY, Arnold SM. 2002. The unfolded protein response in nutrient sensing and differentiation. *Nature Review Molecular Cell Biology* 3: 411-21

7. Ron D, Walter P. 2007. Signal integration in the endoplasmic reticulum unfolded protein response. *Nat Rev Mol Cell Biol* 8: 519-29

8. Malhotra JD, Kaufman RJ. 2007. The endoplasmic reticulum and the unfolded protein response. *Semin Cell Dev Biol* 18: 716-31

9. Zhang W, Feng D, Li Y, Iida K, McGrath B, Cavener DR. 2006. PERK EIF2AK3 control of pancreatic beta cell differentiation and proliferation is required for postnatal glucose homeostasis. *Cell Metab* 4: 491-7

10. Zinszner H, Kuroda M, Wang XZ, Batchvarova N, Lightfoot RT, Remotti H, Stevens JL, Ron D. 1998. CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes Dev* 12: 982-95

11. Oyadomari S, Koizumi A, Takeda K, Gotoh T, Akira S, Araki E, Mori M. 2002. Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. *The Journal of Clinical Investigation* 109: 525-32

12. Song B, Scheuner D, Ron D, Pennathur S, Kaufman RJ. 2008. Chop deletion reduces oxidative stress, improves beta cell function, and promotes cell survival in multiple mouse models of diabetes. *J Clin Invest* 118: 3378-89

13. Rutkowski DT, Arnold SM, Miller CN, Wu J, Li J, Gunnison KM, Mori K, Sadighi Akha AA, Raden D, Kaufman RJ. 2006. Adaptation to ER stress is mediated by differential stabilities of pro-survival and pro-apoptotic mRNAs and proteins. *PLoS Biol* 4: e374

14. Hershey JWB, Merrick WC. 2000. Pathway and mechanism of initiation of protein synthesis. In *Translational control of gene expression*, ed. N Sonenberg, JWB Hershey, M Mathews, pp. 33-88. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press

15. Kapp LD, Lorsch JR. 2004. The molecular mechanics of eukaryotic translation. *Annual Review of Biochemistry* 73: 657-704

16. Hinnebusch AG. 2000. Mechanism and regulation of initiator methionyl-tRNA binding to ribosomes. In *Translational control of gene expression*, ed. N Sonenberg, JWB Hershey, M Mathews, pp. 185-244. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press

17. Algire M. 2005. Pi release from eIF2, not GTP hydrolysis, is the step controlled by start-site selection during eukaryotic translation initiation. *molecular Cell* 20: 251-62

18. Price N, Proud C. 1994. The guanine nucleotide-exchange factor, eIF-2B. [Review]. *Biochimie* 76: 748-60

19. Kimball SR, Fabian JR, Pavitt GD, Hinnebusch AG, Jefferson LS. 1998. Regulation of guanine nucleotide exchange through phosphorylation of eukaryotic initiation factor eIF2alpha. Role of the alpha- and delta-subunits of eiF2b. *J Biol Chem* 273: 12841-5

20. Kimball SR, Mellor H, Flowers KM, Jefferson LS. 1996. Role of translation initiation factor eIF-2B in the regulation of protein synthesis in mammalian cells. *Prog Nucleic Acid Res Mol Biol* 54: 165-96

21. Kimball SR HN, Horetsky RL, Jefferson LS. 1998. Identification of interprotein interactions between the subunits of eukaryotic initiation factors eIF2 and eIF2B. *journal of Biological Chemistry* 273: 3039-44

22. Novoa I, Zeng. H, Harding HP, Ron D. 2001. Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2alpha. *J Cell Biol* 153: 1011-22

23. Ma Y, Hendershot LM. 2003. Delineation of a negative feedback regulatory loop that controls protein translation during endoplasmic reticulum stress. *J Biol Chem* 278: 34864-73

24. He B, Gross M, Roizman B. 1998. The gamma134.5 protein of herpes simplex virus 1 has the structural and functional attributes of a protein phosphatase 1 regulatory subunit and is present in a high molecular weight complex with the enzyme in infected cells. *J Biol Chem* 273: 20737-43

25. Li WW, Alexandre S, Cao X, Lee AS. 1993. Transactivation of the grp78 promoter by Ca2+ depletion. A comparative analysis with A23187 and the endoplasmic reticulum Ca(2+)-ATPase inhibitor thapsigargin. *J Biol Chem* 268: 12003-9

26. Luo S, Baumeister P, Yang S, Abcouwer SF, Lee AS. 2003. Induction of Grp78/BiP by translational block: activation of the Grp78 promoter by ATF4 through and upstream ATF/CRE site independent of the endoplasmic reticulum stress elements. *J Biol Chem* 278: 37375-85

27. Baumeister P, Luo S, Skarnes WC, Sui G, Seto E, Shi Y, Lee AS. 2005. Endoplasmic reticulum stress induction of the Grp78/BiP promoter: activating mechanisms mediated by YY1 and its interactive chromatin modifiers. *Mol Cell Biol* 25: 4529-40

28. Cho DY, Yang GH, Ryu CJ, Hong HJ. 2003. Molecular chaperone GRP78/BiP interacts with the large surface protein of hepatitis B virus in vitro and in vivo. *J Virol* 77: 2784-8

29. Gething MJ. 1999. Role and regulation of the ER chaperone BiP. *Semin Cell Dev Biol* 10: 465-72

30. Munro S, Pelham HR. 1986. An Hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. *Cell* 46: 291-300

31. Yusta B, Baggio LL, Estall JL, Koehler JA, Holland DP, Li H, Pipeleers D, Ling Z, Drucker DJ. 2006. GLP-1 receptor activation improves beta cell function and survival following induction of endoplasmic reticulum stress. *Cell Metab* 4: 391-406

32. Wek RC, Anthony TG. 2006. EXtENDINg beta cell survival by UPRegulating ATF4 translation. *Cell Metab* 4: 333-4

33. Delepine M, Nicolino M, Barrett T, Golamaully M, Lathrop GM, Julier C. 2000. EIF2AK3, encoding translation initiation factor 2-α kinase 3, is mutated in patients with Wolcott-Rallison syndrome. *Nat Genet* 25: 406-9

34. Hinnebusch AG. 2005. Translational regulation of GCN4 and the general amino acid control of yeast. *Annu Rev Microbiol* 59: 407-50

35. Wek RC, Staschke KA, Narasimhan J. 2004. Regulation of the yeast general amino acid control pathway in response to nutrient stress. In *Nutrient-induced responses in eukaryotic cells*, ed. PM Winderickx J and Taylor, pp. 171-99. Berlin Heidelberg: Springer-Verlag

36. Qiu H, Garcia-Barrio MT, Hinnebusch AG. 1998. Dimerization by translation initiation factor 2 kinase GCN2 is mediated by interactions of the C-terminal ribosome binding region and the protein kinase domain. *Mol Cell Biol* 18: 2697-711

37. Bertolotti A, Zhang Y, Hendershot LM, Harding HP, Ron D. 2000. Dynamic interaction of BiP and ER stress transducers in the unfolded protein response. *Nature Cell Biology* 2: 326-32

38. Ma K, Vattem KM, Wek RC. 2002. Dimerization and release of molecular chaperone inhibition facilitate activation of eukaryotic initiation factor-2 kinase in response to endoplasmic reticulum stress. *J Biol Chem* 277: 18728-35

39. Qiu H, Dong J, Francklyn CS, Hinnebusch AG. 2001. The tRNA-binding moiety in GCN2 contains a dimerization domain that interacts with the kinase domain and is required for tRNA binding and kinase activation. *EMBO J* 20: 1425-38

40. Dong J, Qiu H, Garcia-Barrio M, Anderson J, Hinnebusch AG. 2000. Uncharged tRNA activates GCN2 by displacing the protein kinase moiety from a bipartite tRNA-binding domain. *Mol Cell* 6: 269-79

41. Deng J, Harding H, Raught B, Gingras A, Berlanga J, Scheuner D, Kaufman R, Ron D, Sonenberg N. 2002. Activation of GCN2 in UV-irradiated cells inhibits translation. *Curr Biol* 12: 1279-86

42. Jiang H-Y, Wek RC. 2005. GCN2 phosphorylation of eIF2alpha activates NF-kappaB in response to UV irradiation. *The Biochemical journal* 385: 371-80

43. Jiang H-Y, Wek RC. 2005. Phosphorylation of the alpha-subunit of the eukaryotic initiation factor-2 (eIF2alpha) reduces protein synthesis and enhances apoptosis in response to proteasome inhibition. *The Journal of Biological Chemistry* 280: 14189-202

44. Cherkasova VA, Hinnebusch AG. 2003. Translational control by TOR and TAP42 through dephosphorylation of eIF2alpha kinase GCN2. *Genes Dev* 17: 859-72

45. Staschke KA, Dey S, Zaborske JM, Palam LR, McClintick JN, Pan T, Edenberg HJ, Wek RC. 2010. Integration of general amino acid control and target of rapamycin (TOR) regulatory pathways in nitrogen assimilation in yeast. *J Biol Chem* 285: 16893-911

46. Bunpo P, Dudley A, Cundiff JK, Cavener DR, Wek RC, Anthony TG. 2009. GCN2 protein kinase is required to activate amino acid deprivation responses in mice treated with the anti-cancer agent L-asparaginase. *J Biol Chem* 284: 32742-9

47. Jiang HY, Wek RC. 2005. Gcn2 phosphorylation of eIF2α activates NF-κB in response to UV irradiation. *Biochem J* 385: 371-80

48. Karin M, Lin A. 2002. NF-κB at the crossroads of life and death. *Nature Immunology* 3: 221-7

49. Li Q, Verma IM. 2002. NF-κB regulation in the immune system. *Nature Reviews Immunology* 2: 725-34

50. Ghosh S, May MJ, Kopp EB. 1998. NF-κB and rel proteins: Evolutionarily conserved mediators of immune responses. *Annual Review of Immunology* 16: 225-60

51. Han A, Yu C, Lu L, Fujiwara Y, Browne C, Chin G, Fleming P, Leboulch P, Orkin SH, Chen J-J. 2001. Heme-regulated eIF2α kinase (HRI) is required for translational regulation and survival of erythroid precursors in iron deficiency. *EMBO J* 20: 6909-18

52. Chen JJ. 2007. Regulation of protein synthesis by the heme-regulated eIF2alpha kinase: relevance to anemias. *Blood* 109: 2693-9

53. Rafie-Kolpin M, Chefalo PJ, Hussain Z, Hahn J, Uma S, Matts RL, Chen JJ. 2000. Two heme-binding domains of heme-regulated eukaryotic initiation factor-2alpha kinase. N terminus and kinase insertion. *J Biol Chem* 275: 5171-8

54. Chen JJ, Crosby JS, London IM. 1994. Regulation of heme-regulated eIF-2 alpha kinase and its expression in erythroid cells. [Review]. *Biochimie* 76: 761-9

55. Garcia MA, Gil J, Ventoso I, Guerra S, Domingo E, Rivas C, Esteban M. 2006. Impact of protein kinase PKR in cell biology: from antiviral to antiproliferative action. *Microbiol Mol Biol Rev* 70: 1032-60

56. Sadler AJ. 2007. Structure and function of the protein kinase R. *Curr Top Microbiol Immunol* 316: 253-92

57. McKenna SA, Lindhout DA, Shimoike T, Aitken CE, Puglisi JD. 2007. Viral dsRNA inhibitors prevent self-association and autophosphorylation of PKR. *J Mol Biol* 372: 103-13

58. Brand SR, Kobayashi R, Mathews MB. 1997. The Tat protein of human immunodeficiency virus type 1 is a substrate and inhibitor of the interferon-induced, virally activated protein kinase, PKR. *J Biol Chem* 272: 8388-95

59. Yoon CH, Lee ES, Lim DS, Bae YS. 2009. PKR, a p53 target gene, plays a crucial role in the tumor-suppressor function of p53. *Proc Natl Acad Sci U S A* 106: 7852-7

60. Harding HP, Novoa I, Zhang Y, Zeng H, Wek R, Schapira M, Ron D. 2000. Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Molecular Cell* 6: 1099-108

61. Vattem KM, Wek RC. 2004. Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America* 101: 11269-74

62. Wek RC, Cavener DR. 2007. Translational Control and the Unfolded Protein Response. *Antioxid Redox Signal* 9: 2357-71

63. Holcik M, Sonenberg N. 2005. Translational control in stress and apoptosis. *Nat Rev Mol Cell Biol* 6: 318-27

64. Hinnebusch AG, Natarajan K. 2002. Gcn4p, a master regulator of gene expression, is controlled at multiple levels by diverse signals of starvation and stress. *Eukaryot Cell* 1: 22-32

65. Hinnebusch AG, Wek RC, Dever TE, Cigan AM, Feng F, Donahue TF. 1993. Regulation of GCN4 expression in yeast. Gene specific translation control by phosphorylation of eIF-2α. In *Translation Regulation of Gene Expression*, ed. J Ilan, pp. pp. 87-115. New York: Plenum Press

66. Abastado JP, Miller PF, Jackson BM, Hinnebusch AG. 1991. Suppression of ribosomal reinitiation at upstream open reading frames in amino acid-starved cells forms the basis of *GCN4* translational control. *Molecular and Cellular Biology* 11: 486-96

67. Dever TE, Feng L, Wek RC, Cigan AM, Donahue TF, Hinnebusch AG. 1992. Phosphorylation of initiation factor 2 alpha by protein kinase GCN2 mediates gene-specific translational control of GCN4 in yeast. *Cell* 68: 585-96

68. Hinnebusch AG. 1994. Translational control of GCN4: an in vivo barometer of initiation-factor activity. *Trends in Biochemical Sciences* 19: 409-14

69. Harding HP, Zhang Y, Zeng H, Novoa I, Lu PD, Calfon M, Sadri N, Yun C, Popko B, Paules R, Stojdl DF, Bell JC, Hettmann T, Leiden JM, Ron D. 2003. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Mol Cell* 11: 619-33

70. Nishizawa M, Nagata S. 1992. cDNA clones encoding leucine-zipper proteins which interact with G-CSF gene promoter element 1-binding protein. *FEBS Lett* 299: 36-8

71. Peters ea. 2001. ATF-7, a novel bZIP protein, interacts with the PRL-1 protein-tyrosine phosphatase. *Journal of Biological Chemistry* 276: 13718–26

72. Hansen MB, Mitchelmore C, Kjaerulff KM, Rasmussen TE, Pedersen KM, Jensen NA. 2002. Mouse *ATF5*: Molecular cloning of two novel mRNAs, genomic organization, and odorant sensory neuron localization. *Genomics* 80: 344-50

73. Wang H, Lin G, Zhang Z. 2007. ATF5 promotes cell survival through transcriptional activation of Hsp27 in H9c2 cells. *Cell Biol Int* 31: 1309-15

74. Monaco SE, Angelastro JM, Szabolcs M, Greene LA. 2007. The transcription factor ATF5 is widely expressed in carcinomas, and interference with its function selectively kills neoplastic, but not nontransformed, breast cell lines. *Int J Cancer* 120: 1883-90

75. Watatani Y, Kimura N, Shimizu YI, Akiyama I, Tonaki D, Hirose H, Takahashi S, Takahashi Y. 2007. Amino acid limitation induces expression of ATF5 mRNA at the post-transcriptional level. *Life Sci* 80: 879-85

76. Chow LS, Lam CW, Chan SY, Tsao SW, To KF, Tong SF, Hung WK, Dammann R, Huang DP, Lo KW. 2006. Identification of RASSF1A modulated genes in nasopharyngeal carcinoma. *Oncogene* 25: 310-6

77. Angelastro JM, Ignatova TN, Kukekov VG, Steindler DA, Stengren GB, Mendelsohn C, Greene LA. 2003. Regulated expression of ATF5 is required for the progression of neural progenitor cells to neurons. *J Neurosci* 23: 4590-600

78. Nishioka T, Miyai Y, Haga H, Kawabata K, Shirato H, Homma A, Shibata K, Yasuda M. 2009. Novel function of transcription factor ATF5: blockade of p53-dependent apoptosis induced by ionizing irradiation. *Cell Struct Funct* 34: 17-22

79. Li G, Li W, Angelastro JM, Greene LA, Liu DX. 2009. Identification of a novel DNA binding site and a transcriptional target for activating transcription factor 5 in c6 glioma and mcf-7 breast cancer cells. *Mol Cancer Res* 7: 933-43

80. Pascual M, Gomez-Lechon MJ, Castell JV, Jover R. 2008. ATF5 is a highly abundant liver-enriched transcription factor that cooperates with constitutive androstane receptor in the transactivation of CYP2B6: implications in hepatic stress responses. *Drug Metab Dispos* 36: 1063-72

81. Gho JW, Ip WK, Chan KY, Law PT, Lai PB, Wong N. 2008. Re-expression of transcription factor ATF5 in hepatocellular carcinoma induces G2-M arrest. *Cancer Res* 68: 6743-51

82. Angelastro JM, Canoll PD, Kuo J, Weicker M, Costa A, Bruce JN, Greene LA. 2005. Selective destruction of glioblastoma cells by interference with the activity or expression of ATF5. *Oncogene* 25: 907-16

83. Mason JL, Angelastro JM, Ignatova TN, Kukekov VG, Lin G, Greene LA, Goldman JE. 2005. ATF5 regulates the proliferation and differentiation of oligodendrocytes. *Mol Cell Neurosci* 29: 372-80

84. Persengiev SP, Devireddy LR, Green MR. 2002. Inhibition of apoptosis by ATFx: a novel role for a member of the ATF/CREB family of mammalian bZIP transcription factors. *Genes Dev* 16: 1806-14

85. Sheng Z, Li L, Zhu LJ, Smith TW, Demers A, Ross AH, Moser RP, Green MR. 2010. A genome-wide RNA interference screen reveals an essential CREB3L2-ATF5-MCL1 survival pathway in malignant glioma with therapeutic implications. *Nat Med* 16: 671-7

86. Wei Y, Jiang J, Sun M, Chen X, Wang H, Gu J. 2006. ATF5 increases cisplatin-induced apoptosis through up-regulation of cyclin D3 transcription in HeLa cells. *Biochem Biophys Res Commun* 339: 591-6

87. Wei Y, Jiang J, Liu D, Zhou J, Chen X, Zhang S, Zong H, Yun X, Gu J. 2008. Cdc34-mediated degradation of ATF5 is blocked by cisplatin. *J Biol Chem* 283: 18773-81

88. Pati D, Meistrich ML, Plon SE. 1999. Human Cdc34 and Rad6B ubiquitin-conjugating enzymes target repressors of cyclic AMP-induced transcription for proteolysis. *Mol Cell Biol* 19: 5001-13

89. Biason-Lauber A, Lang-Muritano M, Vaccaro T, Schoenle EJ. 2002. Loss of kinase activity in a patient with Wolcott-Rallison syndrome caused by a novel mutation in the EIF2AK3 gene. *Diabetes* 51: 2301-5

90. Senee V, Vattem KM, Delepine M, Rainbow LA, Haton C, Lecoq A, Shaw NJ, Robert J-J, Rooman R, Diatloff-Zito C, Michaud JL, Bin-Abbas B, Taha D, Zabel B, Franceschini P, Topaloglu AK, Lathrop GM, Barrett TG, Nicolino M, Wek RC, Julier C. 2004. Wolcott-Rallison Syndrome: clinical, genetic, and functional study of EIF2AK3 mutations and suggestion of genetic heterogeneity. *Diabetes* 53: 1876-83

91. Harding H, Zeng H, Zhang Y, Jungreis R, Chung P, Plesken H, Sabatini DD, Ron D. 2001. Diabetes mellitus and exocrine pancreatic dysfunction in *Perk* -/- mice reveals a role for translational control in secretory cell survival. *Mol Cell* 7: 1153-63

92. Zhang P, McGrath B, Li S, Frank A, Zambito F, Reinert J, Gannon M, Ma K, McNaughton K, Cavener DR. 2002. The PERK eukaryotic initiation factor 2 alpha kinase is required for the development of the skeletal system, postnatal growth, and the function and viability of the pancreas. *Molecular and Cellular Biology* 22: 3864-74

93. Bobrovnikova-Marjon E, Grigoriadou C, Pytel D, Zhang F, Ye J, Koumenis C, Cavener D, Diehl JA. 2010. PERK promotes cancer cell proliferation and tumor growth by limiting oxidative DNA damage. *Oncogene* 29: 3881-95

94. Scheuner D, Song B, McEwen E, Liu C, Laybutt R, Gillespie P, Saunders T, Bonner-Weir S, Kaufman RJ. 2001. Translational control is required for the unfolded protein response and in vivo glucose homeostasis. *Mol Cell* 7: 1165-76

95. Anthony TG, McDaniel BJ, Byerley RL, McGrath BC, Cavener DR, McNurlan MA, Wek RC. 2004. Preservation of liver protein synthesis during dietary leucine deprivation occurs at the expense of skeletal muscle mass in mice deleted for eIF2 kinase GCN2. *The Journal of Biological Chemistry* 279: 36553-61

96. Zhang P, McGrath BC, Reinert J, Olsen DS, Lei L, Gill S, Wek SA, Vattem KM, Wek RC, Kimball SR, Jefferson LS, Cavener DR. 2002. The GCN2 eIF2alpha kinase is required for adaptation to amino acid deprivation in mice. *Molecular and Cellular Biology* 22: 6681-8

97. Guo F, Cavener DR. 2007. The GCN2 eIF2alpha kinase regulates fatty-acid homeostasis in the liver during deprivation of an essential amino acid. *Cell Metab* 5: 103-14

98. Abraham N, Stojdl DF, Duncan PI, Methot N, Ishii T, Dube M, Vanderhyden BC, Atkins HL, Gray DA, McBurney MW, Koromilas AE, Brown EG, Sonenberg N, Bell JC. 1999. Characterization of transgenic mice with targeted disruption of the catalytic domain of the double-stranded RNA-dependent protein kinase, PKR. *Journal of Biological Chemistry* 274: 5953-62

99. Stojdl DF, Abraham N, Knowles S, Marius R, Brasey A, Lichty BD, Brown EG, Sonenberg N, Bell JC. 2000. The murine double-stranded RNA-dependent protein kinase PKR is required for resistance to vesicular stomatitis virus. *Journal of Virology* 74: 9580-5

100. Williams BR. 1999. PKR: a sentinel kinase for cellular stress. *Oncogene* 18: 6112-20

101. Morel M, Couturier J, Lafay-Chebassier C, Paccalin M, Page G. 2009. PKR, the double stranded RNA-dependent protein kinase as a critical target in Alzheimer's disease. *J Cell Mol Med* 13: 1476-88

102. Jiang H-Y, Wek SA, McGrath BC, Scheuner D, Kaufman RJ, Cavener DR, Wek RC. 2003. Phosphorylation of the alpha subunit of eukaryotic initiation factor 2 is required for activation of NF-kappaB in response to diverse cellular stresses. *Molecular and Cellular Biology* 23: 5651-63

103. Jiang H-Y, Wek SA, McGrath BC, Lu D, Hai T, Harding HP, Wang X, Ron D, Cavener DR, Wek RC. 2004. Activating transcription factor 3 is integral to the eukaryotic initiation factor 2 kinase stress response. *Molecular and Cellular Biology* 24: 1365-77

104. Schroder M, Kaufman RJ. 2005. The mammalian unfolded protein response. *Annu Rev Biochem* 74: 739-89

105. Kilberg MS, Pan YX, Chen H, Leung-Pineda V. 2005. Nutritional control of gene expression: how mammalian cells respond to amino acid limitation. *Annual Review of Nutrition* 25: 59-85

106. Chen H, Pan YX, Dudenhausen EE, Kilberg MS. 2004. Amino acid deprivation induces the transcription rate of the human asparagine synthetase gene through a timed program of expression and promoter binding of nutrient-responsive basic region/leucine zipper transcription factors as well as localized histone acetylation. *J Biol Chem* 279: 50829-39

107. Averous J, Bruhat A, Jousse C, Carraro V, Thiel G, Fafournoux P. 2004. Induction of CHOP expression by amino acid limitation requires both ATF4 expression and ATF2 phosphorylation. *Journal of Biological Chemistry* 279: 5288-97

108. Fawcett TW, Martindale JL, Guyton KZ, Hai T, Holbrook NJ. 1999. Complexes containing activating transcription factor (ATF)/cAMP-responsive-element-binding-protein (CREB) interact with the CCAAT/enhancer-binding protein (C/EBP)-ATF composite site to regulate Gadd153 expression during the stress response. *Biochemical Journal* 339: 135-41

109. Ma Y, Brewer JW, Diehl JA, Hendershot LM. 2002. Two distinct stress signaling pathways converge upon the CHOP promoter during the mammalian unfolded protein response. *J Mol Biol* 318: 1351-65

110. Mathews MB, Sonenberg N, Hershey JWB. 1996. Orgins and Targets of Translational Control. In *Translational Control*, ed. JWB Hershey, MB Mathews, N Sonenberg, pp. 1-30. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press

111. Johannes G, Sarnow P. 1998. Cap-independent polysomal association of natural mRNAs encoding c-myc, PiP, and eIF-4G conferred by internal ribosome entry sites. *RNA* 12: 1500-13

112. Narasimhan J, Staschke KA, Wek RC. 2004. Dimerization is required for activation of eIF2 kinase Gcn2 in response to diverse environmental stress conditions. *J Biol Chem* 279: 22820-32

113. Vallejo M, Ron D, Miller CP, Habener JF. 1993. C/ATF, a member of the activating transcription factor family of DNA-binding proteins, dimerizes with CAAT/enhancer-binding proteins and directs their binding to cAMP response elements. *Proc Natl Acad Sci U S A* 90: 4679-83

114. Watatani Y, Ichikawa K, Nakanishi N, Fujimoto M, Takeda H, Kimura N, Hirose H, Takahashi S, Takahashi Y. 2008. Stress-induced translation of ATF5 mRNA is regulated by the 5'-untranslated region. *J Biol Chem* 283: 2543-53

115. Gjymishka A, Palii SS, Shan J, Kilberg MS. 2008. Despite increased ATF4 binding at the C/EBP-ATF composite site following activation of the unfolded protein response, system A transporter 2 (SNAT2) transcription activity is repressed in HepG2 cells. *J Biol Chem* 283: 27736-47

116. Paluh JL, Orback MJ, Legerton TL, Yanofsky C. 1988. The cross-pathway control gene of *Neurospora crassa*, cpc-1, encodes a protein similar to GCN4 of yeast and the DNA-binding domain of the oncogene v-jun-encoded protein. *Proceedings of the National Academy of Sciences, U.S.A.* 85: 3728-2732

117. Hoffmann B, Valerius O, Andermann M, Braus GH. 2001. Transcriptional autoregulation and inhibition of mRNA translation of amino acid regulator gene *cpcA* of filamentous fungus *Aspergillus nidulans*. *Molecular Biology of the Cell* 12: 2846-57

118. Tripahti G, Wiltshire C, Macaskill S, Tournu H, Budge S, Brown AJP. 2002. Gcn4 co-ordinates morphogenetic and metabolic responses to amino acid starvation in *Candida albicans*. *EMBO Journal* 21: 5448-56

119. Sharova LV, Sharov AA, Nedorezov T, Piao Y, Shaik N, Ko MS. 2009. Database for mRNA half-life of 19 977 genes obtained by DNA microarray analysis of pluripotent and differentiating mouse embryonic stem cells. *DNA Res* 16: 45-58

120. Jean D, Harbison M, McConkey DJ, Ronai Z, Bar-Eli M. 1998. CREB and its associated proteins act as survival factors for human melanoma cells. *J Biol Chem* 273: 24884-90

121. Jean D, Bar-Eli M. 2000. Regulation of tumor growth and metastasis of human melanoma by the CREB transcription factor family. *Mol Cell Biochem* 212: 19-28

122. Al Sarraj J, Vinson C, Thiel G. 2005. Regulation of asparagine synthetase gene transcription by the basic region leucine zipper transcription factors ATF5 and CHOP. *Biol Chem* 386: 873-9

123. Marciniak SJ, Yun CY, Oyadomari S, Novoa I, Zhang Y, Jungreis R, Nagata K, Harding HP, Ron D. 2004. CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. *Genes Dev* 18: 3066-77

124. McCullough KD, Martindale JL, Klotz LO, Aw TY, Holbrook NJ. 2001. Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. *Mol Cell Biol* 21: 1249-59

125. Angelastro JM, Mason JL, Ignatova TN, Kukekov VG, Stengren GB, Goldman JE, Greene LA. 2005. Downregulation of activating transcription factor 5 is required for differentiation of neural progenitor cells into astrocytes. *J Neurosci* 25: 3889-99

126. Dong S, Nutt CL, Betensky RA, Stemmer-Rachamimov AO, Denko NC, Ligon KL, Rowitch DH, Louis DN. 2005. Histology-based expression profiling yields novel prognostic markers in human glioblastoma. *J Neuropathol Exp Neurol* 64: 948-55

CURRICULUM VITAE

Donghui Zhou

EDUCATION:

2004-2010: Ph.D. in Biochemistry and Molecular Biology,
Indiana University, Indianapolis, Indiana
Thesis title The mechanisms regulating the transcription factor ATF5 and its function in the Integrated Stress Response
Thesis advisor: Ronald Wek.

2000-2004: M.S. in Biochemistry,
Peking Union Medical College, Beijing, China
DFMO's Effect on the Growth Characteristics of Human Lung Squamous Carcinoma Cells. Thesis advisor: Muzhen Fan.

1991-1995: B.S. in Pharmacology,
Beijing University of Chinese Medicine, Beijing, China

PROFESSIONAL EXPERIENCE:

1999-2000: Research Assistant
Lab of Biochemistry, China-Japan Friendship Hospital, Beijing

1995-1999: Grants Manager
Department of Scientific Research, China-Japan Friendship Hospital, Beijing

HONORS AND AWARDS:

Best poster by Graduate Student, Indiana University Simon Cancer Center Annual Cancer Research Day. May 8, 2008.

CONFERENCES AND PRESENTATIONS:

Meeting on Molecular Chaperones & Stress Responses at Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, April 30 – May 4, 2008. Phosphorylation of eIF2 directs ATF5 translational control in response to stress conditions, **Zhou D**., Palam L.R., Staschke, K.A., and Wek R.C.

Translational Control, September 3 –7, 2008. Phosphorylation of eIF2 Directs ATF5 Translational Control in Response to Different Stresses, **Zhou D**., Palam L.R., Staschke, K.A., and Wek R.C.

2008 Indiana University Simon Cancer Center Annual Cancer Research Day, May 8, 2008. Phosphorylation of eIF2 Directs ATF5 Translational Control in Response to Diverse Stress Conditions, **Zhou D**., Palam L.R., Staschke, K.A., and Wek R.C.

2007 Biochemistry and Molecular Biology Research Day, September 28, 2007. Transcription factor ATF5 and cellular stress, Zhou D. and Wek R.C.

2008 IU Sigma Xi Research Competition, May 16, 2008. Transcription factor ATF5 and cellular stress, **Zhou D**.

2008 Biochemistry and Molecular Biology Research Day, October 2, 2008. Transcription factor ATF5 and cellular stress, **Zhou D**., Palam L.R., Staschke, K.A., and Wek R.C.

PUBLICATIONS:

Zhou, D., Palam, L.R., Jiang, L., Narasimhan, J., Staschke, K.A. and Wek, R.C. (2008) Phosphorylation of eIF2 directs ATF5 translational control in response to diverse stress conditions. Journal of Biological Chemistry 283, 7064-7073

Dey S., Baird T.D., **Zhou D.**, Palam L.R., Spandau D.F., Wek R.C. (2010) Both transcriptional regulation and translational control of ATF4 is central to the Integrated Stress Response. J Biol Chem. 2010 Aug 23. (Epub ahead of print)