TRANSCRIPTIONAL REGULATION OF ATF4 IS CRITICAL FOR CONTROLLING THE INTEGRATED STRESS RESPONSE DURING eIF2 PHOSPHORYLATION

Souvik Dey

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

May 2012

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

_____

Ronald C. Wek, Ph.D., Chair

_____

Howard J. Edenberg, Ph.D.

Doctoral Committee

_____

Patricia Gallagher, Ph.D.

February 29, 2012

_____

John J. Turchi, Ph.D.

DEDICATION

This thesis is dedicated to my loving wife Arpita Mondal, my father Mr. Subrata Dey and mother Mrs. Keya Dey. Without their care, support and motivation it would have been extremely difficult for me to carry out my doctoral thesis dissertation.

ACKNOWLEDGEMENTS

First and foremost, I am extremely indebted to Dr. Ron Wek for his guidance and mentorship during my graduate career. He inspired me to pursue a career in academics and I hope to continue following his advice and invaluable lessons into the future. I would also like to thank my committee members, Dr. Howard Edenberg, Dr. Patricia Gallagher, and Dr. John Turchi for their invaluable advice in successfully completing my project. I am especially indebted to Sheree Wek for her advice and technical help throughout my graduate career. I would also like to thank the members of the Wek lab including Lakshmi Reddy Palam, Brian Teske, Thomas Baird for their technical advice, training, and friendship. A special thanks to former lab members Dr. Kirk Staschke, Dr. Donghui Zhou and Li Jiang for their technical help.

On a more technical note, I would like to thank Dr. Maria Hatzaglou, Dr. Cornelis Calkhoven and Dr. Dan Spandau for plasmids and cell lines and sharing their experimental expertise.

ABSTRACT

Souvik Dey

TRANSCRIPTIONAL REGULATION OF ATF4 IS CRITICAL FOR CONTROLLING THE INTEGRATED STRESS RESPONSE DURING eIF2 PHOSPHORYLATION

In response to different environmental stresses, phosphorylation of eIF2 (eIF2~P) represses global translation coincident with preferential translation of *ATF4*. ATF4 is a transcriptional activator of the integrated stress response, a program of gene expression involved in metabolism, nutrient uptake, anti-oxidation, and the activation of additional transcription factors, such as CHOP/GADD153, that can induce apoptosis. Although eIF2~P elicits translational control in response to many different stress arrangements, there are selected stresses, such as exposure to UV irradiation, that do not increase *ATF4* expression despite robust eIF2~P. In this study we addressed the underlying mechanism for variable expression of *ATF4* in response to eIF2~P during different stress conditions and the biological significance of omission of enhanced ATF4 function. We show that in addition to translational control, *ATF4* expression is subject to transcriptional regulation. Stress conditions such as endoplasmic reticulum stress induce both transcription and translation of *ATF4*, which together enhance expression of *ATF4* and its target genes in response to eIF2~P. By contrast, UV irradiation represses *ATF4* transcription, which diminishes *ATF4* mRNA available for translation during eIF2~P. eIF2~P enhances cell survival in response to UV irradiation. However, forced expression of *ATF4* and its target gene *CHOP* leads to increased sensitivity to UV irradiation. In this study, we also show

that C/EBPβ is a transcriptional repressor of *ATF4* during UV stress. C/EBPβ binds to critical elements in the *ATF4* promoter resulting in its transcriptional repression. The LIP isoform of C/EBPβ, but not the LAP version is regulated following UV exposure and directly represses *ATF4* transcription. Loss of the LIP isoform results in increased *ATF4* mRNA levels in response to UV irradiation, and subsequent recovery of *ATF4* translation, leading to enhanced expression of its target genes. Together these results illustrate how eIF2~P and translational control, combined with transcription factors regulated by alternative signaling pathways, can direct programs of gene expression that are specifically tailored to each environmental stress.

Ronald C. Wek, Ph.D., Chair

TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS

4E-BP	eIF4E-Binding Protein
ATF3	Activating Transcription Factor-3
ATF4	Activating Transcription Factor-4
ATF5	Activating Transcription Factor -5
bZIP	Basic Leucine Zipper
BCL	B-Cell Lymphoma
BIM	Bcl-2 Interacting Mediator of cell death
CARE	CCAAT-enhancer binding protein Activating transcription factor (C/EBP-ATF) Response Element
CACH	Childhood Ataxia with Central nervous system Hypermyelination
CHOP	C/EBP Homologous Protein
DMEM	Dulbecco's Modified Eagle's Media
DNA-PKc	DNA – Protein Kinase C
DTT	Dithiothreitol
DR5	Death Receptor 5
dsRNA	double-stranded RNA
dsRBMD	double-stranded RNA Binding Motif
EBER	Epstein-Barr Virus Small RNA
eIF2	Eukaryotic Initiation Factor 2
eIF2B	Eukaryotic Initiation Factor-2B
eIF2~P	Eukaryotic Initiation Factor-2 Phosphorylation
ER	Endoplasmic Reticulum
ERO1	ER Oxidoreductase 1

GAP	GTPase-Activating Protein
GDP	Guanosine Diphosphate
GTP	Guanosine-5'-Triphosphate
GCN2	General Control Nonderepressible -2
GDI	GDP-Dissociation Inhibitor
GADD34	Growth Arrest and DNA Damage-inducible protein-34
GRP78	Glucose-Related Protein 78
HisRS	Histidyl-tRNA Synthetase
HRI	Heme Regulated Inhibitor
ISR	Integrated Stress Response
IRE1	Inositol Requiring Enzyme -1
IkBα	Inhibitor of NF-κB alpha
LAP	Liver-Enriched Activating Protein
LIP	Liver-Enriched Inhibitory Protein
Met-tRNA$_i$	Methionyl-Initiator tRNA
MEF	Mouse Embryonic Fibroblast
MMS	Methyl Methane Sulfonate
NASH	Non-Alcoholic Steatohepatitis
NER	Nucleotide Excision Repair
NF-κB	Nuclear Factor -κB
NRF2	Nuclear Factor-like 2
PCR	Polymerase Chain Reaction
PKR	double-stranded RNA-activated Protein Kinase

PEK	Pancreatic eIF2 kinase
PERK	PKR-Like ER kinase
QRT-PCR	Quantitative real time PCR
ROS	Reactive Oxygen Species
RSK2	Ribosomal S6 kinase 2
Runx2	Runt-Related Transcription Factor -2
S6K	S6 Protein Kinase
SNAT2	System A neutral Amino acid Transporter 2
TC	Ternary Complex
TOR	Target-of-Rapamycin
TRB3	Tribbles-Related Protein -3
UPR	Unfolded Protein Response
UTR	Untranslated Region
uORF	upstream Open Reading Frame
WRS	Wolcott-Rallison Syndrome

INTRODUCTION

1. Phosphorylation of eIF2α – a critical event in cellular stress responses

In response to various environmental stress conditions, eukaryotic cells regulate protein synthesis by dampening global translation. This allows the cells to conserve their cellular energy and begin to alleviate the stress damage. Central to this response is a family of protein kinases that phosphorylate the α subunit of eukaryotic initiation factor 2 (eIF2) at serine-51 residue (1). Four different eIF2α kinases have been identified in mammals, including the General control nonderepressible kinase-2 (GCN2), Heme regulated inhibitor (HRI), Double-stranded RNA-activated protein kinase (PKR) and Pancreatic eIF2 kinase (PEK) or PKR-like ER kinase (PERK) (Figure 1) (2). While higher eukaryotes express all four of the eIF2α kinases, yeast *Saccharomyces cerevisiae* contains only a single version, GCN2. The family of eIF2α kinases exhibit sequence homology in their kinase catalytic domains, but diverge significantly in their regulatory regions, thus enabling each to act as a unique sensor during different types of cellular stresses (Figure 3).

Phosphorylation of eIF2α during diverse stress conditions leads to a program of translational and transcriptional regulation known as the Integrated Stress Response (ISR). The ISR is activated by sequential expression of a set of factors that function to alleviate the cellular stress, or alternatively induce apoptosis (2, 3). The ISR can be divided into three major steps. The initial step is the recognition of stress conditions by the stress kinases, leading to phosphorylation of eIF2α (Figure 1). The second step of the ISR involves a decrease in global protein synthesis, coincident with preferential translation of select mRNAs encoding transcription factors, such as ATF4 (4, 5). The

final step of the ISR involves transcriptional expression of the ATF4-target genes, which resolve the stress for cellular survival, or alternatively trigger apoptosis if the stress is chronic and/or the cellular damage is beyond repair. The ISR is activated in response to a myriad of different stress conditions, although there can be unique modulation of the pathway depending on the particulars of the stress arrangement. Each of the three steps of ISR and their key regulatory features will be reviewed in detail in the following chapters.

2. Role of eIF2α~P in disease

Mutations in the ISR signaling can cause disease. Loss of PERK is the basis of patients with Wolcott-Rallison Syndrome (WRS), which features neonatal diabetes, osteoporosis, hepatic and kidney dysfunction, exocrine pancreatic disorders, neutropenia and early death (6-9). Previous study has shown that *GCN2*$^{-/-}$ mice fed on a leucine-deprived diet showed a marked loss of skeletal muscle mass compared to their wild-type littermates, with about 40% of the *GCN2*$^{-/-}$ mice expiring within three days of the nutrient stress (10). PERK and GCN2 have also been shown to play a role in adaptation of solid tumor to hypoxia and nutrient-deprived conditions, respectively, in mouse and human xenograft caner models (11, 12). Loss of PKR in mice has also been shown to lead to increased susceptibility to viral infection (13, 14), while *HRI*$^{-/-}$ mice deprived of iron show enhanced anemia with significant reductions of red blood cells counts, along with compensatory erythroid hyperplasia and increased apoptosis in bone marrow and spleen (15).

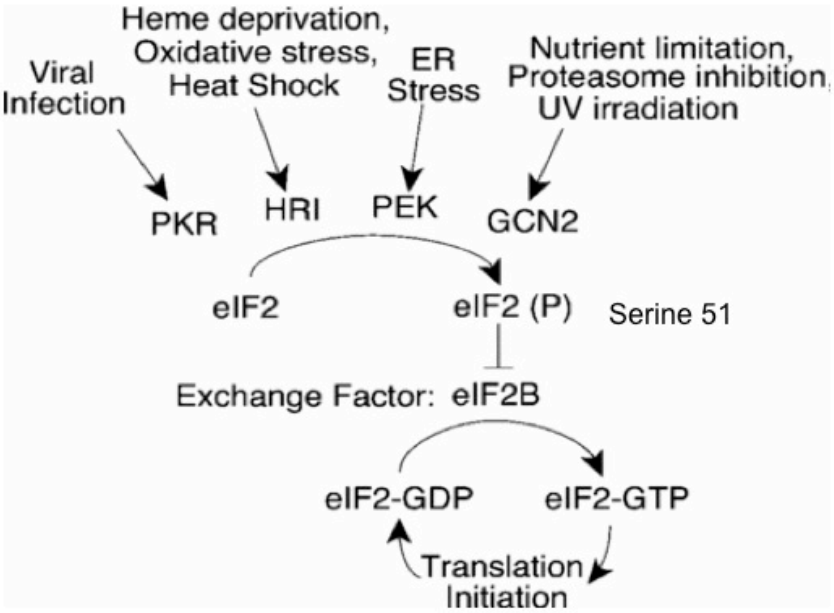


Figure 1. eIF2α kinases regulate translation in response to various cellular stress conditions. In response to diverse environmental stresses, a family of protein kinases, PKR, HRI, PERK and GCN2 phosphorylates eIF2α at the serine-51 residue in response to distinct stress conditions. Phosphorylation of eIF2α reduces the eIF2-GDP to eIF2-GTP exchange by inhibiting the guanine nucleotide exchange factor, eIF2B. Reduced eIF2-GTP levels subsequently lower translation initiation, resulting repression of global protein synthesis.

3. eIF2α~P is critical for regulating cellular translation

The eIF2 consists of three different subunits - α, β and γ, and plays a central role in translation initiation. During translation, eIF2 binds with initiator methionyl-tRNA and GTP to form a ternary complex (eIF2-TC), which then combines with the 40S ribosomal subunit in a pre-initiation complex that associates with the 5'-cap and associated proteins of the target mRNA (16). The small ribosomal complex then scans 5' to 3' along the leader of the mRNA. Once an appropriate initiation codon is found in the mRNA and initiator tRNA bound to this codon is placed into the P site of the ribosome, the 60S ribosomal subunit is recruited to form a translation-competent 80S ribosomal complex. Formation of the 80S subunit is preceded by release of eIF2 combined with GDP, which was hydrolyzed during the initiation process (17, 18). The eIF2-GDP is subsequently recycled to its active GTP form by a guanine nucleotide exchange factor known as eIF2B (Figure 2).

4. Exchange of eIF2-GDP to eIF2-GTP is regulated during cellular stress

The eIF2B consists of α, β, γ, δ and ε subunits. While γ and ε are the catalytic core of eIF2B, the subunits α, β, and δ form its regulatory subunits (19-21). There is sequence similarity between the mammalian subunits α, β, and δ of eIF2B and their yeast counterpart GCN3, GCD7, and GCD3 respectively. Studies *in vitro* and *in vivo* in mammalian and yeast model systems have shown that phosphorylation of eIF2α at serine 51 converts eIF2 from a substrate to a competitive inhibitor of eIF2B (2, 22). As a consequence, eIF2α phosphorylation reduces the levels of the eIF2-TC and subsequent rounds of translation initiation. The reduced global protein synthesis provides cells time

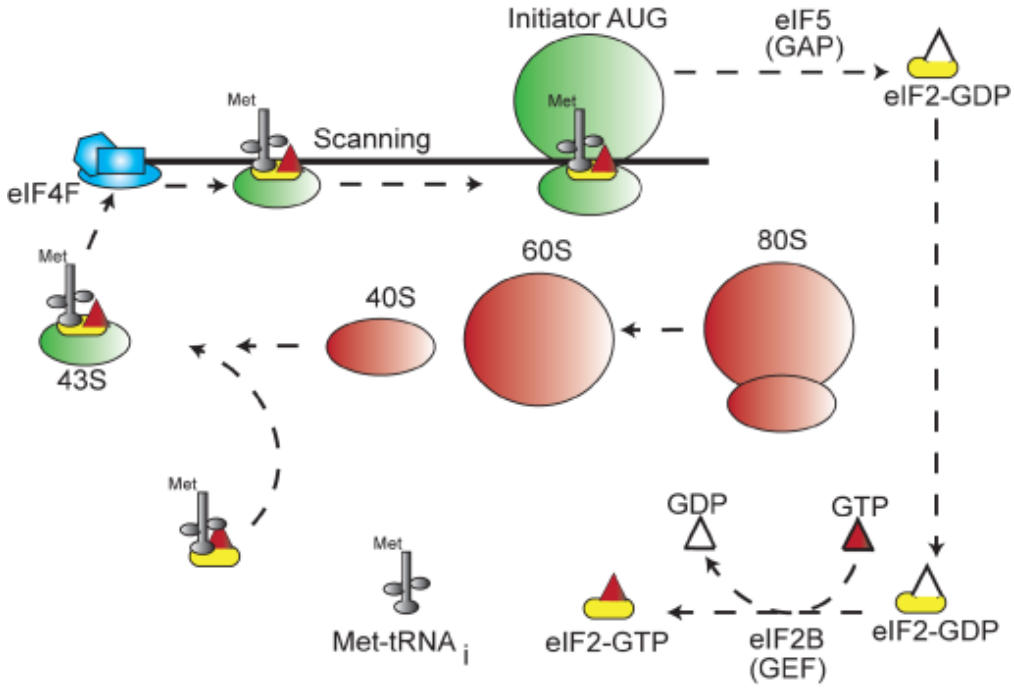


Figure 2. Regulation of eukaryotic translation initiation. The translation initiation factor eIF2 binds with GTP and initiator Met-tRNA$_i^{Met}$ along with the small 40S ribosome, as well as additional translation initiation factors, resulting in a 43S preinitiation complex. The 43S preinitiation ribosomal complex then binds to the 5'-cap structure of mRNAs consisting of the 7'methyl guanosine cap and associated cap binding protein eIF4F. The 43S ribosomal complex along with the associated eIF2 then scans processively 5' to 3' direction along the mRNA until the starting AUG initiation codon is recognized. With the placement of the initiator tRNA bound to the intiator codon in the P site of the ribosome, the 60S ribosome joins to form the competent 80S ribosome, allowing for the elongation phase of protein synthesis. Before the joining of the 60S ribosomal subunit, eIF2-GTP is hydrolyzed to eIF2-GDP and Pi is released, completing the step of translation initiation. The hydrolysis of eIF2-GTP is accelerated by the GTPase activating protein (GAP) eIF5. Inactive eIF2-GDP is converted to its active GTP bound form by the guanine nucleotide exchange factor eIF2B, facilitating subsequent rounds of translation initiation. Phosphorylation of eIF2α converts it from a substrate to an inhibitor of eIF2B. The resulting reduction in eIF2-GTP levels lowers general protein synthesis. This allows the cells to conserve energy and recalibrate the genome by expressing genes responsible for alleviation of the stress, or alternative for triggering cell apoptosis.

to recalibrate gene expression designed to block or remediate cellular damage during stress.

The initiation factor eIF5 is another critical regulator for the nucleotide exchange of eIF2. The eIF5 function as a GTPase-activating protein (GAP) for eIF2, contributing to selection of the start site during the initiation phase of protein synthesis (Figure 2) (23). However recent studies have revealed a new role of eIF5. In yeast, eIF5 was shown to function as a GDP-dissociation inhibitor (GDI), which can stabilize the eIF2-GDP state. A C-terminal domain of eIF5 can bind to eIF2-GDP and inhibit eIF2B function, thus preventing the eIF2-GDP to eIF2-GTP exchange (24). Therefore, eIF5 can contribute to decreased eIF2-GTP levels, aiding the ISR block of global protein synthesis.

5. Dephosphorylation of eIF2α~P

Phosphorylation of eIF2α represses of protein synthesis, which if sustained can contribute to cellular apoptosis. To restore translation, cells have feedback mechanisms in the ISR which includes proteins that can contribute to dephosphorylation of eIF2. These include GADD34 (Ppp1r15a) and CReP (Ppp1r15b), which act as regulatory subunits for the catalytic subunit of protein phosphatase type 1 (PP1c) that facilitates dephosphorylation of eIF2α~P (25). Unlike CReP which is constitutively expressed (26), GADD34 levels are low in unstressed conditions. During stress, *GADD34* is transcriptionally induced by ATF4 (27, 28). Additionally, expression of *GADD34* mRNA is subject to preferential translation in response to eIF2α~P (29). The resulting elevated levels of GADD34 can facilitate PP1c dephosphorylation of eIF2α~P and resumption of protein synthesis (27). Thus dephosphorylation of eIF2α~P provides cells

a mechanism to attenuate translation repression, thus enhancing the synthesis of stress-related mRNAs induced by the ISR.

6. GCN2 facilitates translational control in response to nutrient starvation

As noted above, diverse environmental stress conditions induce eIF2α~P through a family of different protein kinases. Such a wide-range of different stress conditions can lead to enhanced expression of *ATF4* and its target genes, thus activating the Integrated Stress Response (ISR). One of the eIF2α kinases is GCN2 (EIF2AK4) that is present from yeast to mammals and represses translation initiation in response to nutrient starvation (2). GCN2 consists of multiple domains, which contribute to the mechanisms regulating activation of the eIF2α kinase in response to starvation for nutrients. The GCN2 domains include a RWD domain, pseudokinase domain, protein kinase domain, histidyl-tRNA synthetase (HisRS)-related domain, and C-terminal domain that facilitates GCN2 dimerization and its association with ribosomes (Figure 3) (30). The major regulatory region that is important for GCN2 activation is the HisRS-regulated domain. Amino acid starvation leads to accumulation of uncharged tRNAs, which can bind to the HisRS-related domain and alter GCN2 to an activated conformation (31). Activated GCN2 then leads to enhanced GCN2 auto-phosphorylation at the activation loop of the catalytic domain, increasing eIF2α~P and translation of *ATF4* mRNA.

Ribosome association of GCN2 has been suggested to facilitate access of the eIF2α kinase to uncharged tRNA (32). Additionally, the C-terminal domain is suggested to act as an autoinhibitory region by binding to its kinase domain. Upon binding of uncharged tRNA, this inhibitory C terminal domain has been suggested to be released

from the protein kinase domain (33, 34). The pseudo kinase domain is required for eIF2α~P by GCN2. Currently, the mechanistic importance of the pseudo kinase domain is not well understood, but it has been suggested to contribute to the dynamics of the conformation change that occurs during activation of GCN2. Finally, the N-terminal RWD domain is important for direct interaction with a positive acting-regulator GCN1, which is thought to facilitate the delivery of uncharged tRNA to GCN2 (30).

GCN2 is also activated in response to other cytoplasmic stresses such as UV irradiation and proteosome inhibition (35-37). However, the mechanisms for activation of GCN2 in response to these stress conditions are not well defined. One proposed model for GCN2 activation in response to UV is that induced iNOS levels leads to rapid catalysis of L-Arginine to release reactive NO^{*}. This causes depletion of L-Arginine in the cells, which in turn activates GCN2 (38). Alternatively, UV irradiation may reduce the levels of charged tRNA by directly interfering with aminoacyl-tRNA synthetase charging of tRNA or by impeding nuclear export of tRNAs. Reduced proteasome activity has also been suggested to reduce the reclamation of free amino acids from degraded proteins, which may lower the charging of tRNAs.

7. GCN2 functions in conjunction with additional stress pathways to mitigate cell damage

GCN2 interacts with other cellular stress pathways. The serine/threonine kinase TOR acts as a sensor for nutrient condition. TOR is repressed by the drug rapamycin, and in yeast, rapamycin leads to increased GCN2 phosphorylation of eIF2α (39). Furthermore, leucine starvation in livers of *GCN2*$^{-/-}$ mice shows a dramatic reduction in

phosphorylation of TOR target protein, 4E-BP and S6K (40). Along with TOR, GCN2 also has regulatory links with the DNA-damage response kinase, DNA-PK. The activity of DNA-PK was reported to be required for full GCN2 phosphorylation of eIF2α in response to UV irradiation (41). It is suggested that DNA-PK may directly phosphorylate GCN2, contributing to its activation during select stress conditions.

GCN2 can act as a pro-survival factor, or trigger apoptosis, depending on the precise stress arrangement. GCN2 phosphorylation of eIF2α in response to UV irradiation activates cellular survival pathways, such as that directed by NF-κB (36). NF-κB is a key transcriptional factor controlling immune responses, cell proliferation, and apoptosis (42-44). The global translation repression accompanying eIF2α~P significantly reduces the synthesis of IκBα in response to UV irradiation (36). IκBα is an inhibitory protein of NF-κB, binding with the transcription factor and keeping it in an inactive state in the cytosol. IκBα is a labile protein and the lowered synthesis of IκBα following UV irradiation lowers the levels of this inhibitory protein, allowing for enhanced NF-κB entry into the nucleus and increased transcription of its target genes. Loss of either GCN2 or NF-κB (RelA/p65 subunit) can lead to apoptosis following UV exposure (36). However unlike the events occurring during UV stress, it was reported that increased GCN2 phosphorylation of eIF2α upon exposure to drugs that block proteasome function, such as MG132, leads to activation of a pro-apoptotic pathway through ATF4 and its target *CHOP* (37). Therefore, GCN2 can function in combination with various stress pathways to differentially activate genes that dictate cellular survival or apoptosis. As mentioned above, *GCN2*$^{-/-}$ mice are sensitive to leucine starvation, with loss of skeletal muscle to compensate for liver metabolism (10). Recent studies have also

suggested that GCN2 contributes to brain function, specifically the motor functions of the hippocampus and the anterior piriform cortex (45). *GCN2*$^{-/-}$ mice exhibit reduced long-term potentiation (LTP) directed by the hippocampus, as well as reduced learning ability in behavioral tasks, such as conditioned taste aversion (45-49).

8. PERK functions in the unfolded protein response during endoplasmic reticulum stress

PERK (EIF2AK3) is a type 1 ER resident transmembrane protein and eIF2α kinase that is activated in response to accumulation of unfolded proteins in the endoplasmic reticulum. The cytosolic portion of PERK contains the protein kinase domain, while the ER luminal region contains the signal sequence and the regulatory region that senses ER stress and facilitates dimerization between PERK polypeptides (Figure 3). An important regulatory protein that controls the function of PERK is the Glucose related protein 78 (GRP78/BiP), an ER resident chaperone that binds to the N-terminal regulatory region of PERK, maintaining PERK in an inactive state during non-stressed conditions (50). Accumulating misfolded proteins in the stressed ER can titrate off the GRP78 from PERK, allowing PERK to dimerize and trans-autophophorylate (51). As a consequence PERK is activated, leading to enhanced eIF2α~P and repressed global translation, which would reduce further influx of newly synthesized proteins into the stressed ER (52). An alternative model for related ER stress sensor IRE1 (Inositol requiring enzyme 1), is that unfolded proteins can directly bind to the regulatory region of PERK, facilitating enhanced eIF2α~P (52, 53). PERK functions in conjunction with other ER resident factors IRE1 and ATF6 (Activating transcription factor 6), which

contribute to increased transcription of genes involved in the folding, processing, and trafficking of secretory proteins (52). This pathway is collectively referred to as the Unfolded Protein Response (UPR), a stress response pathway that serves to expand the processing capacity of the secretory pathway.

9. HRI directs translational control in erythroid tissues

HRI (EIF2AK1) is an eIF2α kinase that is regulated by the availability of heme in erythroid tissues (54, 55). HRI binds heme at two regions, one at the N terminus of HRI and the other in the insert region within the kinase domain (Figure 3) (56). During non-stressed conditions in erythroid tissues, heme associates with these two sites, rendering HRI inactive. However during heme deprivation, heme is released from HRI, leading to enhanced HRI phosphorylation of eIF2α and reduced translation, which in reticulocytes is predominantly globin synthesis. As heme contains iron, HRI also acts a sensor for cellular iron levels. Absence of HRI in mice leads to cellular sensitivity and apoptosis during heme and iron deprivation, contributing to anemia, with decreased red blood cell counts and compensatory erythroid hyperplasia accompanied by increased apoptosis in the bone marrow and spleen (15).

10. PKR facilitates an anti-viral defense pathway

PKR (EIF2AK2) is expressed ubiquitously in all cells, but is induced upon interferon treatment (14, 57, 58). Activation of PKR occurs in response to binding of double-stranded RNA (dsRNA), which is generated during viral infections. PKR has two dsRNA-binding motifs (dsRBMs) in its N terminus, with a C terminal protein kinase

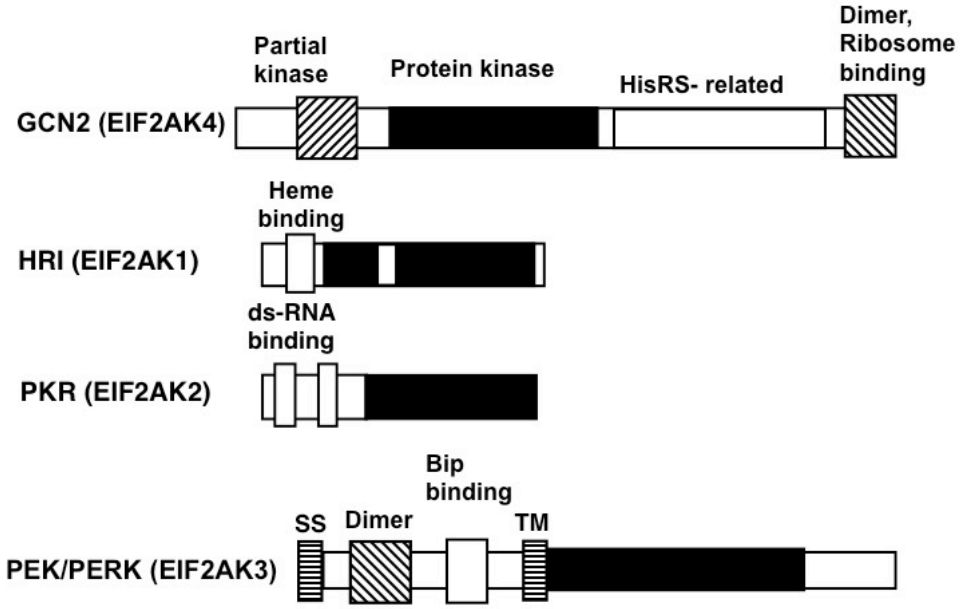


Figure 3. The eIF2α kinase family. There are four kinases in mammalian cells, GCN2, HRI, PKR and PERK (PEK). Each protein kinase is characterized by a conserved protein kinase domain depicted in black, along with divergent regulatory domains that are responsible for recognizing diverse stress condition. As discussed in detail in the text, GCN2 contains a HisRS-related domain that monitors cellular amino acid availability via binding to uncharged tRNA that accumulates during nutrient deprivation. GCN2 also contains a C- terminal region that provides for GCN2 ribosome association and dimerization. HRI has two heme binding domain that serve to regulate HRI in erythroid cells. Viral double-stranded RNA (dsRNA) activates PKR by binding to the two double-stranded RNA binding motifs (dsRBM), blocking cellular translation required for viral replication and proliferation. Endoplasmic reticulum stress activates the eIF2α kinase PEK/PERK. PEK/PERK has a signal sequence (SS) that is important for its entry into the ER lumen, an ER lumenal region that regulates PEK dimerization and association with ER chaperones, such as GRP78, and an ER transmembrane (TM) region. The cytosolic portion of PEK/PERK catalyzes eIF2α~P.

domain (Figure 3) (58). PKR binding to dsRNA causes it to homodimerize, possibly via an RNA bridge, leading to conformational changes and autophosphorylation at the activation loop of PKR (13). Induced PKR then phosphorylates eIF2α, leading to inhibition of protein synthesis, which reduces viral replication and viral infection in neighboring cells (2). Many viruses have developed mechanisms to counteract the effect of translational control directed by PKR. For example, Epstein-Barr virus expresses noncoding RNAs known as Epstein-Barr virus small RNA (EBER) which can bind and block activation of PKR (59). Herpes simplex virus expresses the γ134.5 which is similar in sequence with GADD34 (60). The γ134.5 protein recruits PP1c to dephosphorylate eIF2α~P, thus blocking the host translational control scheme induced during this viral infection (60). Finally, human immunodeficiency virus 1 encodes TAT, a regulatory protein that has high affinity for eIF2, thus diminishing substrate availability for PKR (61).

Apart from being activated by interferon and dsRNA, PKR has been also reported to be induced by ultraviolet A (UVA) irradiation in certain cell types. UVA was suggested to activate PKR by direct ERK2 and RSK2 phosphorylation of Thr-451 in the kinase domain of PKR (62). Furthermore, PKR is suggested to have anti-proliferative and tumor suppressive activities. For example, PKR was reported to be involved in p53-mediated tumor suppression (63).

11. Activation of *ATF4* occurs in response to cellular stresses

ATF4 is a member of the ATF/CREB family of basic leucine zipper (bZIP) transcription factor that regulates genes involved in alleviation of oxidative stress,

differentiation, amino acid synthesis, angiogenesis and intermediary metabolism. The *ATF4* gene is located on chromosome 22 at the locus 22q13. The ATF4 protein is 351 amino acid residues in length, consisting of three functional regions (Figure 4A). The N-terminal region is a p300 binding site, which modulates ATF4 protein stability and transcriptional activation (64). This portion of ATF4 has also been shown to interact with the growth factor regulated kinase RSK2 (65), osteoblast differentiation factor Runx2 (66), CHOP (67) and anti-oxidant factor NRF2 (68). Together these binding partners can modulate ATF4 transcriptional activity. In the middle portion of ATF4 is a βTrCP recognition motif. Phosphorylation of the serine residue in the βTrCP recognition motif DSGXXXS results in the interaction of ATF4 with βTrCP (β transducing repeat containing protein), an F-box containing protein which is part of the receptor for SCF E3 ubiquitin ligase that can facilitates ATF4 degradation by the 26S proteasome (69). Finally, the C-terminal portion of ATF4 contains the DNA binding region with the basic domain and the leucine zipper.

ATF4 can form homo- and heterodimers with members of C/EBP family proteins (70, 71), as well as with AP-1 transcription factors, such as c-Jun and c-Fos (72). This large array of binding partners enables ATF4 to have its diverse array of functions in transcription. Not only does deletion of *ATF4* in MEF cells block effective expression of known downstream ISR targets *CHOP* and *GADD34*, microarray profiling in *ATF4*$^{-/-}$ MEF cells have revealed that ATF4 is responsible for expression of genes involved in amino acid transport, protein synthesis, glutathione synthesis, and anti-oxidation (3). Genes involved in amino acid transport and translation include asparagine synthetase (*ASNS*), cationic amino acid transporter (*Slc7a5*), asparaginyl-tRNA synthetase (*NARS*),

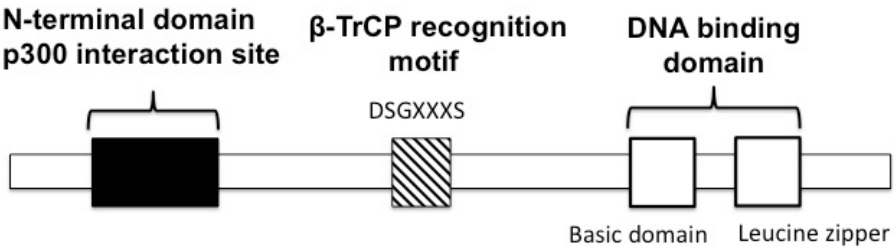
and tryptophan-tRNA synthetase (*WRS*) (3). ATF4 also regulates several detoxifying and redox genes such as ER oxidoreductase 1(*ERO1α*) and heme oxygenase (*HO1*), as well as genes for glutathione synthesis, such as cystathionine γ-lyase (*Cth*), methylenetetrahydrofolate dehydrogenase (*Mthfd*), and the glycine transporter (*Glyt1*) (3). As a result *ATF4*$^{-/-}$ cells are extremely sensitive to amino acid deficiency and oxidative stress (3).

Disruption of *ATF4* results in major developmental and physiological effects in mouse models. *ATF4*$^{-/-}$ mice have smaller body size and are also characterized by delayed hair growth as compared to their wild-type littermates (73). *ATF4*$^{-/-}$ mice develop severe micropthalmia with no lens, anterior chamber, and vitreous body, in the eye (73). Targeted deletion of *ATF4* in mice also causes severe anemia in the fetus due to improper development and function of haematopoietic progenitors (73). Absence of *ATF4* results in reduced osteoblast formation and bone deformation as ATF4 interacts with osteoblast differentiation factor Runx2 (66). However the role of ATF4 in diabetes and obesity is not fully understood. *ATF4*$^{-/-}$ mice are lean and are resistant to age-related and diet-induced obesity, with improved glucose tolerance possibly due to absence of CHOP (74).

12. Phosphorylation of eIF2α increases *ATF4* expression

Though eIF2α~P dampens cellular translation, it can trigger preferential translation of *ATF4* mRNA. The mechanism of preferential translation of ATF4 in mammals is strikingly similar to that of *GCN4*, a transcription factor in the yeast *Saccharomyces cerevisiae*. Like *GCN4*, *ATF4* mRNA consists of multiple upstream

A


N-terminal domain
p300 interaction site
β-TrCP recognition
motif
DSGXXXS
DNA binding
domain
Basic domain
Leucine zipper

B

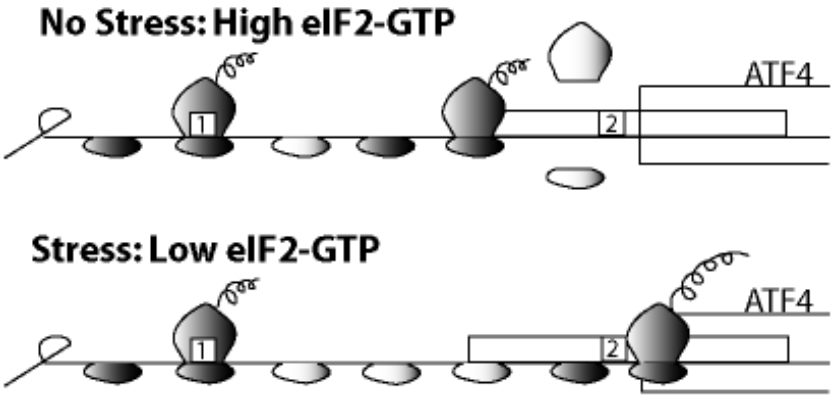

No Stress: High eIF2-GTP
ATF4
1
2
Stress: Low eIF2-GTP
ATF4
1
2

Figure 4.

Figure 4. Preferential translation of ATF4 is induced by eIF2α~P. (A) Schematic representation of the three domains of the bZIP transcription factor ATF4. The N-terminal domain is required for transcriptional activation, while the β-TrCP recognition motif modulates ATF4 protein stability. The DNA binding and dimerization domain are located in the C-terminus of ATF4. (B) The 5-leader of the *ATF4* mRNA has two uORFs that contributes differentially to the preferential translation of ATF4 during eIF2α~P. The uORF1and uORF2 act as positive and negative regulatory elements, respectively. Regulation of ATF4 expression begins with translation of the 5'-proximal uORF1. Following transltion of uORF1, the 40S ribosome is suggested to retain association with the *ATF4* mRNA and resume scanning 5'- to 3'direction along the leader of the *ATF4* transcript. During non-stressed conditions when there is low eIF2α~P, the scanning ribosome rapidly reacquires the eIF2-TC and reinitiates translation at uORF. The uORF2 overlaps out-of-frame with the *ATF4* coding region, and following translation of the uORF2, ribosomes dissociate from the *ATF4* mRNA and there is low *ATF4* expression. However during stress conditions, there is induced eIF2α~P. The resulting low levels of eIF2-GTP cause a delay in the reinitiation of the scanning ribosome. This delay in reinitiation allows the 40S ribosome to bypass the uORF2 initiation codon. During the interval between the initiation codons of uORF2 and the ATF4 ORF, the ribosomes reacquires the eIF2-TC, and translates the *ATF4* coding region.

open reading frames (uORFs) located in the 5'-leader of the mRNA (Figure 4B). The *ATF4* mRNA has two uORFs which contribute differentially to its enhanced translation in response to eIF2α phosphorylation (75). The 5'-proximal uORF1 encodes a polypeptide three amino acids in length, which acts as a 'positive element' by facilitating ribosome scanning and reinitiation at downstream start codons. By contrast, uORF2 overlaps the coding region of *ATF4* and acts as a 'negative element' by blocking translation of the *ATF4* coding region. In non-stressed cells, following translation of uORF1, high levels of eIF2-GTP that occur with low eIF2α~P leads to rapid ribosome reinitiation at the inhibitory uORF2; thus translation of the *ATF4* coding region is blocked and there is low expression of this key ISR transcriptional activator. However under stress conditions, the low availability of eIF2-GTP during eIF2α~P causes a delay in ribosomal reinitiation. Following translation of uORF1, scanning ribosomes bypass the inhibitory uORF2, and instead reinitiate at the *ATF4* coding region causing its increased expression during stress condition (75). Enhanced ATF4 protein then increases the transcription of its target genes in the ISR.

13. eIF2α~P regulates several downstream ISR genes

Though the downstream targets of ISR, including *ATF5*, *CHOP* and *GADD34* are thought to be primarily under ATF4-directed transcriptional regulation, several recent reports have shown that these genes are subject to preferential translation control during eIF2α~P. The 5'-leader of the *ATF5* mRNA has similar uORF architecture as the ATF4 transcription (76). Specifically, the *ATF5* mRNA has two uORFs, and recent studies indicate that eIF2α~P induces ATF5 expression by a mechanism of delayed translation

reinitiation that is similar to that described for *ATF4* (Figure 5A) (76). However the mechanism of *CHOP* mRNA translation regulation is different from *ATF4* and *ATF5* and involves ribosomal bypass of an inhibitory uORF (77). Unlike the abovementioned two mRNAs, *CHOP* mRNA has a single uORF in its 5'-leader, which acts as negative element that blocks scanning ribosomes (Figure 5B). In non-stressed cells with low eIF2α~P and high eIF2-GTP levels, scanning ribosomes initiate at and translate the uORF, which blocks translation of the downstream *CHOP* coding region. The repressing function of the uORF for downstream translation is suggested to be the consequence of the encoded polypeptide sequence, which stalls the ribosome during translation elongation or termination. Therefore, the ribosome is impaired for downstream translation, and the stalled ribosomes can serve as a barrier for subsequent scanning ribosomes in the *CHOP* mRNA. During stress, high eIF2α~P results in a leaky scanning mechanism enabling the ribosome to bypass the inhibitory uORF and instead the scanning ribosome initiates at the *CHOP* start codon (77).

The translation control mechanism for *GADD34* expression is not yet clearly understood. *GADD34* mRNA has two uORFs, with the first uORF1 being poorly translated and the second uORF2 being a repressing element. One complication for the GADD34 is that the uORF arrangement in the 5'-leader can vary with species. For example, in humans there are two uORFs which are separated by 30 nucleotides, while in mice the two uORFs overlap out-of-frame. A study by E. Jan and colleagues is most consistent with a *CHOP* bypass model in which low eIF2-GTP levels causes bypass of the uORF2 resulting in high *GADD34* expression (29).

Another stress induced bZIP transcription factor that is suggested to be under translation control is the C/EBPβ, a factor regulating diverse physiological and metabolic processes, including adipogenesis, immune response, bone and liver function and development (78-80). C/EBPβ is a critical member of the ISR, functioning by hetero-dimerizing with ATF4 and CHOP to regulate the expression of their downstream target genes (81-87). Translation of the *C/EBPβ* mRNA can give rise to three different isoforms, namely LAP (liver enriched activating protein), LAP* (liver enriched activating protein*) and LIP (liver enriched inhibitory protein) (78, 88). The C-terminal bZIP domain is conserved in each of the isoforms, but the LAP/ LAP* contain an N-terminal trans-activation domain, which is missing in the short LIP isoform (Figure 6A). The expression of these three isoforms is a consequence of different sites of translation initiation at four different start codons in the mRNA, designated A, B1, B2 and C (Figure 6A). Translation initiation at the intiation codon designated A expresses LAP*, while the B1 or B2 start codons encodes LAP (88).

Expression of LIP involves another short uORF, which is embedded out-of-frame in the C/EBPβ coding region (Figure 6B). If the scanning ribosome bypasses the 5'-proximal start codon A, there is an option to initiate at this short out-of-frame uORF (designated D start codon). Following translation of ORF-D, ribosomes can resume scanning and reinitiate at the downstream start codon C, yielding the LIP product (88, 89). Thus, translation of the short uORF-D prevents the expression of LAP*. It is not yet well understood whether eIF2α~P plays a role in this mechanism of *C/EBPβ* translation control.

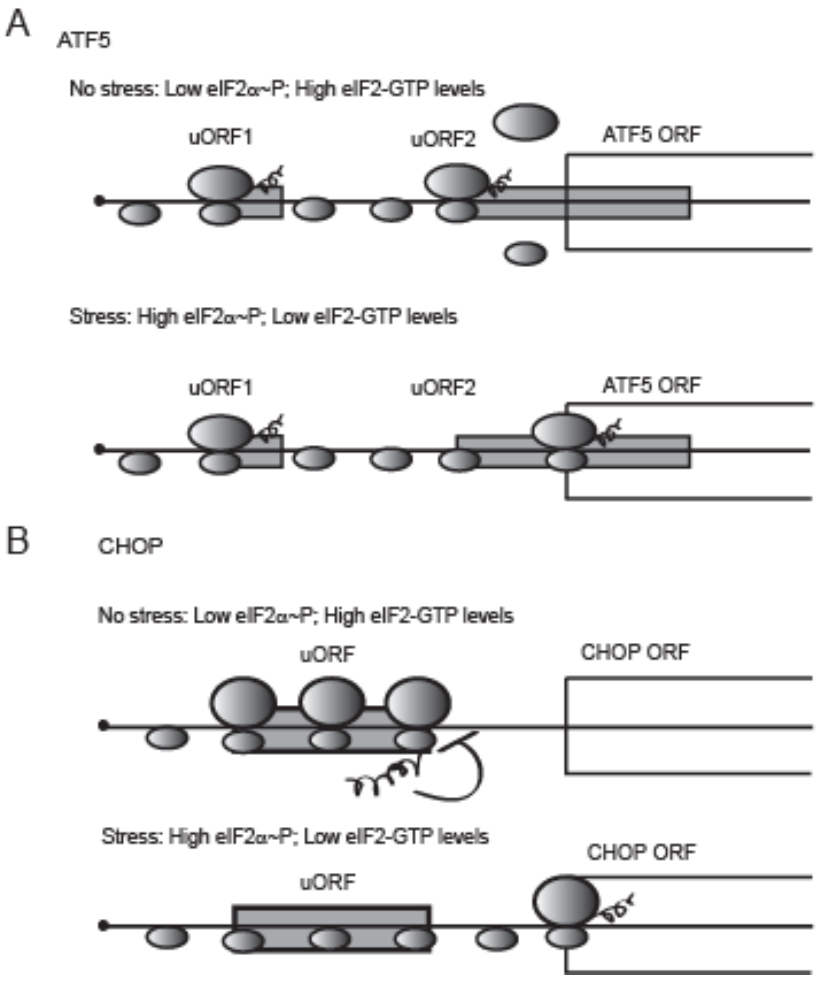


Figure 5. Phosphorylation of eIF2α regulates translation of several ISR genes. (A) *ATF5* is regulated at the translational level through eIF2α~P by a mechanism of delayed translation reinitiation that was described for *ATF4*. The 5'-leader of the *ATF5* mRNA has two conserved upstream ORFs with differential effect towards its expression. Like ATF4, the uORF1 acts as a positive-element and uORF2 is an inhibitor of *ATF5* translation. During non-stressed condition high eIF2-GTP levels allows the ribosome to reinitiate at the uORF2 thus blocking *ATF5* expression. However, low eIF2-GTP levels during stress condition delays the reinitiation of the ribosomes at uORF2. This delay in reinitiation gives the ribosome enough time to initiate at the *ATF5* start codon, enhancing its translation. (B) Regulation of *CHOP* mRNA by eIF2α~P involves a mechanism in which ribosome bypass an inhibitory uORF. In the absence of stress and high eIF2-GTP levels, the scanning ribosome initiates at the uORF, which leads to a ribosome stall, indicated by the "T" symbol, and therefore low *CHOP* translation. During stress, eIF2α~P is thought to allow for the bypass of the unORF due to the weak initiation context. The bypass of the uORF allows for to instead initiate at the *CHOP* start codon, with strong context leading to its increased expression.

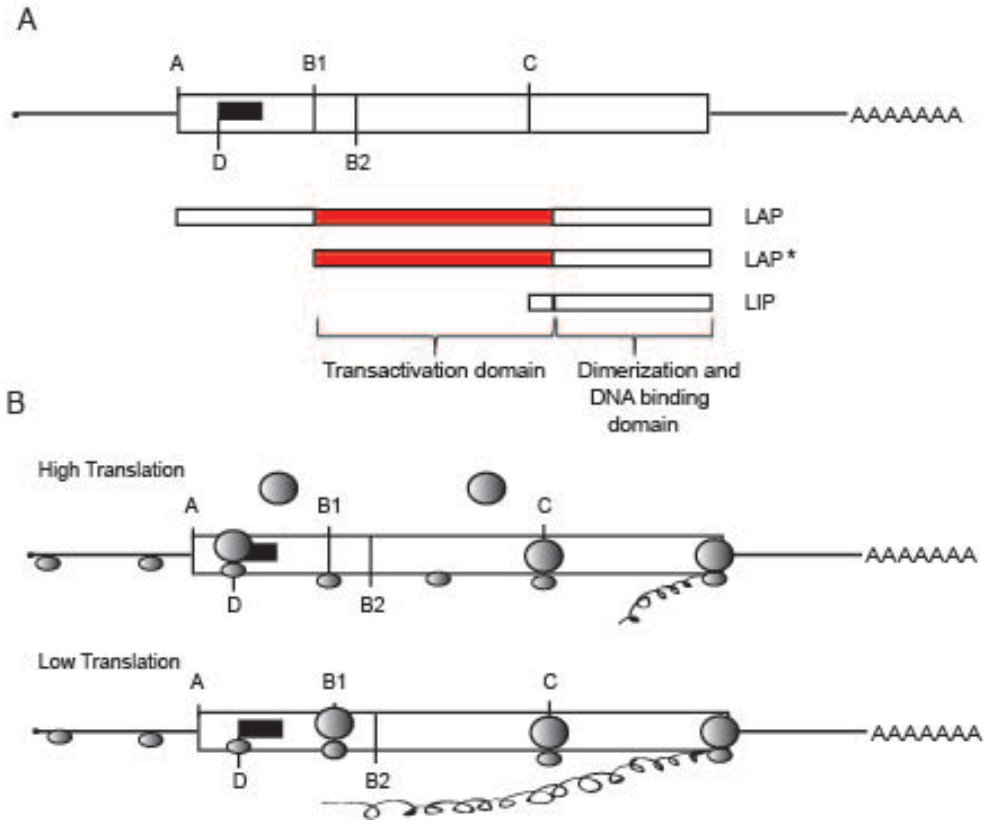


Figure 6. Translation control of *C/EBPβ* regulate synthesis of three isoforms. (A) C/EBPβ isoforms LAP, LAP* and LIP are synthesized from start codons A, B1/B2 and C on the same intron-less mRNA. A regulatory short uORF with start codon D regulates LAP and LIP expression. LAP and LAP* possess transactivation domains in the N-terminus (red box), as well as a DNA binding domain in its C-terminal. LIP only has the C-terminal DNA binding domain, and is considered a repressor of transcription. (B) Translation regulation of *C/EBPβ* is regulated by the short ORF. When ribosomes initiate at start codon D, there is low translation at the start codons B1 and B2, and instead ribosomes can reinitiate at the initiation codon C, leading to high LIP levels. Alternatively, ribosomes can initiate translation directly at A, B, or B1 start codons, leading to the synthesis of LAP or LAP*.

14. ATF4 activates several downstream transcription factors in the ISR

Elevated levels of ATF4 in response to cellular stress leads to increased expression of several downstream bZIP transcription factors, including ATF3, ATF5 and CHOP (2, 76). ATF4 activates these genes by binding to *cis*-acting elements containing the CCAAT- enhancer binding protein activating transcription factor (C/EBP-ATF) response element- often abbreviated as CARE elements, located in the promoters of the targeted genes. The consensus sequence to which ATF4 binds is TGATGxAAx (x indicates any base), half of which is a binding site for the members of the C/EBP family of transcription factors, and the other portion for ATF family members. The composite binding site enables ATF4 to heterodimerize with other bZIP transcription factors and therefore variably induce specific sets of gene promoters in response to different stress conditions (90).

A well-characterized example of ATF4-directed expression is the regulation of asparagine synthetase (*ASNS*), which catalyzes the synthesis of asparagine from aspartate and is induced during both ER stress and amino acid starvation. Following either amino acid deprivation or ER stress condition, ATF4 initially binds to the CARE elements (designated NSRE I and NSRE II) of the *ASNS* promoter through dimerization with C/EBPβ (91, 92). However sustained ATF4 activity following 6 hours of stress leads to increased expression of C/EBPβ and ATF3 proteins which in turn heterodimerize and bind to these CARE elements, thus displacing ATF4 and reducing activation of the *ASNS* promoter. This type of feedback regulation is referred to as the self-limiting regulation model of ATF4 (92).

Self-limiting regulation also occurs with ATF4 transcriptional activation of *TRB3* (93, 94), the human homolog of *Drosophila* tribbles and an important regulator of cellular growth (95). ATF4 dimerizes with CHOP to activate *TRB3* transcription by binding to the three tandem CARE sites in its promoter (93). Increased *TRB3* antagonizes ATF4 activity by physically interacting with ATF4, serving as a negative feedback of the ISR (96, 97).

ATF4 can induce unique patterns of gene expression in response to different stresses. An example of such regulation is of System A neutral amino acid transporter 2 (*SNAT2*), whose transcription is activated by ATF4 in response to nutrient deprivation, but not during ER stress (90). ATF4 dimerizes with ATF3 or C/EBPβ and binds to the C/EBP-ATF composite site in *SNAT2* promoter following both ER stress and amino acid limitation. In response to amino acid starvation, the ATF4 complex binding to SNAT2 promoter recruits the transcription machinery along with increased H3 acetylation resulting in high transcriptional activity (98). However in response to ER stress, the same ATF4 complex fails to recruit transcription machinery along with histone acetylases resulting in no transcriptional activation from the SNAT2 promoter (98). The mechanism by which such ATF4 causes such a differential effect on target genes is still not well understood.

15. Differential regulation of the ISR

Though the eIF2α~P/ATF4/CHOP pathway is induced in response to diverse cellular stresses such as ER stress, proteasome inhibition, nutrient starvation, and oxidative damage, there are certain stress conditions where there is a differential

regulation of the ISR pathway. Following UV irradiation, cells respond by blocking protein synthesis through rapid eIF2α~P. However unlike other stresses, UV stress does not induce *ATF4* and its downstream target *CHOP* (Figure 7) (36, 99). Such differential regulation of the ISR can also be observed during several other pathological conditions. Patients with brain ischemia, as well as mouse ischemic models, were reported to trigger eIF2α~P in cortex in the brain stem and hippocampus, but not trigger *ATF4* or *CHOP* expression (100). This discordant induction of the ISR, which is a reminiscent of UV stress - high eIF2α~P with no *ATF4* expression, has also been reported in livers of patients with Non-Alcoholic Steatohepatitis (NASH) (101). A different kind of differential regulation of ISR is observed during hypertonic conditions inducing osmotic stress in cells. Osmotic stress conditions leads to an increase in eIF2α~P and elevated levels of ATF4 protein (102). However, the expression of downstream targets of ATF4, such as *ATF3* and *CHOP*, are absent (102).

Such variation in the patterns of the induced ISR in response to the diverse stress conditions is further complicated by the variable roles of the eIF2α~P in cell survival. For example as noted above, UV-induced eIF2α~P enhances survival of cells by activating the transcription factor NF-κB by blocking the synthesis of IκBα (36). Additionally, increased eIF2α~P following UV irradiation was reported to lead to preferential translation of genes involved in nucleotide excision repair (NER), including *ERCC1*, *ERCC2* (*XPD*), *ERCC3* (*XPB*), *DDB1*, and *DDB2* (*XPE*) (41). By contrast, eIF2α~P induced by osmotic stress was reported to cause cellular apoptosis by a mechanism involving repressed translation of *BCl-XL*, a pro- survival member of the BCL family (102). It was suggested that eIF2α~P leads to cytoplasmic sequestration of

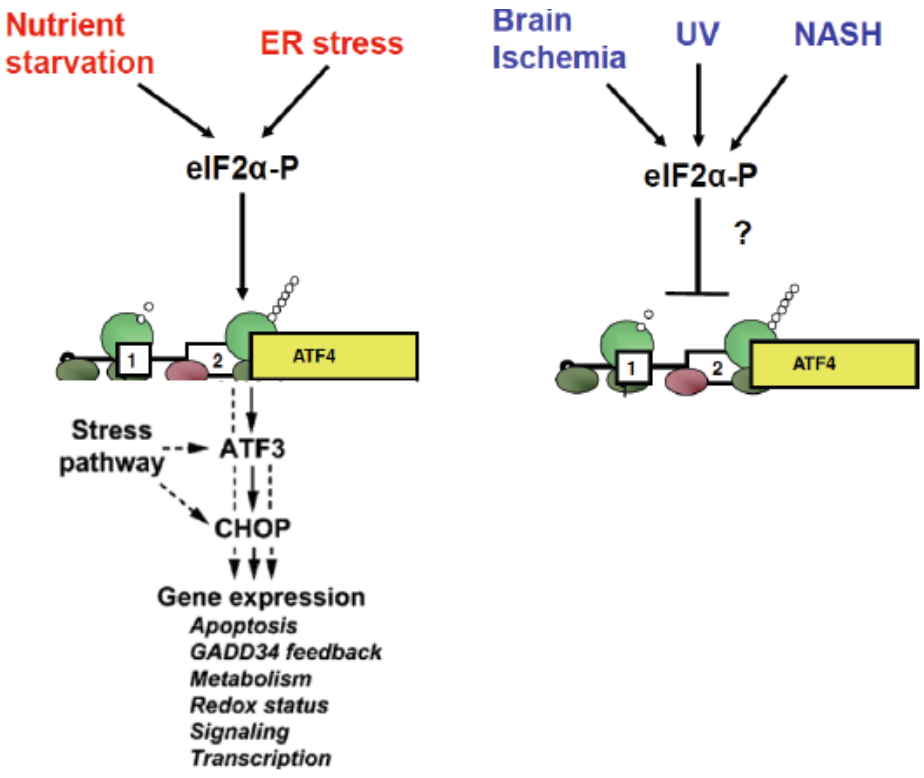


Figure 7. Differential regulation of the Integrated Stress Response. Stress conditions, such as ER stress and nutrient starvation induces eIF2α~P through different protein kinases. Increased eIF2α~P triggers preferential translation of *ATF4*, which in turn activates a cascade of bZIP transcription factors, such as ATF3 and CHOP, which regulates the expression of genes involved in metabolism, signaling, and of the cell redox status (left panel). However, in response to UV irradiation, brain ischemia, and non-alcoholic steatohepatitis (NASH) increased eIF2α~P does not activate the downstream ATF4/CHOP pathway (Right panel). Such differential regulation of ISR was shown to have an important role in determining whether the cell lives or dies in response to the specific stress.

heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) required for IRES-mediated loading of ribosomes during the translation of the BCL-XL mRNA (102). Therefore, the stress context for induction of the ISR can not only change the induction of key regulatory factors in the ISR, but also the mechanistic roles by which eIF2α~P can alter cellular survival in response to different environmental stresses.

16. The role of the ISR in determining cellular fate following stress

Activation of ISR is an important determining factor in directing the survival of cells in response to stress. A central hypothesis in the field states that the nature and the duration of stress conditions determine whether the cell remediates the stress, or alternatively undergoes apoptosis. While ATF4 target genes, such as *ATF5*, have been linked to cellular survival and have been shown to be over-expressed in different types of cancer cells (103-105), other targets, including *CHOP* (106) and *GADD34* (107), are considered to have pro-apoptotic functions. CHOP contributes to cellular apoptosis by repressing the expression of pro-survival factor *BCL2* (108), which can interact with and repress several different pro-apoptotic factors such as BIM, PUMA, and NOXA (109). CHOP also transcriptionally represses the transcription of the BCL2 by interacting with the C/EBPβ isoform LIP and binding to *BCL2* promoter (87). During ER stress, CHOP heterodimerizes with C/EBPα, which then binds to the *BIM* promoter leading to increased BIM expression (110). Decreased levels of BCL2 and increased levels of BIM promote apoptosis by activating cellular caspases.

Another important factor that is controlled by CHOP to induce cell death during ER stress is ER oxidoreductase 1 (*ERO1α*). ERO1α facilitates disulfide bond formation

in newly synthesized proteins as they enter the ER secretory system. During ER stress, CHOP transcriptionally upregulates *ERO1α*, resulting in a hyperoxidative condition in the ER, which can lead to cell death (107). The mechanism by which ERO1α induces cell death is attributed to the activation of the ER calcium release channel IP3R1 (111). Hyperoxidized ER dissociates the inhibitory interaction between IP3R1 and ERp44 (disulphide isomerase) (111). Following chronic ER stress, ERO1α activated IP3R1 releases calcium from the ER to the cytoplasm. Released calcium activates calcium kinase CaMKII, which in turn activates the expression of the NADPH oxidase subunit NOX2 (109). This results in the generation of ROS and cell death. CHOP is also shown to generate ROS production by directly repressing antioxidant genes. CHOP activation of death receptor (*DR5*) (109, 112) and *TRB3* (93) also contributes to cell death following ER stress.

GADD34 and the C/EBPβ isoform LIP have also been shown to induce cell death during ER stress. Over-expression of *GADD34* resumes protein synthesis, which during extended stress conditions is suggested to prematurely restore protein synthesis and overload a stressed ER (107). On the other hand, LIP was shown to induce cell death by stabilizing and facilitating the nuclear localization of CHOP (87). By contrast, over-expression of the LAP isoform of C/EBPβ prevents cell death and promotes tumor cell survival *in vitro* and *in vivo* (113). This suggests that there is a balance between the levels of the LAP and LIP, which is critical for fine-tuning the CHOP activity in apoptosis.

18. Dysregulation of the ISR can lead to disease

As noted earlier, mutations in the eIF2α kinases can lead to disease, such as for *PERK* mutations that lead to Wolcott-Rallison syndrome (6). Mutations in other components of the ISR that cause aberrant eIF2α~P have also been linked to human pathologies. For example, missense mutations in any of the 5 subunits of the guanidine nucleotide exchange factor, eIF2B lead to an autosomal recessive neuropathy known as Childhood ataxia with central nervous system hypomyelination (CACH), also known as the vanishing white matter leukoencephalopathy (114). The eIF2B mutations lead to a partial loss of eIF2B function thus blocking eIF2-GDP to GTP exchange. Reduced eIF2-GTP levels in this physiological condition is suggested to induce the ISR independent of eIF2α~P (115). In a sense, the basal activity of the ISR is constitutively induced. Upon a stress, such as head trauma or infectious disease, CACH patients develop an early rapid impairment of motor and cognitive functions due to loss of white matter in the brain, leading to death. It was suggested that the trauma can induce eIF2α~P, which in combination with the underlying impairment of eIF2B function would hyperactivate the ISR. Hyperactivaion of ISR is suggested to trigger apoptosis in key neural tissues by increasing cellular CHOP levels (114).

Another example of mutations in the ISR that can contribute to human disease involves mutations of *WFS1* (P724L and G695V in exon 8), which cause Wolfram syndrome (116). Wolfram syndrome is a rare juvenile form of diabetes characterized by loss of pancreatic β cells (117). Expression of *WFS1* is induced by ATF4 during ER stress, and loss of WFS1 function is suggested to induce ER stress that causes hyperactivation of *CHOP* and *ERO1α*, which facilitate the death of critical secretory

tissues. The mechanism by which WFS1 acts as a suppressor of ER stress involves its negative regulation of ATF6, an inducer of CHOP. WFS1 stabilizes the E3 ubiquitin ligase HRD1, which in turn degrades ATF6 through the 26S proteasome pathway (118). Thus, WFS1 can modulate the ER stress response by blocking ATF6 activation of target genes.

Loss of downstream effectors of eIF2α~P also can result in disease processes in mice. As discussed earlier, part of this complexity arises because of the fact that conditions such as UV irradiation, brain ischemia and NASH do not increase *ATF4* expression despite of high eIF2α~P. Absence of ATF4 or CHOP in these conditions has been shown to facilitate cellular survival. However in the case of cancer cells, a recent study suggests that the GCN2/ATF4/CHOP pathway is important for tumor growth and survival *in vivo* and *in vitro* (12). As mentioned before, *ATF4*$^{-/-}$ mice develops severe anemia in the fetus along with severe developmental defects like bone deformation and lens formation (73).

Deletion of *CHOP* in mice has shown to increase adiposity with high fatty acid and triglyceride content in serum, but surprisingly does not affect glucose tolerance or insulin insensitivity (119). This *CHOP* phenotype can be explained by the findings that deletion of *CHOP* function improves function of beta cells in several genetic or high fed diet-induced diabetic mice (119, 120). Improvement of beta cell function is due to increased expression of pro-survival UPR genes such as the ER chaperones (GRP94, BIP) (120) and those involved in the ERAD-mode of protein degradation (EDEM1), as well as increase in expression of antioxidant genes (*SOD1* and *SOD2*) (120). Deletion of *CHOP* also decreases the expression of *ERO1*, lowering cellular oxidative damage (107).

Similarly, deletion of *GADD34* (*PPP1r15a*) in mice did not result in any discernible phenotype. However deletion of the constitutively expressed *CReP (PPP1r15b)* resulted in stunted growth and perinatal lethality in mice (121). *CReP*$^{-/-}$ mice display impaired erythropoiesis with a reduced hematocrit and red blood cell count. Deletion of both *GADD34* and *CReP* results in embryonic lethal phenotype; emphasizing the importance of the regulation of eIF2α~P levels for developmental processes (121).

As discussed previously, a range of different environmental stresses has been shown to elicit the ISR. That is not to say that activation of the eIF2α~P/ATF4 pathway and its target is indistinguishable between various stress arrangements. Clearly there can be important differences in gene expression that are required for optimal alleviation of each stress condition. The underlying reasons for the differences in gene expression elicited by eIF2α~P during various stress arrangements can involve the combined action between the ISR and other stress response pathways.

In this thesis we have investigated three important questions. First, what are the underlying mechanisms for variable expression of ATF4 in response to eIF2α~P during different stress conditions? Second, what is the biological significance of the omission of ATF4 function in the ISR? Third, what are the regulators responsible for discordant induction of eIF2α~P and ATF4? Understanding these modes of regulation provides insight into the molecular mechanisms by which cells selectively repress or activate key genes subject to preferential translation, providing the ISR versatility to direct the transcriptome and cell survival during different environmental stress.

METHODS AND MATERIALS

1. Cell Culture and Stress Conditions

ATF4 $^{-/-}$, *CHOP* $^{-/-}$, *C/EBPβ* $^{-/-}$ and *A/A* (eIF2α-S51A) mouse embryonic fibroblast (MEF) cells, along with their wild-type counterparts, were described previously (122-128). MEF cells were cultured in Dulbecco's modified Eagle's media (DMEM, GIBCO, VA), which was supplemented with 1 mM nonessential amino acids (Hyclone, UT), 10% (v/v) fetal bovine serum (Atlanta Biological, GA), 100 units/ml penicillin (Thermo Scientific), and 100 μg/ml streptomycin (Thermo Scientific) at 37°C. *ATF4*$^{-/-}$ cells require additional essential amino acids, along with 55 μM β-mercaptoethanol, to reduce the oxidative stress associated with loss of this transcription factor. Thus, all cell lines were cultured with the same enriched media when compared with *ATF4*$^{-/-}$ cells in the described experiments. *C/EBPβ-ΔuORF* (Delta upstream open reading frame) cells and their wild-type were cultured in DMEM Glutamax (GIBCO) supplemented with 4.5g/L D-glucose, 10% fetal calf serum (Atlanta Biological, GA), 1% HEPES and 100 units/ml penicillin, and 100 μg/ml streptomycin (89).

Human glioblastoma M059K cells were cultured in medium containing a 1:1 mixture of DMEM and Ham's F-12 medium supplemented with 0.05 mM nonessential amino acids and 10% fetal bovine serum (41). Primary normal human keratinocyte cells were isolated from foreskin tissue and cultured in EpiLife medium (Cascade Biologics) supplemented with human keratinocyte growth supplement, (Cascade Biologics) and 1000 units/ml penicillin and 1000 μg/ml streptomycin (Roche Applied Science) (129). Cells were cultured to 70% confluence and irradiated with the indicated dose of UV-C (UV Stratalinker 2400) or UV-B (Xenon Corporation 1000W) followed by further

incubation for the indicated number of hours (36, 129). Alternatively, cells were treated with up to 200 μM methyl methane sulfonate (MMS, Sigma Aldrich) or 1 μM thapsigargin (MP Biomedicals) for up to 6 hours, as indicated. In case of combinatorial treatment, wild-type and C/EBPβ$^{-/-}$ cells were pretreated with 40J/m^2 of UV followed by 1μM thapsigargin after 1 hour and incubated for indicated time points until 6 hrs. In some cases MEF cells were pretreated with 10 μM salubrinal-003 (Enzo Life Sciences) for the indicated number of hours.

To measure the *ATF4* and *C/EBPβ* mRNA half-life, wild-type MEF cells were treated with 1 μM thapsigargin, 40 J/m^2 UV-C irradiation, or no stress. One hour later the cells were treated with 20 μm actinomycin D (Sigma Aldrich A1410). The cells were then cultured for 1, 2, or 4 hours, and *ATF4* and *C/EBPβ* mRNA levels were measured by qRT-PCR, as described below. To determine whether protein synthesis is required for transcriptional regulation of *ATF4* in response to ER stress or UV irradiation, wild-type MEF cells were treated with 50 μg/ml cycloheximide (Sigma Aldrich C1988) for 30 minutes. Cells were then treated with 1 μg/ml thapsigargin, 40 J/m^2 UV-C irradiation, or no stress, and then cultured for an additional 3 or 6 hours. *ATF4* mRNA levels were measured by qRT-PCR following the experimental details highlighted below.

2. Plasmid constructions

Transcriptional regulation of *ATF4* was measured by fusing a 2.5-kb segment with the mouse *ATF4* promoter (chr15:80084614-80085613) to the pGL3 firefly luciferase gene (99). This was accomplished by inserting the *ATF4* promoter region between the NcoI and XhoI restriction sites in the pGL3.1 (Promega, Madison, WI)

expression plasmid, resulting in plasmid P_{ATF4}-*Luc*. *ATF4* translation control was measured by using a previously reported P_{TK}-*ATF4-Luc* plasmid containing a cDNA encoding the 5'-leader of mouse *ATF4* mRNA and *ATF4* start codon downstream of a constitutive *TK* promoter (75). A deletion analysis of the P_{ATF4}-*Luc* plasmid was created by sequential 5'-deletions of 500-base pair segments of the *ATF4* promoter using divergent phosphor-primers to PCR amplify the required construct and subsequent ligation by T4 DNA ligase at room temperature. Additionally, internal deletions of 500 base pair segments removing sequences from -2 to -1.5-kb, -1.5 to -1-kb, -1 to -0.5-kb, and -0.5-kb -1-bp were similarly created in the *ATF4* promoter of the P_{ATF4}-*Luc* plasmid. Deletions of predicted C/EBPβ-binding sites involving the indicated sequences in the *ATF4* promoter were also constructed in the P_{ATF4}-*Luc* plasmid. Plasmids expressing LAP and LIP isoforms of C/EBPβ were described previously (126).

For the transfection analyses, MEF cells were plated at a density of 10^5 cells per well of 35 mm plates (BD Falcon) and grown overnight to 50% cellular confluency. The P_{ATF4}-*Luc* or P_{TK}-*ATF4-Luc* plasmids were cotransfected with control *Renilla* luciferase plasmid (Promega, Madison, WI) at a dilution ratio of 10:1 using FuGENE 6 reagent (Roche Applied Science, Mannheim, Germany). Following 24 hours after transfection, the MEF cells were exposed to vehicle alone, 1 μM thapsigargin, or 40 J/m^2 UVC irradiation, and then cultured for 12 hours. Dual luciferase assays were carried out in triplicates as described by the Promega instruction manual. Luciferase values and their standard deviation were derived as mean of three experiments and statistical significance was calculated with a Student's *t test*.

3. Immunoblot Analysis

Cultured cells treated with or without stress agents were washed twice with ice-cold phosphate buffer- (pH 7.4) saline solution and lysed in a solution containing 50 mM Tris-HCl (pH 7.9), 150 mM NaCl, 0.1% SDS, 100 mM NaF, 17.5 mM glycerol phosphate, and 10% glycerol supplemented with protease inhibitors (100 μM phenylmethylsulfonyl fluoride, 0.15 μM aprotinin, 1 μM leupeptin and 1 μM pepstatin). Lysates were subjected to sonication for 30 seconds and precleared by centrifugation at 12000 rpm at 4°C. Protein concentrations was measured by the Bio-Rad protein quantification kit (Bio-Rad # 500-0114) for detergent lysis, and equal amounts of protein were loaded and subjected to SDS/PAGE, along with low or high molecular weight markers (Bio-Rad# 161-0317). Proteins were transferred to nitrocellulose filters (Whatmann, Germany) and subsequently incubated with TBS-T solution containing 20 mM Tris/HCl (pH 7.9), 150 mM NaCl, and 0.2% Tween 20 supplemented with 4% (w/v) nonfat dried milk for 1hoursfollowed by an overnight incubation with antibodies specific for phosphorylated eIF2α at serine 51, cleaved PARP, or cleaved caspase-3 (Cell Signaling Technologies# 972, 9542 and 9662). ATF4 antibody was prepared against recombinant protein (76). CHOP and β-actin antibodies were obtained from Santa Cruz Biotechnology (sc-7351) and Sigma (A5441), respectively. Antibody which recognizes all isoforms of C/EBPβ (LAP, LAP* and LIP) was obtained from Biolegend (clone 1H7). Monoclonal antibody that recognizes either phosphorylated or nonphosphorylated forms of eIF2α was kindly provided by Dr. Scot Kimball (Pennsylvania State University College of Medicine, Hershey, PA). Filters were washed in the TBS-T solution 3 times, and subsequently incubated with secondary antibody tagged with horseradish peroxidase

and chemiluminescent solution. Proteins bound to antibody in the immunoblots were visualized by exposure to x-ray film (Kodak#178) and by imaging using the LI-COR Odyssey system. Each of the experiments featuring immunoblot analyses were repeated three independent times for validation.

4. Polysome Analyses

Cultured wild-type and *A/A* MEF cells were treated with 1 μM thapsigargin for 6 hours, or subjected to 40 J/m^2 UV-C irradiation and cultured for 6 h. Polysome analysis was carried out as described previously (76). Before lysis, cells were treated with 50 μg/ml cycloheximide for 10 minutes to inhibit translation elongation and preserve large polysomes. Cells were washed twice with an ice-chilled phosphate saline solution containing 50 μg/ml cycloheximide, and cellular lysates were prepared in a solution of 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 100 mM NaCl, and 0.4% Nonidet P-40 supplemented with 50 μg/ml cycloheximide. Lysates were gently passed through a 23-gauge needle followed by a 10-minute incubation on ice to ensure proper lysis. The preparation was then cleared by microcentrifugation (10,000 × g for 10 min) at 4°C and quantified for RNA concentration using a UV spectrophotometer (Nanodrop, Thermo Scientific). Lysates were loaded onto 10-50% sucrose gradients (20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 100 mM NaCl, and 50 μg/ml cycloheximide) and subjected to centrifugation in a Beckman SW-41Ti rotor for 2 hours at 40,000 rpm. Gradients were fractionated using a Biocomp Gradient Station. Absorbance of RNA at 254 nm was recorded by an in-line UV monitor, and polysome to monosome ratios were quantitated by comparing the areas under the recorded peaks.

5. Isolation of RNA and Real Time PCR

MEF cells were treated with the indicated stress conditions and harvested, and total cellular RNA was prepared using TRIzol® reagent (Invitrogen). To remove any DNA contamination, total mRNA from each condition were treated with DNaseI (Promega, Madison, WI) for 30 minutes at 37°C. Single-stranded cDNAs were synthesized using the Taqman reverse transcriptase kit (Applied Biosystems) according to the manufacturer's protocol. Quantitative PCR reactions were carried out with 400 ng of cDNA sample from each reaction using Taqman probes (Applied Biosystems) specific for detection of *ATF4*, *p21*, *CHOP*, *GADD45a*, and β-actin genes in a Roche LightCycler real-time PCR system. Quantitation of the target genes was normalized using the reference 18S rRNA to compensate for inter-PCR variations. Alternatively, quantitations of relative mRNA levels were carried by using SYBR Green PCR mix (Applied biosystems). The primers for detecting *ATF4*, C/*EBPβ* and β-actin through SYBR Green detection methods are as follows: *ATF4* forward primer 5'-GCCGGTTTAAGTTGTGTGCT-3', reverse primer 5'-CTGGATTCGAGGAATGTGCT-3'; C/EBPβ forward primer 5'-CGGGTTTCGGGACTTGAT-3', reverse primer 5'-GCCCGGCTGACAGTTACAC-3'; *β Actin* 5'-TGTTACCAACTGGGACGACA-3', reverse primer 5'-GGGGTGTTGAAGGTCTCAAA-3'. The ATF4 target genes namely *ASNS*, *Cat-1*, and *CHOP* were determined by the following primer sets: *ASNS* forward primer 5'-TTGACCCGCTGTTTGGAATG-3', *ASNS* reverse primer 5'-CGCCTTGTGGTTGTAGATTTCAC-3'; *CAT-1* forward primer 5'-CTTTGGATTCTCTGGTGTCCTGTC-3' and *CAT-1* reverse primer 5'-

GTTCTTGACTTCTTCCCCTGTGG-3'; *CHOP* forward primer 5'-CGGAACCTGAGGAGAGAGTG -3' and *CHOP* reverse primer 5'-CGTTTCCTGGGGATGAGATA -3'. Quantification was carried out using the Light Cycler 480 software (Version 1.2.9.11) to generate C_p values. Values are a representation of three independent experiments, with standard deviations as indicated. Statistical significance was calculated by using the two-tailed Student's *t test*.

6. Luciferase assays

MEF cells were plated at a density of 10^5 cells per well of 35 mm plates (BD Falcon) and grown overnight to establish the desired density of 50% cellular confluency. The P_{ATF4}-*Luc* or P_{TK}-*ATF4-Luc* plasmids were cotransfected with control Renilla luciferase at a dilution ratio of 10:1 using FuGENE 6 reagent (Roche Applied Science). Following 24 hours after transfection, the MEF cells were exposed to vehicle alone, 1 μM thapsigargin, or 40 J/m^2 UV-C irradiation, and then cultured for 12 hours. Dual luciferase assays (Promega, Madison, WI) were carried out in triplicates as described by the Promega instruction manual. Luciferase values and their standard deviation were derived as mean of three experiments and statistical significance was calculated with a Student's *t test*.

7. Cell Survival Assays

Cells were plated in 96-well plates and treated with 40 J/m^2 UV-C irradiation, followed by culture incubation for the indicated times. Selected cultures were pretreated with 10 μM salubrinal-003(Sal-003) for 6 hours, followed by UV irradiation and then

incubated for the indicated times. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed by using the CellTitre 96 nonradioactive cell proliferation assay kit (Promega, Madison, WI) to measure the number of viable cells, as described in the manufacturer's protocol. Clonogenic assays involved plating cells at a density of 500 cells/ well in a 60-mm plate. 48 hours after seeding, cells were then treated with UV-C irradiation and Sal-003, as indicated, and the cells were cultured for a period of 7 to 10 days. Cells were fixed with a solution containing 10% methanol and 10% acetic acid and stained with 0.4% crystal violet. Colonies were counted by using the AlphaImager system from Innotech and plotted from three independent experiments. Statistical significance was calculated using the Student's *t test*.

8. Chromatin immunoprecipitation

C/EBPβ$^{-/-}$ MEF cells, and a wild-type counterpart, were plated in 15 cm plates (BD Falcon) and grown overnight to about 60% confluency. Cells were treated with 40 J/m^2 of UV-C, followed by 6 hours incubation, 1 μM thapsigargin for 6 hours, or to no stress. Chromatin immunoprecipitations were performed with the SimpleChIP® Enzymatic Chromatin IP kit (Cell Signaling Technology #9003) following the manufacturer's protocol. Immunoprecipitation reactions were carried by using antibodies against C/EBPβ (Biolegend clone 1H7), Histone 3 (D2B12) (Cell Signaling Technology #4620), and rabbit IgG (Cell Signaling Technology #4620). Immunoprecipitated DNA samples were then analyzed by qRT-PCR. The primers for the segment of DNA analyzed for C/EBPβ binding designated P1, P2, and P3 were as follows: P1 forward

primer 5'-GGGACTGGAGAGTTAGGTTCG-3' and reverse primer 5'-TGTTTAAGTGACTCACAC-3', P2 forward primer 5'-AAGGCTTGAGAGCCAACTGA-3' and reverse primer 5'-TTCCTCCAGTTCAGCGATTT-3', and P3 forward primer 5'-TCGGTTCTGGAAACAACAAA-3' and reverse primer 5'-GTCACACCTGCCATCTCTTG-3'. Values are represented as the mean from three independent experiments, with standard deviations as indicated. Statistical significance was calculated by using the two-tailed Student's *t test*.

RESULTS

1. Both transcriptional regulation and translational control of *ATF4* are central to the Integrated Stress Response

1.1 UV irradiation induces eIF2α~P without activation of *ATF4* and *CHOP*

UV irradiation has been reported to induce translation control by eIF2α~P without activating its target genes, *ATF4* and *CHOP* (36). To test this model, we measured the levels of eIF2α~P in MEF cells in response to increasing doses of UV-C irradiation (Figure 8A). The levels of eIF2α~P were determined by immunoblot analysis using a polyclonal antibody that specifically recognizes the translation initiation factor that is phosphorylated at serine 51. eIF2α~P was detected with a dose range between 20 and 80 J/m^2 of UV-C, with a maximum at 40 J/m^2. Levels of eIF2α~P at 40 J/m^2 UV-C irradiation were comparable with that measured in cells treated with 1 μM thapsigargin, a well-established inducer of ER stress (Figure 8A). Phosphorylated eIF2α was detected by 1 hour after exposure to 40 J/m^2 UV-C and was sustained for 6 hours (Figure 8B). Importantly, neither ISR regulators, ATF4 or CHOP were appreciably induced in response to UV irradiation, whereas both were highly expressed during ER stress (Figure 8, A and B). By comparison, expression of the p53 target gene p21 was enhanced in response to UV irradiation, but not during ER stress. We also carried out a dose- and time-dependent analysis of the ISR during treatment of the MEF cells with MMS, an alkylating agent that can damage DNA, and found induction of both ATF4 and CHOP proteins (Figure 8, C and D).

We also observed increased eIF2α~P without induced expression of the ISR genes in two different cultured human cell types (glioblastoma cell line M059K and

primary human keratinocytes) treated with UV-C irradiation (Figure 9, A and B). By contrast, in response to thapsigargin treatment, both of the human cells showed increased ATF4 and CHOP levels accompanied by eIF2α~P. Finally, we addressed whether UV-B irradiation elicits a similar discordant induction of the ISR. MEF and normal human keratinocyte cells were treated with increasing doses of UV-B irradiation, and although there were significant levels of eIF2α~P, there was no induction of ATF4 and CHOP (Figure 9, C and D). These results indicate that both UV-C and UV-B irradiation significantly increase eIF2α~P without activation of the central ISR regulators, ATF4 and CHOP.

1.2 eIF2α~P by UV irradiation reduces global protein synthesis

eIF2α~P is a potent repressor of translation initiation. We examined the effects of UV-induced eIF2α~P on global protein synthesis by sucrose gradient analyses of polysomes. UV-C irradiation of MEF cells significantly reduced polysomes, coincident with increased free ribosomes and monosomes, indicating repressed translation initiation (Figure 10). By comparison, there was a substantial restoration of polysomes in UV-C irradiated *A/A* MEF cells expressing a mutant form of eIF2α with alanine substituted for the phosphorylated serine 51. This result supports the model that eIF2α~P is the primary mediator of global translation repression in response to UV irradiation. This central idea is also true for ER stress, with eIF2α~P being required for a robust reduction in translation initiation (Figure 10).

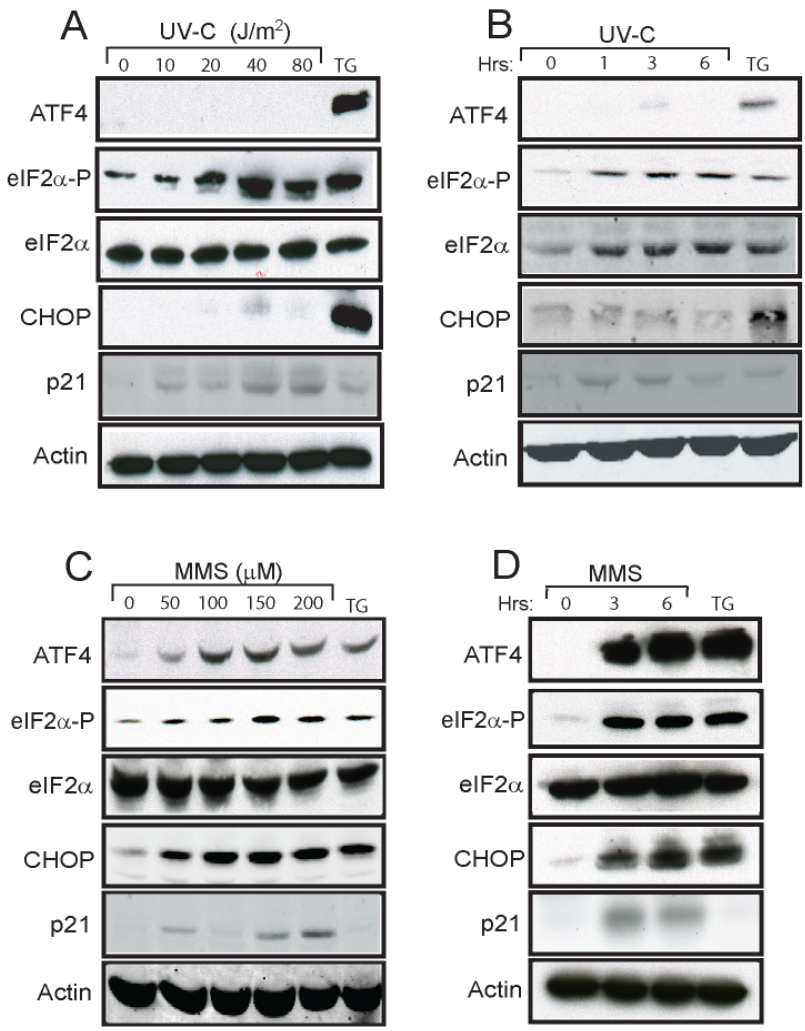


Figure 8. UV irradiation elicits eIF2α~P in the absence of induced ATF4 and CHOP. (A) Wild-type MEF cells were treated with the indicated dosage of UV-C irradiation and then incubated in the culture medium for 6 hours. (B) Alternatively, the MEF cells were treated with 40 J/m^2 UV-C irradiation and then cultured for up to 6 hours, as indicated. Lysates were prepared from the UV-C irradiated cells, and the levels of ATF4, phosphorylated eIF2α, total eIF2α, p21, and β-actin were measured by immunoblot analysis using antibody specific to each protein. As a control, cells were subjected to ER stress elicited by 1 μM thapsigargin (TG) for 6 hours, and immunoblot analyses were carried out on the cell lysates. (C) MEF cells were treated with up to 200 μM MMS for 6 hours, as indicated. (D) Alternatively, the cells were exposed to 100 μM MMS for up to 6 hours. Lysates were prepared from the treated cells and the levels of the indicated proteins were measured by immunoblot analysis. Results shown in each panel are representative of three independent experiments.

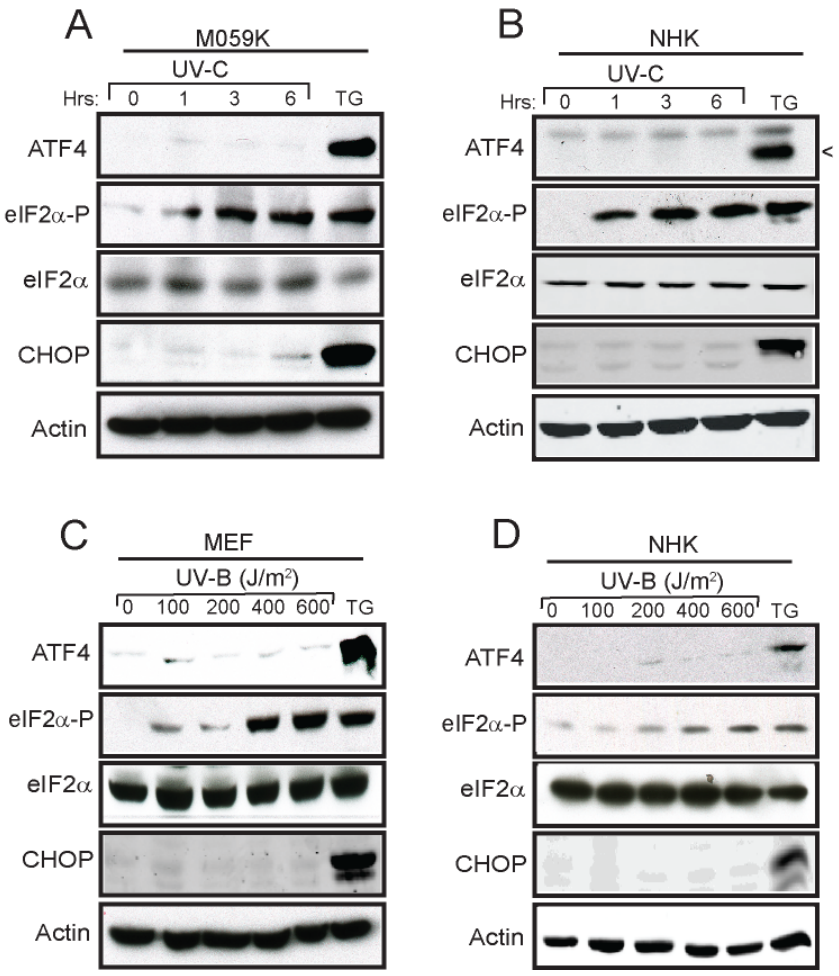


Figure 9. UV-C and UV-B irradiation induces eIF2α~P in different cell types. Human glioblastoma cells M059K (A) and primary human keratinocytes NHK cells (B) were treated with 40 J/m^2 UV-C irradiation and then cultured for up to 6 hours. As a control, cells were also exposed to 1μM thapsigargin (TG) for 6 hours. Lysates were prepared from the stressed human cells and the levels of indicated proteins were measured by immunoblot analysis. Wild-type MEF cells (C) and human NHK keratinocytes (D) were treated with increasing dosages of UV-B irradiation, cultured for up to 6 hours, and the indicated proteins were measured by immunoblot analyses. Each panel is representative of three independent experiments.

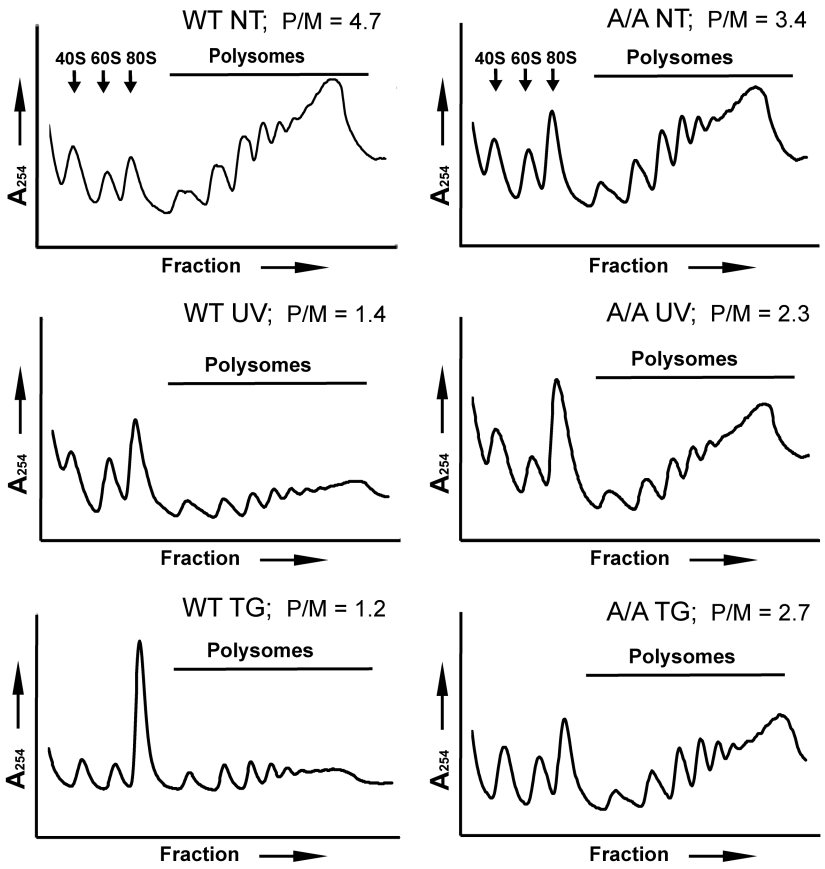


Figure 10. Phosphorylation of eIF2α reduces translation initiation in response to UV irradiation or ER stress. Wild-type MEF cells (WT) and a mutant version expressing the non-phosphorylated eIF2α-S51A (*A/A*) were treated with 40 J/m^2 UV-C irradiation (UV), followed by culture incubation for 6 hours, or to no stress treatment (NT). Alternatively, the MEF cells were subjected to ER stress by treatment with 1 μM thapsigargin (TG) for 6 hours. Lysates were prepared and were subjected to centrifugation in a 10%-50% sucrose gradient. Polysome profiles were generated with absorbance values as measured at 254 nm. Arrows indicate peaks corresponding to 40S and 60S ribosomal subunits or 80S monosomes, and the line highlights polysomal fractions. Polysomes to monosome (P/M) ratios were determined by calculating the area under the indicated peaks.

1.3 *ATF4* mRNA is lowered in response to UV irradiation

Both UV and ER stresses elicited eIF2α~P and repressed translation initiation, yet there is differential regulation of ATF4, with ER stress triggering enhanced ATF4 protein levels, whereas in response to UV irradiation, ATF4 is absent. The loss of ATF4 protein in response to UV irradiation may be due to altered regulation of the synthesis and/or turnover of *ATF4* mRNA or protein. *ATF4* mRNA levels were measured in MEF cells during the ER and UV stress conditions. Treatment with thapsigargin led to almost a 3-fold increase in *ATF4* mRNA levels, whereas UV-C irradiation led to a lowering of *ATF4* mRNA in a dose-dependent fashion, with significant reductions in the transcript levels in response to exposure to 40 J/m^2 UV-C irradiation (Figure 11A). Treatment with MMS, a condition that led to elevated ATF4 protein levels along with eIF2α~P, elicited a modest increase in *ATF4* mRNA levels. UV irradiation did not lead to a general reduction in mRNA levels, as the amount of actin mRNA remained unchanged for up to 6hoursafter exposure to 40 J/m^2 UV-C irradiation (Figure 11, A and B). The amount of *p21* and *GADD45a* transcripts, both known to be induced by genotoxic stress, were increased 3-fold or greater (Figure 11B). The kinetics of the *ATF4* mRNA changes are gradual, with lowered *ATF4* transcript levels 2hoursafter UV irradiation and a further reduction 4hoursafter the UV stress (Figure 11, B and C). By contrast, in MEF cells exposed to thapsigargin there was a continuous enhancement in *ATF4* mRNA levels, with a 3-fold increase after 4hoursof ER stress (Figure 11C). These results indicate that changes in *ATF4* transcript levels are an important reason for differential expression of *ATF4* in response to UV and ER stress. UV-C irradiation leads to selective reduction in *ATF4* mRNA levels, coincident with low ATF4 protein levels despite a robust eIF2α~P.

1.4 *ATF4* mRNA is short-lived independent of stress

Reduced *ATF4* mRNA in response to UV irradiation can be due to an increase in mRNA decay or repressed transcription. To address whether the half-life of *ATF4* mRNA is decreased in response to UV irradiation, we utilized the transcription blocking capabilities of actinomycin D. MEF cells were treated with UV-C irradiation, thapsigargin, or no stress, and 1hourslater 20 μM actinomycin D was added to the cells, which were then incubated for an additional period of 1, 2, or 4 h. Total RNA was isolated, and subsequently *ATF4* mRNA was measured by qRT-PCR (Figure 11, C and D). We anticipated that if the *ATF4* mRNA half-life is a significant factor in the lowered levels of *ATF4* transcript in response to UV irradiation that the turnover of *ATF4* mRNA would be considerably greater than that measured in cells treated with thapsigargin or no stress. Although *ATF4* mRNA was short-lived, with a half-life of ~ 3 hours, there was not a significant difference in *ATF4* transcript turnover in the MEF cells treated with UV irradiation, thapsigargin, or no stress (Figure 11D). These results indicate that the decay of *ATF4* mRNA does not change between different stress arrangements and is not the regulatory switch for reduced mRNA levels in response to UV irradiation.

1.5 *ATF4* transcription is repressed in response to UV irradiation

We propose that *ATF4* transcription is repressed in response to UV irradiation, leading to low levels of *ATF4* mRNA available for preferential translation in response to eIF2α~P. To test this model, a 2.5-kb insert encompassing the *ATF4* promoter was fused to a firefly luciferase reporter gene. The resulting P_{ATF4}-*Luc* reporter, which did not encode the 5′-leader region of *ATF4* mRNA required for translational control in response

to eIF2α~P, was introduced into MEF cells and subjected to ER or UV stress. After thapsigargin treatment there was about a 4-fold increase of luciferase activity, consistent with the idea that enhanced *ATF4* mRNA in response to ER stress was due to increased transcription (Figure 12A). Exposure to 40 J/m^2 UV-C resulted in a 3-fold reduction of luciferase activity, indicating that UV irradiation leads to transcriptional repression of *ATF4*.

We next addressed whether *ATF4* translational control can occur during UV-C irradiation and eIF2α~P if the *ATF4* transcript is available. We analyzed an *ATF4-Luc* fusion reporter, which contained the 5′-leader of the *ATF4* mRNA expressed from a constitutive thymidine kinase (TK) promoter (75). MEF cells transfected with the P_{TK}-*ATF4-Luc* plasmid were treated with thapsigargin or UV irradiation. In response to either stress conditions, there was a significant increase in luciferase activity compared with non-treated cells (Figure 12B). By comparison a similar reporter with mutations in the uORF1 that block preferential translation of *ATF4* led to low levels of luciferase expression independent of stress. Finally, mutations in both uORF1 and uORF2 in the 5′-leader of the *ATF4* mRNA remove the underlying translation control in response to eIF2α~P (75). In this case, there were high levels of luciferase activity in response to the stress and non-stressed conditions, consistent with the idea that the luciferase was subject to the constitutive transcription from the thymidine kinase promoter. These results indicate that if *ATF4* mRNA is present in response to UV-C irradiation that the transcript is subject to preferential translation.

Our results indicate that *ATF4* is transcriptionally regulated in response to UV-C and ER stress, suggesting that there is a transcriptional repressor(s) and activator(s) that

contribute to *ATF4* expression. To address the nature of these transcriptional regulators, we stressed the MEF cells with thapsigargin or UV-C irradiation in the presence or absence of cycloheximide. We reasoned that if the transcriptional regulators were present before stress and were subject to allosteric regulation or signaling that cycloheximide would not affect the changes in *ATF4* mRNA levels in response to ER or UV stress. Alternatively, if the activities of the proposed transcriptional repressor(s) and activator(s) relied directly or indirectly on synthesis for their regulation in response to the stress conditions, cycloheximide would block the changes in *ATF4* mRNA in response to these stress conditions. In both stress conditions, treatment with cycloheximide significantly blocked the changes in *ATF4* mRNA levels (Figure 12C). There was a 3-fold increase in the levels of the *ATF4* transcripts after 6 hours of the thapsigargin exposure, whereas there were minimal changes in cells with the combined thapsigargin and cycloheximide treatment. Similarly, 6 hours after the UV insult there was a 3-fold decrease in the levels of *ATF4* mRNA. By contrast, this reduction was blocked in cells when cycloheximide was combined with UV-C irradiation (Figure 12C). As a control we measured *p21* mRNA levels 6 hours after UV-C irradiation, and similar to the qRT-PCR measurements in Figure 11B, there was a 5-fold increase in *p21* mRNA levels compared with no treatment. This increase in *p21* transcript levels after UV-C exposure was not significantly changed when cycloheximide was combined with UV irradiation, which is consistent with the idea that induction of *p21* transcription by genotoxic stress involves signaling events activating the p53 transcription factor (130). These results suggest that there are transcription factors that are synthesized after stress or are controlled by co-regulators expressed during stress, which are central to ATF4 transcriptional control.

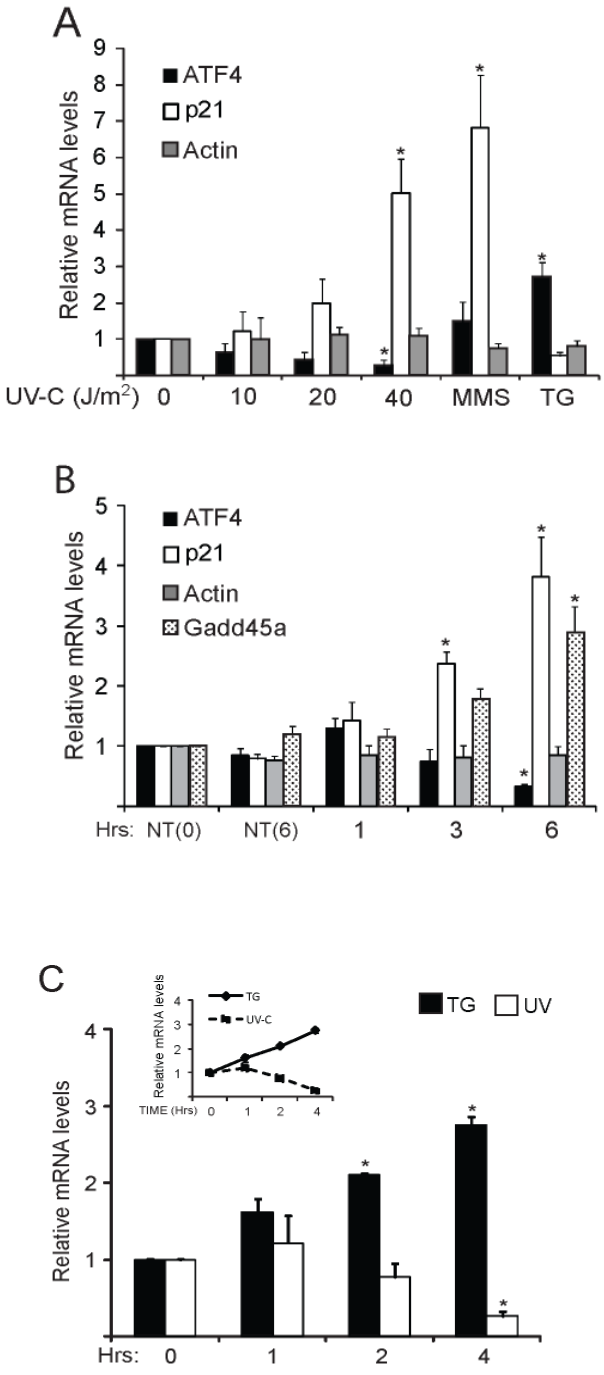

A
Relative mRNA levels
ATF4
p21
Actin
UV-C (J/m2) 0 10 20 40 MMS TG
B
Relative mRNA levels
ATF4
p21
Actin
Gadd45a
Hrs: NT(0) NT(6) 1 3 6
C
Relative mRNA levels
TG
UV
TG
UV-C
TIME (Hrs)
Hrs: 0 1 2 4

Figure 11 A-C.

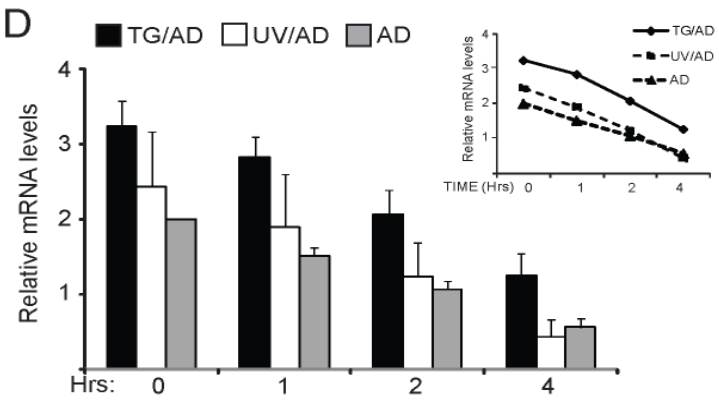


Figure 11. Levels of *ATF4* mRNA are reduced in response to UV irradiation. (A) MEF cells were treated with the indicated doses of UV-C irradiation, and subsequenly incubated in the culture medium for 6 hrs. Alternatively cells were treated with either 100 μM MMS or 1 μM thapsigargin (TG) for 6 hours. Total RNA was isolated from the samples and the levels of *ATF4*, *p21* and *β-Actin* mRNAs were measured by qRT-PCR. Values were plotted as fold change compared to the no treatment control (0). (B) MEF cells were treated with 40 J/m^2 UV-C, and then incubated in the culture medium for up to 6 hrs, as indicated. NT (0) indicates cells not treated with UV-C stress, and NT (6) indicated a mock treated cell preparation. Transcript levels were measured by qRT-PCR for *ATF4*, *p21*, *GADD45a*, and *β-Actin*, as indicated. (C) Levels of *ATF4* mRNA were measured in MEF cells were treated with 40 J/m^2 UV-C irradiation, and then incubated for in culture medium for 1, 2, or 4 hours, as indicated. Cells not subjected to stress are indicated as 0. Additionally, *ATF4* transcript levels were measured in cells that were exposed to 1 μM thapsigargin (TG) for up to 4 hours. (D) Measurement of the half-life of *ATF4* mRNA was carried out by first subjecting MEF cells to 1 μM thapsigargin (TG) for 1 hour or to 40 J/m^2 UV-C (UV), followed by 1 hour of incubation. To halt transcription, cells were treated with 20 μM of actinomycin D (UV+AD or TG+AD), and then cultured for up to 4 hours. Alternatively, cells were treated with actinomycin D alone (AD). Control experiments with MEFs treated with only 1 μM thapsigargin or 40J/m^2 UV-C (C) were also performed. *ATF4* mRNA levels were measured by qRT-PCR at the indicated times, and the panels are presented as averages ± S.D. of three independent experiments, with each measurement performed in triplicate (* $p<0.05$).

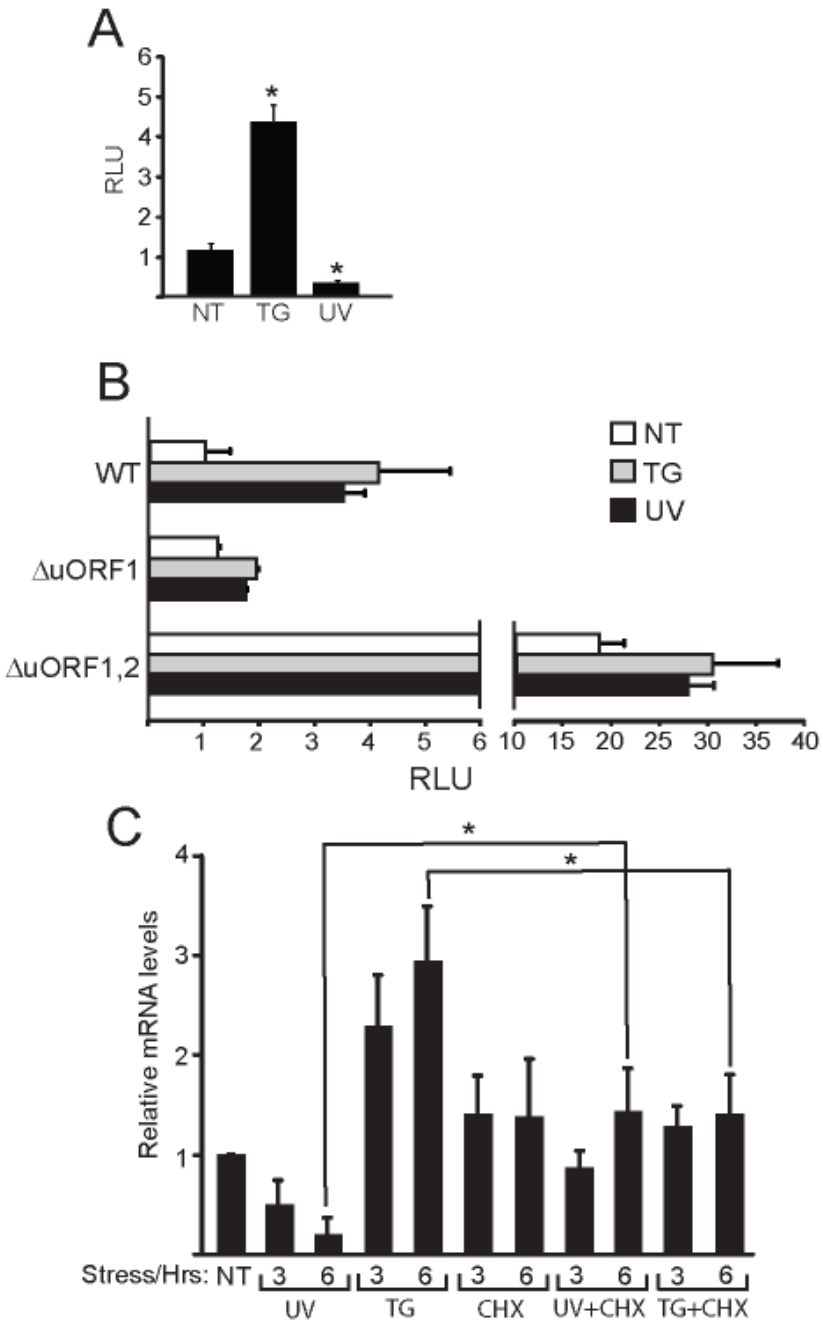

A
RLU
NT
TG
UV
B
WT
ΔuORF1
ΔuORF1,2
NT
TG
UV
RLU
C
Relative mRNA levels
Stress/Hrs: NT
UV
TG
CHX
UV+CHX
TG+CHX

Figure 12.

Figure 12. *ATF4* transcription is regulated during stress. (A) A 2.5-kb segment of the *ATF4* promoter was fused to a firefly luciferase reporter and assayed for expression in MEF cells treated with 1 μM thapsigargin (TG) for 6 hours, 40 J/m^2 UV-C, followed by culture incubation for 6 hours, or to no stress treatment (NT). The firefly luciferase activity was assayed as described in the "Materials and methods," and the relative light units (RLU) are presented relative to the non-stressed cells. (B) Wild-type and mutant versions of the 5'-leader sequences of the *ATF4* mRNA that mediates translational control was inserted between the constitutive thymidine kinase promoter and the firefly luciferase reporter gene. MEF cells were co-transfected with the P_{TK}-*ATF4-Luc* plasmid and a control Renilla luciferase plasmid. The transfected cells were treated with 1 μM thapsigargin, 40 J/m^2 UV-C or no stress agent, as indicated in the figure legend. The mutant versions of the 5'-leader of the *ATF4* transcript include a mutation in the initiation codon of uORF1 (ΔuORF1), abolishing the positive-acting element for translational control. Alternatively the mutations were present in the initiation codons for both uORFs (ΔuORF1, 2). For clarity, the histograms are represented in two different scales. (C) Protein synthesis in wild-type MEF cells were blocked by pretreatment with 50 μg of cycloheximide (CHX) for 30 minutes followed by treatment with UV (UV+CHX) or thapsigargin (TG+CHX) stress for the indicated periods of time. Control experiments were carried out by treating cells only with 40 J/m^2 UV-C (UV), thapsigargin (TG), cycloheximide (CHX), or no stress (NT). Levels of ATF4 mRNA were measured by qRT-PCR. Panels A, B, and C illustrate experimental averages ± S.D. from three independent experiments (* $p<0.05$).

1.6 eIF2α~P is important for cell survival in response to UV-C

We next addressed the role of eIF2α~P in the resistance to UV-C irradiation and the functional consequences of the absence of induced *ATF4* expression. The *A/A* MEF cells expressing the non-phosphorylated version of eIF2α (S51A) showed lowered cell survival in response to UV irradiation. There was a decrease in the number of surviving *A/A* cells as judged by the MTT assay, with a 2.5-fold reduction 24 hours after exposure to 40 J/m^2 UV-C (Figure 13A). By comparison, wild-type MEF cells showed only a modest 20% reduction in cell count after the UV insult. Long term clonogenic survival assays also showed over a 3-fold decrease in surviving *A/A* cells after UV irradiation, whereas the wild-type did not show significant reductions (Figure 13B). Finally, *A/A* cells showed measureable cleavage of PARP and caspase-3, markers of apoptosis, after 8hoursof exposure to UV-C (Figure 13C). These studies indicate that eIF2α~P significantly contributes to cell survival after UV irradiation.

We wished to determine whether expression of *ATF4*, and its downstream target *CHOP*, have negative consequences on cell survival following UV stress. To address this question, we used a derivative of the drug salubrinal, a selective inhibitor of eIF2α~P dephosphorylation (131), to induce ATF4 and CHOP along with eIF2α~P without adding any cellular stress. This drug has been used to precondition cells in response to oxidizing stress, providing for a heightened ISR gene expression that provides for increased resistance to stress conditions (132). Wild-type MEF cells were pretreated with salubrinal-003, a derivative of salubrinal that is more potent and soluble, for 6 hours, which is sufficient for inducing fivefold levels of eIF2α~P and its downstream targets *ATF4* and *CHOP*. After this pretreatment, salubrinal was removed from the media, and

the cells were then exposed to 40 J/m^2 UV- C. We found that the combined salubrinal and UV-irradiation significantly reduced survival of the wild-type cells as judged by the clonogenic assay (Figure 14A). Salubrinal alone did not have any negative consequence on wild-type cells, although *A/A* cells showed a partial decrease in cell survival, suggesting that this drug may have consequences beyond eIF2α~P. Importantly, deletion of either *ATF4* or *CHOP* restored cell survival in response to the combined salubrinal and UV treatment (Figure 14A). These results indicate that although eIF2α~P contributes to resistance to UV-C irradiation, activation of ATF4 and the downstream CHOP is detrimental to survival.

We also carried out immunoblot analyses of the wild-type, *ATF4*$^{-/-}$, and *CHOP*$^{-/-}$ cells 3 hours and 24 hours after the UV irradiation. Early in the UV stress response, there were measurable increases in ATF4 and CHOP protein in the wild-type cells following the combined salubrinal and UV treatments, as compared to UV-C irradiation alone, which yielded induced eIF2α~P but no detectable ATF4 and CHOP (Figure 14B). As expected, *ATF4*$^{-/-}$ cells displayed no expression of ATF4, or its target CHOP, early in the salubrinal and UV treatment regimen. *CHOP*-deficient cells displayed elevated ATF4 protein, but no CHOP. After an extended 24 hr period following the salubrinal and UV treatment, there was measureable cleavage of PARP and caspase 3 in the wild-type cells, supporting a role for apoptosis in the reduced cell death (Figure 14C). These apoptotic markers were not detectable in the *ATF4*$^{-/-}$ and *CHOP*$^{-/-}$ cells subjected to salubrinal and UV irradiation. It is noted that *CHOP* expression is robust in the wild-type cells 24 hours after the treatment with salubrinal and UV, or with salubrinal alone. This suggests that *CHOP* expression alone is not sufficient to trigger cell death, but rather the timing and

duration of CHOP expression may be critical for the sensitivity of cells to UV irradiation. Furthermore, *ATF4*$^{-/-}$ cells expressed measureable CHOP levels 24 hours after treatment with salubrinal alone or the combined salubrinal and UV irradiation. This indicates that during extended stress conditions, *CHOP* can be expressed independent of ATF4.

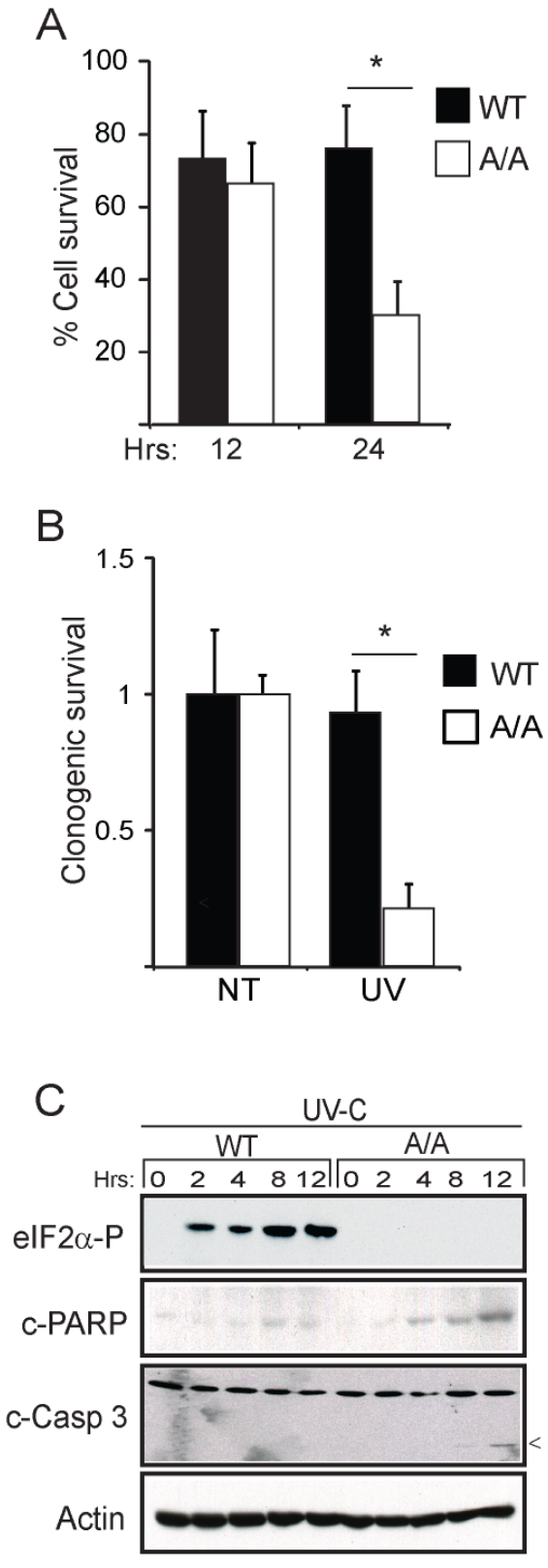

A
100
80
60
40
20
% Cell survival
*
WT
A/A
Hrs: 12 24
B
1.5
1
0.5
Clonogenic survival
*
WT
A/A
NT
UV
C
UV-C
WT
A/A
Hrs: 0 2 4 8 12 0 2 4 8 12
eIF2α-P
c-PARP
c-Casp 3
Actin

Figure 13. Phosphorylation of eIF2α provides for resistance to UV irradiation. (A) Wild-type (WT) and *A/A* (eIF2α-S51A) MEF cells treated with 40 J/m^2 UV-C irradiation, and cultured for 12 or 24 hours. The number of viable cells were then determined by the MTT assay. (B) The percentage of surviving cells following exposure to 40 J/m^2 UV-C was determined by the clonogenic survival assay. NT indicates cell not treated with UV stress. The results in panels A and B corresponds to the mean ± S.D. derived from three independent experiments and is normalized to the no treatment control. (C) Cells were subjected to the UV-C stress, cultured for up to 12 hours, as indicated, and phosphorylated eIF2α, β-actin, and apoptotic markers- cleaved caspase 3 and PARP were measured by immunoblot. "0" represents lysates not subjected to the UV stress.

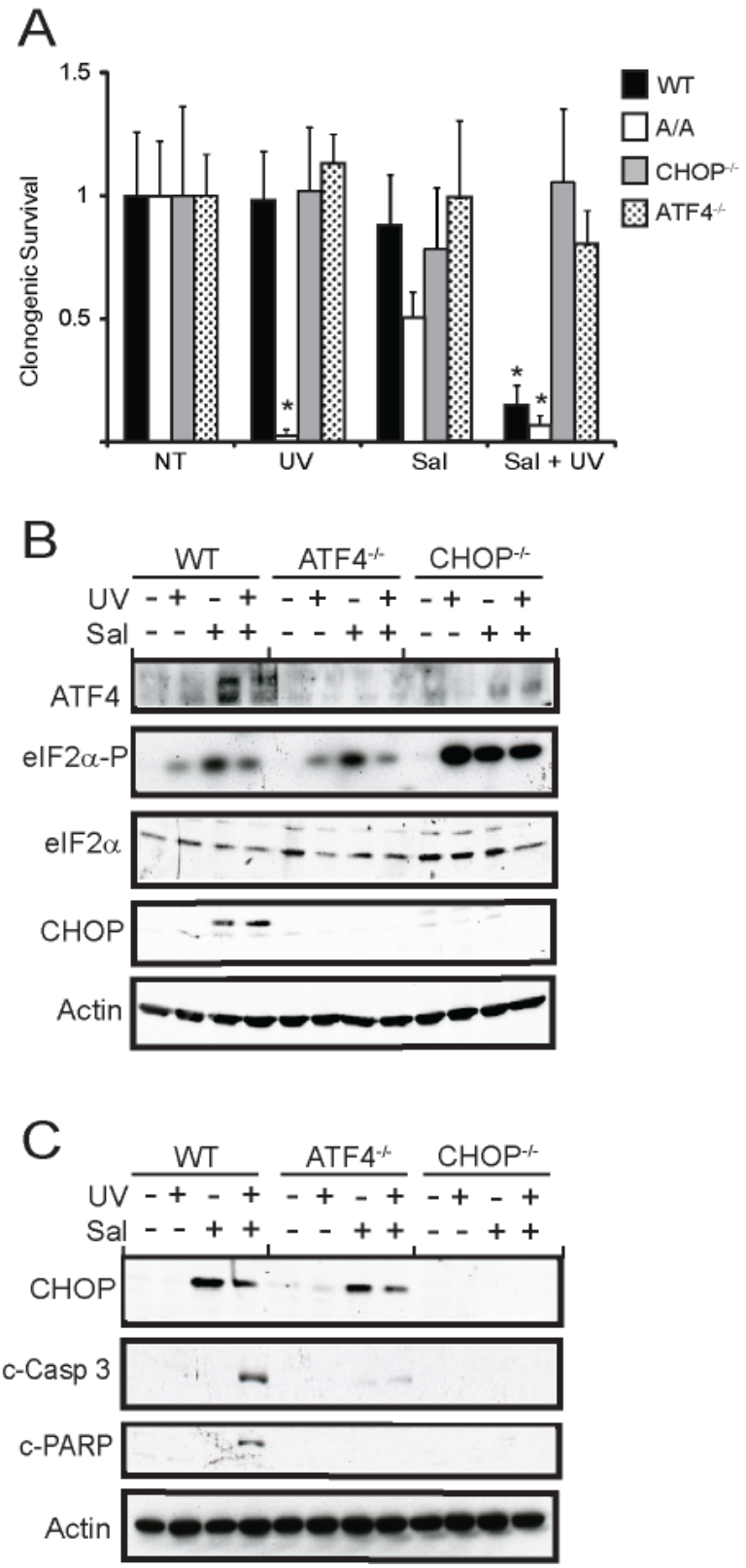

A
1.5
1
0.5
Clonogenic Survival
WT
A/A
CHOP-/-
ATF4-/-
NT
UV
Sal
Sal + UV
B
WT
ATF4-/-
CHOP-/-
UV - + - + - + - + - + - +
Sal - - + + - - + + - - + +
ATF4
eIF2α-P
eIF2α
CHOP
Actin
C
WT
ATF4-/-
CHOP-/-
UV - + - + - + - + - + - +
Sal - - + + - - + + - - + +
CHOP
c-Casp 3
c-PARP
Actin

Figure 14. Expression of *ATF4* and *CHOP* elicited by pretreatment with salubrinal reduces viability of cells during UV stress. (A) Wild-type (WT), *A/A* (eIF2α-S51A), *ATF4*$^{-/-}$, and *CHOP*$^{-/-}$ MEF cells were treated with 10 μM of salubrinal-003 (Sal) for 6 hrs. After pretreatment with salubrinal, the cells were washed , and then treated with 40 J/m^2 UV-C (UV) irradiation. Alternatively, cells were subjected to only the salubrinal pretreatment, UV-C irradiation, or no stress treatment (NT). Survival of the stressed wild-type and mutant MEF were measured by clonogenic assays, which are represented as the mean ± S.D. derived from three experiments. Values for each are normalized to the no treatment controls. (B) Induction of the ISR in the wild-type, *ATF4*$^{-/-}$, and *CHOP*$^{-/-}$ MEF cells after 3 hours of the stress arrangements was validated by immunblot analysis by using antibodies specific for phosphorylated eIF2α, total eIF2α, ATF4, CHOP and β-Actin. (C) CHOP, β-actin, and apoptotic markers- cleaved caspase 3 and PARP were measured by immunoblot in the wild-type, *ATF4*$^{-/-}$ and *CHOP*$^{-/-}$ following 24 hours of the stress regimen.

2. Transcriptional repression of *ATF4* by C/EBPβ

2.1 *ATF4* expression is significantly reduced in response to UV irradiation despite robust eIF2α~P

Low *ATF4* expression following UV-C irradiation has been attributed to its transcriptional repression, which diminishes the levels of *ATF4* mRNA available for preferential translation during eIF2α~P (99). These central ideas are illustrated in wild-type MEF cells treated with 40 J/m^2 of UV-C irradiation, or with 1μM thapsigargin, an inducer of ER stress that enhances both the transcriptional and translational expression of *ATF4* (99). *ATF4* mRNA levels as measured by qRT-PCR were lowered about 3-fold 6 hours following UV irradiation, while ER stress enhanced *ATF4* transcript levels by over 2-fold compared to the non-treated cells (Figure 15A). When UV-C irradiation was combined with thapsigargin treatment there was no induction of *ATF4* mRNA levels, in fact following 6 hours of this combined stress regimen there was a significant reduction in *ATF4* transcripts. This expression pattern of ATF4 indicates that the repressing effects of UV stress are dominant during the progression of the stress response.

We also measured expression of ATF4 protein by immunoblot analysis in response to UV and ER stress. While both stress treatments increase eIF2α~P, only thapsigargin treatment increased ATF4 protein levels, as well as its downstream target gene product CHOP (Figure 15B). Combined treatment of the MEF cells with UV-C and thapsigargin led to induced eIF2α~P, but by 6 hours of the stress regimen there was minimal ATF4 protein expression and some reduction in CHOP levels, as compared to thapsigargin alone (Figure 15B). It is noted that at 3 hours of the combined stress treatment there was some expression of ATF4 protein. At this early time point, *ATF4*

mRNA levels were not yet lowered, suggesting some availability of transcript for preferential translation. These protein measurements support the idea that although UV irradiation induces eIF2α~P, this stress condition serves to repress *ATF4* expression.

The changes in *ATF4* mRNA levels are suggested to be the consequence of transcriptional regulation as wild-type MEF cells containing the luciferase reporter P_{ATF4}-*Luc*, which includes 2.5-kb of the *ATF4* promoter, showed over a 2-fold increase in activity during ER stress (Figure 15C). By comparison, UV-C irradiation reduced the P_{ATF4}-*Luc* activity to less than 50% of the non-treated cells (Figure 15C). Taken together, these results show that *ATF4* mRNA is transcriptionally repressed in response to UV irradiation, leading to low levels of ATF4 protein despite enhanced eIF2α~P.

2.2 The *ATF4* promoter contains elements responsible for transcriptional repression

To determine the underlying mechanism for repression of the *ATF4* promoter in response to UV irradiation, we processively deleted 500-bp segments from the 5'-end of the 2.5-kb of *ATF4* promoter included in the P_{ATF4}-*Luc* plasmid (Figure 16A). The resulting reporter plasmids were transfected into wild-type MEF cells and were treated with 40 J/m^2 of UV-C. A 2-fold repression of luciferase activity was seen in cells containing plasmids deleted for the promoter sequences from -2.5 to -1-kb, although there were some significant increases in the P_{ATF4}-*Luc* expression in the non-treated cells with some of the deletion constructs (Figure 16A). Importantly, deletion of the segment from -1 to -0.5 -kb relieved the repression of P_{ATF4}-*Luc* expression, with no change in luciferase activity following UV treatment, suggesting that this portion of the *ATF4* promoter facilitated transcription repression.

In parallel to the processive 5'-deletions of the *ATF4* promoter, we also carried out internal 500-bp deletions in the 2.5-kb promoter of the P_{ATF4}-*Luc* reporter (Figure 16B). Whereas deletions from -2 to -1.5 -kb and -1.5 to -1.0 -kb retained repression of the luciferase reporter, removal of -1 to -0.5 -kb changed the UV stress from a repressing signal to one that activates, with almost a 4-fold increase in luciferase activity upon UV-C irradiation (Figure 16B). The internal deletion from -0.5-kb to -1bp removed core promoter sequences, leading to minimal P_{ATF4}-*Luc* expression in both UV irradiated and non-treated cells.

The *ATF4* promoter sequences from -1.0 to -0.5 -kb are suggested to be central for repression of *ATF4* in response to UV irradiation. Interestingly, included within this promoter segment are two predicted C/EBPβ-binding elements situated at -950 to -935 (TAAATAGCAATCAAT) and from -874 to -859 (TTGCAAATAATCACT) that reside in this portion of the *ATF4* promoter. We constructed smaller internal deletions that removed either predicted C/EBPβ-binding sequence. A deletion from -1,000 to -875 -bp led to over a 2-fold increase in P_{ATF4}-*Luc* expression in response to UV irradiation, which recapitulated the key findings from the larger -1.0 to -0.5 -kb internal deletion (Figure 16B). Deletion of the -875 to -789 -bp region triggered high basal luciferase expression, which was retained even with UV stress. These results suggest that there are key regulatory elements in the *ATF4* promoter, from approximately -1,000 to -789 -bp, which facilitate repression during in response to UV irradiation.

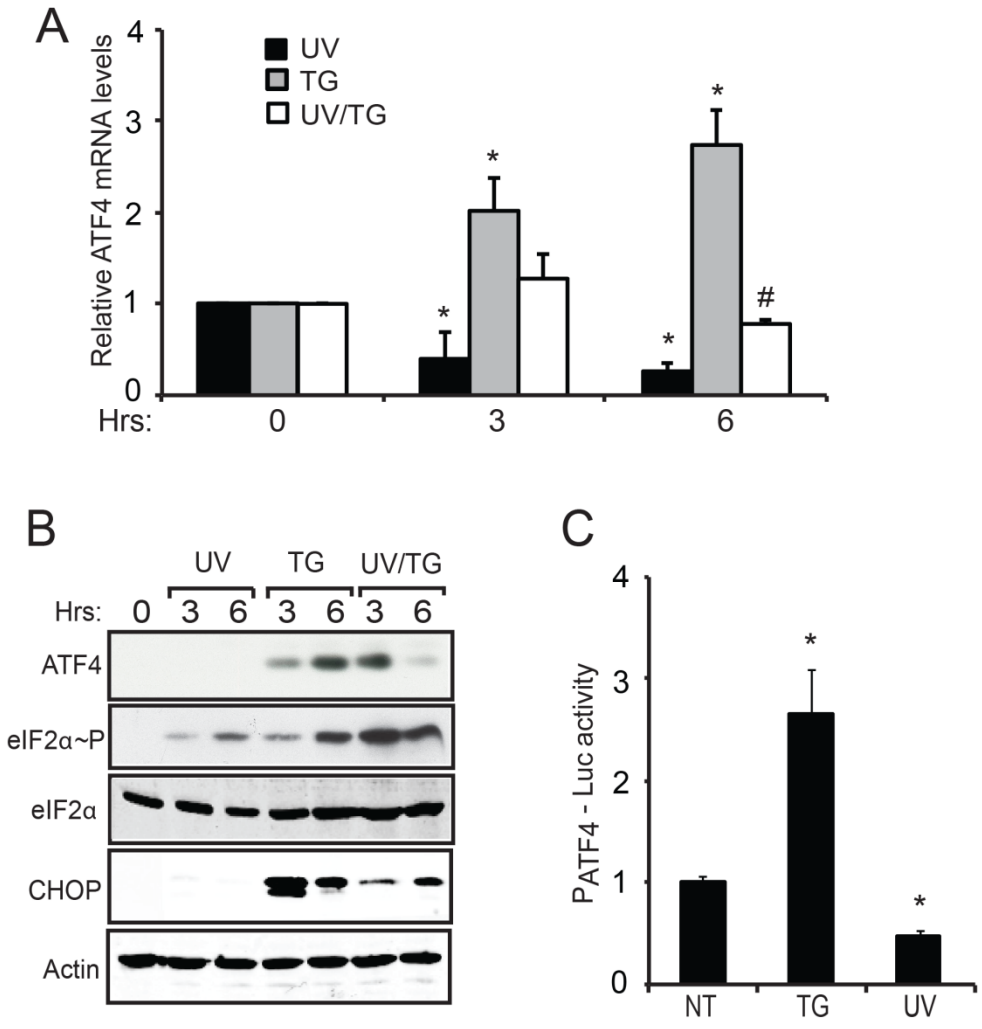

A
Relative ATF4 mRNA levels
UV
TG
UV/TG
Hrs:
0
3
6
B
UV
TG
UV/TG
Hrs: 0 3 6 3 6 3 6
ATF4
eIF2α~P
eIF2α
CHOP
Actin
C
PATF4 - Luc activity
NT
TG
UV

Figure 15.

Figure 15. Expression of *ATF4* is blocked during UV irradiation despite increased eIF2α~P. (A) Wild-type MEF cells were treated either with 40 J/m^2 of UV-C (UV) or 1 μM thapsigargin (TG) and were cultured for 3 hours or 6 hours, as indicated. Alternatively cells were treated with UV-C irradiation for an hour, followed by thapsigargin (UV/TG) for indicated time. The 0 hours represents no stress treatment. Total mRNA was then isolated from the cells and the levels of *ATF4* mRNA were measured by qRT-PCR. Values obtained are fold change compared to the no treatment control. Each experiment was performed three independent times, with per error bars representing the S.D. The "*" indicates significance with $p<0.05$ compared to non-treated control. The "#" symbol indicates a significant difference between the UV and ER stress treatments after 6 hours. (B) Protein lysates were prepared from wild-type MEF cells treated with the conditions as indicated for (A). Levels of ATF4, eIF2α~P, total eIF2α, CHOP and β-actin were measured by immunoblot analysis using antibodies specific to the indicated proteins. (C) The P_{ATF4}-Luc reporter plasmid containing 2.5-kb of the *ATF4* promoter was transfected into the wild-type MEF cells along with a control *Renilla* luciferase plasmid. The transfected cells were treated with 1 μM thapsigargin, 40 J/m^2 of UV-C, or no not treated (NT) as indicated. Firefly luciferase activity was measured as described in the "Materials and methods" and the luciferase activity relative to the NT preparation is presented in the histogram, along with the S.D.

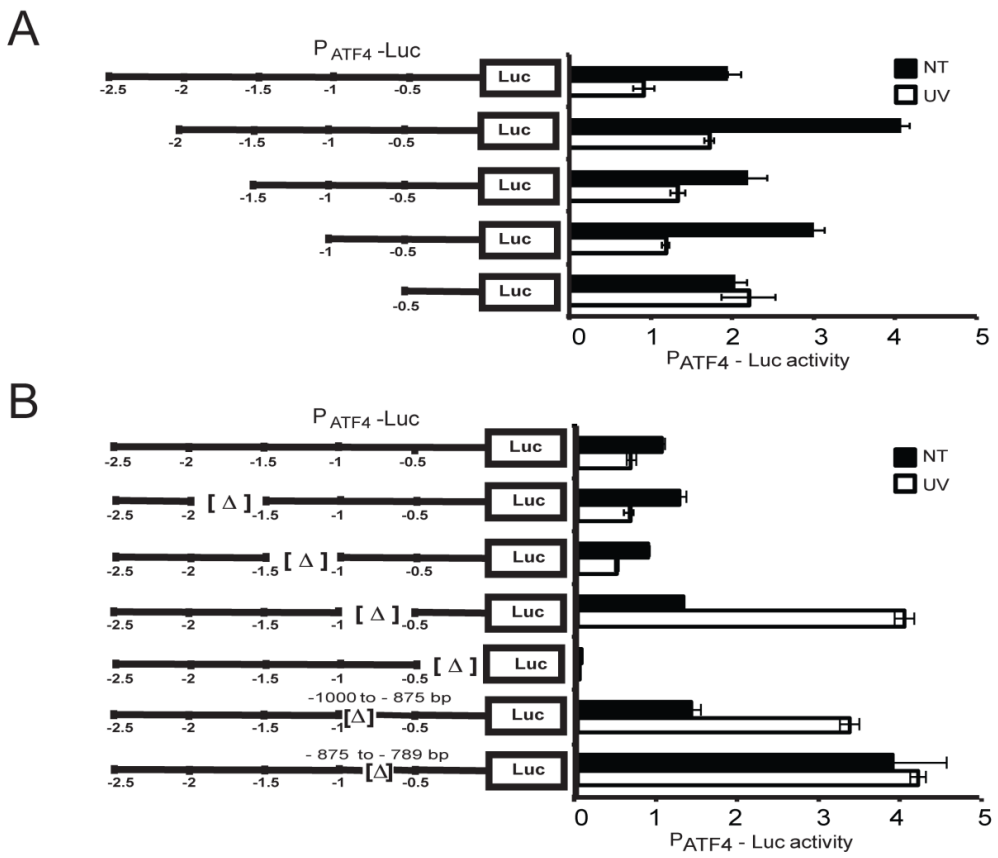


Figure 16. The *ATF4* promoter contains critical elements for repression in response to UV irradiation. (A) The *P_{ATF4}-Luc* reporter plasmid, containing 2.5-kb of the *ATF4* promoter, was transfected into wild-type MEF cells, and following UV irradiation (UV) or no treatment (NT), luciferase activity was measured. In parallel, 0.5-kb segments were sequentially deleted from the 5'-end of the *ATF4* promoter in the *P_{ATF4}-Luc* reporter and analyzed for activity in the wild-type cells treated with UV-C or no treatment. P_{ATF4}-Luc activity is presented, along with the S.D. (B) Internal 0.5-kb deletions were also constructed in the *P_{ATF4}-Luc* reporter, and transfected into wild-type MEF cells, followed by exposure to UV-C irradiation, or no treatment. Furthermore, smaller deletion constructs within the -1 to -0.5 -kb region of the *ATF4* promoter and assayed in wild-type cells the presence or absence of UV stress.

2.3 C/EBPβ represses the *ATF4* promoter

Our promoter deletion analyses suggested that C/EBPβ contributes to transcriptional repression of *ATF4*. To test this idea, we treated *C/EBPβ*$^{-/-}$ MEF cells, and its wild-type counterpart, with UV-C irradiation or thapsigargin and measured the *ATF4* transcript and protein levels. The *C/EBPβ*$^{-/-}$ MEF cells showed a 4-fold increase in *ATF4* mRNA levels 6 hours following 40J/m^2 of UV treatment compared to non-treated cells (Figure 17A). By comparison, wild-type and *CHOP*$^{-/-}$ MEF cells showed reduced amounts of *ATF4* transcript in response to UV irradiation. Each of these cell lines had increased *ATF4* mRNA levels in response to ER stress; in fact the *C/EBPβ*$^{-/-}$ cells showed almost a 7-fold enhancement of *ATF4* mRNA upon thapsigargin treatment, which was modestly, albeit significantly, higher than wild-type cells (Figure 17A). Previously, we noted that the repressing effects of UV irradiation were dominant in combination stress treatments in wild-type cells (Figure 15A and B). However in *C/EBPβ*$^{-/-}$ cells, the combination of UV and thapsigargin treatments increased *ATF4* mRNA to levels similar to that measured for ER stress alone (Figure 17B).

Immunoblot analyses of ATF4 protein from lysates prepared from *C/EBPβ*$^{-/-}$ cells treated with UV-C irradiation also showed increased ATF4 levels, whereas ATF4 protein was absent in the similarly stressed wild-type cells (Figure 17C). During ER stress, increased ATF4 protein was observed in both wild-type and *C/EBPβ*$^{-/-}$ cells. As expected, neither LIP nor LAP forms of C/EBPβ were present in the *C/EBPβ* deleted cells. Levels of CHOP protein were significantly diminished in the *C/EBPβ*$^{-/-}$ cells during ER stress compared to wild-type, and absent in the mutant cells during UV irradiation (Figure 17C). It was reported that C/EBPβ can dimerize with CHOP,

allowing for increased stability and nuclear targeting of CHOP (87). Therefore, C/EBPβ can regulate multiple steps in the ISR, including both regulation of *ATF4* transcription and the function of its downstream effector. This experiment is consistent with our measurements of *ATF4* mRNA, indicating that C/EBPβ serves to repress *ATF4* expression.

To determine if C/EBPβ directly binds *in vivo* to the regulatory elements in the *ATF4* promoter, we carried out ChIP analysis using antibody that specifically recognizes both LIP and LAP isoforms of C/EBPβ. We considered three sites in the *ATF4* promoter, designated P1 that includes the two predicted C/EBPβ binding sites (-978 to -800), along with two flanking regions designated P2 (-628 to -470) and P3 (-334 to -194) (Figure 18A). There was specific C/EBPβ binding at the P1 segment, which was absent using control antibody (Figure 18B). The C/EBPβ association at the P1 portion of the *ATF4* promoter was enhanced in MEF cells following UV irradiation when *ATF4* mRNA levels are low, and there was decreased binding of C/EBPβ to the P1 segment during ER stress, when *ATF4* transcript levels are elevated. As expected, no significant C/EBPβ binding to the P1 segment of the *ATF4* promoter was found in the *C/EBPβ*$^{-/-}$ cells (Figure 18C). Additionally, C/EBPβ was not associated with the P2 or P3 portions of the *ATF4* promoter in the ChIP experiments using either C/EBPβ-specific or control antibodies. As an additional control, histone H3 Lys4-specific antibody was observed to bind equally to a portion of the *RPL30* gene in either wild-type or *C/EBPβ*$^{-/-}$ cells independent of stress arrangements (Figure 18B and C). These results support the model that C/EBPβ binds to a specific segment of the *ATF4* promoter following UV stress, resulting in repression of transcription.

2.4 Expression of the *C/EBPβ* isoforms is differentially regulated in response to UV and ER stress

Measurements of *C/EBPβ* mRNA levels in response to either UV or ER stress showed increase in transcript levels 2 hours after the initiation of the stress, with UV irradiation eliciting the large enhancement in *C/EBPβ* expression (Figure 19A). Interestingly, measurements of the turnover of *C/EBPβ* mRNA after treatment of the wild-type MEF cells with actinomycin D, a potent inhibitor of RNA polymerase II, prior to the stress exposure indicated that UV increased the half-life of the *C/EBPβ* transcripts to greater than 10 hours (Figure 19B and C). By comparison, the half-life of *C/EBPβ* mRNA was ~2 hours in the cells treated with thapsigargin or no stress agent. Together, these studies suggest that UV and ER stresses differentially affect the expression the *C/EBPβ*.

We next measured C/EBPβ proteins, which can exist as three isoforms: LAP and LAP*, which include an extended amino terminus, and LIP, which is devoid of this transcriptional activation region and has been observed to inhibit transcription (78-80, 133). To determine whether any significant change occurs in the expression of the C/EBPβ isoforms following thapsigargin treatment or UV irradiation, we treated wild-type and *C/EBPβ*$^{-/-}$ MEF cells with these stress arrangements for up to 24 hours. ER stress resulted in a sharp decrease in LIP expression following 3 and 6 hours of thapsigargin exposure, in an agreement with an earlier report (Figure 20A) (126). With longer ER stress, at 8 hours of the treatment regimen, the LIP levels returned to that measured for the non-stressed cells (Figure 20A and C). By contrast, following UV irradiation the amount of LIP remained largely unchanged, with a modest increase

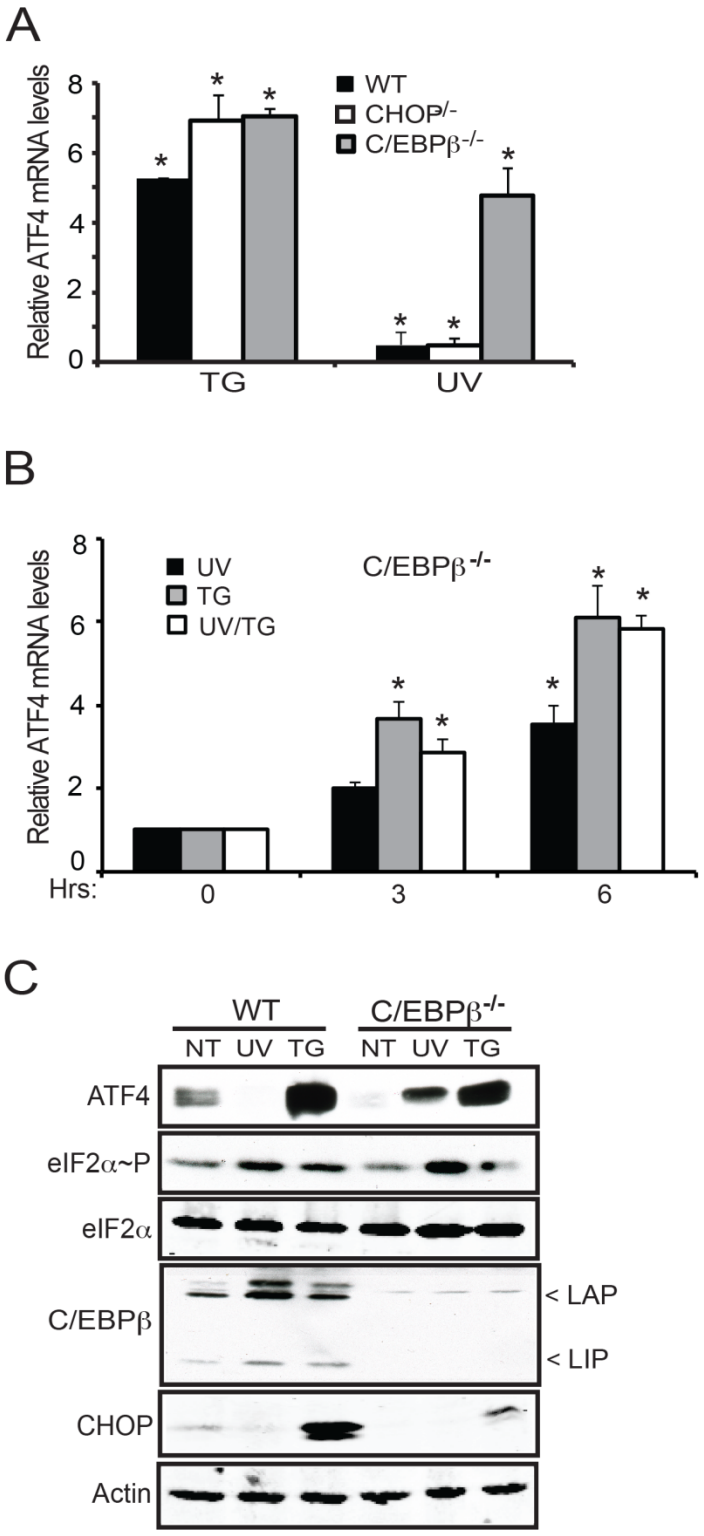

A
Relative ATF4 mRNA levels
8
6
4
2
0
WT
CHOP-/-
C/EBPβ-/-
TG
UV
B
Relative ATF4 mRNA levels
UV
TG
UV/TG
C/EBPβ-/-
Hrs:
0
3
6
C
WT
C/EBPβ-/-
NT UV TG
NT UV TG
ATF4
eIF2α~P
eIF2α
C/EBPβ
< LAP
< LIP
CHOP
Actin

Figure 17. C/EBPβ is required for reduced *ATF4* mRNA in response to UV irradiation. Wild-type, *CHOP*$^{-/-}$ and *C/EBPβ*$^{-/-}$ MEF cells were treated with 1 μM thapsigargin (TG) or with 40 J/m^2 of UV-C (UV) and cultured for 6 hours. (A) The levels of *ATF4* mRNA were measured by qRT-PCR, and the fold-change in the transcript levels is represented relative to cells not treated with stress, with the S.D. indicated. (B) *C/EBPβ*$^{-/-}$ MEF cells were either treated with UV or thapsigargin, or incubated in combination (UV/TG), and cultured for 3 or 6 hours. Values are relative to the no-treatment control (0), and the S.D. is indicated. (C) Protein lysates were prepared from wild-type and *C/EBPβ*$^{-/-}$ MEF cells subject to UV irradiation, thapsigargin, or no treatment (NT), and the indicated protein levels were measured by immunoblot analysis. The LAP and LIP isoforms of C/EBPβ are indicated to the right of the panel. Results are representative of three independent experiments. "*" indicates significance, with $p < 0.05$.

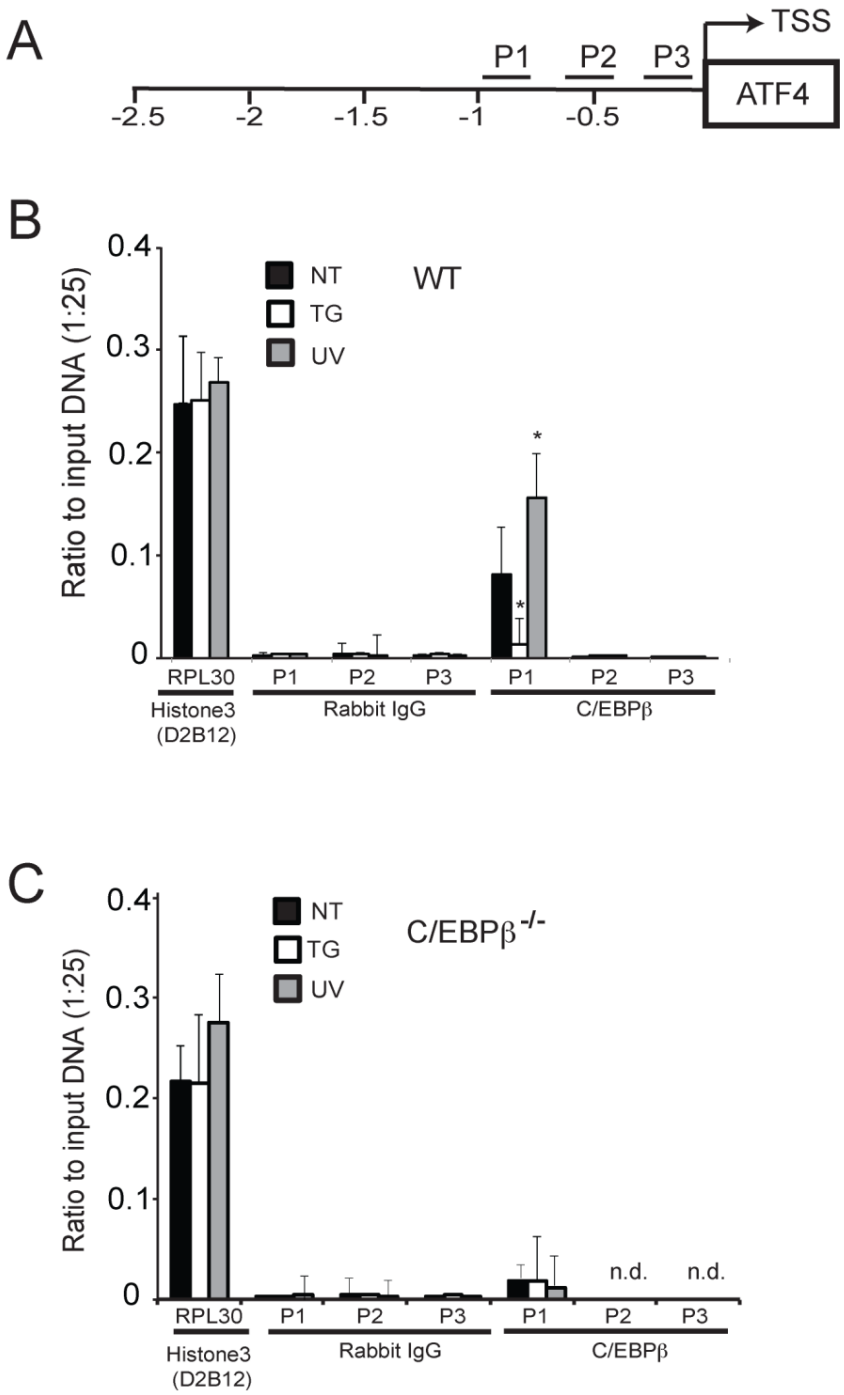

A
TSS
P1
P2
P3
ATF4
-2.5
-2
-1.5
-1
-0.5
B
WT
NT
TG
UV
Ratio to input DNA (1:25)
0.4
0.3
0.2
0.1
0
*
*
RPL30
P1
P2
P3
P1
P2
P3
Histone3
(D2B12)
Rabbit IgG
C/EBPβ
C
C/EBPβ -/-
NT
TG
UV
Ratio to input DNA (1:25)
0.4
0.3
0.2
0.1
0
n.d.
n.d.
RPL30
P1
P2
P3
P1
P2
P3
Histone3
(D2B12)
Rabbit IgG
C/EBPβ

Figure 18.

Figure 18. C/EBPβ binds to the specific elements in the *ATF4* promoter. (A) Schematic representation of the regions of *ATF4* promoter analyzed for C/EBPβ binding by ChIP. The region designated P1 includes sequences -978 to -800 bp, and P2 and P3 represent regions -628 to -470 bp and -334 to -194 bp, respectively. (B) Six hours after exposure to 40 J/m^2 UV-C (UV), 1 μM thapsigargin (TG) or no treatment (NT), wild-type (WT, B) and *C/EBPβ*$^{-/-}$ (C) cells were analyzed by ChIP analyses for C/EBPβ binding to the P1, P2, and P3 regions of the *ATF4* promoter. The immunoprecipitated DNA was analyzed by qRT-PCR with primer sets specific for each promoter region. C/EBPβ indicates that antibody specific to this transcription factor was used in the ChIP assay, while Rabbit IgG antibody was used as a control. ChIP analyses were also carried out with the positive control histone H3 Lys4 (D2B12) antibody and was analyzed by primer sets for *RPL30* as provided by the manufacturer. Data is represented as a ratio of the input sample (1:25) and is the mean and S.D. of three different experiments. "*" indicates significance, with $p < 0.05$. "n.d.' indicates that the C/EBPβ binding to the P2 and P3 was not detected in the *C/EBPβ*$^{-/-}$ cells.

following 6 hours of the stress (Figure 20B). The levels of LAP were largely unchanged throughout the 24 hour time course of thapsigargin treatment, with some increase after 6 hours of exposure to UV irradiation (Figure 20A and B). Together these results indicate that expression of LIP, a known transcriptional inhibitor, is differentially regulated during UV and ER stress. UV irradiation can enhance C/EBPβ expression by stabilizing *C/EBPβ* mRNA, and the levels of the LIP isoform is present throughout UV stress, a condition where *ATF4* transcription is repressed. By contrast, LIP levels are sharply diminished during the early ER stress, a phase where there is induction of *ATF4* transcription.

2.5 LIP is a potent repressor of *ATF4* transcription

Our studies suggested that the LIP isoform of C/EBPβ can function as a repressor of *ATF4* transcription. We addressed this idea by two experimental approaches. First, we transfected plasmids specifically expressing either the LIP or LAP isoforms into the *C/EBPβ*$^{-/-}$ MEF cells and measured the activity of the *ATF4* promoter using the P_{ATF4}-*Luc* reporter. In cells expressing only LAP (Figure 21A), there was about a 2-fold increase in P_{ATF4}-Luc activity following UV irradiation (Figures 21B). This level of induced luciferase activity was similar to that measured in the *C/EBPβ*$^{-/-}$ cells transfected with the P_{ATF4}-*Luc* expression vector alone. Expression of P_{ATF4}-*Luc* was also increased over 2-fold upon thapsigargin treatment, independent of LAP expression (Figure 21B). By comparison, expression of LIP in the *C/EBPβ*$^{-/-}$ cells repressed the *ATF4* promoter, with a 50% reduction in luciferase activity with UV irradiation (Figure 21A and C). Interestingly, expression of LIP also blocked the activation of the *ATF4* promoter.

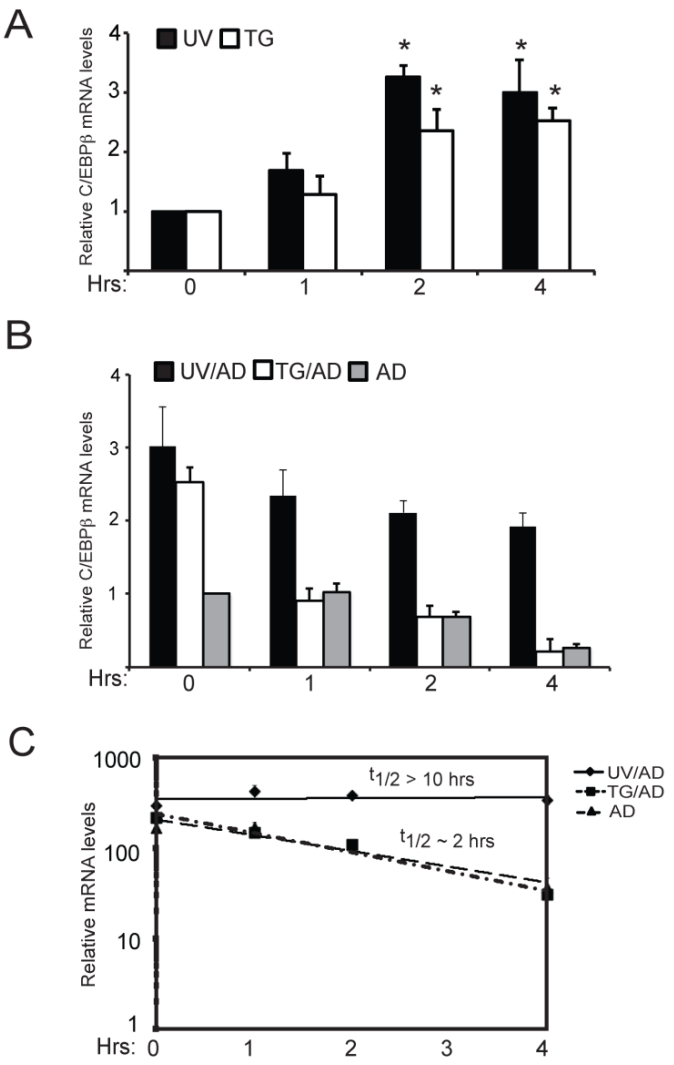


Figure 19. *C/EBPβ* mRNA is stabilized following UV treatment. (A) Wild-type MEF cells were exposed to 1 μM Thapsigargin (TG) or 40 J/m^2 of UV-C (UV) and cultured for up to 4 hours as indicated. RNA was prepared from these cells and *C/EBPβ* mRNA levels were measured by qRT-PCR. The amount of C/EBPβ transcript is presented relative to the no-treatment control (0), and the S.D. is indicated. "*" indicates significance, with $p<0.05$. (B) Measurements of *C/EBPβ* mRNA half-life were carried out by first treating cells with UV irradiation or thapsigargin. One hour after the initiation of the stress regimen, transcription was blocked by treating the cells with 20 μM actinomycin D (UV/AD or TG/AD) and cultured up to 4 additional hours. Alternatively, cells were treated with actinomycin D (AD) alone. *C/EBPβ* mRNA levels were measured by qRT-PCR at the indicated time intervals. Values are representative of the mean, along with the S.D. (C) The half-life of the *C/EBPβ* mRNA for each of the stress arrangements was determined by plotting the transcript levels versus the length of time of the actinomycin D treatment in a semi-logarithmic graph.

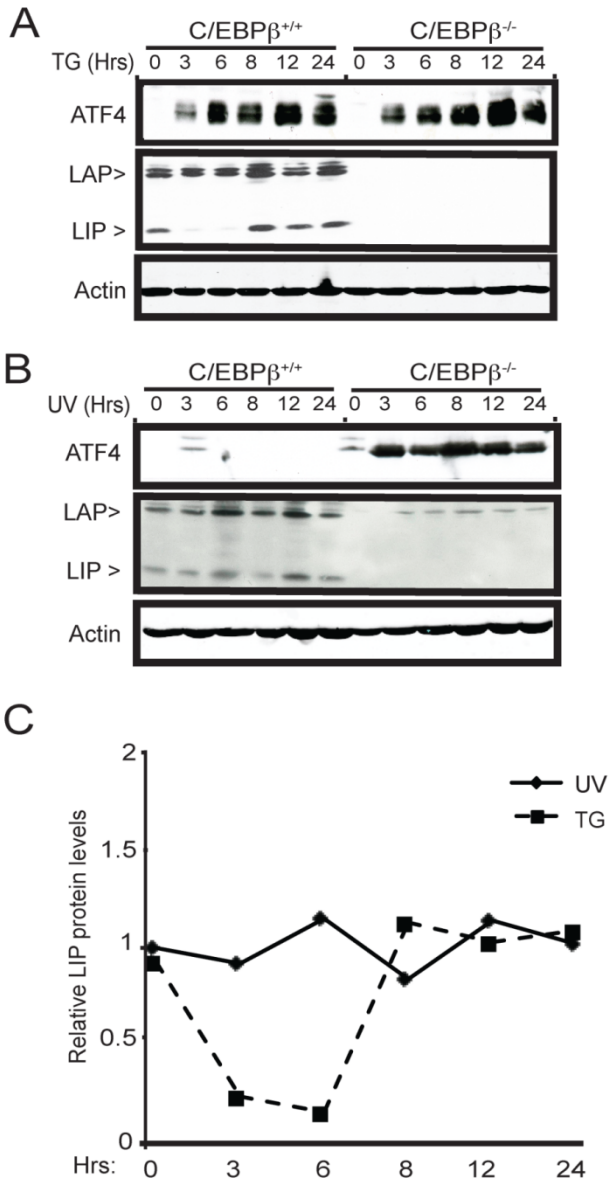


Figure 20. The LIP and LAP isoforms of C/EBPβ are differentially expressed during UV and ER stress. *CEBPβ*$^{-/-}$ MEF cells, and its wild-type counterpart, were treated with 1μM thapsigargin (TG) (Panel A) or with 40 J/m^2 of UV-C (UV) (Panel B) and were cultured for up to 24 hours, as indicated. Protein lysates were prepared from the treated cells, and the levels of ATF4, LIP, LAP, and actin were measured by immunoblot analyses. Each panel is representative of three independent experiments. (C) The levels of the LIP isoform of C/EBPβ were quantified by densitometry and represented as relative levels of the LIP band as compared to the no treatment control (0).

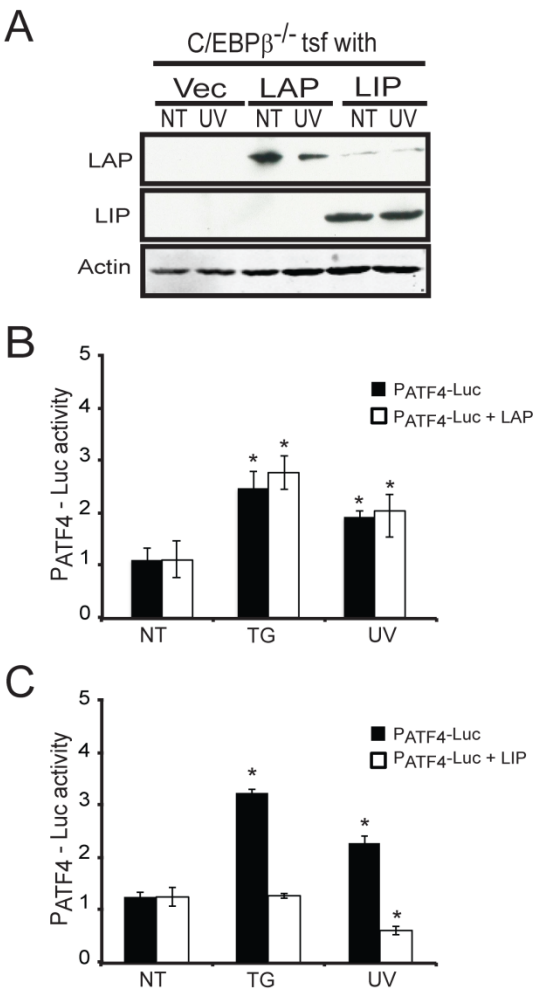


Figure 21. LIP is a potent repressor of *ATF4* transcription. (A) *C/EBPβ*$^{-/-}$ MEF cells were co-transfected with the P_{ATF4}*-Luc* reporter and plasmids specifically expressing either the LIP or LAP isoforms of C/EBPβ, or the parent vector (Vec). The transfected cells were treated with UV-C irradiation (UV) or no treatment (NT), and the levels of LAP, LIP, and β-actin were measured by immunoblot analyses. Levels of P_{ATF4}-Luc activity were also measured in the cells expressing LAP (P_{ATF4}-Luc + LAP) (B) or LIP (P_{ATF4}-Luc + LIP) (C), as compared to the cells containing P_{ATF4}-Luc and the expression vector alone (P_{ATF4}-Luc). Luciferase activity is presented in the histograms, with the luciferase activity in the non-treated wild-type cells being represented as a value of 1. Values were derived from three independent experiments, with the S.D. indicated.

in response to ER stress (Figure 21C); suggesting that our observation that absence of LIP expression in the early phase of ER stress is critical for induction of *ATF4* mRNA (Figure 20A).

Our second experimental approach to address the role of LIP in the repression of *ATF4* transcription centered on previous reports that a short uORF in the *C/EBPβ* mRNA is essential for expression of the LIP isoform (88, 89). This uORF can inhibit translation initiation of LAP and instead enhance ribosomal scanning and subsequent reinitiation at the downstream LIP (Figure 6). As a consequence MEF cells containing a mutation in the initiation codon of the uORF (*C/EBPβ-ΔuORF*) lose the ability to express LIP, resulting in only LAP and LAP* being present in the cells (89). We transfected the P_{ATF4}-*Luc* reporter into the *C/EBPβ*$^{-/-}$ and *C/EBPβ-ΔuORF* MEF cells, along with the wild-type counterpart, and measured the *ATF4* promoter activity in response to either treatment with UV irradiation or thapsigargin. In response to the UV stress, luciferase activity was increased by over 2-fold in *C/EBPβ*$^{-/-}$ and *C/EBPβ-ΔuORF* cells, whereas P_{ATF4}-*Luc* expression was sharply decreased in the wild-type cells (Figure 22A). There was significant increase though modestly difference in *ATF4* promoter activity in both mutant and wild-type MEF cells during ER stress. As LIP levels were significantly reduced following ER stress (Figure 20A), this would suggest that diminished LIP is a contributor to increased levels of ATF4 mRNA during stress, such as that afflicting the ER. The levels of *ATF4* mRNA in the *C/EBPβ-ΔuORF* MEF cells were significantly enhanced in response to UV irradiation, which was similar to that found in the *C/EBPβ*$^{-/-}$ cells (Figure 22B). By comparison, the wild-type cells showed a sharp reduction in *ATF4* transcripts following UV stress (Figure 22B). During ER stress, the *C/EBPβ*$^{-/-}$, *C/EBPβ-ΔuORF*,

and wild-type cells each showed over a 4-fold increase in *ATF4* mRNA levels.

We also measured ATF4 protein in *C/EBPβ-ΔuORF* and their wild-type isogenic MEF cells and found that there was an increase in ATF4 protein following 3 and 6 hours of the UV irradiation (Figure 22C). In the matched wild-type MEF cells, we found only low levels of *ATF4* expression after 3 hours of the UV stress. As expected, LIP was absent in the *C/EBPβ-ΔuORF* cells. Furthermore, CHOP protein was significantly diminished in *C/EBPβ–ΔuORF* cells in response to UV irradiation, whereas during ER stress there were similar levels of induced CHOP in both the wild-type and *C/EBPβ–ΔuORF* cells. As noted earlier C/EBPβ can dimerize with CHOP, enhancing its stability and transcriptional activity (87). This finding suggests that LIP, but not LAP, facilitates CHOP function.

Finally, we measured preferential translation of *ATF4* in the wild-type, *C/EBPβ*$^{-/-}$, and *C/EBPβ–ΔuORF* cells in which eIF2α~P was induced by either UV or ER stress (Figure 22D). The *ATF4* translational control was determined by transfecting into these cells a previously described plasmid encoding the 5'-leader of the *ATF4* mRNA that includes the uORFs between the constitutive *TK* promoter and the firefly luciferase reporter (75). There was a significant increase in the luciferase activity in each cell line in response to either UV irradiation or thapsigargin (Figure 22D). This indicates that C/EBPβ is not required for preferential translation of *ATF4* in response to eIF2α~P, and if the *ATF4* mRNA is available following UV irradiation, there will be high levels of synthesized ATF4 protein. Taken together these experiments demonstrate that LIP is critical for repression of *ATF4* transcription in response to UV irradiation. Furthermore, LAP is not required for activation of the *ATF4* promoter in response to ER stress.

2.6 Loss of the C/EBPβ isoform LIP increases expression of ATF4-target genes

The absence of LIP led to increased *ATF4* mRNA and protein levels in both *C/EBPβ*$^{-/-}$ and *C/EBPβ-ΔuORF* MEF cells in response to UV irradiation. We next wanted to determine if expression of key ATF4-target genes in the ISR also increased with the elevated *ATF4* expression in the LIP-deficient cells. We measured three well-characterized ISR genes: asparagine synthetase (*ASNS*) that catalyzes the conversion of aspartate to asparagine, the cationic amino acid transporter 1 (*CAT-1*), and *CHOP*, which encodes a bZIP transcription factor that can facilitate apoptosis (Figure 23) (52, 81, 134, 135). As illustrated in Figures 23A, B, and C, ATF4 was required for full induction of each of these mRNAs in response to ER stress. Next we measured *ASNS*, *CAT-1*, and *CHOP* mRNAs in the wild-type, *C/EBPβ*$^{-/-}$ and *C/EBPβ-ΔuORF* MEF cells treated with UV irradiation or thapsigargin. The levels of each of these three transcripts were not induced in either wild-type or *C/EBPβ*$^{-/-}$ cells when treated with UV, whereas *ASNS* and *CAT-1* mRNAs increased significantly by 6 hours in the *C/EBPβ-ΔuORF* cells (Figure 23D and E). Transcription of these genes was uniformly increased among these MEF cells in response to ER stress. These results suggest that increased ATF4 protein levels lead to increased expression of two of its key target genes. The fact that these genes were induced in *C/EBPβ-ΔuORF* cells, but not the *C/EBPβ*$^{-/-}$ version, is consistent with reports that LAP heterodimerizes with ATF4 and facilitates transcription of ISR promoters, such as *ASNS* (81, 82). The levels of *CHOP* mRNA did not significantly increase in the *C/EBPβ-ΔuORF* cells response to UV-C irradiation (Figure 23F). Absence of CHOP esxpression was only observed following UV-C irradiation and not following ER stress suggests that there are multiple mechanisms by which CHOP can be regulated following

various stress conditions. As will be highlighted further in the discussion section, this observation is consistent with previous reports that like *ATF4,* prior treatment with UV blocks induced *CHOP* transcription by other stress treatments that enhance eIF2α~P and *ATF4* expression.

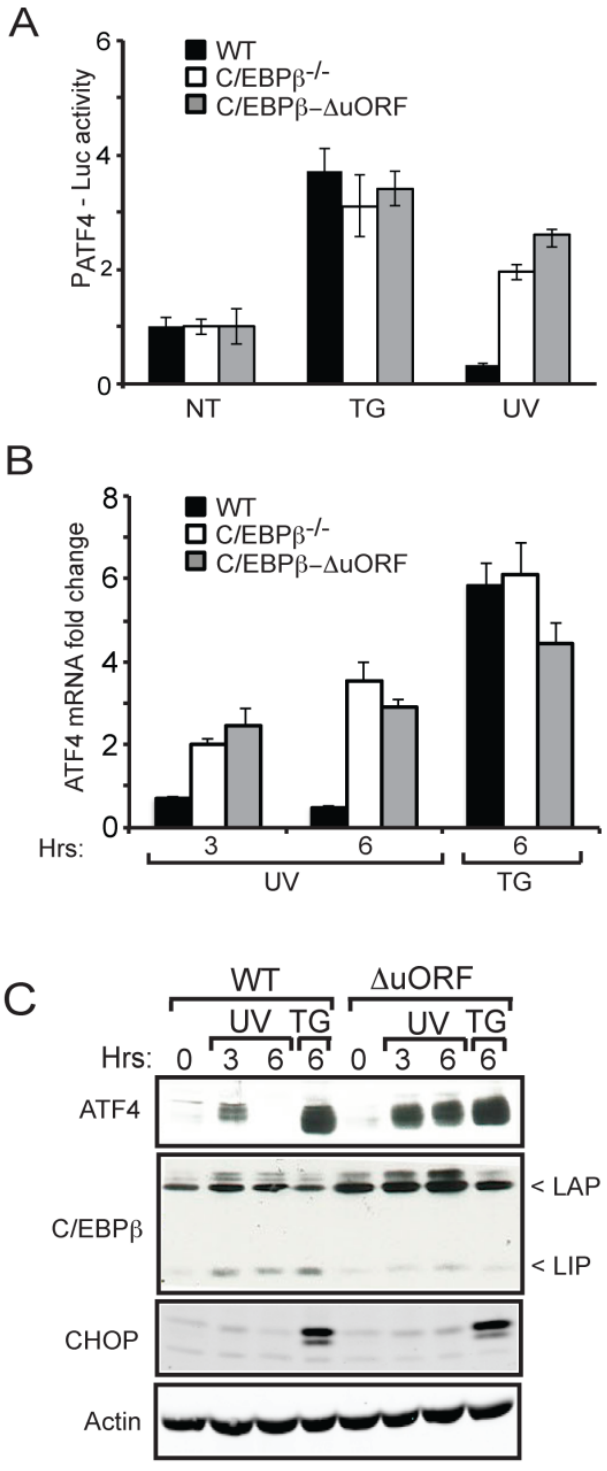

A
WT
C/EBPβ-/-
C/EBPβ–ΔuORF
PATF4 - Luc activity
0
2
4
6
NT
TG
UV
B
WT
C/EBPβ-/-
C/EBPβ–ΔuORF
ATF4 mRNA fold change
0
2
4
6
8
Hrs:
3
6
6
UV
TG
C
WT
ΔuORF
UV TG
UV TG
Hrs: 0 3 6 6 0 3 6 6
ATF4
C/EBPβ
< LAP
< LIP
CHOP
Actin

Figure 22A-D

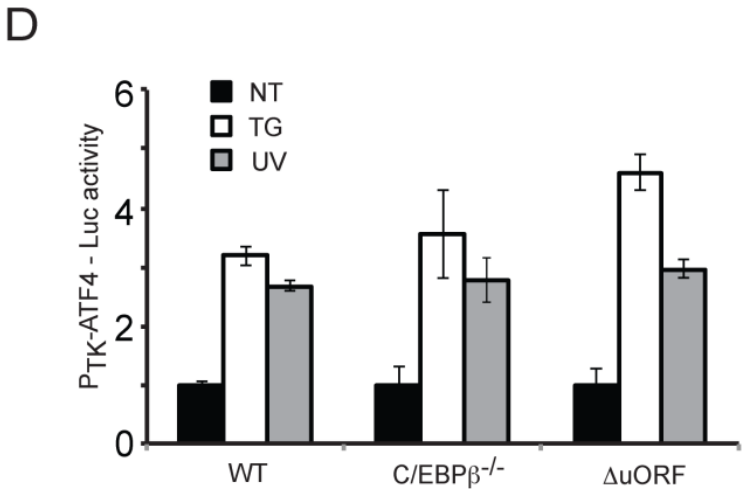


Figure 22. Loss of LIP in *C/EBPβ–ΔuORF* cells alleviates repression of *ATF4* transcription. (A) Wild-type, *C/EBPβ*$^{-/-}$ and *C/EBPβ–ΔuORF* MEF cells were transfected with the P_{ATF4}*-Luc*, and treated with either with 1 μM thapsigargin (TG), 40 J/m^2 of UV-C (UV), or no stress treatment (NT). P_{ATF4}*-Luc* expression was measured and is represented in the histogram, with the non-treated cells being represented as a value of 1. Values were determined from three independent experiments, with the S.D. as indicated. (B) The wild-type (WT), *C/EBPβ*$^{-/-}$, and *C/EBPβ–ΔuORF* cells were treated with UV or thapsigargin stress for up to 6 hours, and the levels of *ATF4* mRNA were determined qRT-PCR. Mean values are presented in the histograms, along with the S.D. indicated. (C) Alternatively the levels of the indicated proteins in the stressed wild-type (WT) and *C/EBPβ–ΔuORF* (ΔuORF) cells were measured by immunoblot analyses. The 0 time indicates no stress treatment. (D) Levels of *ATF4* translational control were measured in wild-type, *C/EBPβ*$^{-/-}$, and *C/EBPβ–ΔuORF* cells that were transfected with the P_{TK}*-ATF4-Luc* reporter. Following UV or thapsigargin treatment, luciferase activity was measured and presented in the histograms relative to the no stress treatment (NT) with a value of 1. The luciferase measurements were from three independent experiments, with the S.D. indicated.

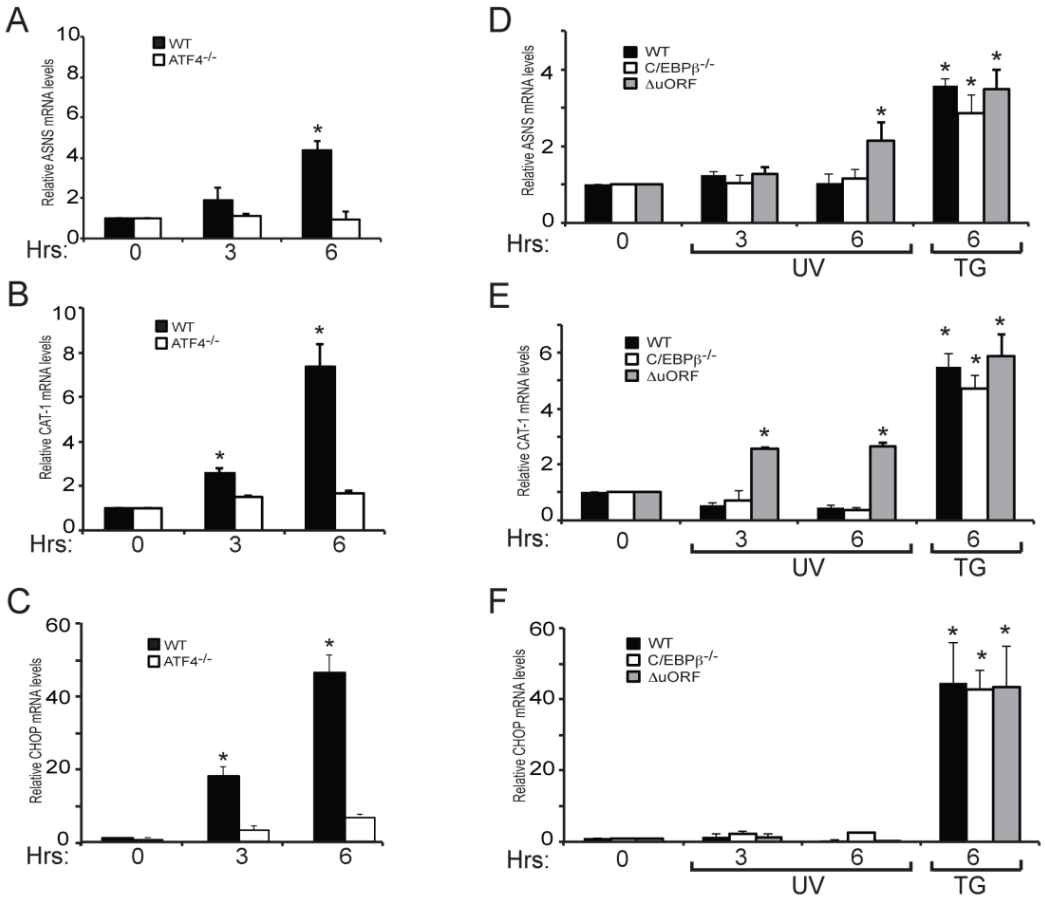


Figure 23. Alleviation of *ATF4* repression in *C/EBPβ–ΔuORF* cells causes increased expression of ATF4-target genes in response to UV irradiation. Wild-type and *ATF4*$^{-/-}$ MEF cells were treated with 1 μM Thapsigargin for up to 6 hours, and the levels of *ASNS* (A), *CAT-1* (B), and *CHOP* (C) mRNAs were measured by qRT-PCR. Wild-type, *C/EBPβ*$^{-/-}$ and *C/EBPβ–ΔuORF* cells were treated with 40 J/m^2 of UV-C (UV) or Thapsigargin (TG), and cultured for up to 6 hours, as indicated. The levels of *ASNS* (D), *CAT-1* (E), and *CHOP* (F) mRNAs were measured by qRT-PCR. Values are presented relative to the no treatment controls (0), and the S.D. for each is indicated. "*" indicates significance, with $p<0.05$.

DISCUSSION

1. ATF4 is transcriptionally repressed following UV irradiation

This study addressed the regulatory mechanisms governing the variable *ATF4* expression in response to eIF2α~P and different stress conditions. From our experimental results, we draw four central conclusions. First, eIF2α~P is induced by UV-B and UV-C irradiation in several different mammalian cell types (Figures 8 and 9), and this phosphorylation event leads to a reduction in global translation initiation (Figure 10). This finding is consistent with earlier studies that showed that UV irradiation can enhance GCN2 phosphorylation of eIF2α (35, 36).

The second conclusion is that expression of *ATF4* in response to environmental stress involves changes in mRNA levels. For example, *ATF4* mRNA levels are lowered 3-fold in response to UV stress, while ATF4 transcript levels are significantly increased during ER stress (Figure 11). *ATF4* transcriptional regulation is the key step for the changes in *ATF4* mRNA levels in response to UV and ER stress conditions (Figure 12A). *ATF4* mRNA is also subject to rapid turnover, with a half-life of about 3 hours, but this is independent of stress conditions (Figure 11D). The labile nature of *ATF4* mRNA renders it more sensitive to changes in transcriptional regulation elicited by stress conditions. For example, transcriptional repression in response to UV irradiation, coupled with the constitutive short half-life, facilitates reduced levels of *ATF4* mRNA during this stress condition. These central ideas are further supported by earlier reports that observed increased *ATF4* transcript levels in response to ER stress (5, 138, 139). Additionally, it was reported that *ATF4* mRNA levels are elevated in response to amino acid starvation (140). These indicates that ATF4 is regulated by transcriptional and translational control.

The third conclusion is that the combined transcriptional and translational control of *ATF4* provides for versatility in regulating the ISR gene expression. In response to UV irradiation, the lowered availability of the *ATF4* transcript significantly reduces translation, thus down regulating ATF4 induction in ISR. Therefore, there does not appear to be an inherent inability to elicit preferential *ATF4* mRNA translation in response to eIF2α~P and UV stress. This was illustrated by our finding that constitutive transcription of the ATF4-luciferase reporter from the thymidine kinase promoter led to preferential translation during UV irradiation (Figure 12B). Consistent with this idea, mutation of uORF1 in the 5'-leader of the ATF4 mRNA, which is required for reintiation of translating ribosomes and preferential translation, blocked expression of the luciferase reporter. During ER stress, there is elevated transcription of *ATF4*, which enhances the amount of *ATF4* transcript available for preferential translation by eIF2α~P, therefore amplifying expression of *ATF4* and the ISR. These results indicate that the combination of transcription regulation with translation control allows for genes marked for preferential translation by eIF2α~P to be selectively induced in response to a range of environmental stresses. The ISR is not constricted to a specific program of gene expression, but rather can tailor it for a given stress condition.

The fourth central conclusion is that the absence of ATF4 expression appears to be advantageous for cells during UV stress (Figure 14). Clearly eIF2α~P and reduction in translation initiation facilitates resistance to UV irradiation (Figure 13). However, the versatility of the ISR has provided for selective loss of expression of *ATF4* and its ISR target genes. Pretreatment with salubrinal, an inhibitor of eIF2α~P dephosphorylation that enhances the eIF2α~P/ATF4 pathway, typically provides for heightened resistance to

stress conditions, such as those triggered by oxidizing agents (131, 139, 141-143). However, this pretreatment regimen renders cells much more susceptible to UV irradiation (Figure14). Importantly, this UV sensitivity is alleviated by deletion of either *ATF4* or *CHOP* (Figure 14). During the ISR, elevated CHOP levels for an extended period are thought to elicit gene expression that triggers apoptosis (107, 108, 120, 144). These findings suggest that cells encountering UV stress are hypersensitive to expression of *CHOP*.

2. C/EBPβ represses *ATF4* transcription

This study provides mechanistic insight into how *ATF4* expression is repressed in response to UV irradiation despite there being induced eIF2α~P. Consistent with the first portion of this thesis (99), transcription of *ATF4* is repressed following UV irradiation, and therefore there are low levels of *ATF4* mRNA available for translation (Figure 15). This differs from environmental stresses that increase ATF4 synthesis, such as those afflicting the ER, where there is activation of *ATF4* transcription, thus further enhancing the levels of *ATF4* mRNA for preferential translation by eIF2α~P (Figure 15). Central to the repression of *ATF4* transcription is the LIP isoform of C/EBPβ (Figures 18, 21, and 22). The *ATF4* promoter contains elements that bind C/EBPβ, and this association is enhanced following UV irradiation (Figure 18). Sequential 5'-truncations, as well as internal deletions, of the *ATF4* promoter indicate that sequences situated between -1000 and -789 facilitate repression of *ATF4* transcription in response to the UV stress (Figure 16). Within this repressing region of the *ATF4* promoter are binding sites for C/EBPβ, which encodes three isoforms of the bZIP transcriptional regulator, LAP, LAP* and LIP,

produced by differential selection of initiation codons during translation (88, 89). Deletion of *C/EBPβ* also negated the inhibition of *ATF4* transcription following UV irradiation, turning this stress condition instead into an inducer of *ATF4* mRNA and protein expression (Figure 17).

The shorter version of C/EBPβ isoform LIP, contains a bZIP domain that is important for DNA binding, but is missing the N-terminal activation domain. Hence, LIP is a documented repressor of transcription (78-80, 133). Two lines of evidence support the idea that the LIP version of C/EBPβ represses the transcription of *ATF4*. First, expression of LIP, but not LAP, restored repression of *ATF4* transcription in *C/EBPβ*$^{-/-}$ cells subjected to UV irradiation (Figure 21). Second, *ATF4* transcription was not repressed upon UV irradiation in *C/EBPβ-ΔuORF* cells, which express the LAP and LAP* isoforms, but not LIP (Figure 22). In fact UV stress in the *C/EBPβ-ΔuORF* cells led to a significant increase of *ATF4* mRNA and protein levels in response to UV irradiation.

Increased *ATF4* expression in the *C/EBPβ-ΔuORF* cells subjected to UV irradiation resulted in enhanced transcription of ISR target genes, *ASNS* and *CAT-1* (Figure 23 A-C). However, it is noteworthy that there was no increase in the ATF4-target gene *CHOP* in *C/EBPβ-ΔuORF* cells following the UV stress. Previously, Schmitt-Ney and Habener (136) reported that UV irradiation is a potent repressor of *CHOP* expression, and like *ATF4,* prior treatment with UV irradiation blocks induced *CHOP* transcription by other stress treatments, such ER stress and nutrient deprivation. Central to this repression is the first exon of *CHOP*, as inclusion of this region of the *CHOP* gene into a reporter containing the *CHOP* promoter represses transcription in

response to UV irradiation (136). This would explain the absence of induced *CHOP* transcription in the *C/EBPβ-ΔuORF* cells where ATF4 is activated by UV stress, i.e. the ATF4-targeted activation of *CHOP* transcription is blocked by additional regulatory factors that can function through via exon region of *CHOP*. Furthermore, this suggests that while LIP is a potent repressor of *ATF4* in response to UV irradiation, this isoform of C/EBPβ does not directly contribute to repression of *CHOP*. In fact during ER stress, LIP can dimerize with CHOP, facilitating the stabilization and nuclear targeting of CHOP (87).

3. The combination of transcriptional and translational control allows for differential expression of ISR target genes

We propose that a combination of transcriptional regulation and translational control of ATF4 underlies the ability of the ISR to differentially express ATF4 depending on the precise stress condition, and that this process is central to cell survival (Figure 24). Although this model is specifically highlighted for *ATF4*, it would apply to other genes subject to translational control in response to eIF2α~P. Expression of genes slated for preferential translation in the ISR can be enhanced or blocked depending on its transcription status. Enhanced transcript availability would insure a greater level of preferential translation, which together would lead to higher levels of ATF4 protein and activity, as illustrated by the degree of red color in the gradient depiction in figure 24. A range of *ATF4* expression appears to be central for alleviation of different stress arrangements. In most cases, including nutrient and ER stress, ATF4 activity provides for resistance to the stress insults, while in others, such as UV irradiation, ATF4 is

suggested to be harmful. The detrimental properties of ATF4 resides at least in part on its ability to induce *CHOP*, which is suggested to elicit apoptosis through repression of the pro-survival gene *BCL2* and induction of pro-apoptotic genes, such as *BIM*, *DR5*, and those tied to autophagy (108, 112, 145). In addition to UV irradiation, lowered *ATF4* expression during heightened eIF2α~P has been reported during brain ischemia and non-alcoholic steatohepatitis (100, 101), suggesting that the dampened expression of *ATF4* in the gradient model can be applied to a number of stress arrangements.

The idea of a combination of transcriptional regulation and translational control of *ATF4* redefines some features of the ISR. ATF4 was defined as a common downstream target, which integrates signaling from different eIF2α kinases (52). In this respect the mammalian ISR builds on and elaborates upon the earlier concept of the general amino acid control in yeast. This yeast pathway features the ability of different nutritional stresses to activate GCN2 phosphorylation of eIF2α and preferential translation of GCN4, a "master regulator" of genes involves in amino acid metabolism and the salvaging and uptake of nutrients (30, 146). It is noted that UV irradiation has been reported to enhance *GCN4* translation in yeast, possibly in a GCN2-independent manner (147). Therefore, regulation of GCN4 by UV irradiation in yeast may differ from the ISR in mammalian cells.

The model featuring combined transcriptional regulation and translation control indicates that eIF2α~P in response to various stress arrangements does not lead to default activation of *ATF4*. Rather, there are additional target genes activated by eIF2α~P that play a major role in alleviating stress damage. In some cases these additional target genes may function in conjunction with ATF4, whereas in others they may function in

the absence of the ATF4 transcriptional activator. These additional target genes could include those subject to preferential translation in response to eIF2α~P through 5′-leader configurations in their mRNAs. For example, several DNA repair enzymes were reported to be subject to preferential translation by eIF2α~P after UV stress (41). These preferentially translated genes include *ERCC1*, *ERCC5*, and *DDB1*, and the uORFs in the 5′-leaders of these encoded transcripts were suggested to be important to maintain elevated expression after UV irradiation. However, in these cases eIF2α~P in the absence of UV irradiation did not appear to be sufficient for translational control, suggesting that additional signaling pathways and proteins may be involved. It is also noted that in this earlier study that UV irradiation did not enhance expression of luciferase activity expressed from a transfected vector that included the 5′-leader of *ATF4* mRNA (41). This result appears to differ from our findings in Figure 12B. We are currently uncertain as to the underlying reason for this difference, but it may reflect the different cell type (HeLa cells) or the UV-B irradiation used in the earlier study.

Repressed translation by UV irradiation also reduces the synthesis of key labile regulatory proteins, such as IκBα (36), which can relieve its repression of NF-κB. Activation of NF-κB would then enhance the transcription of diverse target genes, such as those involved in inflammation and the regulation of apoptosis. In this respect, the ISR can be viewed as a collection of eIF2α kinases that recognize various stress arrangements activating different combinations of target gene modules that can provide for stress resistance.

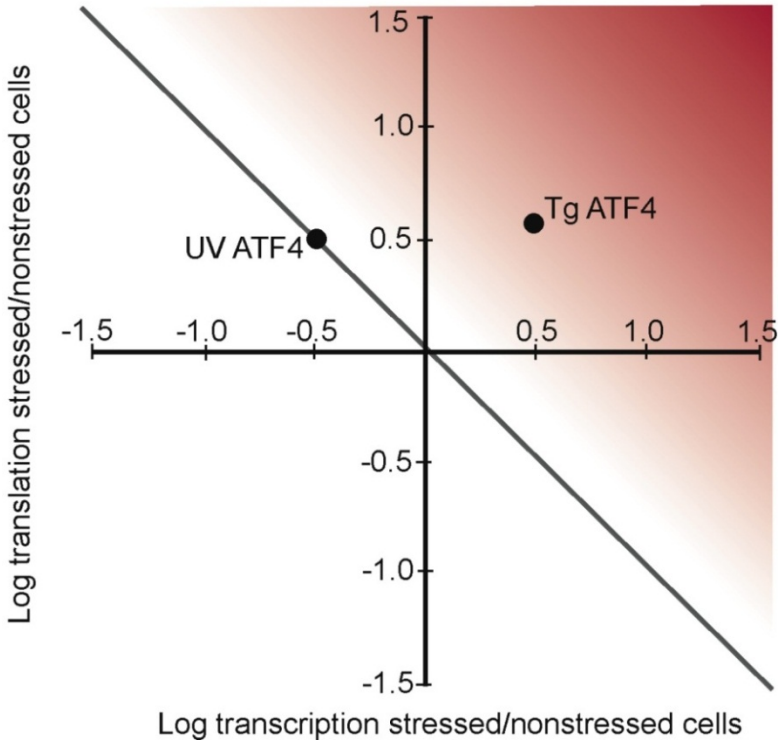


Figure 24. Model depicting proposed transcriptional and translation control of *ATF4* expression and the ISR. The y-axis represents the log of the levels of mRNA translation in stressed cells compared to non-stressed, while the x-axis represents the log of the transcription levels of a given gene in stressed cells relative to non-stressed. The diagonal line represents the levels of coupled transcription and translation that are proposed to yield no change in ATF4 protein levels. The red gradient depicts the levels of transcriptional and translational control that would enhance ATF4 protein levels and induce its target ISR genes. The solid circles represent the combined transcription and translation of *ATF4* in response to UV irradiation (UV) or ER stress elicited by Thapsigargin treatment (TG).

4. Regulation of *ATF4* transcription in response to various stress conditions

C/EBPβ contributes to cell proliferation and differentiation processes, along with cellular stress responses (78-80). During the course of these processes, the activity of C/EBPβ can be regulated at multiple levels, including transcription, mRNA stability, protein phosphorylation, as well as translation, which can lead to differential selection of start codons (78-80, 87-89, 126, 148-155). Consistent with these ideas, *C/EBPβ* mRNA is stabilized by UV irradiation (Figure 19), and the levels of LIP protein are abundant upon UV irradiation, but not during the early phases of ER stress (Figure 20) (87, 154). *C/EBPβ* mRNA was reported to be stabilized by HuR, a protein that binds to AU-rich elements in the 3'-untranslational regions of the mRNAs, which provides a mechanism for decreased decay of *C/EBPβ* transcripts in response to selected environmental stresses, such as UV irradiation (149). Changes in *C/EBPβ* mRNA levels, as well as stress signaling is likely to be central for the levels of LIP and LAP expression.

Regarding transcriptional activation of ATF4, stresses shown to enhance *ATF4* mRNA include ER stress (138, 139, 156), such as that induced by thapsigargin, starvation for amino acids (140), oxidative stress (157, 158), and resistance to anticancer agents (159, 160). We are just beginning to understand the underlying mechanisms by which these stress conditions can induce *ATF4* mRNA. In the case of oxidative stress, the transcription factor NRF2 was reported to bind to the *ATF4* promoter and enhance its expression in response, which can alleviate stress damage and facilitate angiogenesis (157, 158). The transcription factor CLOCK was also reported to bind to the *ATF4* promoter, resulting in enhanced *ATF4* expression that can provide resistance to the anti-cancer drugs cisplatin and etoposide (161). Finally, PDX1, a pancreas-specific

transcription factor, activate the *ATF4* promoter upon ER stress response in islet β-cells (162). Together these findings suggest that many different transcription factors can bind to the *ATF4* promoter and modulate the levels of *ATF4* mRNA. Some of these transcription factors are inhibitors; triggering discordant induction of eIF2α~P and *ATF4* expression upon selected environmental stresses, while others are activators, amplifying *ATF4* expression in the ISR. Furthermore, there can be tissue-specific regulation of ATF4 during certain stress conditions. As a consequence, multiple stress pathways can control the induction ATF4 by eIF2α~P, insuring that the levels of ATF4 and its ISR-target genes are tailored for a given stress condition.

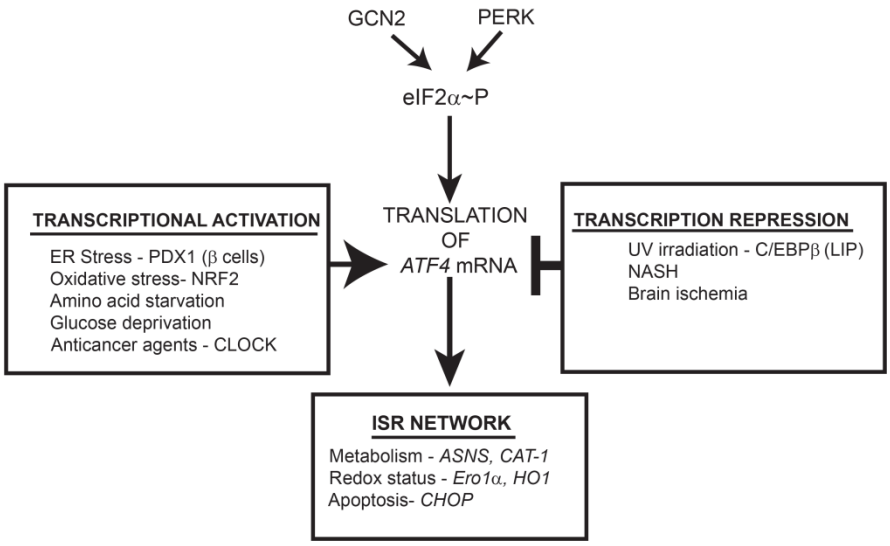


Figure 25. A combination of transcriptional and translational control of *ATF4* directs the gene expression program of the ISR. The eIF2 kinase, GCN2 is activated by nutritional deprivation or UV irradiation, while PERK is regulated by ER stress. The resulting induced eIF2α~P can lead to preferential translation of *ATF4* by a mechanism involving delayed ribosome reinitiation, which allows for ribosomes to bypass an inhibitory uORF in the *ATF4* mRNA. Activation of *ATF4* transcription by many different stresses enhances the amount of *ATF4* mRNA available for translation in response to eIF2α~P. Transcription factors that activate the *ATF4* promoter include PDX1 in islet β-cells of the pancreas upon ER stress, NRF2 in response to oxidative stress, and CLOCK, which facilitates resistance to anticancer agents, cisplatin and etoposide. As a consequence, there will be enhanced levels of ATF4 that directly activate the transcription of ISR-target genes involved in metabolism, the redox status of cells, and the regulation of apoptosis. Examples of target genes for each ISR category are illustrated. Alternatively, the *ATF4* promoter can be repressed by a different set of stress conditions. The LIP isoform of C/EBPβ directly facilitates repression of *ATF4* transcription in response to UV irradiation. This would result in low levels of *ATF4* mRNA available for preferential translation during UV stress despite high levels of eIF2α~P, thus lowering the expression of the ATF4-target genes in the ISR.

REFERENCES

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

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

3. 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

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

5. 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. *Mol Cell* 6: 1099-108

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

7. Harding HP, Zeng H, Zhang Y, Jungries 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

8. 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

9. Senee V, Vattem KM, Delepine M, Rainbow LA, Haton C, Lecoq A, Shaw NJ, Robert JJ, 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

10. 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. *J Biol Chem* 279: 36553-61

11. Bi M, Naczki C, Koritzinsky M, Fels D, Blais J, Hu N, Harding H, Novoa I, Varia M, Raleigh J, Scheuner D, Kaufman RJ, Bell J, Ron D, Wouters BG, Koumenis C. 2005. ER stress-regulated translation increases tolerance to extreme hypoxia and promotes tumor growth. *EMBO J* 24: 3470-81

12. Ye J, Kumanova M, Hart LS, Sloane K, Zhang H, De Panis DN, Bobrovnikova-Marjon E, Diehl JA, Ron D, Koumenis C. 2010. The GCN2-ATF4 pathway is critical for tumour cell survival and proliferation in response to nutrient deprivation. *EMBO J* 29: 2082-96

13. 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

14. Garcia MA, Meurs EF, Esteban M. 2007. The dsRNA protein kinase PKR: virus and cell control. *Biochimie* 89: 799-811

15. Han AP, Yu C, Lu L, Fujiwara Y, Browne C, Chin G, Fleming M, Leboulch P, Orkin SH, Chen JJ. 2001. Heme-regulated eIF2alpha kinase (HRI) is required for translational regulation and survival of erythroid precursors in iron deficiency. *EMBO J* 20: 6909-18

16. Sonenberg N, Hinnebusch AG. 2009. Regulation of translation initiation in eukaryotes: mechanisms and biological targets. *Cell* 136: 731-45

17. Kapp LD, Lorsch JR. 2004. The molecular mechanics of eukaryotic translation. *Annu Rev Biochem* 73: 657-704

18. Maag D, Fekete CA, Gryczynski Z, Lorsch JR. 2005. A conformational change in the eukaryotic translation preinitiation complex and release of eIF1 signal recognition of the start codon. *Mol Cell* 17: 265-75

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

20. 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: 12841-45

21. 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

22. Kimball SR, Heinzinger NK, Horetsky RL, Jefferson LS. 1998. Identification of interprotein interactions between the subunits of eukaryotic initiation factors eIF2 and eIF2B. *J Biol Chem* 273: 3039-44

23. Singh CR, Lee B, Udagawa T, Mohammad-Qureshi SS, Yamamoto Y, Pavitt GD, Asano K. 2006. An eIF5/eIF2 complex antagonizes guanine nucleotide exchange by eIF2B during translation initiation. *Embo J* 25: 4537-46

24. Jennings MD, Pavitt GD. 2010. eIF5 has GDI activity necessary for translational control by eIF2 phosphorylation. *Nature* 465: 378-81

25. Brush MH, Weiser DC, Shenolikar S. 2003. Growth arrest and DNA damage-inducible protein GADD34 targets protein phosphatase 1 alpha to the endoplasmic reticulum and promotes dephosphorylation of the alpha subunit of eukaryotic translation initiation factor 2. *Mol Cell Biol* 23: 1292-303

26. Jousse C, Oyadomari S, Novoa I, Lu P, Zhang Y, Harding HP, Ron D. 2003. Inhibition of a constitutive translation initiation factor 2alpha phosphatase, CReP, promotes survival of stressed cells. *J Cell Biol* 163: 767-75

27. 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

28. 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

29. Lee YY, Cevallos RC, Jan E. 2009. An upstream open reading frame regulates translation of GADD34 during cellular stresses that induce eIF2alpha phosphorylation. *J Biol Chem* 284: 6661-73

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

31. Wek SA, Zhu S, Wek RC. 1995. The histidyl-tRNA synthetase-related sequence in the eIF-2 alpha protein kinase GCN2 interacts with tRNA and is required for activation in response to starvation for different amino acids. *Mol Cell Biol* 15: 4497-506

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

33. Qiu H, Dong J, Hu C, 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

34. 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

35. Deng J, Harding HP, Raught B, Gingras AC, Berlanga JJ, Scheuner D, Kaufman RJ, Ron D, Sonenberg N. 2002. Activation of GCN2 in UV-irradiated cells inhibits translation. *Curr Biol* 12: 1279-86

36. Jiang HY, Wek RC. 2005. GCN2 phosphorylation of eIF2alpha activates NF-kappaB in response to UV irradiation. *Biochem J* 385: 371-80

37. Jiang HY, 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. *J Biol Chem* 280: 14189-202

38. Lu W, Laszlo CF, Miao Z, Chen H, Wu S. 2009. The role of nitric-oxide synthase in the regulation of UVB light-induced phosphorylation of the alpha subunit of eukaryotic initiation factor 2. *J Biol Chem* 284: 24281-8

39. 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

40. Kim J, Guan KL. 2011. Amino acid signaling in TOR activation. *Annu Rev Biochem* 80: 1001-32

41. Powley IR, Kondrashov A, Young LA, Dobbyn HC, Hill K, Cannell IG, Stoneley M, Kong YW, Cotes JA, Smith GC, Wek R, Hayes C, Gant TW, Spriggs KA, Bushell M, Willis AE. 2009. Translational reprogramming following UVB irradiation is mediated by DNA-PKcs and allows selective recruitment to the polysomes of mRNAs encoding DNA repair enzymes. *Genes Dev* 23: 1207-20

42. Karin M, Ben-Neriah Y. 2000. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu Rev Immunol* 18: 621-63

43. Li Q, Verma IM. 2002. NF-kappaB regulation in the immune system. *Nat Rev Immunol* 2: 725-34

44. Pahl HL. 1999. Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene* 18: 6853-66

45. Hao S, Sharp JW, Ross-Inta CM, McDaniel BJ, Anthony TG, Wek RC, Cavener DR, McGrath BC, Rudell JB, Koehnle TJ, Gietzen DW. 2005. Uncharged tRNA and sensing of amino acid deficiency in mammalian piriform cortex. *Science* 307: 1776-8

46. Costa-Mattioli M, Sonenberg N. 2006. Translational control of long-term synaptic plasticity and memory storage by eIF2alpha. *Crit Rev Neurobiol* 18: 187-95

47. Costa-Mattioli M, Gobert D, Harding H, Herdy B, Azzi M, Bruno M, Bidinosti M, Ben Mamou C, Marcinkiewicz E, Yoshida M, Imataka H, Cuello AC, Seidah N, Sossin W, Lacaille JC, Ron D, Nader K, Sonenberg N. 2005. Translational control of hippocampal synaptic plasticity and memory by the eIF2alpha kinase GCN2. *Nature* 436: 1166-73

48. Costa-Mattioli M, Gobert D, Stern E, Gamache K, Colina R, Cuello C, Sossin W, Kaufman R, Pelletier J, Rosenblum K, Krnjevic K, Lacaille JC, Nader K, Sonenberg N. 2007. eIF2alpha phosphorylation bidirectionally regulates the switch from short- to long-term synaptic plasticity and memory. *Cell* 129: 195-206

49. Maurin AC, Jousse C, Averous J, Parry L, Bruhat A, Cherasse Y, Zeng H, Zhang Y, Harding HP, Ron D, Fafournoux P. 2005. The GCN2 kinase biases feeding behavior to maintain amino acid homeostasis in omnivores. *Cell Metab* 1: 273-7

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

51. 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

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

53. Korennykh AV, Egea PF, Korostelev AA, Finer-Moore J, Zhang C, Shokat KM, Stroud RM, Walter P. 2009. The unfolded protein response signals through high-order assembly of Ire1. *Nature* 457: 687-93

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

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

56. 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

57. Roberts LO, Jopling CL, Jackson RJ, Willis AE. 2009. Viral strategies to subvert the mammalian translation machinery. *Prog Mol Biol Transl Sci* 90: 313-67

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

59. 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

60. 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

61. 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

62. Zykova TA, Zhu F, Zhang Y, Bode AM, Dong Z. 2007. Involvement of ERKs, RSK2 and PKR in UVA-induced signal transduction toward phosphorylation of eIF2alpha (Ser(51)). *Carcinogenesis* 28: 1543-51

63. 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

64. Ameri K, Harris AL. 2008. Activating transcription factor 4. *Int J Biochem Cell Biol* 40: 14-21

65. Yang X, Matsuda K, Bialek P, Jacquot S, Masuoka HC, Schinke T, Li L, Brancorsini S, Sassone-Corsi P, Townes TM, Hanauer A, Karsenty G. 2004. ATF4 is a substrate of RSK2 and an essential regulator of osteoblast biology; implication for Coffin-Lowry Syndrome. *Cell* 117: 387-98

66. Xiao G, Jiang D, Ge C, Zhao Z, Lai Y, Boules H, Phimphilai M, Yang X, Karsenty G, Franceschi RT. 2005. Cooperative interactions between activating transcription factor 4 and Runx2/Cbfa1 stimulate osteoblast-specific osteocalcin gene expression. *J Biol Chem* 280: 30689-96

67. Gachon F, Gaudray G, Thebault S, Basbous J, Koffi JA, Devaux C, Mesnard J. 2001. The cAMP response element binding protein-2 (CREB-2) can interact with the C/EBP-homologous protein (CHOP). *FEBS Lett* 502: 57-62

68. He CH, Gong P, Hu B, Stewart D, Choi ME, Choi AM, Alam J. 2001. Identification of activating transcription factor 4 (ATF4) as an Nrf2-interacting protein. Implication for heme oxygenase-1 gene regulation. *J Biol Chem* 276: 20858-65

69. Lassot I, Segeral E, Berlioz-Torrent C, Durand H, Groussin L, Hai T, Benarous R, Margottin-Goguet F. 2001. ATF4 degradation relies on a phosphorylation-dependent interaction with the SCF(betaTrCP) ubiquitin ligase. *Mol Cell Biol* 21: 2192-202

70. 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

71. Vinson CR, Hai T, Boyd SM. 1993. Dimerization specificity of the leucine zipper-containing bZIP motif on DNA binding: prediction and rational design. *Genes Dev* 7: 1047-58

72. Hai T, Curran T. 1991. Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proc Natl Acad Sci U S A* 88: 3720-4

73. Masuoka HC, Townes TM. 2002. Targeted disruption of the activating transcription factor 4 gene results in severe fetal anemia in mice. *Blood* 99: 736-45

74. Seo J, Fortuno ES, 3rd, Suh JM, Stenesen D, Tang W, Parks EJ, Adams CM, Townes T, Graff JM. 2009. Atf4 regulates obesity, glucose homeostasis, and energy expenditure. *Diabetes* 58: 2565-73

75. Vattem KM, Wek RC. 2004. Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. *Proc Natl Acad Sci U S A* 101: 11269-74

76. Zhou D, Palam LR, Jiang L, Narasimhan J, Staschke KA, Wek RC. 2008. Phosphorylation of eIF2 directs ATF5 translational control in response to diverse stress conditions. *J Biol Chem* 283: 7064-73

77. Palam LR, Baird TD, Wek RC. 2011. Phosphorylation of eIF2 facilitates ribosomal bypass of an inhibitory upstream ORF to enhance CHOP translation. *J Biol Chem* 286: 10939-49

78. Nerlov C. 2007. The C/EBP family of transcription factors: a paradigm for interaction between gene expression and proliferation control. *Trends Cell Biol* 17: 318-24

79. Ramji DP, Foka P. 2002. CCAAT/enhancer-binding proteins: structure, function and regulation. *Biochem J* 365: 561-75

80. Tsukada J, Yoshida Y, Kominato Y, Auron PE. 2011. The CCAAT/enhancer (C/EBP) family of basic-leucine zipper (bZIP) transcription factors is a multifaceted highly-regulated system for gene regulation. *Cytokine* 54: 6-19

81. Kilberg MS, Pan YX, Chen H, Leung-Pineda V. 2005. Nutritional control of gene expression: how mammalian cells respond to amino acid limitation. *Annu Rev Nutr* 25: 59-85

82. 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

83. Siu F, Chen C, Zhong C, Kilberg MS. 2001. CCAAT/enhancer-binding protein-beta is a mediator of the nutrient-sensing response pathway that activates the human asparagine synthetase gene. *J Biol Chem* 276: 48100-7

84. Bruhat A, Averous J, Carraro V, Zhong C, Reimold AM, Kilberg MS, Fafournoux P. 2002. Differences in the molecular mechanisms involved in the transcriptional activation of the CHOP and asparagine synthetase genes in response to amino acid deprivation or activation of the unfolded protein response. *J Biol Chem* 277: 48107-14

85. 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

86. 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. *Biochem J* 339: 135-41

87. Chiribau CB, Gaccioli F, Huang CC, Yuan CL, Hatzoglou M. 2010. Molecular symbiosis of CHOP and C/EBP beta isoform LIP contributes to endoplasmic reticulum stress-induced apoptosis. *Mol Cell Biol* 30: 3722-31

88. Calkhoven CF, Muller C, Leutz A. 2000. Translational control of C/EBPalpha and C/EBPbeta isoform expression. *Genes Dev* 14: 1920-32

89. Wethmar K, Begay V, Smink JJ, Zaragoza K, Wiesenthal V, Dorken B, Calkhoven CF, Leutz A. 2010. C/EBPbetaDeltauORF mice--a genetic model for uORF-mediated translational control in mammals. *Genes Dev* 24: 15-20

90. Kilberg MS, Shan J, Su N. 2009. ATF4-dependent transcription mediates signaling of amino acid limitation. *Trends Endocrinol Metab* 20: 436-43

91. Zhong C, Chen C, Kilberg MS. 2003. Characterization of the nutrient-sensing response unit in the human asparagine synthetase promoter. *Biochem J* 372: 603-9

92. Su N, Kilberg MS. 2008. C/EBP homology protein (CHOP) interacts with activating transcription factor 4 (ATF4) and negatively regulates the stress-dependent induction of the asparagine synthetase gene. *J Biol Chem* 283: 35106-17

93. Ohoka N, Yoshii S, Hattori T, Onozaki K, Hayashi H. 2005. TRB3, a novel ER stress-inducible gene, is induced via ATF4-CHOP pathway and is involved in cell death. *EMBO J* 24: 1243-55

94. Ord D, Ord T. 2005. Characterization of human NIPK (TRB3, SKIP3) gene activation in stressful conditions. *Biochem Biophys Res Commun* 330: 210-8

95. Ord D, Meerits K, Ord T. 2007. TRB3 protects cells against the growth inhibitory and cytotoxic effect of ATF4. *Exp Cell Res* 313: 3556-67

96. Ord D, Ord T. 2003. Mouse NIPK interacts with ATF4 and affects its transcriptional activity. *Exp Cell Res* 286: 308-20

97. Jousse C, Deval C, Maurin AC, Parry L, Cherasse Y, Chaveroux C, Lefloch R, Lenormand P, Bruhat A, Fafournoux P. 2007. TRB3 inhibits the transcriptional activation of stress-regulated genes by a negative feedback on the ATF4 pathway. *J Biol Chem* 282: 15851-61

98. 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

99. Dey S, Baird TD, Zhou D, Palam LR, Spandau DF, Wek RC. 2010. Both transcriptional regulation and translational control of ATF4 are central to the integrated stress response. *J Biol Chem* 285: 33165-74

100. Kumar R, Krause GS, Yoshida H, Mori K, DeGracia DJ. 2003. Dysfunction of the unfolded protein response during global brain ischemia and reperfusion. *J Cereb Blood Flow Metab* 23: 462-71

101. Puri P, Mirshahi F, Cheung O, Natarajan R, Maher JW, Kellum JM, Sanyal AJ. 2008. Activation and dysregulation of the unfolded protein response in nonalcoholic fatty liver disease. *Gastroenterology* 134: 568-76

102. Bevilacqua E, Wang X, Majumder M, Gaccioli F, Yuan CL, Wang C, Zhu X, Jordan LE, Scheuner D, Kaufman RJ, Koromilas AE, Snider MD, Holcik M, Hatzoglou M. 2010. eIF2alpha phosphorylation tips the balance to apoptosis during osmotic stress. *J Biol Chem* 285: 17098-111

103. 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

104. 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

105. 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

106. 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

107. 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

108. 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

109. Tabas I, Ron D. 2011. Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. *Nat Cell Biol* 13: 184-90

110. Puthalakath H, O'Reilly LA, Gunn P, Lee L, Kelly PN, Huntington ND, Hughes PD, Michalak EM, McKimm-Breschkin J, Motoyama N, Gotoh T, Akira S, Bouillet P, Strasser A. 2007. ER stress triggers apoptosis by activating BH3-only protein Bim. *Cell* 129: 1337-49

111. Li G, Mongillo M, Chin KT, Harding H, Ron D, Marks AR, Tabas I. 2009. Role of ERO1-alpha-mediated stimulation of inositol 1,4,5-triphosphate receptor activity in endoplasmic reticulum stress-induced apoptosis. *J Cell Biol* 186: 783-92

112. Yamaguchi H, Wang HG. 2004. CHOP is involved in endoplasmic reticulum stress-induced apoptosis by enhancing DR5 expression in human carcinoma cells. *J Biol Chem* 279: 45495-502

113. Meir O, Dvash E, Werman A, Rubinstein M. 2010. C/EBP-beta regulates endoplasmic reticulum stress-triggered cell death in mouse and human models. *PLoS One* 5: e9516

114. Pavitt GD. 2005. eIF2B, a mediator of general and gene-specific translational control. *Biochem Soc Trans* 33: 1487-92

115. Kantor L, Harding HP, Ron D, Schiffmann R, Kaneski CR, Kimball SR, Elroy-Stein O. 2005. Heightened stress response in primary fibroblasts expressing mutant eIF2B genes from CACH/VWM leukodystrophy patients. *Hum Genet* 118: 99-106

116. Fonseca SG, Fukuma M, Lipson KL, Nguyen LX, Allen JR, Oka Y, Urano F. 2005. WFS1 is a novel component of the unfolded protein response and maintains homeostasis of the endoplasmic reticulum in pancreatic beta-cells. *J Biol Chem* 280: 39609-15

117. Sandhu MS, Weedon MN, Fawcett KA, Wasson J, Debenham SL, Daly A, Lango H, Frayling TM, Neumann RJ, Sherva R, Blech I, Pharoah PD, Palmer CN, Kimber C, Tavendale R, Morris AD, McCarthy MI, Walker M, Hitman G, Glaser B, Permutt MA, Hattersley AT, Wareham NJ, Barroso I. 2007. Common variants in WFS1 confer risk of type 2 diabetes. *Nat Genet* 39: 951-3

118. Fonseca SG, Ishigaki S, Oslowski CM, Lu S, Lipson KL, Ghosh R, Hayashi E, Ishihara H, Oka Y, Permutt MA, Urano F. 2010. Wolfram syndrome 1 gene negatively regulates ER stress signaling in rodent and human cells. *J Clin Invest* 120: 744-55

119. Ariyama Y, Shimizu H, Satoh T, Tsuchiya T, Okada S, Oyadomari S, Mori M. 2007. Chop-deficient mice showed increased adiposity but no glucose intolerance. *Obesity (Silver Spring)* 15: 1647-56

120. 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

121. Harding HP, Zhang Y, Scheuner D, Chen JJ, Kaufman RJ, Ron D. 2009. Ppp1r15 gene knockout reveals an essential role for translation initiation factor 2 alpha (eIF2alpha) dephosphorylation in mammalian development. *Proc Natl Acad Sci U S A* 106: 1832-7

122. Jiang HY, 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. *Mol Cell Biol* 23: 5651-63

123. Jiang HY, 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. *Mol Cell Biol* 24: 1365-77

124. Zinszner H, Kuroda M, Wang X, 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

125. 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

126. Li Y, Bevilacqua E, Chiribau CB, Majumder M, Wang C, Croniger CM, Snider MD, Johnson PF, Hatzoglou M. 2008. Differential control of the CCAAT/enhancer-binding protein beta (C/EBPbeta) products liver-enriched transcriptional activating protein (LAP) and liver-enriched transcriptional inhibitory protein (LIP) and the regulation of gene expression during the response to endoplasmic reticulum stress. *J Biol Chem* 283: 22443-56

127. 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. *Mol Cell Biol* 22: 3864-74

128. 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. *Mol Cell Biol* 22: 6681-8

129. Lewis DA, Yi Q, Travers JB, Spandau DF. 2008. UVB-induced senescence in human keratinocytes requires a functional insulin-like growth factor-1 receptor and p53. *Mol Biol Cell* 19: 1346-53

130. Vousden KH. 2002. Activation of the p53 tumor suppressor protein. *Biochim Biophys Acta* 1602: 47-59

131. Boyce M, Bryant KF, Jousse C, Long K, Harding HP, Scheuner D, Kaufman RJ, Ma D, Coen DM, Ron D, Yuan J. 2005. A selective inhibitor of eIF2alpha dephosphorylation protects cells from ER stress. *Science* 307: 935-9

132. Robert F, Kapp LD, Khan SN, Acker MG, Kolitz S, Kazemi S, Kaufman RJ, Merrick WC, Koromilas AE, Lorsch JR, Pelletier J. 2006. Initiation of protein synthesis by hepatitis C virus is refractory to reduced eIF2.GTP.Met-tRNA(i)(Met) ternary complex availability. *Mol Biol Cell* 17: 4632-44

133. Hata K, Nishimura R, Ueda M, Ikeda F, Matsubara T, Ichida F, Hisada K, Nokubi T, Yamaguchi A, Yoneda T. 2005. A CCAAT/enhancer binding protein beta isoform, liver-enriched inhibitory protein, regulates commitment of osteoblasts and adipocytes. *Mol Cell Biol* 25: 1971-9

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

135. Hatzoglou M, Fernandez J, Yaman I, Closs E. 2004. Regulation of cationic amino acid transport: the story of the CAT-1 transporter. *Annu Rev Nutr* 24: 377-99

136. Schmitt-Ney M, Habener JF. 2000. CHOP/GADD153 gene expression response to cellular stresses inhibited by prior exposure to ultraviolet light wavelength band C (UVC). Inhibitory sequence mediating the UVC response localized to exon 1. *J Biol Chem* 275: 40839-45

137. Wang XZ, Lawson B, Brewer JW, Zinszner H, Sanjay A, Mi LJ, Boorstein R, Kreibich G, Hendershot LM, Ron D. 1996. Signals from the stressed endoplasmic reticulum induce C/EBP-homologous protein (CHOP/GADD153). *Mol Cell Biol* 16: 4273-80

138. Adachi Y, Yamamoto K, Okada T, Yoshida H, Harada A, Mori K. 2008. ATF6 is a transcription factor specializing in the regulation of quality control proteins in the endoplasmic reticulum. *Cell Struct Funct* 33: 75-89

139. Lu PD, Jousse C, Marciniak SJ, Zhang Y, Novoa I, Scheuner D, Kaufman RJ, Ron D, Harding HP. 2004. Cytoprotection by pre-emptive conditional phosphorylation of translation initiation factor 2. *EMBO J* 23: 169-79

140. Siu F, Bain PJ, LeBlanc-Chaffin R, Chen H, Kilberg MS. 2002. ATF4 is a mediator of the nutrient-sensing response pathway that activates the human asparagine synthetase gene. *J Biol Chem* 277: 24120-7

141. Oh YK, Shin KS, Yuan J, Kang SJ. 2008. Superoxide dismutase 1 mutants related to amyotrophic lateral sclerosis induce endoplasmic stress in neuro2a cells. *J Neurochem* 104: 993-1005

142. Lewerenz J, Maher P. 2009. Basal levels of eIF2alpha phosphorylation determine cellular antioxidant status by regulating ATF4 and xCT expression. *J Biol Chem* 284: 1106-15

143. Nakka VP, Gusain A, Raghubir R. 2010. Endoplasmic reticulum stress plays critical role in brain damage after cerebral ischemia/reperfusion in rats. *Neurotox Res* 17: 189-202

144. Marciniak SJ, Ron D. 2006. Endoplasmic reticulum stress signaling in disease. *Physiol Rev* 86: 1133-49

145. Rouschop KM, van den Beucken T, Dubois L, Niessen H, Bussink J, Savelkouls K, Keulers T, Mujcic H, Landuyt W, Voncken JW, Lambin P, van der Kogel AJ, Koritzinsky M, Wouters BG. 2010. The unfolded protein response protects human tumor cells during hypoxia through regulation of the autophagy genes MAP1LC3B and ATG5. *J Clin Invest* 120: 127-41

146. Natarajan K, Meyer MR, Jackson BM, Slade D, Roberts C, Hinnebusch AG, Marton MJ. 2001. Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast. *Mol Cell Biol* 21: 4347-68

147. Engelberg D, Klein C, Martinetto H, Struhl K, Karin M. 1994. The UV response involving the Ras signaling pathway and AP-1 transcription factors is conserved between yeast and mammals. *Cell* 77: 381-90

148. Chen C, Dudenhausen E, Chen H, Pan YX, Gjymishka A, Kilberg MS. 2005. Amino-acid limitation induces transcription from the human C/EBPbeta gene via an enhancer activity located downstream of the protein coding sequence. *Biochem J* 391: 649-58

149. Bergalet J, Fawal M, Lopez C, Desjobert C, Lamant L, Delsol G, Morello D, Espinos E. 2011. HuR-mediated control of C/EBPbeta mRNA stability and translation in ALK-positive anaplastic large cell lymphomas. *Mol Cancer Res* 9: 485-96

150. Piwien-Pilipuk G, MacDougald O, Schwartz J. 2002. Dual regulation of phosphorylation and dephosphorylation of C/EBPbeta modulate its transcriptional activation and DNA binding in response to growth hormone. *J Biol Chem* 277: 44557-65

151. Timchenko LT, Salisbury E, Wang GL, Nguyen H, Albrecht JH, Hershey JW, Timchenko NA. 2006. Age-specific CUGBP1-eIF2 complex increases translation of CCAAT/enhancer-binding protein beta in old liver. *J Biol Chem* 281: 32806-19

152. Timchenko NA, Wang GL, Timchenko LT. 2005. RNA CUG-binding protein 1 increases translation of 20-kDa isoform of CCAAT/enhancer-binding protein beta by interacting with the alpha and beta subunits of eukaryotic initiation translation factor 2. *J Biol Chem* 280: 20549-57

153. Timchenko LT, Iakova P, Welm AL, Cai ZJ, Timchenko NA. 2002. Calreticulin interacts with C/EBPalpha and C/EBPbeta mRNAs and represses translation of C/EBP proteins. *Mol Cell Biol* 22: 7242-57

154. Li B, Si J, DeWille JW. 2008. Ultraviolet radiation (UVR) activates p38 MAP kinase and induces post-transcriptional stabilization of the C/EBPdelta mRNA in G0 growth arrested mammary epithelial cells. *J Cell Biochem* 103: 1657-69

155. Chen C, Dudenhausen EE, Pan YX, Zhong C, Kilberg MS. 2004. Human CCAAT/enhancer-binding protein beta gene expression is activated by endoplasmic reticulum stress through an unfolded protein response element downstream of the protein coding sequence. *J Biol Chem* 279: 27948-56

156. Armstrong JL, Flockhart R, Veal GJ, Lovat PE, Redfern CP. 2010. Regulation of endoplasmic reticulum stress-induced cell death by ATF4 in neuroectodermal tumor cells. *J Biol Chem* 285: 6091-100

157. Afonyushkin T, Oskolkova OV, Philippova M, Resink TJ, Erne P, Binder BR, Bochkov VN. 2010. Oxidized phospholipids regulate expression of ATF4 and VEGF in endothelial cells via NRF2-dependent mechanism: novel point of convergence between electrophilic and unfolded protein stress pathways. *Arterioscler Thromb Vasc Biol* 30: 1007-13

158. Miyamoto N, Izumi H, Miyamoto R, Bin H, Kondo H, Tawara A, Sasaguri Y, Kohno K. 2011. Transcriptional regulation of activating transcription factor 4 under oxidative stress in retinal pigment epithelial ARPE-19/HPV-16 cells. *Invest Ophthalmol Vis Sci* 52: 1226-34

159. Levenson VV, Davidovich IA, Roninson IB. 2000. Pleiotropic resistance to DNA-interactive drugs is associated with increased expression of genes involved in DNA replication, repair, and stress response. *Cancer Res* 60: 5027-30

160. Tanabe M, Izumi H, Ise T, Higuchi S, Yamori T, Yasumoto K, Kohno K. 2003. Activating transcription factor 4 increases the cisplatin resistance of human cancer cell lines. *Cancer Res* 63: 8592-5

161. Igarashi T, Izumi H, Uchiumi T, Nishio K, Arao T, Tanabe M, Uramoto H, Sugio K, Yasumoto K, Sasaguri Y, Wang KY, Otsuji Y, Kohno K. 2007. Clock and ATF4 transcription system regulates drug resistance in human cancer cell lines. *Oncogene* 26: 4749-60

162. Sachdeva MM, Claiborn KC, Khoo C, Yang J, Groff DN, Mirmira RG, Stoffers DA. 2009. Pdx1 (MODY4) regulates pancreatic beta cell susceptibility to ER stress. *Proc Natl Acad Sci U S A* 106: 19090-5

CURRICULUM VITAE

Souvik Dey

EDUCATION:

2006-2012: Ph.D. in Biochemistry and Molecular Biology,
Indiana University, Indianapolis, Indiana
Thesis title: Transcriptional regulation of ATF4 is critical for controlling the Integrated Stress Response during eIF2 phosphorylation
Thesis advisor: Ronald Wek.

2002-2006: B.Tech. in Biotechnology,
West Bengal Institute of Technology, India

PROFESSIONAL EXPERIENCE:

2005-2006: Research internship
Department of Microelectronics, Saha Institute of Nuclear Physics
Department of Atomic Energy, Govt. of India, India
Project advisor: Dr. K. Bhowmik

2005: Summer research internship
Haffkine Institute of Training, Research and Development, India
Project advisor: Dr. R. Deshmukh

HONORS AND AWARDS:

2009-2010: Graduate student representative
Department of Biochemistry and Molecular Biology
Indiana University, Indianapolis, Indiana

2009-2010: Jack Davis Award for best graduate student seminar
Department of Biochemistry and Molecular Biology
Indiana University, Indianapolis, Indiana

CONFERENCES AND PRESENTATIONS:

Translational Control, Cold Spring Harbor Laboratory, NY, September 13–17, 2010. Both transcriptional regulation and translational control of ATF4 are central to the integrated stress response, **Dey S.,** Baird T.D., Zhou D., Palam L.R., Spandau D.F., and Wek R.C.

Biochemistry and Molecular Biology Research Day, Indiana University, February 19, 2010. Differential regulation of Integrated Stress pathway in response to genotoxic stress, **Dey S.,** Zhou D., and Wek R.C.

Translational Control, Cold Spring Harbor Laboratory, NY, September 3–7, 2008. Integration of General Control and Nitrogen Regulatory Pathways during Nutrient Stress in Yeast, Staschke K.A., **Dey S.,** Wek R.C.

Deptartment of Medical and Molecular genetics, Indiana University School of Medicine, December 15th 2009. Differential regulation of Integrated Stress Pathway in response to DNA damage, **Dey S**.

Biochemistry and Molecular Biology Research Day, Indiana University, October 2, 2008. Differential regulation of Integrated Stress pathway in response to DNA damage, **Dey S.,** Staschke, K.A., and Wek R.C.

PUBLICATIONS:

Dey S., Baird T.D., Zhou D., Palam L.R., Spandau D.F., Wek R.C. Both transcriptional regulation and translational control of ATF4 are central to the integrated stress response. *J Biol Chem.* 2010 Oct 22; 285(43):33165-74.

Staschke K.A., **Dey S.,** Zaborske J.M., Palam L.R., McClintick J.N., Pan T., Edenberg H.J., Wek R.C. Integration of general amino acid control and target of rapamycin (TOR) regulatory pathways in nitrogen assimilation in yeast. *J Biol Chem.* 2010 May28; 285(22):16893-911.

Dey S., Savant S., Hatzoglou M., Calkhoven C.F., Wek R.C. Transcriptional repression of *ATF4* by C/EBPβ differentially regulates the Integrated Stress Response. Manuscript submitted, 2012.