TRANSCRIPTIONAL REGULATION OF THE SODIUM-COUPLED NEUTRAL AMINO ACID TRANSPORTER (SNAT2) GENE BY AMINO ACID AVAILABILITY

By

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To my family and friends

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I thank my mentor, Dr. Kilberg, for his guidance in helping me to become a better scientist. I thank my committee members for their support, helpful suggestions, and commentary on my work. I thank other members of the Kilberg laboratory, both past and present, for their help and friendship. I also thank my family and my friends for all their love and support.

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LIST OF ABBREVIATIONS

AAR	amino acid response
AARE	amino acid responsive element
ASNS	asparagine synthetase
ATF	activating transcription factor
BMK1	big mitogen-activated protein kinase 1
bp	base pair
C/EBP	CCAAT/enhancer-binding protein
c-Maf	cellular homologue of the avian viral oncogene v-maf which induces musculoaponeurotic fibrosarcoma
CDK	cyclin-dependent kinase
ChIP	chromatin immunoprecipitation
CHOP	CCAAT/enhancer-binding protein homologous protein
eIF2α	eukaryotic translation initiation factor 2A
EMSA	electromobility shift assay
ERK	extracellular signal-regulated kinase
GADD34	growth arrest and DNA-damage-inducible 34
GCN	general control non-derepressible
GDP	guanosine diphosphate
GTF	general transcription factor
GTP	guanosine triphosphate
HAT	histone acetyltransferase
JNK	c-Jun N-terminal kinase
LAP	liver-enriched activating protein
LIP	liver-enriched inhibitory protein

MAPK	mitogen-activated protein kinase
MEF	mouse embryonic fibroblast
MEK	mitogen-activated or extracellular signal-regulated protein kinase kinase
mRNA	messenger RNA
NSRE	nutrient sensing responsive element
nt	nucleotide
p-	phosphorylated
Pol II	RNA Polymerase II
qPCR	quantitative polymerase chain reaction
RT-qPCR	reverse transcriptase quantitative polymerase chain reaction
sem	standard error of the means
SNAT2	sodium-coupled neutral amino acid transporter gene member 2
SV40	Simian virus 40
TAF	TBP-associated factor
TBP	Tata-box binding protein
uORF	upstream open reading frame
VEGF	vascular endothelial growth factor

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Limited intake of nutrients, especially amino acids, can alter general growth and development as well as disease states such as diabetes and solid tumor growth. The transcription of many genes, including the Na^+-coupled neutral amino acid transporter, SNAT2, is increased by amino acid limitation through the Amino Acid Response (AAR) pathway. Previous analysis of the SNAT2 gene structure identified a 9 bp region in the first intron that acts as a transcriptional enhancer upon amino acid starvation. Evidence from *in vitro* studies suggested that members of the ATF and C/EBP families regulate SNAT2 gene transcription by interacting with this intronic amino acid responsive element (AARE). The purpose of the current study was to determine the specific transcription factors responsible for regulating SNAT2 induction through its AARE, and to establish the mechanism by which these proteins regulate the SNAT2 gene. Chromatin immunoprecipitation (ChIP) analysis of the AARE region established that the transcription factors ATF4, ATF3, C/EBPβ, and C/EBPα bind the AARE in a specific temporal order reflective of the transcription activity. The kinetics of RNA polymerase II and general transcription factor binding to the SNAT2 promoter region was consistent with the increased transcription, but binding of the Mediator complex did not increase at the SNAT2 promoter or AARE. ATF4 and C/EBPβ knockout cell lines and siRNA-ChIP assays demonstrated that ATF4

enhanced but was not required for SNAT2 induction and that C/EBPβ repressed SNAT2 transcription. siRNA assays to knockdown subunits of the Mediator complex determined that increased recruitment was not necessary for SNAT2 transcription induction. Given that both ATF4 and C/EBPβ are regulated by various MAPK pathways, cellular inhibition of these pathways was performed and demonstrated that only the MEK MAPK pathway is required for SNAT2 transcription induction by amino acid limitation through the AAR signaling pathway. Furthermore, inhibition of the MEK pathway prevented phosphorylation of eIF2α by the AAR, and conversely, blocking the AAR through knockout of GCN2 or p-eIF2α prevented increased MEK signaling. These data establish that there is an interdependence between the MEK and AAR signaling pathways in HepG2 cells during amino acid limitation. Overall, these results demonstrate that SNAT2 transcriptional regulation is mediated through a complex mechanism of timed activator and repressor recruitment to the AARE through activation of both the AAR and MEK signaling pathways.

CHAPTER 1
INTRODUCTION

Characteristics of System A Mediated Amino Acid Transport

The activity of System A was initially described in Ehrlich tumor cells by Oxender and Christensen (Oxender and Christensen, 1963). Further studies with different cell lines have determined that System A activity is Na^+- dependent, pH sensitive, and preferentially transports neutral amino acids with small or unbranched sidechains (Kilberg et al., 1985). To specifically study the transport of System A, a non-metabolizable amino acid analog, 2-(methylamino)-isobutyric acid (MeAIB), can be used as a substrate or a competitive inhibitor, thus preventing interference from other transport systems (Kilberg et al., 1980). Transport competition studies between MeAIB and the other amino acids allowed for the determination of amino acid substrate specificity for System A. Specifically, alanine, glycine, serine, and proline all exhibit 50% or greater total uptake by System A, while histidine exhibited approximately 15% total uptake (Kilberg, 1986). The amino acids leucine, isoleucine, lysine, and arginine all displayed little or no transport via System A. The substrate specificity of System A is thought to be physiologically important; for example, the most actively transported amino acids are also the most efficient gluconeogenic precursors (Kilberg et al., 1985).

Hormonal and Adaptive Regulation of System A

The regulation of System A by a wide spectrum of hormones is well documented in the scientific literature. Epidermal growth factor (EGF) inhibited transport of System A in rat hepatocytes, and also partially blocked System A induction by glucagon (Kilberg et al., 1985). Platelet-derived growth factor (PDGF) also exhibited inhibition of System A similar to that of EGF, whereas an isoform of insulin-like growth factor (IGF) in rat hepatocytes was shown to increase System A transport (Auberger et al., 1983). Glucagon stimulation of System A has been

well characterized, and transporter activity increases after 30-90 minutes of hormone addition (Kilberg, 1986). Stimulation of System A activity by insulin, through a phosphoinositide-3-kinase (PI3K) dependent wortmannin-sensitive mechanism, occurs via translocation of the transporter protein from endosomal vesicles to the plasma membrane (Hyde et al., 2002). Increasing ceramide concentration levels antagonized insulin signaling by blocking System A protein translocation to the plasma membrane (Hyde et al., 2005). Along with transport stimulation by cAMP (Kilberg and Neuhaus, 1977), it is obvious that System A is tightly regulated by various hormones.

Another well-documented feature of System A is its ability to respond to changes in cellular amino acid availability. This adaptive regulation can be blocked by inhibitors of RNA and protein synthesis, suggesting that System A transport in response to amino acid limitation is dependent on active transcription and translation (Fong et al., 1990). Recently, Ling et al. determined that the System A response to amino acid limitation consists of an acute phase and a chronic phase (Ling et al., 2001). The acute phase is the result of preformed transporter proteins being recruited from an intracellular protein pool to the plasma membrane. The chronic phase, however, is caused by an induction of gene expression, which was blocked by actinomycin D and cycloheximide. Recent reviews have provided several mechanistic models proposing the System A transporter itself may be a nutrient sensor and actually induce cellular signaling cascades. Hyde et al. have proposed that the System A transporter is actually a dual function transporter and receptor, thus developing the term "amino acid transceptor" (Hyde et al., 2007). Their data suggest that in amino acid complete conditions, the System A protein can act as a repressor of adaptive regulation through an unknown pathway. In amino acid limiting conditions, the System A protein would induce multiple cellular signaling pathways, through a

conformation change in transporter orientation, to allow for the cellular adaptive response to progress.

System A Transport in Transformed or Cancerous Cell Lines

System A mediated, neutral amino acid transport has been shown to be preferentially upregulated in every cancerous and tumor cell line tested. Experiments performed in breast tumor cell lines demonstrated that System A transport was induced 2- to 4-fold in estrogen receptor-positive tumor cells stimulated by estrogen (Bhattacharyya et al., 2006). Quiescent mouse fibroblasts demonstrated low rates of System A active transport, but when these cells were chemically transformed with methylcholanthrene, System A activity was considerably increased (Bhattacharyya et al., 2006). The first demonstration of System A upregulation in virally transformed cells was by Pardee's laboratory which showed that SV40-transformed 3T3 mouse fibroblasts display several fold higher System A transport activity than in the parental 3T3 cells (Foster and Pardee, 1969). The Kilberg laboratory and others have also shown that a positive correlation exists between cell growth rate and System A transport, and that the effect from cell growth can be separated from transformation (Handlogten and Kilberg, 1988; Borghetti et al., 1980). When System A activity was monitored in SV40 transformed fetal rat hepatocytes, it was determined that System A transport activity was 8- to 10-fold higher in the transformed cells compared to the quiescent cells (Handlogten and Kilberg, 1988). Interestingly, System A shows a two-fold increase during early G1 of the cell cycle and returns to basal levels during mitosis. The System A relationship to the cell cycle has also been demonstrated during rat partial hepatectomy, in which an increase in System A activity occurs after surgery in unison with the initiation of cell replication (Walker and Whitfield, 1978), and inhibition of System A retards liver regeneration (Freeman et al., 1999). All of these observations clearly show that System A transport plays a specific role in cellular transformation and cancer.

Given that System A transport activity is highly upregulated in cancerous and virally transformed cells, it was hypothesized that this transporter system may be a useful tool for tumor imaging by positron emission tomography (PET). Metabolic tumor imaging looks to take advantage of increased protein synthesis, requiring induced amino acid transport, in cancerous cells by monitoring these cells abundance and growth with radiolabeled amino acids (Jager et al., 2001). Initial studies were performed with the amino acids methionine and tyrosine, neither of which is highly transported by System A, due to convenient radiolabeled synthesis (Jager et al., 2001; Mackenzie and Erickson, 2004). Besides its upregulation in cancerous cells, the System A transporter is a useful candidate for PET imaging because it preferentially transports the non-metabolizable amino acid analogue MeAIB. Original studies in rat brain tumors demonstrated that radiolabeled 2-amino isobutric acid (AIB) uptake enhanced tumor visualization by PET, but this analogue is not completely specific for System A (Uehara et al., 1997). More recently, PET scanning by Sutinen et al. with MeAIB demonstrated higher uptake in tumor tissue compared to normal tissue (Sutinen et al., 2001; Sutinen et al., 2003). These investigators noted that MeAIB PET imaging may be useful for tumor imaging, especially in brain tissue where low levels of System A activity were detected in normal tissue.

Genes Responsible for System A Transport Activity

Although the System A transporter system was one of the first mammalian amino acid transporter systems to be characterized, the genes responsible for producing System A proteins, the SNAT family, were difficult for researchers to identify. Cloning of the first SNAT transporters occurred by chance through their genetic sequence homology to another set of transporter proteins, the vesicular inhibitory amino acid transporters. Several researchers eventually identified three genes encoding proteins with characteristics similar to System A transport activity (Sugawara et al., 2000; Yao et al., 2000), which have recently been renamed as

SNAT1 (ATA1, SAT1), SNAT2 (ATA2, SAT2), and SNAT4 (ATA3) (Mackenzie and Erickson, 2004). Two other genes, SNAT3 and SNAT5, were actually determined to be responsible for the amino acid transport activity termed System N. System N differs from System A in that it prefers transport of glutamine and histidine and it also countertransports hydrogen ions (Mackenzie and Erickson, 2004). All three System A encoding SNAT genes are located in tandem on human chromosome 12, and display similar substrate specificities. SNAT1 is expressed mainly in brain, retina, and heart tissue, and is believed to have a prominent function in neuronal signaling through glutamine transport. SNAT4 expression is liver and placenta specific, and was surprisingly determined to also be a partially imprinted gene (Mizuno et al., 2002; Smith et al., 2003). Though SNAT4 is expressed biallelically in adult liver tissue, its expression in placenta was exclusively from the paternal allele. Contrary to its counterparts, SNAT2 is expressed in all cell types, though its highest expression is in brain, muscle, lung and placental tissue.

It first was demonstrated by Gazzola et al. that SNAT2 is the primary isoform induced by adaptive regulation and that there exists a direct relationship between System A activity and SNAT2 mRNA expression (Gazzola et al., 2001). Recent mechanistic models of System A transport activity during amino acid starvation have also demonstrated the SNAT2 protein is responsible for this activity (Ling et al., 2001; Hyde et al., 2007). For SNAT2 specifically, its genomic sequence spans over ~14.5 kilobases and consists of 16 exons. The protein start site is located in exon 2 (+1479 relative to the putative transcription start site), so the entire first exon and part of the second exon are part of the 5'-untranslated region of the mRNA (Palii et al., 2004). The protein stop codon is within the first few base pairs of exon 16 providing a large 3'-untranslated region. This gene structure is preserved in many known eukaryotic genomes

including rat, mouse, and chimp (Figure 1-1) (Palii et al., 2004). Since the cloning of the mouse and human SNAT2 genes by our laboratory (Palii et al., 2004), regulation by amino acid limitation has begun to be characterized, and has been contrasted with other extensively studied amino acid regulated genes.

Adaptive Regulation of Gene Expression by Amino Acid Limitation

Regulation in *Saccharomyces cerevisiae*

Gene expression in response to amino acid limitation has been extensively investigated in the yeast *Saccharomyces cerevisiae*. These studies have determined that there exists a general control mechanism (GCN, general control non-derepressible) in which many genes are transcriptionally upregulated in response to limitation of a single amino acid (Hope and Struhl, 1985). The adaptive response to amino acid limitation in yeast begins with initiation of a cellular signaling cascade (Figure 1-2) (Hinnebusch, 1997). When cellular amino acid content is limiting, the kinase GCN2 initiates this pathway through recognition and binding to any one of the uncharged tRNA molecules. GCN2 binding to uncharged tRNA causes its dimerization with another GCN2 protein and autophosphorylation at specific threonine residues (Hinnebusch, 2005). The GCN1/GCN20 complex interacts with GCN2 to enhance its kinase activity, which phosphorylates the alpha subunit of eukaryotic translation initiation factor 2 (eIF2α) at residue serine 51. During non-starvation conditions, the guanine nucleotide exchange factor eIF2B exchanges eIF2α-bound guanosine diphosphate (GDP) for guanosine triphosphate (GTP) (Figure 1-2) (Hinnebusch, 1997). The eIF2α-GTP complex is responsible for delivering the charged initiator tRNA for methionine to the 40 S ribosomal subunit. During amino acid starvation, however, phosphorylation of the eIF2α subunit inhibits the ability of eIF2B to exchange GTP for GDP on eIF2α, therefore slowing initiation of translation (Hinnebusch, 1997). In addition, the phosphorylated eIF2α-GDP complex has a higher affinity for eIF2B than the non-phosphorylated

complex, further impairing GDP to GTP exchange on the non-phosphorylated eIF2α subunit. The widespread consequence of eIF2α phosphorylation is suppression of global cellular protein synthesis. Though general protein production decreases, specific mRNAs, such as the GCN4 mRNA, are able to escape this downregulation through a ribosomal scanning mechanism.

The protein GCN4 was determined to be ultimately responsible for increased transcription through the amino acid response (AAR) pathway, because this protein is preferentially translated during amino acid starvation conditions. GCN4 is a member of the basic-region leucine zipper (bZIP) family of transcriptional activators known to bind DNA directly (Hope and Struhl, 1987). GCN4 binds as a homodimer to the consensus palindromic sequence TGACTCA, which was discovered in the promoter sequence of many genes it regulates (Hope and Struhl, 1987). The translational regulation of GCN4 is governed by a series of upstream open reading frames (uORFs) preceding the actual translation start site needed to produce GCN4 protein (Figure 1-2) (Hinnebusch, 1997). Under conditions where all amino acids are present, translational initiation begins at the first uORF on the GCN4 mRNA, and then the ribosome continues scanning until the necessary proteins are efficiently reassembled at an intermediate uORF which overlaps the start site for production of the GCN4 protein itself. Thus, when cells are nutritionally replete, the actual GCN4 coding sequence is weakly translated. Under amino acid starvation conditions, however, the ribosomal complex assembly is much less efficient and after translation of the first uORF, the complex is not reassembled until the 40-S ribosomal subunit has reached the actual start site of the GCN4 protein (Hinnebusch, 1997). Thus, high levels of GCN4 protein synthesis accumulate only in amino acid limiting conditions.

Microarray analysis of a wild type yeast strain after histidine deprivation demonstrated approximately 1000 genes to be transcriptional upregulated when compared to a non-starved

control strain (Natarajan et al., 2001). From these 1000 genes, transcriptional induction of 539 genes was directly dependent on GCN4 as demonstrated using GCN4 overexpressing and knockout strains. In contrast, approximately the same number of genes required GCN4 for repression of their transcriptional activation. Genes regulated by GCN4 include not only amino acid biosynthetic genes, but also vitamin and purine biosynthetic genes, peroxisomal genes, protein kinases and phosphatases, transcription factors, ribosomal proteins, and glycogen homeostasis genes. These results led Natarajan et al. to designate GCN4 as a "master regulator of gene expression" (Natarajan et al., 2001).

Regulation in Mammalian Systems

In the mammalian system, an activating transcription factor, ATF4, is considered the functional counterpart to GCN4, and was recently determined to be translationally regulated in a manner similar to GCN4 (Lu et al., 2004; Vattem and Wek, 2004). Other studies in the mammalian system have demonstrated a GCN2-peIF2α stress inducible signaling cascade that mirrors its yeast counterpart (Harding et al., 2000). Micrroarray studies have further characterized that ATF4 is responsible for increased expression of many genes, although the whole genome has not been screened (Harding et al., 2003). eIF2α can be phosphorylated by multiple mammalian kinases depending on the initial stress signal, but in yeast GCN2 is the eIF2α kinase. Determining which ATF4 inducible genes are regulated specifically by amino acid deprivation will be useful for characterization of the mammalian AAR pathway.

In mammalian cell systems, specific amino acid regulated genes have been extensively characterized by functional studies involving deletion/mutagenesis of cis-elements, *in vitro* binding by electromobility shift analysis (EMSA), and *in vivo* binding by chromatin immunoprecipitation (ChIP). All of these genes exhibited activation by ATF4. One of the first amino acid responsive genes to be well characterized was asparagine synthetase, ASNS, which is

responsible for the conversion of aspartate and glutamine into asparagine and glutamate (Richards and Kilberg, 2006). The promoter region of the ASNS gene contains two genomic sites which are necessary for gene induction by amino acid regulation. The first site is at –68 to –60 nts upstream from the transcription start site, and contains the sequence 5'-TGATGAAAC-3' (Nutrient Sensing Response Element, NSRE1), whereas the second site at –48 to –43 nts contains the sequence 5'-GTTACA-3' (NSRE2) (Barbosa-Tessmann et al., 1999; Guerrini et al., 1993). These sequences have been termed nutrient-sensing response elements, instead of amino acid response elements (AAREs), because they mediate regulation of the ASNS gene by multiple nutrient signaling pathways. It is of interest that the first site is considered a C/EBP-ATF composite binding site, containing half the consensus sequence for activating transcription factor (ATF) binding proteins (TGATG) and another half for CCAAT/enhancer-binding protein (C/EBP) binding proteins (nAAn) (Fawcett et al., 1999).

Like the ASNS NSRE1, all mammalian AARE sequences identified so far are also C/EBP-ATF composite sites. Both ATF and C/EBP proteins are DNA-binding bZIP proteins which are known to homo- and heterodimerize with other group members (Hai and Curran, 1991). The DNA-binding site for ATF proteins was identified as "TGACGT (C/A) (G/A)," (Ameri and Harris, 2008), similar to the first half of the AARE consensus sequence, while the C/EBP consensus binding sequence was originally identified as the palindrome "ATTGCGCAAT," (Johnson, 1993), similar to the second half of the AARE consensus sequence. Although the ATF name for its family members implies these factors activate transcription, studies have established that some of these members, particularly the protein ATF3, actually repress transcription (Wolfgang et al., 1997; Arai et al., 2006; Chen et al., 2004). Numerous studies have demonstrated that modification of ATF and C/EBP proteins (i.e., phosphorylation, etc.) can

affect how these proteins regulate transcription through DNA binding (Averous et al., 2004; Lee and Kim, 2006; Yang et al., 2004). Amino acid limitation regulates the specific ATF and C/EBP family members ATF3, ATF4, and C/EBPβ at the transcriptional level (Pan et al., 2007; Chen and Kilberg, 2006), ATF4 at the translational level (Vattem and Wek, 2004), and causes phosphorylation of the proteins ATF2 and C/EBPβ (Averous et al., 2004; Thiaville et al., 2008a).

It has been shown by both electromobiliity shift assay (EMSA) and chromatin immunoprecipitation (ChIP) analysis that the ASNS NSRE1 binds the transcription factors ATF4, ATF3, and C/EBPβ (Chen et al., 2004; Siu et al., 2002; Siu et al., 2001). This region of the gene also exhibited increased binding of RNA Polymerase II (Pol II) and changes in histone acetylation in response to amino acid limitation. These data were compiled to develop a working model for the transcriptional regulation of ASNS (Figure 1-3) (Chen et al., 2004). During Phase I of this model, 0–4 h after amino acid decline, ATF4 binding rapidly increases at the ASNS promoter and histone acetylation increases due to recruitment of a yet unidentified histone acetyltransferases (HAT) thus, ultimately leading to binding of general transcription factors and Pol II (Chen et al., 2004). During Phase II in which amino acid deprivation continues (4-12 hours), the rate of transcription declines. In parallel, ATF3 and C/EBPβ binding at the ASNS promoter increase whereas ATF4 binding decreases. Therefore, Phase I and ATF4 binding is associated with active gene transcription, whereas Phase II and ATF3-C/EBPβ binding is associated with a decrease in gene transcription. It remains to be determined if this model will hold true for all amino acid regulated genes. Data related to this question will be presented in later chapters.

During the analysis of ASNS, two other genes, C/EBPβ homologous protein (CHOP) and SNAT2, were determined to contain an AARE element, with the core sequence of 5'-

TGATGCAAT-3' (Bruhat et al., 2000; Palii et al., 2004), two nucleotides different than NSRE1 (5'-TGATGCAAC-3'). Whereas the AARE for CHOP resides within the promoter region (-302 to -310), the SNAT2 AARE is surprisingly located within the first intron of the gene (+709 to +717). Two other highly conserved genomic sequence elements are also located in close proximity to the SNAT2 AARE. A 9 nt region containing a CAAT motif, therefore, named the CAAT box, is located upstream of the SNAT2 AARE, and another 9 nt region that is purine-rich, the PuR box, is located downstream of the AARE (Figure 1-4) (Palii et al., 2004). All of these elements are highly conserved among mammalian species. Functional assays have demonstrated that the AARE is absolutely required for response to amino acid limitation, whereas deletion of the CAAT-box only decreased induction by approximately 40% (Palii et al., 2004). Mutagenesis of the PuR box had no effect on regulated transcription, but did induce the basal (amino acid fed) rate significantly, and therefore, may act as a repressor site.

SNAT2 amino acid regulation was contrasted to that of ASNS and it was determined that there are fundamental molecular differences between these two genes' adaptive response (Bain et al., 2002). In response to amino acid limitation, it was reported that the SNAT2 mRNA increase is independent of protein synthesis, whereas ASNS mRNA induction is completely dependent on protein synthesis. The SNAT2 gene is not induced by glucose starvation, whereas the ASNS gene has repeatedly been shown to display induction in response to glucose limitation. SNAT2 mRNA induction is highest when the cellular conditions are depleted of all twenty amino acids, whereas ASNS mRNA induction increases the greatest in response to limitation of a single amino acid. These essential differences between ASNS and SNAT2 transcriptional regulation, as well as the different AARE sequences and genomic locations with reference to the promoter,

may be related to a different composition or temporal order of transcription factor binding to each gene. Data related to this issue will also be presented in this report.

Recent functional assays and EMSA analysis have demonstrated that the SNAT2 AARE is regulated by ATF and C/EBP transcription factors (Palii et al., 2006). Individual over-expression of six different ATF transcription factor family members in conjunction with a reporter construct containing the SNAT2 promoter and AARE (–512/+770) plus the luciferase reporter gene demonstrated that reporter gene activity increased significantly in both amino acid complete and amino acid limiting conditions only when ATF4 was overexpressed. Under amino acid starvation conditions, overexpressed ATF2 showed only a slight activity increase, while ATF3-full length repressed gene activity. The reporter construct was also used in conjunction with overexpression of either C/EBPα or C/EBPβ transcription factors, which both increased reporter gene activity significantly in both MEM and amino acid limiting conditions. Dr. Palii also used EMSA analysis to demonstrate that the transcription factors ATF4, C/EBPα, and C/EBPβ bind the SNAT2 AARE. Initial EMSA investigations established that three specific protein complexes bound the SNAT2 AARE, and that these complexes were induced upon amino acid limitation. Through EMSA competition and supershift analysis, C/EBPα and C/EBPβ demonstrated specific binding to the SNAT2 AARE in both fed and starved conditions, whereas ATF4 only bound under amino acid limiting conditions. This transcription factor specific analysis of the SNAT2 AARE *in vitro* allowed for further characterization of these specific transcription factors' regulation of SNAT2 *in vivo,* as described below.

Regulation in Whole Animal Models

Adaptive regulation in animal models has been demonstrated at both the behavioral and molecular levels in rats. Rats provided a diet limiting in any essential amino acid will detect and reject this diet well before they are satiated, based on comparison control feeding studies

(Koehnle et al., 2004). Though the rejection response was fast, 90% of rats stopped eating the deficient diet within 20 min, it was not fast enough to be based on taste alone (Gietzen et al., 2007). Further studies demonstrated that a specific region of the brain, the anterior piriform cortex (APC), was responsible for the rejected dietary response through induction of the GCN2-peIF2α signaling pathway (Hao et al., 2005). This observation was elegantly demonstrated by the fact that mice with a brain specific deletion of GCN2 do not reject an amino acid deficient diet, because they can no longer detect the deficiency (Gietzen et al., 2007)

Given that the AAR signaling pathway is induced *in vivo*, induction of its downstream target genes has also been demonstrated to occur *in vivo*. Microarray analysis of mRNA from liver tissue of rats fed a protein deficient diet showed differential regulation of 281 genes when compared to control (Endo et al., 2002). Interestingly, two genes characterized extensively in mammalian cell culture systems, ASNS and CHOP, were demonstrated to be upregulated by 6.9 and 2.5 fold, respectively. Other studies using liver from rats fed a protein limiting diet have demonstrated that ATF4 protein increases *in vivo* as well as mRNA levels for SNAT2 and C/EBPβ (Thiaville et al., 2008a). Overall, the amino acid responsive pathway is an important biologically relevant mechanism for coping with cellular stress due to protein or amino acid limitation.

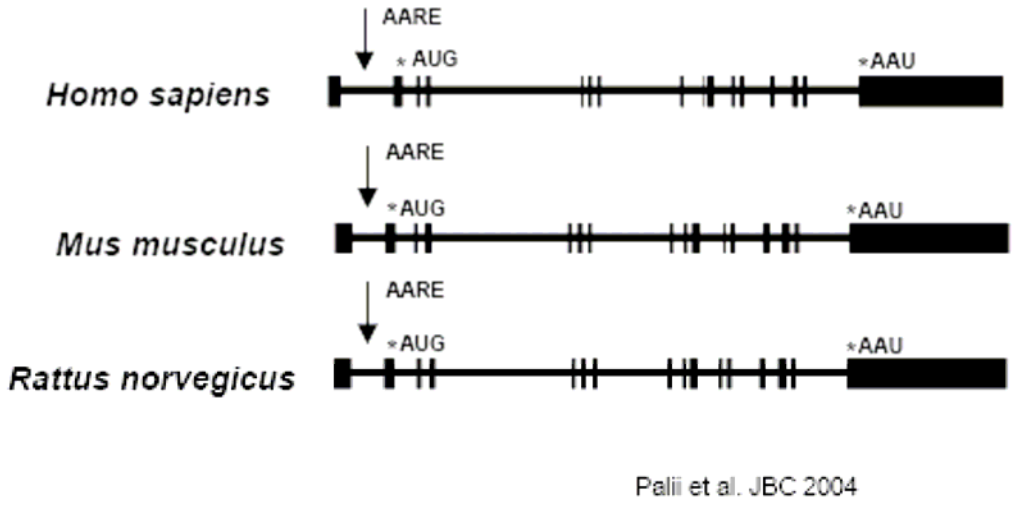

Figure 1-1. Conserved Structure of the SNAT2 gene. Depicted is an alignment of the SNAT2 chromosome 12 genomic region from human (*Homo sapiens*), mouse (*Mus musculus),* and rat (*Rattus norvegicus*) species. The figure was taken from Palii et al JBC 2004.

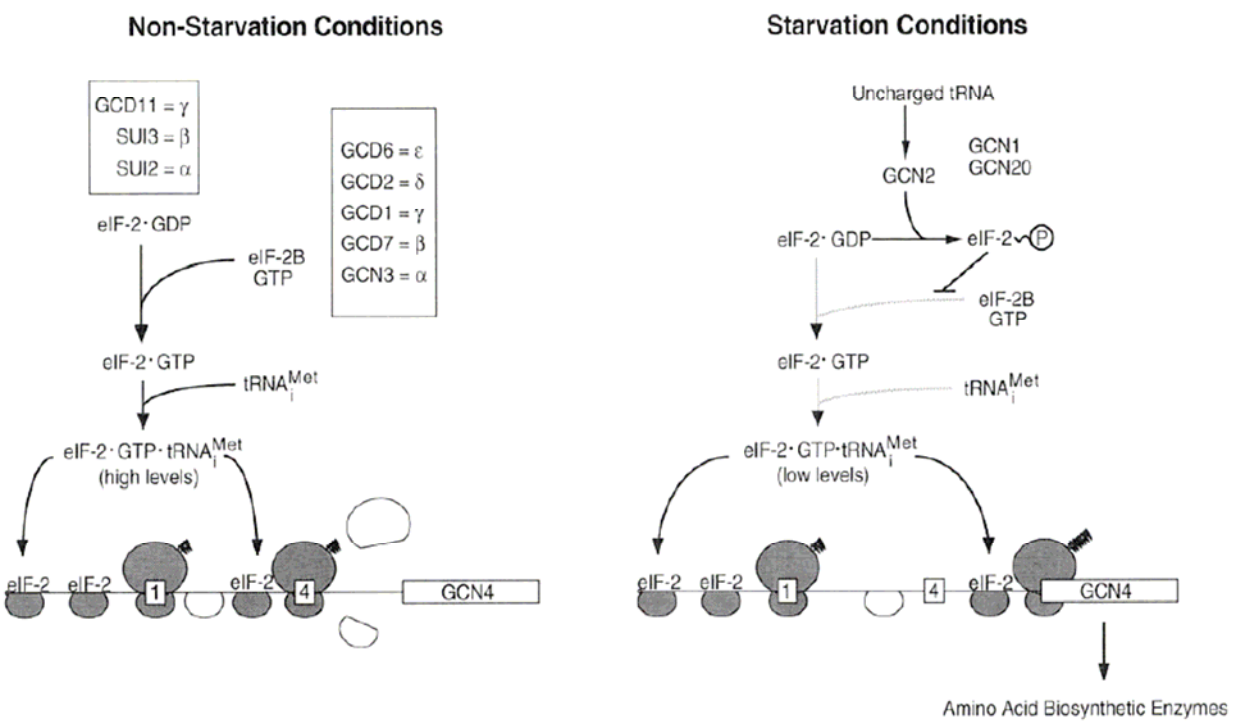

Figure 1-2. A model for GCN4 translational control in *Saccharomyces cerevisiae* by the mechanism of eIF2α phosphorylation. The model depicts translational control of the GCN4 gene in both complete and amino acid starvation conditions. The α, β, and γ subunits of the eIF2 complex are encoded by yeast genes SUI2, SUI3, and GCD11, respectively. The subunits of eIF2B are encoded by their boxed yeast genomic names. 40 S ribosomal subunits are represented as small circles and 60 S ribosomal subunits are represented as large circles. When these two circles are together, this graphic represents the 80 S ribosomal subunit. Boxes labeled 1 and 4 represent uORFs one and four on the GCN4 mRNA. See text of Chapter 1 for further details. The figure was taken from Hinnebusch JBC 1997.

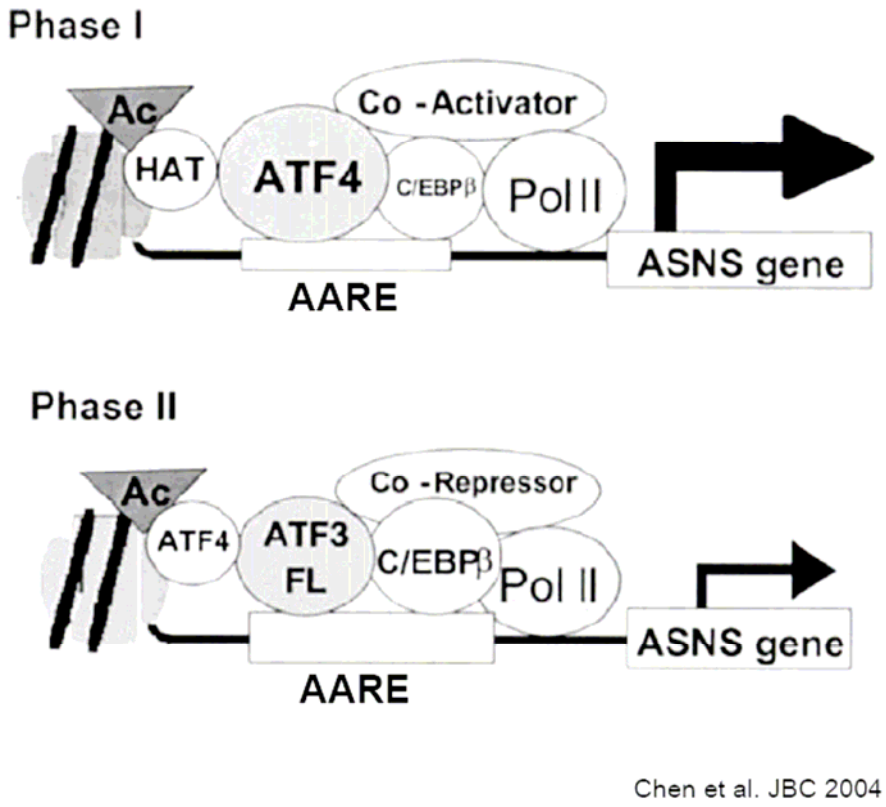

Figure 1-3. A model for transcriptional regulation of the ASNS gene by amino acid limitation. The figure demonstrates a two-phased model of amino acid regulation of the ASNS gene through its AARE element. During Phase I, the activation phase, ATF4 binding increases at the AARE and gene transcription increases. During Phase II, the repression phase, ATF3-FL and C/EBPβ binding increases at AARE while ATF4 binding is diminished, thus, repressing transcription of the ASNS gene. This figure was adapted from Chen et al. JBC 2004.

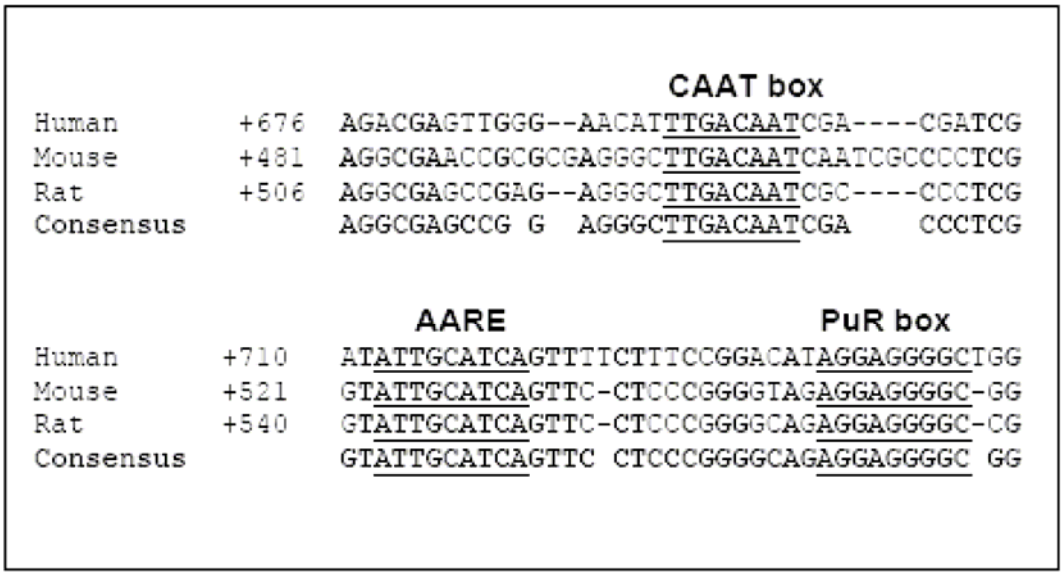

Figure 1-4. Characterization of the SNAT2 intronic enhancer region. The SNAT2 intron 1 genomic region from human (*Homo sapiens*), mouse (*Mus musculus),* and rat (*Rattus norvegicus*) species was aligned to determine the species conservation for the CAAT box, AARE, and PuR box elements. This chart also depicts the 9 bp sequence for each element. These data were taken from Palii et al. JBC 2004.

CHAPTER 2
MATERIALS AND METHODS

Cell Culture

All cell types were maintained at 37ºC in an atmosphere of 5% CO_2 and 95% air. HepG2 human hepatoma cells were cultured in modified Eagle's MEM (minimal essential medium; pH 7.4) (Mediatech, Herndon, VA), supplemented with 1x non-essential amino acids, 2 mM glutamine, 100 μg/mL streptomycin sulfate, 100 units/mL penicillin G, 0.25 μg/mL amphotericin B and 10% (v/v) FBS (fetal bovine serum). MCF7 human mammary adenocarcinoma cells, human embryonic kidney (HEK) 293T cells, and all mouse embryonic fibroblasts (MEF) from wild type, GCN2 knockout mice (Drs. Heather Harding and David Ron), C/EBPβ knockout mice (Dr. Peter Johnson), ATF4 knockout mice (Dr. Tim Townes), eIF2α-S/S knockout, and eIF2α-A/A (S51A) knock-in mice (Dr. Randal Kaufman) were cultured in Dulbecco's modified Eagle's medium (DMEM) with glutamine (Mediatech), supplemented with 1X non-essential amino acids, 10% fetal bovine serum, 100 μg/mL streptomycin sulfate, 100 units/mL penicillin G, and 0.25 μg/mL amphotericin B.

For nutrient deprivation experiments, all cells were given fresh medium and serum 12 h prior to initiating treatment to ensure that no nutrient deprivation took place prior to the start of experimental incubations. Nutrient deprivation was performed by either treating cells with fresh medium lacking the amino acid histidine and containing 10% (v/v) dialyzed FBS, or by culturing cells in the presence of 2 mM histidinol. HisOH competitively blocks binding of histidine to its uncharged tRNA and therefore generates induction of the AAR pathway by causing an increased pool of cellular uncharged tRNA (Hansen et al., 1972). For these experiments, control cells were incubated in the appropriate amino acid complete medium. For amino acid deprivation in the presence of kinase inhibitors, the following inhibitors were purchased from Sigma-Aldrich and

diluted in DMSO to be used at the respective final concentrations: 50 μM PD98059 (MEK), 10 μM U0126 (MEK), 10 μM SB203580 (p38), and 20 μM SB600125 (JNK). All cell lines were pre-treated with the specified inhibitor in complete medium conditions for 1 h, and then incubated with inhibitor in either complete medium or nutrient deprivation medium. Cells not treated with inhibitor but incubated in either complete or nutrient deprivation medium were also used as controls.

For induction of estrogen responsive genes, MCF7 cells were deprived of most hormones for 48 h before being treated by addition of β-estradiol (E2) to the culture medium. To remove all hormones, MCF7 cells were washed twice in phosphate-buffered saline (PBS), (137 mM sodium chloride, 2.7 mM potassium chloride, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4), and incubated in DMEM lacking phenol red (Gibco) with 10% (v/v) charcoal-dextran treated serum (Gibco). β-estradiol was purchased from Sigma-Aldrich. An initial stock solution was made by dissolving 5 mg of β-estradiol in 1 mL of one hundred percent ethanol under slight heating (5 min in a 37ºC H_2O bath) to reach solubility. This stock was diluted further in one hundred percent ethanol and then added to the culture medium to reach a final concentration of 100 nM. An equal volume of ethanol was also added to the control cells as vehicle and the final percent was 0.027.

RNA Isolation and Real Time Quantitative PCR (RT-qPCR)

Total mRNA was isolated from all cell lines using the Qiagen RNeasy kit with optional on-column DNase treatment to eliminate any trace amounts of DNA (Qiagen). RNA concentrations were measured by the optical density using the 260 nm spectrophotometer reading, and then all RNA samples were diluted in H_2O to a final concentration of 20 ng/μL. To measure either the steady state mRNA or transcription activity of all genes, real-time quantitative PCR (RT-qPCR)

was performed using the DNA Engine Opticon 2 system (Bio-Rad) and SYBR Green chemistry (ABI). For quantification of real-time PCR data, a relative standard curve method was used as follows. In order to generate a standard curve, RNA samples from 8 h starved extracts were pooled and diluted to a concentration of 400 ng/5 μL. Serial dilutions of one-half concentration were performed starting with the 400 ng sample to give six tubes with final concentrations of 400 ng, 200 ng, 100 ng, 50 ng, 25 ng, and 12.5 ng (all per 5 μL volume). This type of standard was made independently for each cell line used. For individual RT-qPCR reactions, 5 μL of both standards and samples were run in duplicate wells to ensure accuracy. The master mix used for all RT-qPCR experiments consists of 12.5 μL 2 X SYBR Green PCR Master Mix (ABI 4309155), 0.125 μL MultiScribe Reverse Transcriptase (ABI 4311235), 0.125 μL RNase Inhibitor (ABI N808-0119), 1.25 μL sense primer (5 μM), 1.25 μL antisense primer (5 μM), and 4.75 μl H_2O for a total volume of 20 μL. The final reaction volume per well is 25 μL. RNA reaction mixtures were incubated at 48^{o}C for 30 min followed by 95ºC for 15 min and amplification of 35 cycles at 95ºC for 15 s, and then XºC for 60 s. Table 2-1 illustrates all the primer sets used and the specific annealing temperature (XºC) for each primer set. To establish that a single product was amplified during the reaction, melting curves were generated for each reaction by a stepwise increase of the temperature from 55 to 95ºC and measurements were taken at every degree change. Reactions were also run without reverse transcriptase to ensure that there was no DNA amplification. The primers used for mRNA amplification are generally located inside one exon or amplify two adjacent exons. The primers used to measure transcription activity span an exon-intron junction and assay for hnRNA amplification based on a method described by Lipson and Baserga (Lipson and Baserga, 1989). To quantify all data, RT-qPCR

was done in duplicate with samples from at least three independent experiments and data are graphed as the means ± the standard error of the means.

Chromatin Immunoprecipitation (ChIP) and Analysis by Quantitative Real Time PCR (qPCR)

ChIP analysis was performed according to a modified protocol of Upstate Biotechnology, Inc. For all experiments, cells were seeded into 150-mm dishes and grown for 36 h prior to any experimental induced treatments. HepG2 cells were seeded at 1.5 x 10^7/150-mm dish in complete MEM with 3 dishes per treatment condition. C/EBPβ wild type and knock-out cells were seeded at 6 x 10^6/150-mm dish in complete DMEM with 5 dishes per treatment condition. MCF7 cells were seeded at 5 x 10^6/150-mm dish in complete DMEM or DMEM lacking phenol red with 10% (v/v) charcoal-dextran treated serum with 4 dishes per treatment condition. All cells were transferred to fresh medium 12 h before treatment. Cells were then treated with reagents and for the specific time period indicated in each figure. After treatment, protein-DNA was cross-linked by adding formaldehyde directly to the culture medium to a final concentration of 1% and then stopped 10 min later by adding 2 M glycine to a final concentration of 0.125 M. Cross-linked chromatin was sheared to 500-800 bp in length by sonication using a Sonic Dismembrator (Model 60, Fisher Scientific Co.) for five bursts of 40 s at power 10 with 2-min cooling on ice between each burst. Total sonicated chromatin was diluted into aliquots equivalent to approximately 1 x 10^7 cells, and these extracts were incubated with 2-10 μg of primary antibody overnight at 4°C. A rabbit anti-chicken IgG was used as the nonspecific antibody control. Table 2-2 lists of all ChIP antibodies used with relevant data. Either protein A-Sepharose beads (Amersham Biosciences) or protein G-Sepharose beads (Zymed) were used to precipitate the antibody-bound complexes. All immunoprecipitations with antibodies produced from goat serum were precipitated with protein G-Sepharose beads while antibodies produced

from rabbit serum were precipitated with either protein A-Sepharose or protein G-Sepharose beads. Beads were incubated in a blocking solution (3% bovine serum albumin, 0.05% sodium azide, and protease inhibitor in TE pH 8.0) as a 50:50 bead to solution slurry overnight at 4°C. After incubation, 60 µL of the bead slurry was added to each chromatin-antibody aliquot and incubated at 4°C with rotation for 2-3 h. Antibody-bead complexes were pelleted and resuspended in a series of wash buffers, each incubated for a 5 min rotation at 4°C, in a volume of 1 mL. Wash buffers, in order of washes, were: low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl pH 8.0); high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris-HCl pH 8.0); LiCl buffer (0.25 M LiCl, 1% NP 40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.0); and TE buffer (1 mM EDTA, 10 mM Tris-HCl pH 8.0). After the final wash, antibody-bead complexes were resuspended in 65°C elution buffer (1% SDS, 0.1 M $NaHCO_3$), and incubated at 37°C for 30 min with vigorous shaking. The DNA fragments in the immunoprecipitated complex were released by reversing the cross-linking at 65°C for either 5 h or overnight and purified using a QIAquick PCR purification kit (Qiagen) and eluting all samples in 100 µL TE (Ambion). Purified, immunoprecipitated DNA was routinely visualized by ethidium bromide staining after gel electrophoresis to ensure the average DNA fragment size was 500-800 bp.

To measure the amount of DNA precipitated by the ChIP procedure, quantitative PCR (qPCR) was performed using the DNA Engine Opticon 2 system (Bio-Rad) and SYBR Green chemistry. For quantification of qPCR data, a relative standard curve method was used. To generate a standard curve, total purified DNA from input ChIP extracts was pooled and diluted by serial dilutions to either one-fourth or one-half concentrations to give six tubes with final concentrations of 1/4, 1/16, 1/64, 1/128, 1/256, and 1/512 dilutions (all per 5 µL volume). This

type of standard was made independently for each cell line used. For individual qPCR reactions, 5 µL of both standards and samples were run in duplicate wells to ensure accuracy. The master mix used for all qPCR experiments consisted of 12.5 µL 2 X SYBR Green PCR Master Mix (ABI 4309155), 1.25 µL sense primer (5 µM), 1.25 µL antisense primer (5 µM), and 5 µL H_2O (Ambion) for a total volume of 20 µL. The final reaction volume per well was 25 µL. qPCR reaction mixtures were incubated at 50ºC for 2 min followed by 95ºC for 15 min and amplification of 35 cycles at 95ºC for 15 s, and then XºC for 60 s. Table 2-3 illustrates all the primers sets used and the specific annealing temperature (XºC) for each primer set. To establish that a single product was amplified during the reaction, melting curves were generated for each reaction by a stepwise increase of the temperature from 55 to 95ºC and measurements were taken at every degree change. The results are expressed as the ratio to input DNA. Samples from at least three independent immunoprecipitations were analyzed, and the means ± sem of the means between conditions were graphed.

Protein Isolation and Immunoblotting

For whole cell protein extracts, untreated and treated adherent cells were washed with 1X PBS and collected in sample dilution buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% Bromophenol Blue, 5% 2-mercaptoethanol) containing a 1X concentration of protease and phosphatase inhibitors (Roche). Protein concentrations were determined using a modified Lowry method (Markwell et al., 1978). For immunoblotting, 30 µg of protein/lane was loaded onto Precast Tris-HCl polyacrylamide gels (Bio-Rad), and then electrotransferred onto polyvinylidene difluoride membranes (Bio-Rad). Membranes were stained with Fast Green FCF (Sigma) to ensure equal loading by incubating for 1 to 2 min in Fast Green stain solution (0.1% Fast Green FCF, 50% methanol, 10% acetic acid) followed by repeated 5 min rinses in Destain

Solution (50% methanol, 10% acetic acid). Stained membranes were blocked with 5-10% blocking solution (5-10% (w/v) Carnation non-fat dry milk, 30 mM Tris-Base pH 7.5, 0.1% (v/v) Tween-20, and 200 mM NaCl) for 1 h at room temperature depending on the antibody to be used for probing. Each primary antibody was used at a dilution between 1:200 and 1:1000 (v/v) in 5-10% blocking solution, and incubated with membranes overnight at 4ºC with rotation. The blots were washed 5 x 5 min in 5-10% blocking solution and then incubated with the appropriate secondary antibody (rabbit, goat, etc.) conjugated to horseradish perioxidase at a 1:5,000 to 1:20,000 dilution (v/v) for 1-2 h at room temperature with rotation. The blots were then washed for 5 x 5 min in 5-10% blocking solution and 2 x 5 min in freshly made TBS/Tween (30 mM Tris-Base, 0.1% Tween-20, and 200 mM NaCl pH 7.5). The bound secondary antibody was detected using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech) and exposing the blot to Biomax MR film (Kodak). For quantification of immunoblots, the program UN-SCAN-IT gel Version 6.1 was used. Quantification was performed on at least three blots, and was accomplished by selecting the sample band area and normalizing to a control band area (normally either time zero or two hour MEM) for each individual blot. The background area was also subtracted from each individual blot. Values obtain for individual blots were then averaged and graphed with the standard error of the means.

Transient Transfection and Luciferase Assay

The SNAT2 –512/ +770-Firefly luciferase plasmid was generated in the pGL3-basic plasmid (Promega) by placing nt -512/ +770 of the human SNAT2 gene in front of the Firefly luciferase reporter gene (courtesy of Dr. Stela Palii). The ASNS -173/ +51-Firefly luciferase plasmid was generated in the pGL3-basic plasmid (Promega) by placing nt -173/ +51 of the human ASNS gene in front of the Firefly luciferase reporter gene (courtesy of Dr. Hong Chen). Luciferase vectors containing either the wild type or mutated mouse ATF4 uORFs upstream of

the luciferase gene were kindly supplied by Dr. R. Wek (Vattem and Wek, 2004). The plasmid containing rat ATF4 cDNA driven by the cytomegalovirus promoter was kindly provided by Dr. J. Alam (He et al., 2001).

For transient transfection, HepG2 cells were seeded in 24-well plates at a density of 1.5 x 10^5 cells per well in MEM and cultured for 16 h. Cells were transfected for 3 h with Superfect transfection reagent (Qiagen) at a ratio of 6:1 (Superfect to DNA) according to the manufacturer's protocol using one μg of the luciferase reporter construct per well. Where noted, 100 ng of the rATF4 construct was co-transfected with the luciferase reporter plasmid. After transfection and a subsequent 24 h recovery in complete MEM, cells were then incubated for an additional 8 h in either fresh MEM or MEM minus histidine. The cells were then rinsed once in PBS and lysed in 100 μL passive lysis buffer (Promega) for 15 min at room temperature with shaking. After exposure to a freeze-thaw cycle (frozen at –80^{o}C), cells were assayed for luciferase activity according to the manufacturer's protocol (Promega), using 100 μL reaction reagent per 20 μL of sample. The data are expressed as the averages ± standard deviation of at least 3 assays, and each experiment was repeated with multiple batches of cells.

Short Interfering RNA (siRNA) Transfection

The following single-targeting sequence human siRNAs were purchased from Dharmacon, Inc.: ATF4 siRNA (# D-005125-07), C/EBPβ siRNA (# D-006423-01), Cyclophilin B siRNA (# D-001136-01), PPP1R15A (GADD34) siRNA (# D-004442-02), siControl Non-Targeting siRNA #2 (# D-001210-02), and siGLO Red (# D-001630-02). All transfections were performed with the DharmaFECT 4 transfection reagent. For starvation and inhibitor experiments with HepG2 cells, the cells were seeded in 12-well plates at a density of 2.5 x 10^5 cells per well in MEM and grown for 16 h. Transfection was performed according to Dharmacon's instructions using 3 μL of DharmaFECT 4 and 100 nM per well final siRNA concentration for all siRNAs

except C/EBPβ siRNA for which 80 nM final concentration was used. HepG2 cells were treated with transfection reagent for 24 h then rinsed with PBS, given fresh MEM, and cultured another 24 h. Medium was then removed and replaced with control MEM, starvation media, or media plus inhibitor. RNA or protein was isolated at specific times and analyzed by RT-qPCR or immunoblotting, respectively.

For siRNA-ChIP experiments, HepG2 cells were seeded into 150 mm dishes at a density of 1.0×10^7 cells per dish in MEM and transfection was performed using 30 μL of DharmaFECT 4 and 100 nM per plate final siRNA concentration. All other transfection and incubation procedures were the same as described above, and the ChIP protocol was performed as previously described.

For siRNA-luciferase experiments, HepG2 cells were seeded into 24 well plates at a density of 0.8×10^5 cells per well in MEM, and 12-16 h later siRNA transfection was performed for 24 h using 1.5 μL of DharmaFECT 4 and 100 nM per plate final siRNA concentration. The following day, cells were transfected for 3 h with SuperFect-luciferase plasmid cocktail according to the transient transfection procedure described in the previous section. The next day, 48 h after initial siRNA treatment, cells were treated in either MEM or MEM minus histidine for 8 h, lysed, and analyzed according to the Luciferase Assay protocol described in the previous section.

For knockdown of Mediator subunits, the following SMARTpool multi-targeting sequence human siRNAs were purchased from Dharmacon, Inc.: CRSP2 (MED14) siRNA (# M-011928-00), CRSP3 (MED23) siRNA (# M-013220-00), CRSP6 (MED17) siRNA (# M-006312-01), CRSP9 (MED7) siRNA (# M-017313-00), MED6 siRNA (# DM-019963-00), MED31 siRNA (# M-027282-01), PPARBP (MED1) siRNA (# M-004126-03), and siControl Non-Targeting

siRNA Pool (# D-001210-02). All transfections were performed with the DharmaFECT 4 transfection reagent. For starvation experiments with MCF7 cells, the cells were seeded in 6-well plates at a density of 3.0 x 10^5 cells per well in DMEM and grown for 16 h. For β-estradiol experiments with MCF7 cells, the cells were seeded in 6-well plates at a density of 5.0 x 10^5 cells per well in DMEM minus phenol red with 10% charcoal stripped FBS and grown for 16 h. For both conditions, transfection was performed according to Dharmacon's instructions using 3 μL of DharmaFECT 4 and 100 nM per well final siRNA concentration. MCF7 cells were treated with transfection reagent for 24 h then rinsed with PBS, given fresh medium, and cultured another 24 h. Medium was then removed and replaced with either the appropriate control DMEM, starvation medium, or medium plus β-estradiol. RNA or protein was isolated at specific times and analyzed by RT-qPCR or immunoblotting, respectively.

Table 2-1. Primer sets and annealing temperatures for RT-qPCR

Primer Pair	Gene Region	Primer Sequences	Annealing Temperature (^{o}C)
Human			
ASNS mRNA, human	+13723 to +13784 (exon 7)	FP 5′-GCAGCTGAAAGAAGCCCAAGT-3′ RP 5′-TGTCTTCCATGCCAATTGCA-3′	60
ASNS transcription activity, human	+19577 to +19653 (intron 12 to exon 13)	FP 5′-CCTGCCATTTTAAGCCATTTTGC-3′ RP 5′-TGGGCTGCATTTGCCATCATT-3′	58
ATF4 mRNA, human	+1154 to +1221	FP 5′-TGAAGGAGTTCGACTTGGATGCC-3′ RP 5′-CAGAAGGTCATCTGGCATGGTTTC-3′	60
BIP transcription activity, human	+2661 to +2759 (exon 6 to intron 6)	FP 5′-CCGTGGCATAAACCCAGATGAA-3′ RP 5′-TGCTGCGATGATGACCTACCTGTA-3′	60
C/EBPβ mRNA, human	+967 to +1054	FP 5′-CAGGTCAAGAGCAAGGCCAAGA-3′ RP 5′-TGCGCACGGCGATGTTGT-3′	60
Cyclophilin B mRNA, human	+502 to +575	FP 5′-TGGCACAGGAGGAAAGAGCATCTA-3′ RP 5′-CAGGCCCGTAGTGCTTCAGTTTG-3′	60
ERK1 mRNA, human	+220 to +311	FP 5′-GCGCTACACGCAGTTGCAGTACA-3′ RP 5′-TGATGGCCACGCGAGTCTTG -3′	60
ERK2 mRNA, human	+1212 to +1311	FP 5′-CGCCGAAGCACCATTCAAGTT-3′ RP 5′-TCCTGGCTGGAATCTAGCAGTCTCT-3′	60
GADD34 mRNA, human	+1372 to +1472	FP 5′-CACTCCTGCTACAGGTGTCT-3′ RP 5′-TCGGCTGATCCTGTATCA-3′	60
GAPDH mRNA, human	+1382 to +1442	FP 5′-TTGGTATCGTGGAAGGACTC-3′ RP 5′-ACAGTCTTCTGGGTGGCAGT-3′	60
MED1 mRNA, human	+1288 to +1355	FP 5′-CTTCCTGGTCAGCAGCACTGCTAT-3′ RP 5′-TGTAGACTTCGGCCATCTGGAAGAG-3′	59.1
MED6 mRNA, human	+346 to +416	FP 5′-TATCAGGCACCAGACTTGGGATCA-3′ RP 5′-GCTGACTGAATACCATGCACTGCA-3′	58
MED7 mRNA, human	+140 to +235	FP 5′-TCAAGAAGGCTTAGCTCCCAAG-3′ RP 5′-GGGCGGATGATAAGATCA-3′	60.4
MED14 mRNA, human	+528 to +625	FP 5′-GCCTCGTTAGCTAGAGATGCT-3′ RP 5′-AGCCGTGGGTAAGATCCA-3′	60.4
MED17 mRNA, human	+1818 to +1909	FP 5′-TGTCACAGCACCAGGTACA-3′ RP 5′-AGGTCCAAGTCCCACATG-3′	59.1
MED23 mRNA, human	+566 to +658	FP 5′-ACAAGGGTGTTCGAGATC-3′ RP 5′-CCAGAAGCTGCTGTACAA-3′	55
MED31 mRNA, human	+357 to +445	FP 5′-TCCGAAAGGAGCTGGTGAA-3′ RP 5′-CATCCGCTTCCGGGAATA-3′	59.1

Table 2-1. Continued

Primer Pair	Gene Region	Primer Sequences	Annealing Temperature (^{o}C)
pS2 transcription activity, human	+3076 to +3146 (intron 1 to exon 2)	FP 5′-CATCAAGGAATTCAGCCCACAACT-3′ RP 5′-GGGCCACTGTACACGTCTCTGAAA -3′	60
SNAT2 mRNA, human	+8243 to +8319 (exon 10)	FP 5′-GTGTCCTGTGGAAGCTGCTTTGA-3′ RP 5′-CAGGTACAAGAGCTGTTGGCTGTGT-3′	60
SNAT2 transcription activity, human	+2205 to +2299 (exon 4 to intron 4)	FP 5′-GCAGTGGAATCCTTGGGCTTTC-3′ RP 5′-CCCTGCATGGCAGACTCACTACTTA-3′	63
VEGF mRNA, human	+4 to +91	FP 5′-AGCTCCAGAGAGAAGTCGAGGAAGA-3′ RP 5′-TCACTTTGCCCCTGTCGCTTT-3′	60
Mouse			
ASNS mRNA, mouse	+10892 to +11197 (exon 4 to exon 5)	FP 5′-CATGCCATCTATGACAGCGTGGA-3′ RP 5′-CGCAGATTGTTCTTCACGGTCTCT-3′	60.8
ASNS transcription rate, mouse	+11125 to +11198 (intron 4 to exon 5)	FP 5′-CTTGTTTGTGGCGGTTCCATTTAC-3′ RP 5′-ACGCAGATTGTTCTTCACGGTCTC-3′	60
GAPDH mRNA, mouse	+738 to +879	FP 5′-GCCTTCCGTGTTCCTACCC-3′ RP 5′-CCTCAGTGTAGCCCAAGATGC-3′	59
SNAT2 mRNA, mouse	+4225 to +4430 (exon 5 to exon 6)	FP 5′-CTCCTCCTCAAGACTGCCAACGA-3′ RP 5′-TTCCAGCCAGACCATACGCCTTA-3′	60
SNAT2 transcription rate, mouse	+7977 to +8049 (exon 13 to intron 13)	FP 5′-GTCACCCTCACGGTCCCAGTAGTTA-3′ RP 5′-GCATACCCATAGCTGTCGCAGAAGT-3′	61

Table 2-2. Antibodies used for ChIP and/or Immunoblotting

Antibody	Catalogue Number	Company	Animal in which antibody was produced
Acetylated H3 (specific for acetylated Lys-9 and Lys-14)	06–599	Millipore (formerly Upstate)	rabbit
Acetylated H4 (recognizes acetylated H4 at Lys-5, -8, -12, and -16)	06-866	Millipore (formerly Upstate)	rabbit
Actin	A2066	Sigma-Aldrich	rabbit
ATF2	sc-187	Santa Cruz Biotechnology, Inc.	rabbit
ATF3	sc-188	Santa Cruz Biotechnology, Inc.	rabbit
ATF4	sc-200	Santa Cruz Biotechnology, Inc.	rabbit
ATF4 (2)	Custom made by Kilberg laboratory	CoCalico Biologicals, Inc.	rabbit
C/EBPα	sc-61	Santa Cruz Biotechnology, Inc.	rabbit
C/EBPβ	sc-150	Santa Cruz Biotechnology, Inc.	rabbit
C/EBPβ phosphorylated at threonine 235	3084	Cell Signaling Technology	rabbit
C/EBPδ	sc-636	Santa Cruz Biotechnology, Inc.	rabbit
C/EBPε	sc-158	Santa Cruz Biotechnology, Inc.	rabbit
c-Maf	sc-10017	Santa Cruz Biotechnology, Inc.	goat
cdk8	sc-5612	Santa Cruz Biotechnology, Inc.	rabbit
eIF2α	9722	Cell Signaling Technology	rabbit
eIF2α phosphorylated at serine 51	9721	Cell Signaling Technology	rabbit
MED1 (TRAP220)	sc-5334	Santa Cruz Biotechnology, Inc.	goat
MED6	sc-9434	Santa Cruz Biotechnology, Inc.	goat
MED23 (CRSP130)	sc-12454	Santa Cruz Biotechnology, Inc.	goat
Normal rabbit IgG (non-specific IgG)	sc-2027	Santa Cruz Biotechnology, Inc.	rabbit
RNA Pol II	sc-899	Santa Cruz Biotechnology, Inc.	rabbit
TAF1/TAFII250 (TFIID)	sc-17134	Santa Cruz Biotechnology, Inc.	goat
TAF9 (TFIID)	sc-1248	Santa Cruz Biotechnology, Inc.	goat
TBP (TFIID)	sc-204	Santa Cruz Biotechnology, Inc.	rabbit
TFIIA-γ	sc-5316	Santa Cruz Biotechnology, Inc.	goat
TFIIB	sc-274	Santa Cruz Biotechnology, Inc.	rabbit
TFIIE	sc-237	Santa Cruz Biotechnology, Inc.	rabbit

Table 2-3. Primer sets and annealing temperatures for qPCR

Primer Pair	Gene Region (bp)	Primer Sequences	Annealing Temperature (^{o}C)
Human			
ASNS coding region, human	+13723 to +13784 (exon 7)	FP 5′-GCAGCTGAAAGAAGCCCAAGT-3′ RP 5′-TGTCTTCCATGCCAATTGCA-3′	60
ASNS promoter/AARE, human	-87 to -22	FP 5′- TGGTTGGTCCTCGCAGGCAT-3′ RP 5′- CGCTTATACCGACCTGGCTCCT-3′	61.4
ATF3 AARE, human	-59 to +35	FP 5′-GCCGCCAGCCTGAGGGCTAT-3′ RP 5′-CGAGAGAAGAGAGCTGTGCA-3′	60
C/EBPβ AARE, human	+1607 to +1671	FP 5′- CGCAACCCACGTGTAACTGTCAG-3′ RP 5′- CAGCAACAAGCCCGTAGGAACA-3′	60
CHOP AARE, human	-216 to -159	FP 5′-AAGCCTCGTGACCCAAAGC-3′ RP 5′-GTCGCTCCCTCTCGCTAG-3′	60
pS2 promoter, human	-167 to -30	FP 5′-CAAGATGACCTCACCACATG-3′ RP 5′-GAGCGTTAGATAACATTTGCC-3′	58
SNAT2 AARE, human	+672 to +745	FP 5′-GGGAAGACGAGTTGGGAACATTTG-3′ RP 5′-CCCTCCTATGTCCGGAAAGAAAAC-3′	60
SNAT2 coding region, human	+1428 to +1495 (exon 2)	FP 5′-GACAAAAGGAGCCCAGCGCTACT-3′ RP 5′-TCGGCCTTCTTCATGCTAAGCA-3′	60
SNAT2 coding region (2), human	+8243 to +8319 (exon 10)	FP 5′-GTGTCCTGTGGAAGCTGCTTTGA-3′ RP 5′-CAGGTACAAGAGCTGTTGGCTGTGT-3′	60
SNAT2 promoter, human	+12 to +108	FP 5′-GCCGCCTTAGAACGCCTTTC-3′ RP 5′-TCCGCCGTGTCAAGGGAA-3′	62
SNAT2 upstream gene region, human	-987 to -882	FP 5′-TGTTTCCTGCAAGGGAGTGASGTCT-3′ RP 5′-GGGACCCTTCGCTGTGGAAA-3′	60
VEGF AARE, human	+1626 to +1705	FP 5′-CTCTGCCCAGTGCTAGGAGGAATT -3′ RP 5′-CAGGGGCTTCTCTCCAGGCTAAA -3′	60
Mouse			
ASNS promoter/AARE, mouse	-151 to -83	FP 5′-TCGGCCCCAGGATGCACGT-3′ RP 5′-TGGCCCGCAGTGCTGACGA-3′	62.5
SNAT2 AARE, mouse	+414 to +505	FP 5′-ATCGGGTCTTGTGCCTCGAAA-3′ RP 5′-ATACCGAGGGGCGATTGATTGT-3′	59.1
SNAT2 promoter, mouse	-117 to -27	FP 5′-GGAGCCCAGGTGCTGTTGATACA-3′ RP 5′-GCTTCCCGAGCGCGAGTTATA-3′	59.9

CHAPTER 3
IDENTIFICATION BY CHROMATIN IMMUNOPRECIPITATION (CHIP) ANALYSIS OF THE TRANSCRIPTION FACTORS THAT BIND THE SNAT2 PROMOTER AND INTRONIC AMINO ACID RESPONSE ELEMENT (AARE)

Introduction

Transcriptional Regulation by General Transcription Factors (GTFs)

Control of gene expression in eukaryotic cells is tightly regulated through transcription of messenger RNA (mRNA), particularly during the event of transcription initiation. Though the RNA Polymerase II (Pol II) holoenzyme complex is responsible for all actual mRNA synthesis, it is unable to efficiently and accurately transcribe a DNA template *in vitro* and *in vivo* without the assistance of other protein complexes (Kornberg, 1996). The precise temporal coordination of binding of these general transcription factor (GTF) complexes to their DNA binding sites and/or to each other during transcription initiation fine tunes regulation of the transcription process. The GTF complexes TFIIB, TFIID, TFIIE, TFIIF, and TFIIH are required for efficient Pol II transcription *in vitro*, while the TFIIA complex aids in enhancing active transcription (Roeder, 1996).

Initiation of transcription begins with the TFIID complex, composed of the TATA box-binding protein (TBP) and ten TAF (TBP-associated factor) proteins, and its recognition of core binding elements in a genomic promoter region (Lagrange et al., 1998). The TBP subunit recognizes and binds to the minor groove of the TATA-box DNA sequence, approximately 25-30 bp upstream of the transcription start site (TSS), causing a sharp bend in the DNA structure. Two other DNA binding elements, the downstream promoter element (DPE) located approximately 30 bp downstream of the TSS and the initiator-binding element (Inr) directly upstream of the TSS, are bound by one or more of the TAF subunits (Lagrange et al., 1998). Other TAF subunits are believed to enhance transcription through various mechanisms such as

histone acetylation and phosphorylation of other TFII complexes. More recently, genome-wide scanning of eukaryotic promoters has revealed that not all actively transcribed genes contain a consensus TATA-box within the proper location of their promoters (Trinklein et al., 2003; Kim et al., 2005). This observation suggests that the TBP subunit may possibly bind other DNA sequences or there exists other mechanisms of regulating the first crucial step in transcription initiation.

After TFIID assembles at the promoter region, its binding is stabilized by association between its TBP subunit and the protein TFIIB. TFIIB's ability to stabilize this large complex is caused by a conformational change in the TFIIB protein upon TFIID complex binding. A subset of promoters also contain a TFIIB recognition element (BRE) located immediately upstream of the TATA-box (Kornberg, 2007). Controversy surrounds the effect of TFIIB binding to the BRE, as it has been demonstrated to both enhance and inhibit transcription, suggesting further specific regulation at certain promoters (Lagrange et al., 1998).

Pol II association with the promoter region occurs only after stabilization of the TFIID and TFIIB complexes on the promoter itself. Given that the role of Pol II is to transcribe DNA in a sequence independent manner, Pol II associates with DNA only through contact with other protein complexes and not with any known consensus sequence. Its initial presence at the promoter region is through an independent interaction with TFIIF, the protein complex that transports Pol II to the promoter region. Although TFIIF brings Pol II in stable contact with the TFIID-TFIIB protein complex, transcription cannot begin until the factors TFIIE and TFIIH are present (Kornberg, 1999). The main role of TFIIE is the recruitment of TFIIH and stimulation of its catalytic abilities, although it can interact with specific transcriptional activator proteins thus allowing for gene specific regulation (Hampsey, 1998). The last GTF recruited to the pre-

initiation complex (PIC), TFIIH contains subunits with both ATP-dependent helicase activity and kinase activity. Thus TFIIH, along with stimulation from TFIIE, allows for Pol II promoter escape by opening the promoter region (helicase activity) and phosphorylating the Pol II protein complex (kinase activity). Once Pol II has moved from the highly regulated phase of transcription initiation, the elongation phase begins from which progressive rounds of mRNA synthesis follow. As their name suggests, GTF-regulated transcription of all eukaryotic mRNA synthesis has been previously portrayed as occurring similarly on all genes. However, recent evidence suggests that through interaction and modification by different protein activator and repressor complexes, GTF-driven transcription can be enhanced or repressed at specific genes due to specific stimuli (i.e., cellular stress) (Li et al., 2007a; Pedersen et al., 2001). New experimental evidence also suggests that specific subunits within a GTF complex are specifically regulated and perform promoter specific tasks (Yu et al., 2008).

Transcriptional Regulation by Histone Acetylation

Although an ordered recruitment of GTFs is necessary for regulated transcription, the GTFs are unable to associate with nucleosome compacted DNA. The nucleosome is a 210 kDa multi-protein complex consisting of a histone octamer around which 147 bp of DNA are wrapped (Luger, 2006). Thorough crystal structure analysis by the Luger laboratory has demonstrated the composition of the histone octamer to be two copies each of the histone proteins H2A, H2B, H3, and H4. Thus, long regions of DNA are compacted by repeated nucleosome wrapping of 147 bp DNA sections separated by 10-60 bp regions of free DNA. Though histone packaging of DNA is physically necessary to contain the entire genome within the cellular nucleus, compacted DNA is not easily transcribed thus designating the nucleosome as a transcription inhibitory complex. To initiate transcription through these regions, a change in the nucleosome architecture of the DNA must occur. Experimental evidence from yeast genetics

has demonstrated that nucleosomes can be either completely removed from a region of DNA or slide up and down a region of DNA (Jessen et al., 2006). This mechanism allows for both exposure of a DNA binding site and open chromatin through which Pol II can transcribe. Specific protein complexes, termed nucleosome-remodeling complexes, are responsible for altering nucleosomes in an ATP-dependent manner (Armstrong, 2007). Assembly and disassembly of the nucleosome complex by histone chaperons is another mechanism for changing chromatin structure. Evidence from crystal structure analysis demonstrates that two H2A-H2B dimers can be removed, independently, from the octamer complex thus leaving an intact H3-H4 tetramer on the DNA (Luger, 2006). These smaller histone protein complexes allow for either active or repressive transcription through post-translational modification of their histone tail regions, a 30-35 non-DNA bound stretch of mostly basic residues at the amino-terminal of each histone protein. An area of current intense research, it has been demonstrated that both lysine and arginine residues on the histone tails are frequently methylated while acetylation occurs only at lysine residues (Peterson and Laniel, 2004; Li et al., 2007b). While histone acetylation is generally associated with active transcription and histone methylation with repressive transcription, there are documented cases where the reverse is true. Likewise, phosphorylation of serine and theronine residues on the histone tails also occurs and is generally associated with specific cellular processes such as DNA repair and mitosis. The effects of these modifications on transcription are even more complex given that residues can be either mono-, di-, or tri-methylated, and that specific combinations of histone modifications can have different effects. Further evidence is necessary to determine which of these modifications are associated with gene specific transcription and which lend to a more general control of global transcription.

Transcriptional Regulation by the Mediator Complex

Though not originally isolated with the GTFs, the Mediator complex has emerged as a second complex believed by many investigators to be required for all Pol II transcription (Takagi and Kornberg, 2006). First identified in *Saccharomyces cerevisiae*, this 32 subunit multiprotein complex was isolated by its interaction with both transcriptional activators and the pre-initiation complex, specifically Pol II (Bjorklund and Kim, 1996; Kim et al., 1994). Evidence for its requirement during transcription initiation *in vivo* was demonstrated by temperature sensitive knockout of its subunits in yeast, specifically MED22 and MED17, causing global repression of all Pol II transcription (Linder et al., 2006). Temperature sensitive mutation of other Mediator subunits in yeast also exhibits milder gene specific effects on transcription regulation that can be either positive or negative with regard to gene expression (Lee and Kim, 1998; Balciunas and Ronne, 1995). Mediator's proposed requirement during transcription initiation is to function as a bridge between distal activator proteins and promoter bound GTFs effectively "mediating" the signal of transcriptional activation to the promoter. Mediator was established to be recruited to and bind preferentially to activators at their DNA elements and only associate with Pol II upon transcription activation (Takagi and Kornberg, 2006). Electron microscopy analysis of the Mediator complex has lead to the grouping of different subunits into either the "head," "middle," or "tail" regions of the three global domains observed (Boube et al., 2002). Through biochemical analysis, the head domain is believed to modulate Pol II function through direct interaction, while the tail domain functions as a "signal-sensor" through interaction with gene specific protein activators. The function of the middle region is less clear, though it may organize signaling between the head and tail domains. Until recently, most information about the Mediator complex was derived from studies using yeast as a model system. Many Mediator-like complexes were isolated in mammalian cells and named for the activator with which they

associated (i.e., cofactor required for Sp1 activation (CRSP), thyroid hormone receptor-associated proteins (TRAP), vitamin D receptor-interacting proteins (DRIP), etc). Recently, the Conaway laboratory used a new technique called multidimensional protein identification technology (Mud-PIT), an immunoprecipitation and mass spectroscopy technique, to demonstrate that all of these complexes were the same complex, and that this complex was homologous to the yeast Mediator complex (Sato et al., 2004b; Bourbon et al., 2004).

New data has since emerged both supporting and disputing Mediator's role as a general transcription factor. Fan et al. have demonstrated that there is not always a correlation between Pol II and Mediator recruitment at highly active genes, such as ribosomal and glycolytic genes (Fan et al., 2006). These authors demonstrated that Mediator is recruited to promoters in an activator-specific manner, although not all activators seem to recruit Mediator. Likewise, Lee and Lis have demonstrated that two components of the Mediator complex, MED17 and MED22, deemed critical for Mediator function, are not required for activated gene expression of specific copper-inducible genes (Lee and Lis, 1998). In contrast, temperature sensitive knockout of two Mediator subunits in yeast, specifically MED22 and MED17, did cause global repression of all Pol II transcription (Linder et al., 2006). Although Mediator is able to enhance both basal and activator induced transcription at specific genes (Zhang et al., 2005), it is unknown if Mediator is required for transcription from amino acid responsive genes in mammalian cells. Previous experiments have demonstrated that the yeast GCN4 protein does interact with the yeast Mediator complex at genomic promoters during amino acid limitation in *Saccharomyces cerevisiae* (Zhang et al., 2004; Park et al., 2000). However, the functional human counterpart for GCN4, the ATF4 protein, has not been demonstrated to interact specifically with the human Mediator complex during any inducible stress condition.

Monitoring Transcriptional Regulation through Use of the Chromatin Immunoprecipitation (ChIP) Technique

Chromatin immunoprecipitation (ChIP) has emerged as the standard molecular biology technique used to demonstrate that a specific DNA region interacts with a specific protein *in vivo*. The basis for this experiment is simply to stably crosslink nuclear protein to DNA, sonicate the DNA into soluble fragments, immunoprecipitate specific protein-DNA complexes, then amplify the DNA by qPCR (Boyd and Farnham, 1999; Farnham, 2002). ChIP analysis, as a technique, is powerful because it permits analysis of endogenous genomic regions in their endogenous chromatin environment in living cells. The relative levels of protein binding to DNA in living cells can be measured quantitatively over an experimental time frame of several hours, days, or weeks using this technique. Though the technique itself is very powerful, in practicality, this technique must be optimized at several experimental points to be useful. Unlike its *in vitro* counterpart, the electrophoretic mobility shift assay (EMSA), which uses relatively short defined oligonucleotides, ChIP specificity of the identified binding site is directly related to sonication efficiency, and thus, DNA fragment size. Another caveat of this technique is its dependence on reliable and specific antibodies. A third requirement is the ability to amplify DNA in a quantitative manner through the selection of sequence specific primers that amplify in a linear manner. Given that the main technique used in this chapter is ChIP, these factors have been considered and optimized as much as experimentally possible.

Results

Amino Acid Deprivation Induces SNAT2 Gene Expression and Transcription Activity

Previous Northern blot results from the Kilberg laboratory demonstrated that SNAT2 gene expression was induced by amino acid deprivation (Bain et al., 2002). To determine the pattern of SNAT2 gene expression by a more quantitative method, RT-qPCR was performed on RNA

extracts from HepG2 cells that had been deprived of the amino acid histidine over a time course of 24 hours (Figure 3-1). The time course demonstrates that SNAT2 steady state mRNA begins to increase following two hours of amino acid deprivation. Total SNAT2 mRNA content peaks at eight hours of amino acid deprivation, resulting in an approximately four-fold maximal induction, and the amount of mRNA declines slightly before reaching a plateau at twenty-four hours. SNAT2 mRNA levels in basal or MEM conditions are relatively unchanged for the first 12 hours of treatment. During the 16 to 24 hour time period, the SNAT2 mRNA levels increase significantly, presumably because the HepG2 cells are beginning to become nutrient deprived.

To determine whether the increase in SNAT2 mRNA was caused by either increased mRNA transcription or mRNA stabilization, primers that flanked an intron-exon boundary of the SNAT2 gene were used to measure SNAT2 transcription activity by RT-qPCR (Figure 3-1). Similar to its pattern of amino acid induced steady state mRNA, SNAT2 transcription activity increased after two hours, reached a maximum at eight hours, and decreased slightly before stabilizing at 24 hours. The SNAT2 transcription activity in the MEM condition also closely reflected the pattern shown for steady state mRNA. Figure 3-1 also includes the pattern of GAPDH mRNA expression to demonstrate approximately equal levels of total RNA were present in all samples. The data clearly demonstrate that increased mRNA transcription from the SNAT2 gene contributes to the increase in SNAT2 steady state mRNA during amino acid deprivation.

Specific Members of the ATF and C/EBP Protein Families Bind to the SNAT2 AARE during Amino Acid Limitation

Given that SNAT2 transcription was induced by amino acid deprivation, chromatin immunoprecipitation (ChIP) analysis of the SNAT2 gene was performed to determine the proteins responsible. Initial experiments focused on two specific genomic regions, the SNAT2

promoter and the SNAT2 intronic AARE enhancer element. These two regions are approximately 700 bp apart, so while a few overlapping fragments may remain after cellular sonication, designed to produce 400-800 bp fragments, it is still possible to distinguish between binding of proteins specific for either site. ChIP for Pol II demonstrated binding at both the SNAT2 promoter and the AARE, though binding at the promoter was much stronger when compared to the AARE (Figure 3-2). Pol II binding increased significantly at the SNAT2 gene after two hours of amino acid starvation and peaked between eight and 12 hours. The pattern of increased Pol II binding to the SNAT2 gene was also similar to the pattern of SNAT2 transcription activity. Previous EMSA analysis from the Kilberg laboratory had demonstrated that specific members of the ATF and C/EBP families could bind to the SNAT2 AARE sequence *in vitro* (Palii et al., 2006). Based on those data, binding of ATF and C/EBP family members to the SNAT2 gene was screened by ChIP analysis, and proteins that bound the gene *in vivo* are shown in Figure 3-2. ATF4 binding to the SNAT2 AARE increased after only one hour of amino acid deprivation, peaked at two hours, and then gradually declined. This pattern of binding is consistent with the model of nutrient regulation that proposes ATF4 is an activator of gene expression through AARE binding. In contrast, both ATF3 and C/EBPβ did not show increased binding to the SNAT2 AARE until four to eight hours of amino acid deprivation. This binding peaked between 12 to 16 hours, coinciding with the decrease in SNAT2 transcription activity, and then even the association of these two factors began to decline. Given that the working model of amino acid-dependent regulation of ASNS proposes that ATF3 and C/EBPβ are transcriptional repressors, this result further supports the model's conclusions. While C/EBPα displayed positive binding to the SNAT2 AARE, it demonstrated no clear increase or decrease in binding during the time course tested. ChIP analysis also demonstrated that though

the transcription factors ATF4, ATF3, C/EBPα, and C/EBPβ bind to the SNAT2 AARE, none of them are ever bound to the SNAT2 promoter. "Positive binding" for all proteins was based on the criteria of having a ratio value above the level of the non-specific antibody control (n/s IgG), and by producing a ratio value above the level of binding to a control region of the SNAT2 gene. An example of the latter control is presented in Figure 3-3 for which the SNAT2 exon 10 region was assayed for protein binding by qPCR. This region is approximately 8000 bp from the SNAT2 AARE and is not known to contain any protein binding elements. Thus, any ratio value not significantly different from these two values was considered to be non-specific binding to the SNAT2 AARE or promoter. ATF and C/EBP family members that did not bind to the SNAT2 AARE region are shown in Figure 3-4. Ratio values for the binding of the transcription factors ATF2, C/EBPγ, and C/EBPε to the SNAT2 AARE after eight hours of amino acid starvation did not fulfill the above requirements and were concluded not to bind the SNAT2 AARE. Though only one time point is shown, a time course of binding for each of these factors was tested at least one time.

Specific Members of the ATF and C/EBP Protein Families Bind to Many Functional AARE-Containing Genes during Amino Acid Limitation

Given that transcription factor binding to the SNAT2 AARE supported the proposed working model of amino acid-dependent regulation of ASNS, ChIP analysis was used to further test this model by monitoring ATF4, C/EBPβ, ATF3, and Pol II binding to other known functional AARE-containing genes (Figure 3-5). The genes ATF3, C/EBPβ, vascular endothelial growth factor (VEGF), and CCAAT/enhancer-binding protein homologous protein (CHOP) were all previously demonstrated to contain functional genomic AARE elements required for their transcription induction by amino acid starvation (Pan et al., 2007; Chen et al., 2005; Abcouwer et al., 2002; Bruhat et al., 2002). Both ATF4 and Pol II binding peaked at the AARE regions of

most genes between two and four hours of amino acid limitation, and then flattened out or slightly declined after eight hours of starvation. These observations are consistent with the idea that ATF4 functions to activate transcription by binding to AARE regions, and that these regions demonstrate maximum transcription, through measured Pol II binding, when ATF4 presence is highest. ATF3 binding to all AARE regions, while inconsistent between two and four hours, produced a consistent maximum binding after eight hours of amino acid limitation. Likewise, C/EBPβ binding to the AARE regions began to increase at four hours, but was maximum after eight hours of amino acid limitation. Maximal binding of both ATF3 and C/EBPβ to the AARE after eight hours of starvation, when both ATF4 and Pol II AARE binding has flattened or decreased, is consistent with the model that these proteins function as repressors of AARE-mediated transcription. Though the magnitude of transcription factor binding differed for each individual gene, the overall pattern of transcription factor binding is reproducible for all six AARE-containing genes. Thus, the model of amino acid-dependent regulation of the ASNS gene can also be applied to ATF3, C/EBPβ, VEGF, and CHOP, and extrapolated to predict that it is likely that newly identified genes containing a functional AARE will display a similar pattern of transcription factor recruitment.

General Transcription Factor (GTF) Binding to the SNAT2 Promoter Increases during Amino Acid Limitation

GTF binding to the SNAT2 gene was measured by ChIP analysis to determine if the binding increased at the promoter and if there was any interaction with the SNAT2 AARE enhancer region (Figure 3-6). The binding of TFIIA, TFIIB, TFIIE, and two subunits of the TFIID complex (TAF1 and TAF9) to the SNAT2 promoter and AARE were assessed. The dashed line on the graph represents the pattern for SNAT2 transcription activity (taken from Figure 3-1, starvation induced 0-8 h data), which has been superimposed on all the graphs. All

of the GTF subunits bound the SNAT2 promoter, and their binding increased during amino acid deprivation. The pattern of TFIIB and TFIIE binding most closely resembled the pattern of SNAT2 transcription activity. None of the GTFs displayed equivalent levels of binding between the SNAT2 promoter and AARE regions. TFIIE was the only GTF to display significant ratio values for binding at the SNAT2 AARE, demonstrating a low level of positive binding that is consistent with its role in early transcription elongation (Conaway et al., 2000). However, based on the disparity between the ratio binding values for the SNAT2 promoter and AARE regions, the results show that the GTF complexes are not actively recruited to the AARE region.

General Acetylation of Histone H3 on the SNAT2 Gene Increases during Amino Acid Limitation

Acetylation of specific histone residues is necessary to alter chromatin structure, thus allowing for binding of regulatory protein factors at specific genomic sequences (Li et al., 2007b). Previous studies demonstrated that acetylation of histones H3 and H4 occurred at the ASNS AARE region during amino acid deprivation (Chen et al., 2004). Thus, ChIP analysis of the SNAT2 gene was performed using antibodies to acetylated residues on either histone H3 or H4 that are known to associate with active transcription. Figure 3-7 demonstrates binding of acetylated histone H3 (Acetyl H3) or acetylated histone H4 (Acetyl H4) to both the SNAT2 promoter and AARE region at two and eight hours after amino acid starvation. The two hour time point was chosen, because that is the first time that SNAT2 transcription activity is different between the MEM and MEM – His condition. Eight hours was chosen because it is at the peak of SNAT2 transcription activity and ATF4 binding. Acetylation of histone H3 increased only at the SNAT2 promoter for the first two hours, but then increased at both the SNAT2 promoter and AARE region after 8 hours of amino acid starvation. Acetylation of histone H4 did not increase at either the SNAT2 promoter or the AARE region during amino acid starvation, which is in

contrast from the ASNS promoter (Chen et al., 2004). Overall, MEM levels of acetylation for histone H3 and H4 were high when compared to the non-specific IgG control (Figure 3-2) suggesting a generally open chromatin structure.

To test the SNAT2 genomic range of histone H3 acetylation altered by amino acid starvation, primers were designed to amplify regions of the gene approximately 700 bp upstream of the promoter region and 700 bp downstream of the AARE region. Figure 3-8 demonstrates the fold induction of histone acetylation after eight hours of amino acid starvation at these four different regions of the SNAT2 gene. The results document that increased acetylation of histone H3 by amino acid deprivation is specific for the promoter and AARE regions of the SNAT2 gene.

Increased Binding of the Mediator Complex to the SNAT2 Gene Is Not Required for Induced SNAT2 Transcription

The data of Fig 3-2 and 3-6 demonstrate that none of the transcription factors that bind the AARE enhancer also bind the SNAT2 promoter, and the proteins that bind the SNAT2 promoter do not bind the SNAT2 AARE. Based on the theory that the proteins bound at a distal enhancer and the promoter region must interact when transcription is induced, a bridging protein complex may be necessary to provide cross-talk between these two genomic regions. The Mediator complex has been reported to be a general transcription complex that bridges promoter and enhancer interacting elements (Takagi and Kornberg, 2006), thus its binding to the SNAT2 gene was measured by ChIP. In HepG2 hepatoma cells, a ChIP time course was first performed with antibodies to two of the Mediator subunits, MED1 and MED23, to determine their binding to the SNAT2 AARE and promoter regions (Figure 3-9). Neither of the subunits showed positive binding to the SNAT2 gene when compared to the values for the non-specific IgG control (Figure 3-2), nor was there a pattern of starvation-induced association, as seen with the GTFs

(Figure 3-6). To ensure that the antibodies were of good quality and that the ChIP assay was optimized for analysis of Mediator complex binding, ChIP control experiments were performed in MCF7 breast cancer cells by monitoring induction of the pS2 gene by β-estradiol. Several laboratories have demonstrated that specific subunits of the Mediator complex bind to the promoter of the pS2 gene during treatment with β-estradiol in MCF7 cells (Zhang et al., 2005; Burakov et al., 2002). ChIP was performed in MCF7 with antibodies to the Mediator subunits MED1, MED23, and CDK8, and binding was measured by qPCR analysis of the pS2 promoter (Figure 3-10a). Pol II was used as a positive control for gene induction and non-specific IgG was used again as a negative control. After treatment with β-estradiol for two hours, there was clearly an increase in Mediator subunit binding to the pS2 promoter. Given that the antibodies and protocol have produced a positive result, the ChIP for Mediator subunits at the SNAT2 gene was repeated in MCF7 cells (Figure 3-10b). For this set of experiments, two hours was chosen and the SNAT2 promoter, AARE, and a region upstream of the SNAT2 promoter were assayed for Mediator binding. At all three genomic locations, the association of Mediator was quite small. However, when the ratio to input levels of binding at the SNAT2 promoter and the AARE are considered, the values were larger than the upstream region, so it is possible that some association of the Mediator complex with the SNAT2 promoter may occur. Unlike the pattern seen with GTF recruitment, however, there was no induced binding of the Mediator complex to the SNAT2 promoter or AARE after amino acid starvation. As a positive control to show that the SNAT2 gene was in fact induced by amino acid starvation in the MCF7 cells, binding of Pol II to the SNAT2 promoter was demonstrated to increase during treatment (Figure 3-10b).

Given the negative binding results for Mediator illustrated by Figures 3-9 and 3-10, a second method was used to confirm the ChIP data. Knockdown of the Mediator subunits by

siRNA in MCF7 cells was performed under various stress conditions and either mRNA or transcription activity of specific genes was assayed by RT-qPCR. Figure 3-11 is a series of RT-qPCR controls to establish the mRNA content for each Mediator subunit was reduced by treatment with a specific siRNA. For each graph, the control condition (cells treated with non-targeting control siRNA) has been set to one and the siMediator condition has been set as the fold change of mRNA knockdown relative to the control. Every subunit tested displayed at least a 50% knockdown in total mRNA after 48 hours of treatment with some subunits displaying a 90% reduction in total mRNA. A complementary control experiment, Figure 3-12, established the mRNA levels for each Mediator subunit in both basal and stress conditions to demonstrate that the gene expression for the subunits of the Mediator complex was not induced by the experimental treatment, amino acid starvation or β-estradiol treatment.

Given the effective knockdown of each Mediator subunit, SNAT2 transcription activity was assayed in both MEM and MEM-histidine conditions in cells treated with either control siRNA or siRNA specific for the individual Mediator subunits. The data demonstrate that depletion of the Mediator complex had no effect on either SNAT2 basal or induced transcription activity (Figure 3-13). To establish that depletion of one subunit of the large multi-protein Mediator complex can in fact alter transcription activity, the transcription activity of the pS2 gene in either basal or β-estradiol induced conditions was measured in the absence of the selected Mediator subunits. Figure 3-14 demonstrates that knockdown of the Mediator subunits MED1, MED6, MED7, MED17, and MED31 caused an approximately 50% reduction in pS2 induced transcription activity with a p-value less than or equal to 0.05. For the Mediator subunits MED1 and MED31, knockdown of these subunits also inhibits basal transcription of the pS2 gene (p-value less than or equal to 0.05). Analysis of both Figures 3-13 and 3-14

established that the Mediator complex was necessary for induced transcription of the pS2 gene by β-estradiol, but it was not needed for induced transcription of SNAT2 by amino acid deprivation.

Conclusions

Through development and improvement in molecular biology techniques such as ChIP and qPCR, a global picture of regulated gene expression during transcription initiation *in vivo* has emerged. However, the mechanisms of activator-induced gene transcription during specific cellular stresses or stimuli has yet to be completely resolved. Initially, transcriptional regulation of the stress caused by amino acid starvation was characterized by induced gene expression of the ASNS and CHOP genes (Chen et al., 2004; Bruhat et al., 1997). Both genes were demonstrated to contain similar 9 bp nutrient response elements capable of inducing transcriptional activation (Kilberg et al., 2005). Recently, many laboratories have demonstrated that other genes containing a similar nutrient responsive element exhibit induced gene expression during amino acid limitation (Palii et al., 2004; Sato et al., 2004a; Fernandez et al., 2003; Pan et al., 2007). Though functional assays and EMSA analysis of the ASNS and CHOP genes provided evidence of possible DNA binding proteins at these nutrient responsive elements, the first *in vivo* evidence was provided through ChIP analysis of the ASNS gene (Chen et al., 2004). By combining both RT-qPCR and ChIP analysis of the ASNS gene, a model of nutritional regulation of gene expression was proposed by the Kilberg laboratory. This proposed model was used as the basis for analyzing SNAT2 gene regulation during amino acid limitation with the purpose of further validating and extending the model of nutrient regulation. The present results demonstrate that SNAT2 transcription activity was induced two hours after initiating amino acid limitation, and the pattern of transcription activity and mRNA accumulation closely mirror each other. In contrast, ASNS transcription activity was demonstrated to be rapidly induced by amino

acid starvation, within 30 minutes, and was not reflected in the slower pattern of mRNA accumulation (Chen et al., 2004). It was possible that, given their difference in nutrient responsive elements and flanking DNA region, the SNAT2 gene requires unique transcription factors or signaling proteins for its induction by nutrient stress than those present at the ASNS gene.

ChIP analysis of the SNAT2 gene for ATF and C/EBP proteins demonstrated that AARE-associated binding of ATF4, ATF3, and C/EBPβ increases upon amino acid deprivation. While increased ATF4 binding occurred early during treatment and was associated with transcription induction, ATF3 and C/EBPβ binding increased later and was associated with transcriptional repression. These data strengthen the Kilberg laboratory model, based on the ASNS gene, that ATF4 initially binds the AARE to activate transcription whereas ATF3 and C/EBPβ are repressor proteins that bind later to further regulate and depress transcription (see the Introduction chapter for an extensive review). Given that the SNAT2 AARE is located approximately 700 bp downstream of the promoter, further data was collected to determine if the AARE proteins were recruited to the promoter or if recruited promoter factors also associated with the AARE. ChIP analysis demonstrated that while Pol II and the GTFs were recruited to the promoter during amino acid deprivation, they were not present at the AARE. Likewise, the AARE binding proteins were not present at the SNAT2 promoter. Thus, it was proposed that a bridging protein or protein complex must be necessary for conveying signals between the AARE activator binding proteins and promoter bound proteins. While extensive experimental analysis was performed, it seems unlikely that this bridging complex is the Mediator complex. Given that both ChIP and siRNA analysis were able to verify the Mediator requirement for β-estradiol induction of the pS2 gene, there was no evidence to suggest that Mediator is required for SNAT2

gene induction. Given that the distance between the SNAT2 promoter and enhancer is 700 bp, it is possible that the starvation-recruited GTF complexes themselves could function as bridging components. Recent data has shown that the gamma subunit of the TFIIA protein complex can bridge interaction between the promoter and ATF4 activator bound regions of the osteocalcin gene (Yu et al., 2008). Lewis and Reinberg have also suggested that, in metazoans, a subset of genes may use TFIID instead of Mediator as a bridging complex (Lewis and Reinberg, 2006). Thus it may be necessary to perform a more extensive ChIP analysis to screen for multiple subunits of each GTF on the SNAT2 gene to determine if one of these subunits could be responsible for bridging the promoter and enhancer regions. The present Mediator data is also surprising given the fact that some *in vitro* yeast studies have suggested the Mediator complex is required for all Pol II transcription (Kornberg, 2007). However, there is also evidence in yeast demonstrating that for many highly active genes, like those for ribosomal genes and glycolytic enzymes, Pol II transcription can take place without the presence of Mediator (Fan et al., 2006; Lee and Lis, 1998). Therefore, another bridging complex may be necessary for activated transcription of the SNAT2 gene. Conversely, transcription factor binding at the SNAT2 AARE may cause localized acetylation at the SNAT2 promoter by an unknown histone acetyltransferase, thus allowing chromatin opening and GTF binding without the need for a protein-based bridging complex.

While transcription factor binding was similar for the ASNS and SNAT2 AAREs, histone acetylation at these regions was different for the two genes. While general acetylation of histone H4 at the ASNS AARE was increased approximately four-fold between one to eight hours of amino acid limitation (Chen et al., 2004), there was no increase of histone H4 acetylation at the SNAT2 promoter or AARE after amino acid starvation. Though this result indicates some gene

specificity in histone acetylation during amino acid starvation, a more thorough ChIP of individual H4 residues will be necessary to draw a strong conclusion. Acetylation of histone H3 increased after starvation at both the ASNS and SNAT2 AAREs, though the timing of this event was different between the two genes. Overall, increased histone acetylation appears to be coordinated with induced transcription during amino acid starvation, though fining tuning for each gene's expression may be regulated by slight differences in the acetylation of specific histone residues.

All of the data presented demonstrate a finely coordinated timing of transcription factor recruitment to the SNAT2 gene that results in induced transcription activity during the early hours of amino acid deprivation. Further detailed analysis of each individual component of the SNAT2 gene transcription machinery will be necessary to develop a complete model of its regulation during nutrient deprivation. Furthermore, contrasting this new data with the existing ASNS model of gene regulation will be useful to gain insight into the differences and similarities between AARE-containing gene expression during amino acid deprivation.

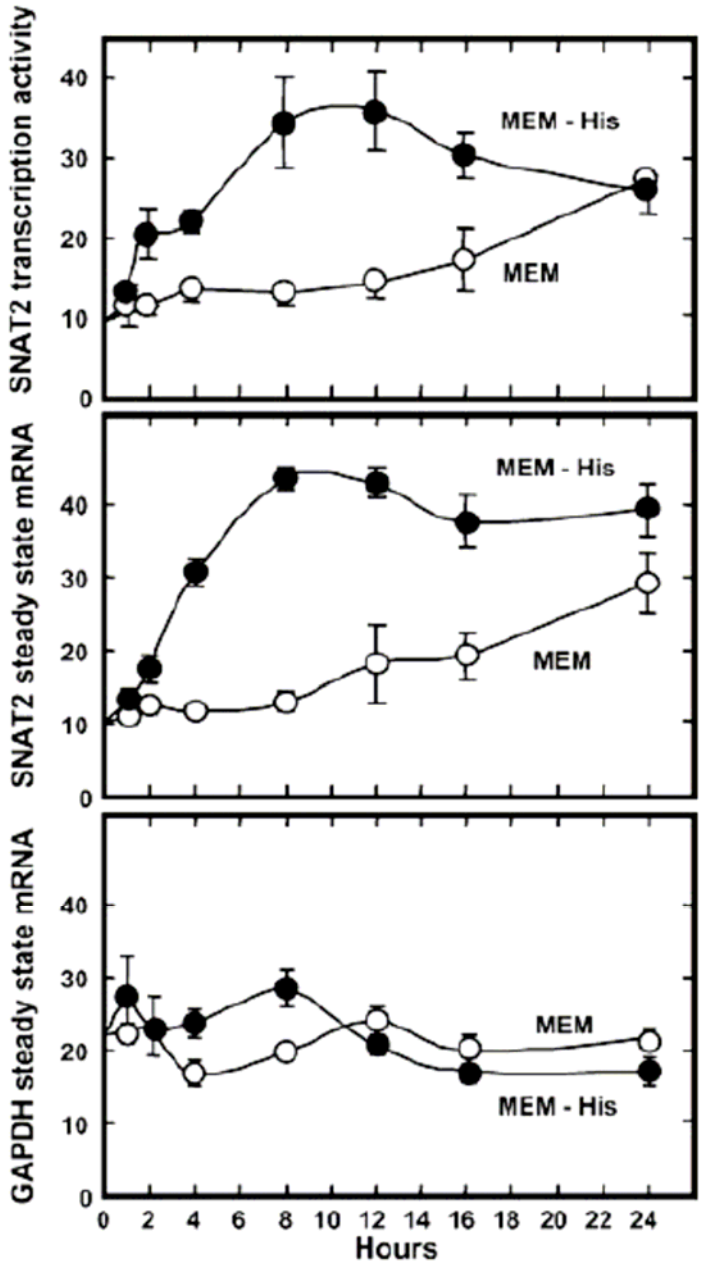

Figure 3-1. SNAT2 transcription activity and steady state mRNA increase during amino acid deprivation. HepG2 cells were treated with either complete MEM or MEM lacking histidine (MEM- His) over a period of 24 hours, and at the indicated times total RNA was isolated. Specific primers were used to measure either SNAT2 transcription activity or steady state mRNA by real time PCR. As a loading control, GAPDH mRNA was measured by RT-qPCR. RNA for each data point represents three samples and RT-qPCR for each data point was performed in duplicate. The data are presented as the averages ± the standard error of the means (sem). This figure was taken from Palii et al. Biochem J. 2006.

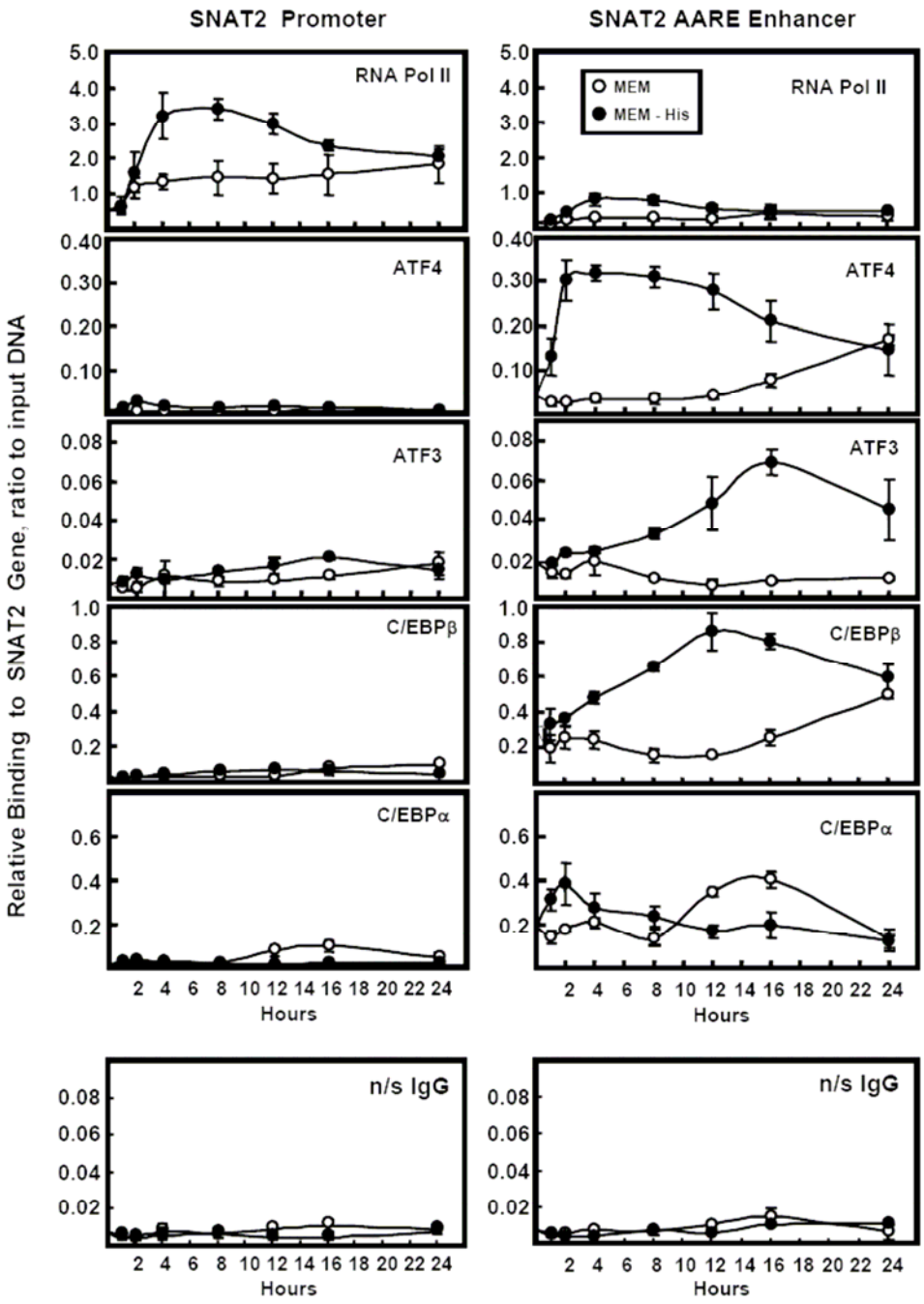

Figure 3-2. Binding of ATF and C/EBP proteins to the SNAT2 AARE, but not the promoter, increases after amino acid limitation. HepG2 cells were treated with either complete MEM (open circles) or MEM lacking histidine (closed circles) for the indicated times, and ChIP analysis was performed as described in Chapter 2 (Materials and Methods). Data are presented as the ratio of the qPCR signal for immunoprecipitated DNA to the qPCR signal for total DNA (input). Each data point represents three samples and the qPCR was performed in duplicate. The data are presented as the average ± sem. This figure was adapted from Palii et al. Biochem J. 2006.

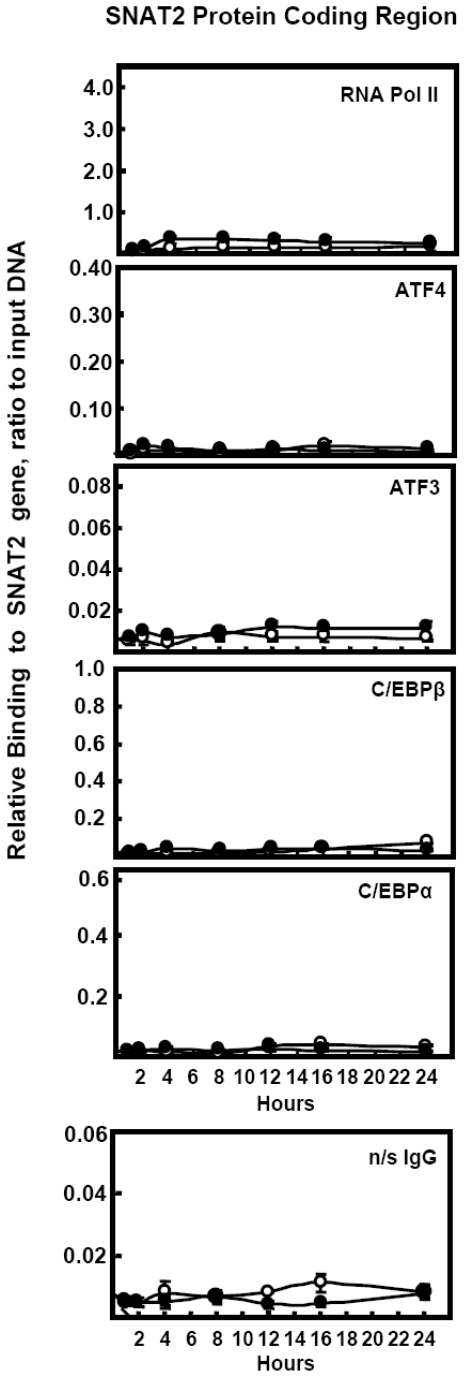

Figure 3-3. ATF and C/EBP proteins do not bind to the SNAT2 protein-coding region after amino acid deprivation. HepG2 cells were treated with either complete MEM (open circles) or MEM lacking histidine (closed circles) for the indicated times, and ChIP analysis was performed. Data are presented as the ratio of the qPCR signal for immunoprecipitated DNA to the qPCR signal for total DNA (input). Each data point represents three samples and the qPCR was performed in duplicate. The data are presented as the average ± sem.

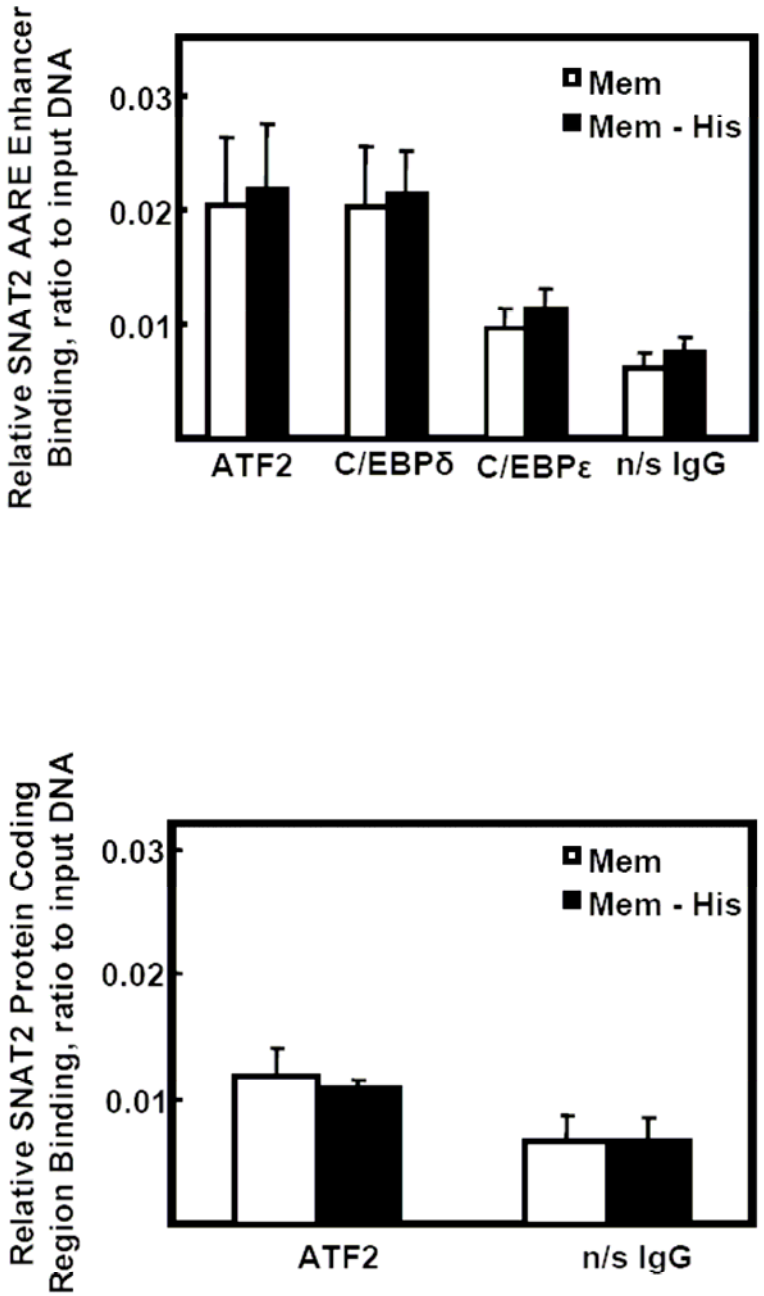

Figure 3-4. Amino acid deprivation does not lead to binding of ATF2, C/EBPδ, and C/EBPε to the SNAT2 AARE. HepG2 cells were treated with either complete MEM (white boxes) or MEM lacking histidine (black boxes) for eight hours, and ChIP analysis was performed. Data are presented as the ratio of the qPCR signal for immunoprecipitated DNA to the qPCR signal for total DNA (input). Each data point represents three samples and the qPCR was performed in duplicate. The data are presented as the average ± sem.

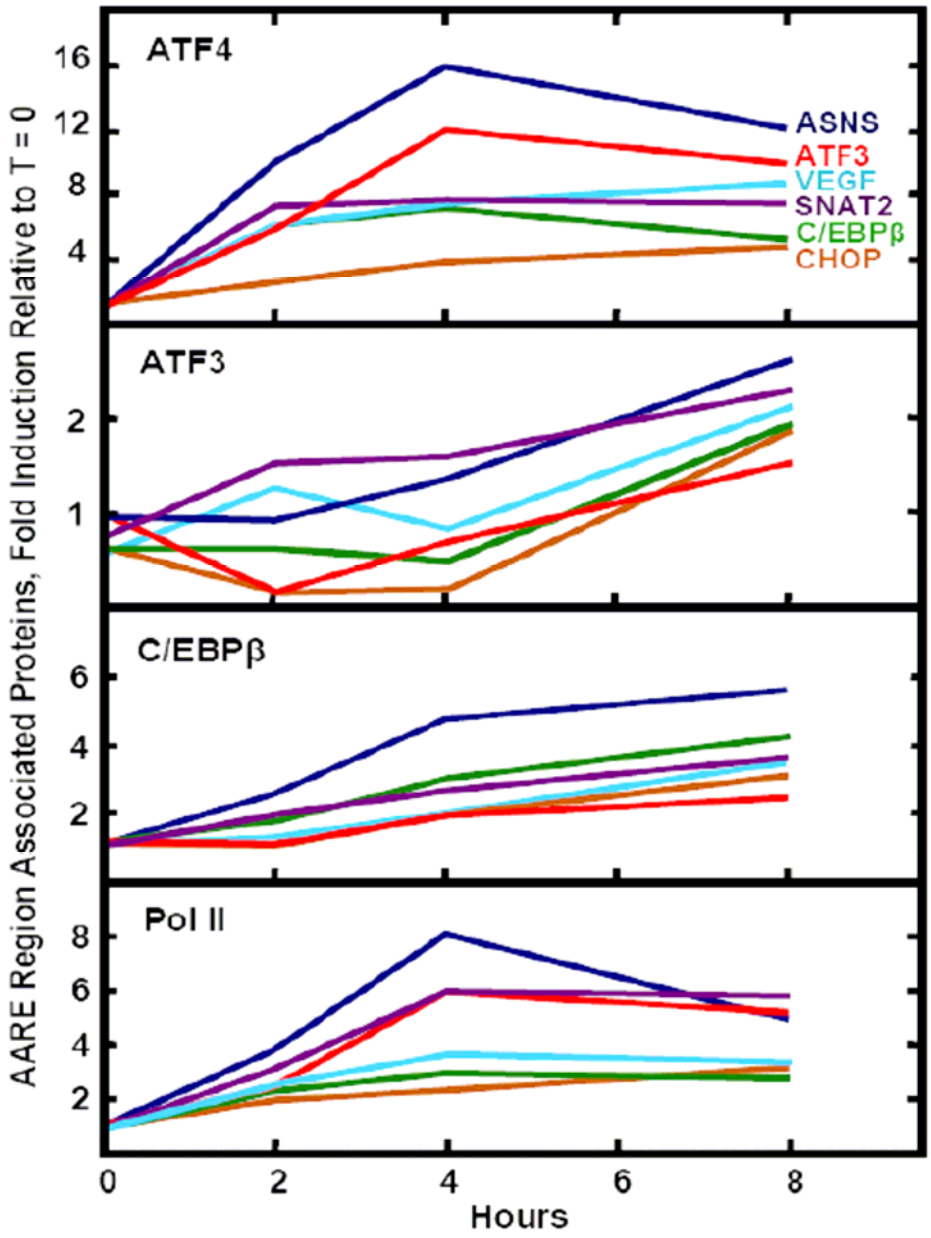

Figure 3-5. Amino acid deprivation causes a predictable pattern of ATF4, ATF3, C/EBPβ, and Pol II binding to functional AARE-containing genes. HepG2 cells were treated with either complete MEM or MEM lacking histidine for 2, 4, or 8 h, and ChIP analysis was performed with primers specific for the AARE region of each gene (listed in Table 2-3). qPCR data for individual immunoprecipitations were normalized to total DNA (input), and are presented as the ratio of the qPCR signal for 2, 4, or 8 hr relative to time zero (T=0). Each data time point represents three samples and the qPCR was performed in duplicate. The data are presented as the average minus the sem for clarity. Each colored line represents data for a single specific AARE-containing gene corresponding to the individual colored gene name labels. This figure was adapted from Pan et al. Biochem J. 2007.

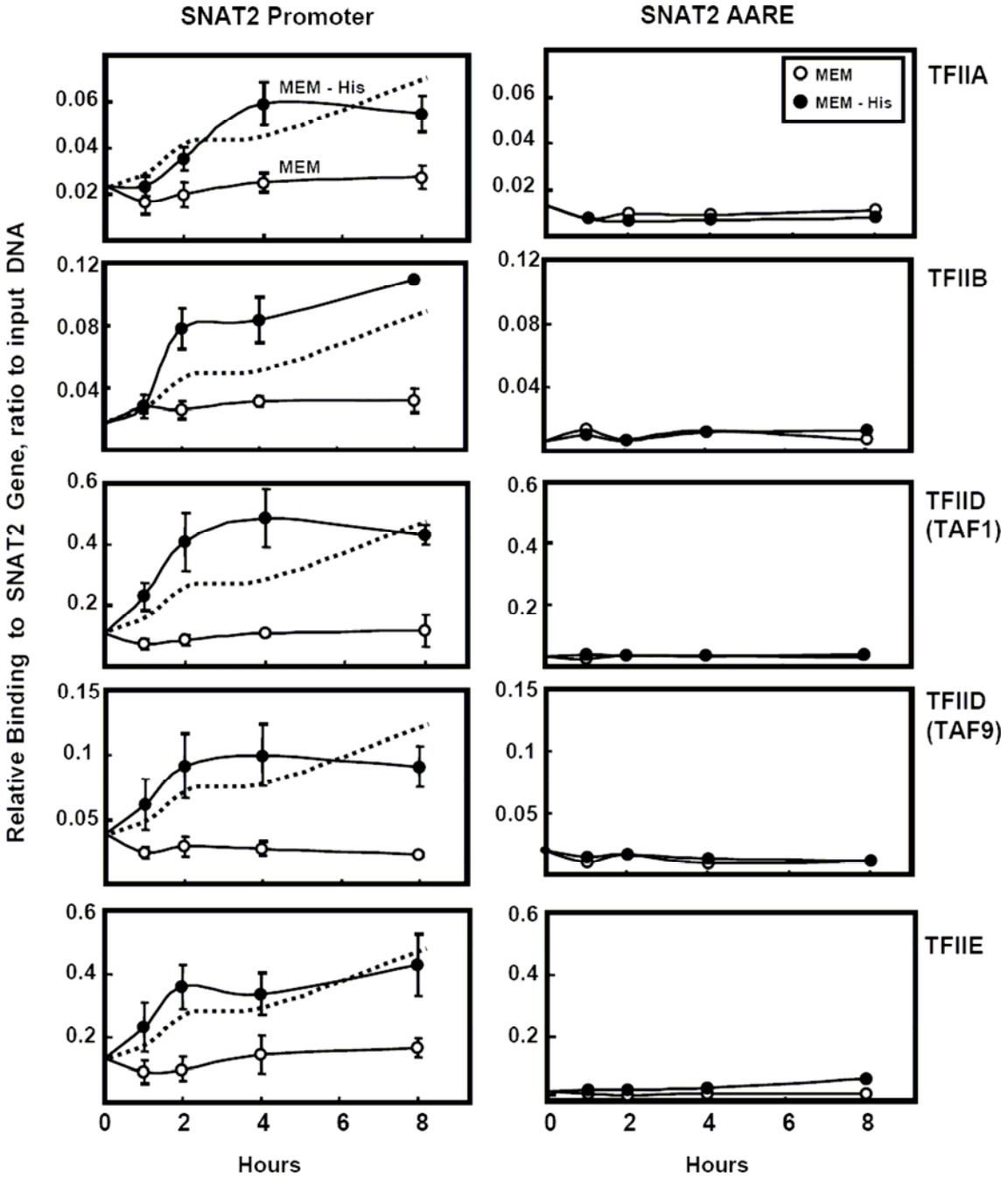

Figure 3-6. Increased GTF binding to the SNAT2 promoter, but not the AARE, occurs after amino acid limitation. HepG2 cells were treated with either complete MEM (open circles) or MEM lacking histidine (closed circles) for the indicated times, and ChIP analysis was performed. Data are presented as the ratio of the qPCR signal for immunoprecipitated DNA to the qPCR signal for total DNA (input). Each data point represents three samples and the qPCR was performed in duplicate. The data are presented as the average ± sem.

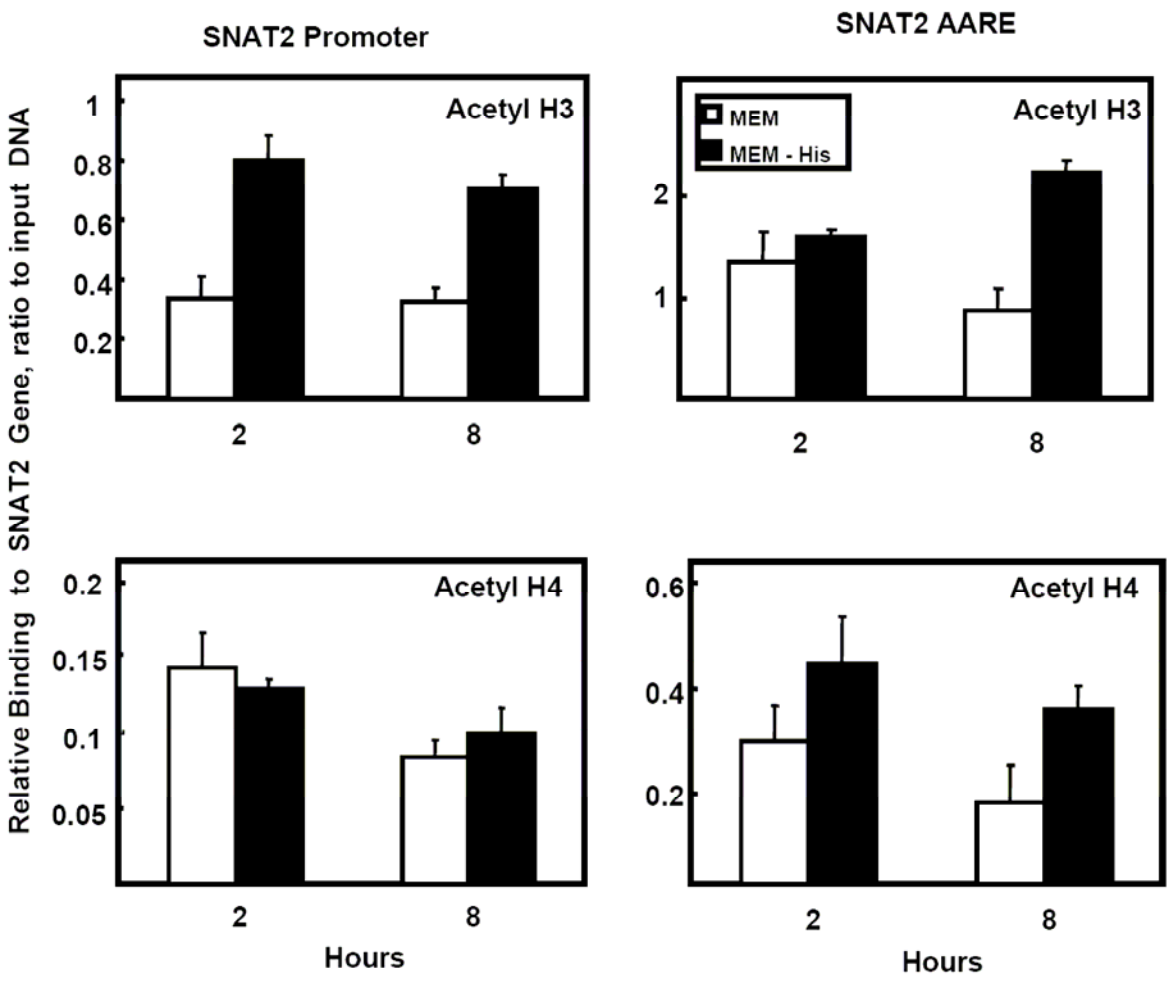

Figure 3-7. Amino acid limitation causes increased acetylation of histone H3, but not histone H4, at the SNAT2 promoter and AARE. HepG2 cells were treated with either complete MEM (white boxes) or MEM lacking histidine (black boxes) for either 2 or 8 hours, and ChIP analysis was performed. Data are presented as the ratio of the qPCR signal for immunoprecipitated DNA to the qPCR signal for total DNA (input). Each data point represents three samples and the qPCR was performed in duplicate. The data are presented as the average ± sem.

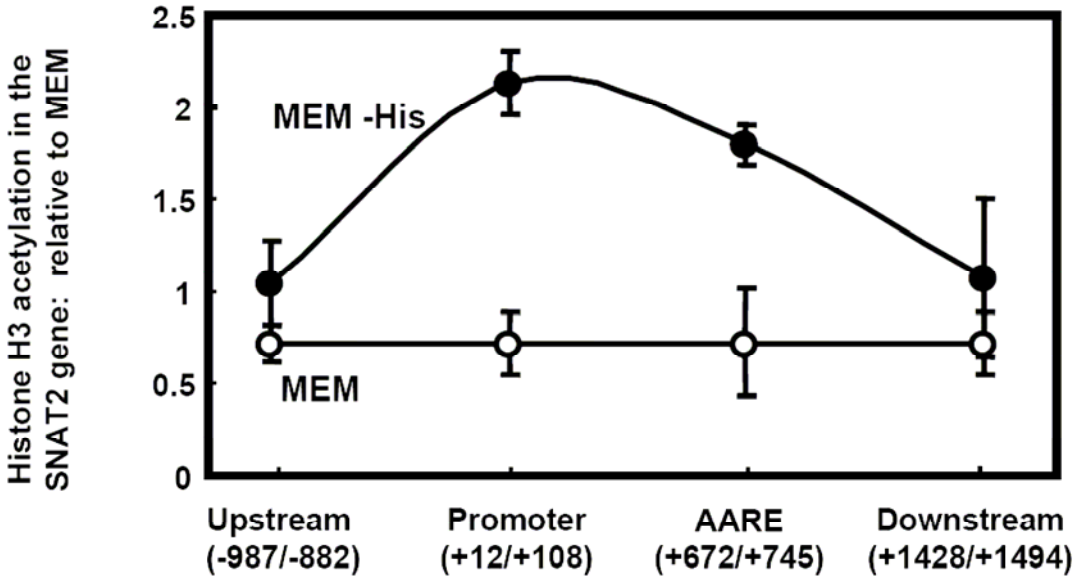

Figure 3-8. Increased acetylation of histone H3 occurs specifically at the SNAT2 promoter and AARE regions. HepG2 cells were treated with either complete MEM (open circles) or MEM lacking histidine (closed circles) for 8 hours, and ChIP analysis was performed. Data are presented as the fold change of binding in MEM –HIS condition relative to its own MEM condition. Each data point represents three samples and the qPCR was performed in duplicate. The data are presented as the average ± sem.

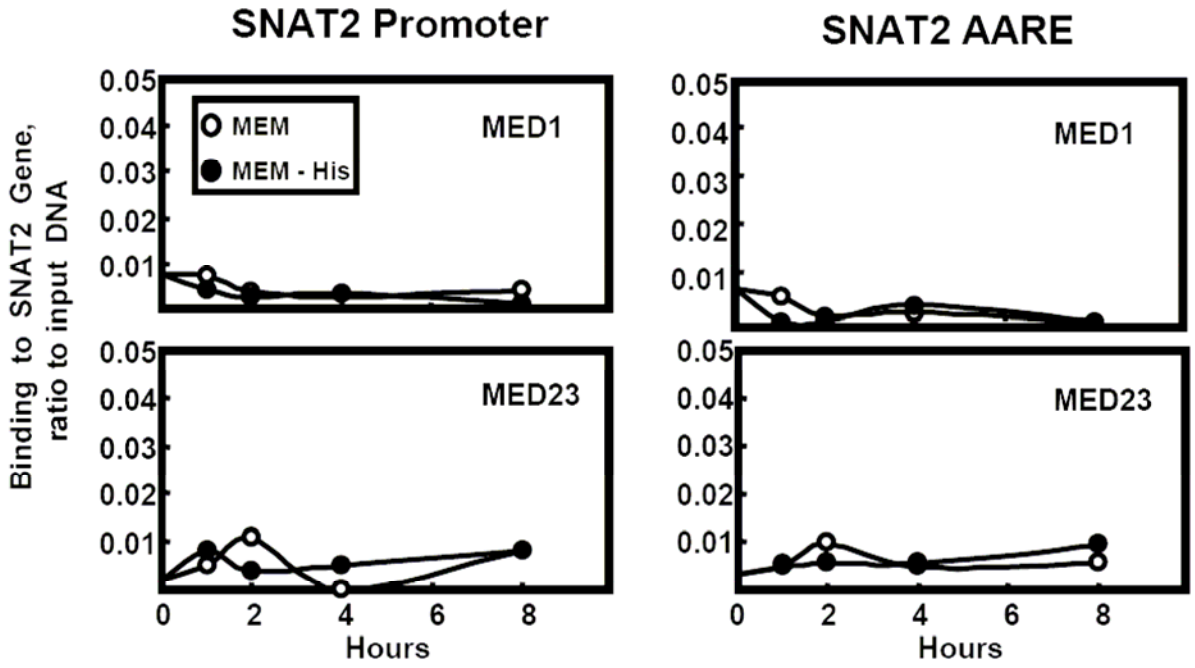

Figure 3-9. No increased binding of the Mediator subunits MED1 and MED23 to the SNAT2 gene occurs after amino acid limitation. HepG2 cells were treated with either complete MEM (open circles) or MEM lacking histidine (closed circles) for the indicated times over a period of eight hours, and ChIP analysis was performed. Data are presented as the ratio of the qPCR signal for immunoprecipitated DNA to the qPCR signal for total DNA (input). Presented is a single experiment with qPCR analysis performed in duplicate.

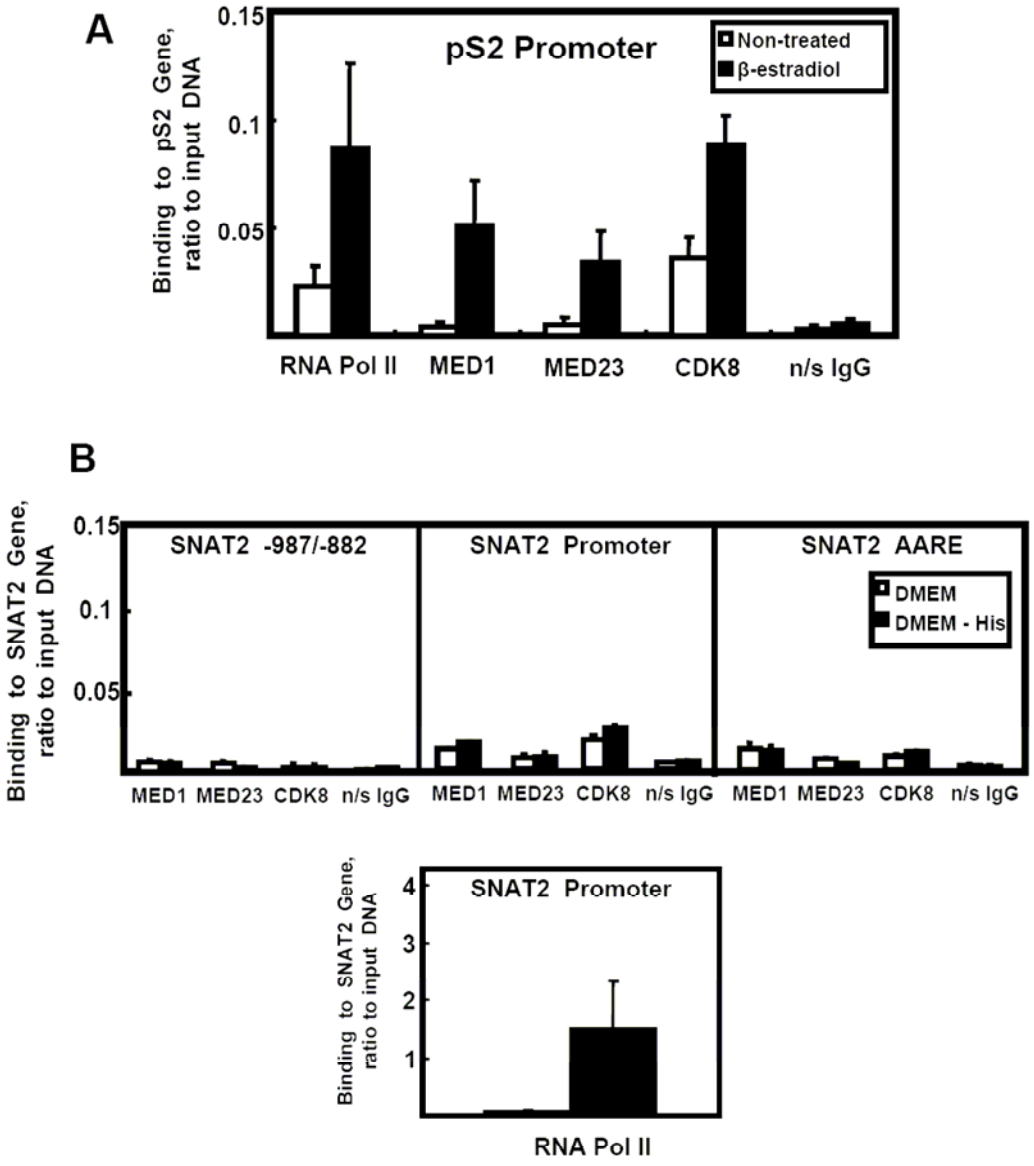

Figure 3-10. Increase in binding of the Mediator subunits occurs on the pS2 promoter but not on the SNAT2 gene. (A) MCF7 cells were treated with either complete DMEM (white boxes) or DMEM plus 100 nM β-estradiol (E2) (black boxes) for 2 hours, and ChIP analysis was performed. The data in the left panel are presented as the ratio of the qPCR signal for immunoprecipitated DNA to the qPCR signal for total DNA (input). The data in the right panel are presented as percent of the MEM control, thus each E2 value was divided by its corresponding DMEM value. (B) MCF7 cells were treated with either complete DMEM (white boxes) or DMEM lacking histidine (black boxes) for 2 hours, and ChIP analysis was performed. Data are presented as the ratio of the qPCR signal for immunoprecipitated DNA to the qPCR signal for total DNA (input). For both A and B, each data point represents three samples and the qPCR was performed in duplicate. The data that are presented as ratio to input binding are the average qPCR value ± sem.

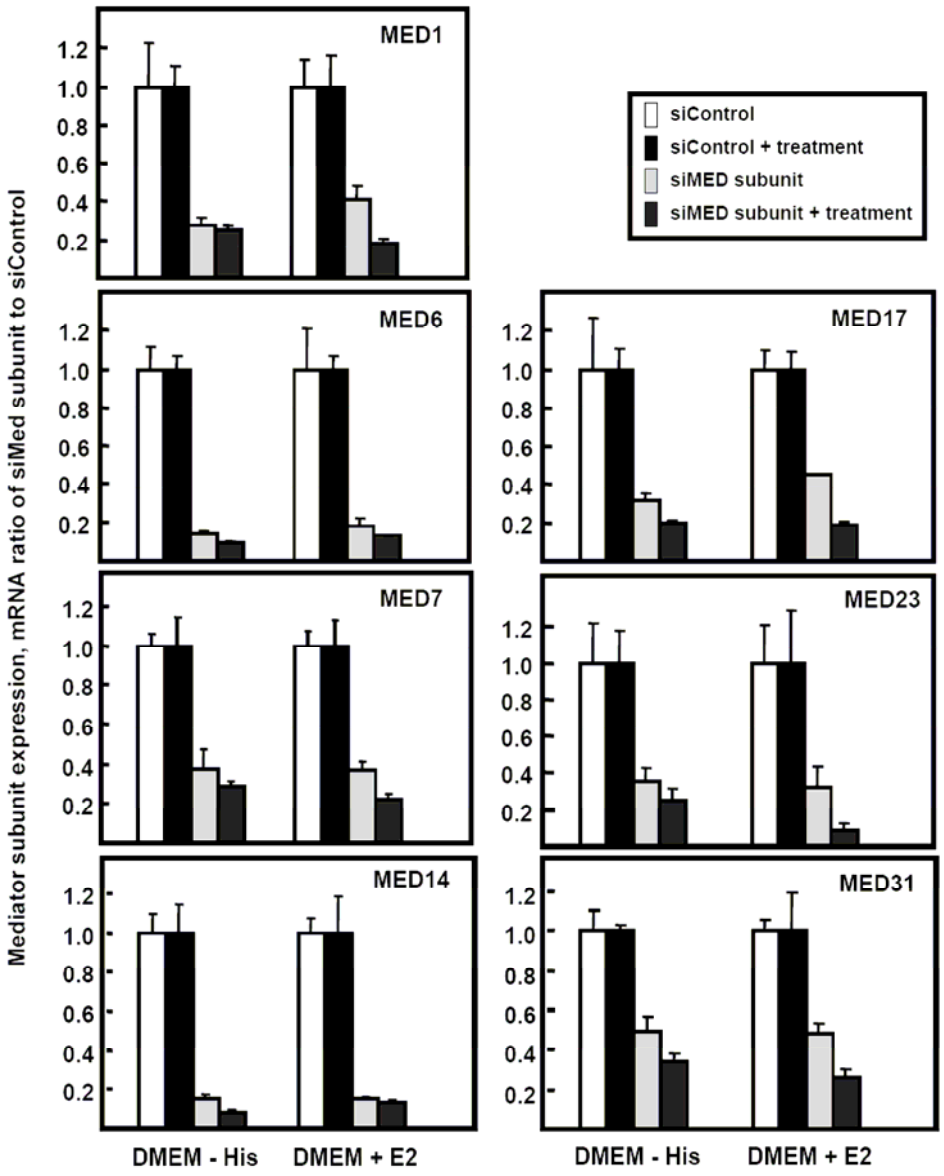

Figure 3-11. RT-qPCR analysis of Mediator subunit steady state mRNA after histine limitation or E2 treatment during siRNA knockdown. MCF7 cells were treated with either a control siRNA or a siRNA specific to a subunit of the Mediator complex. After a 48 h siRNA treatment and incubation, cells were treated with either complete DMEM, DMEM lacking histidine, or DMEM plus 100 nM β-estradiol (E2) for 4 hours, and total RNA was isolated. RT-qPCR for each Mediator subunit in both siRNA control and siRNA knockdown of that subunit was assayed. The values for the Mediator subunit mRNA in siControl conditions have been set to 1, and the data are presented as the amount of the siMediator subunit mRNA in knockdown treatment relative to control treatment. Each data point represents three samples and the RT-qPCR was performed in duplicate. The data are presented as the average ± sem.

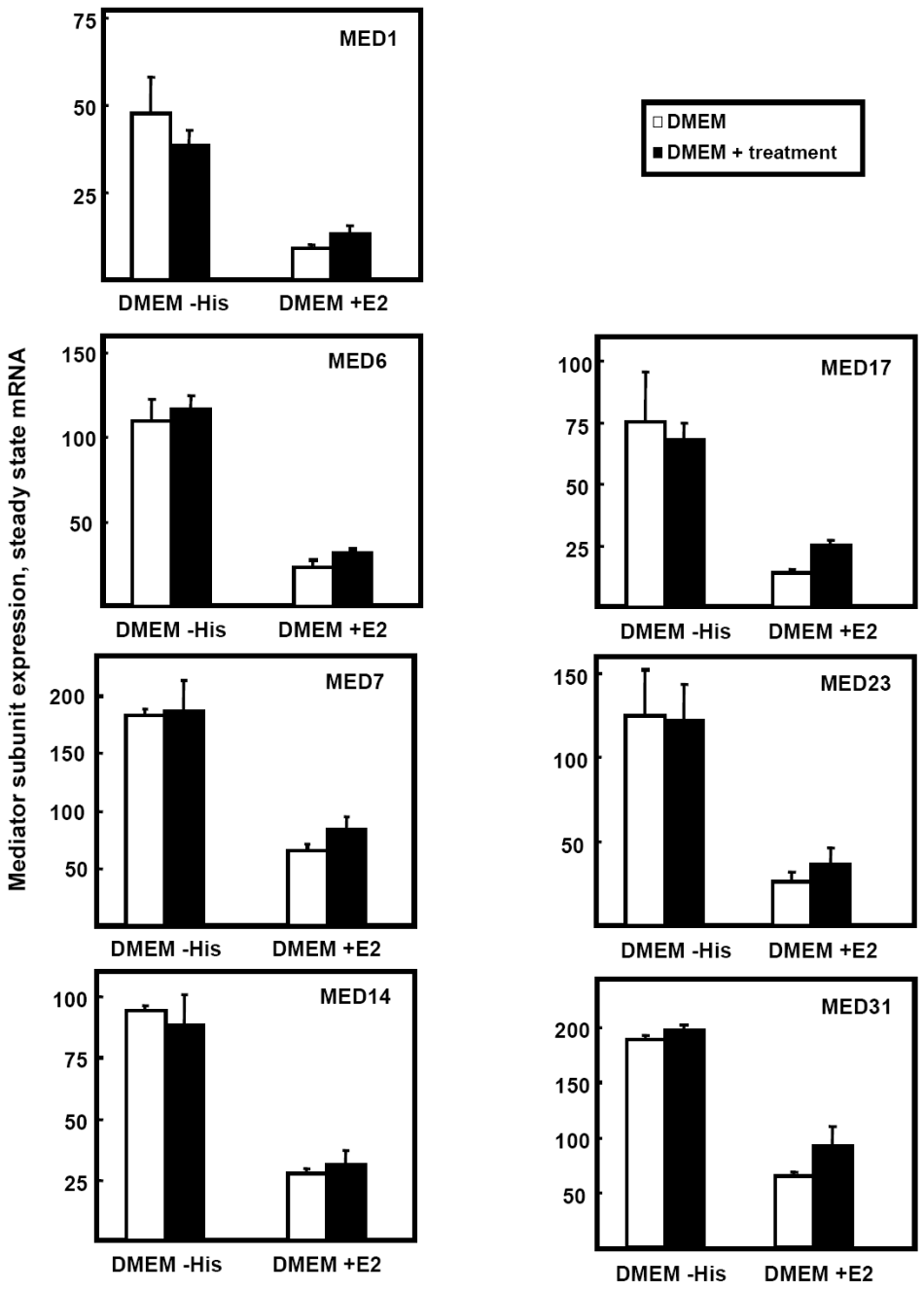

Figure 3-12. Gene expression of specific Mediator subunits was not increased after amino acid limitation or β-estradiol treatment. MCF7 cells were treated with either complete DMEM, DMEM lacking histidine, or DMEM plus 100 nM β-estradiol (E2) for 4 hours, and total RNA was isolated. Specific primers were used to measure the steady state mRNA of individual Mediator subunits by RT-qPCR and data are graphed as relative values to each other. RNA for each data point represents three samples and the RT-qPCR for each data point was performed in duplicate. The data are presented as the average ± sem.

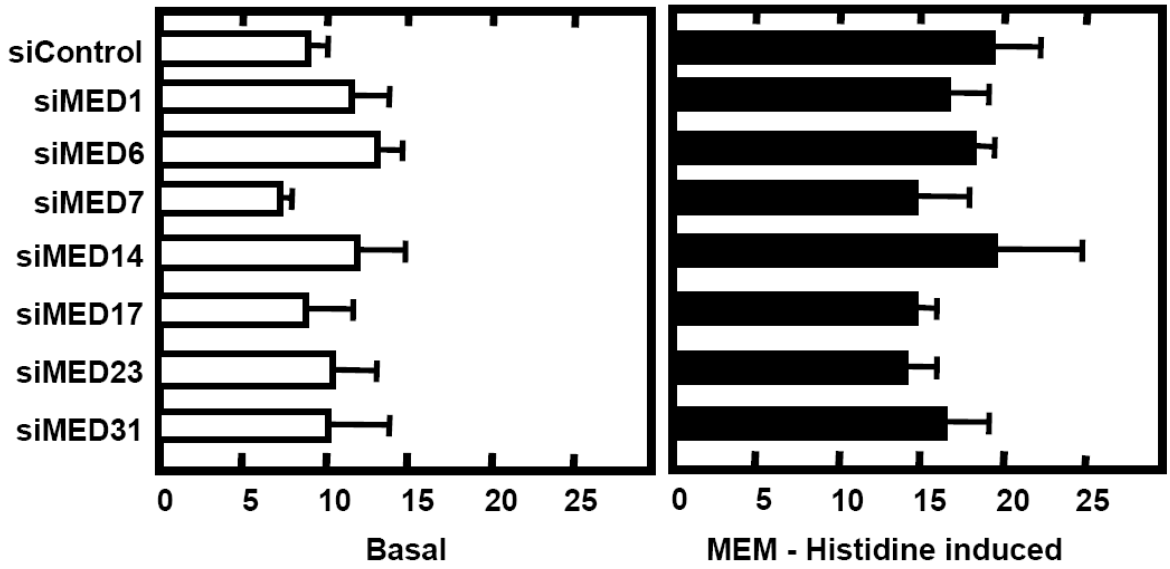

Figure 3-13. Knockdown of specific subunits of the Mediator complex does not affect SNAT2 transcription activity. MCF7 cells were treated with either a control siRNA or a siRNA specific to a subunit of the Mediator complex. After a 48 h siRNA treatment and incubation, cells were incubated in either DMEM or DMEM lacking histidine for 4 hours, and total RNA was isolated. RT-qPCR for SNAT2 transcription activity was performed, and the data are graphed as relative values. Each data point represents three samples and the RT-qPCR was performed in duplicate. The data are presented as the averages ± sem.

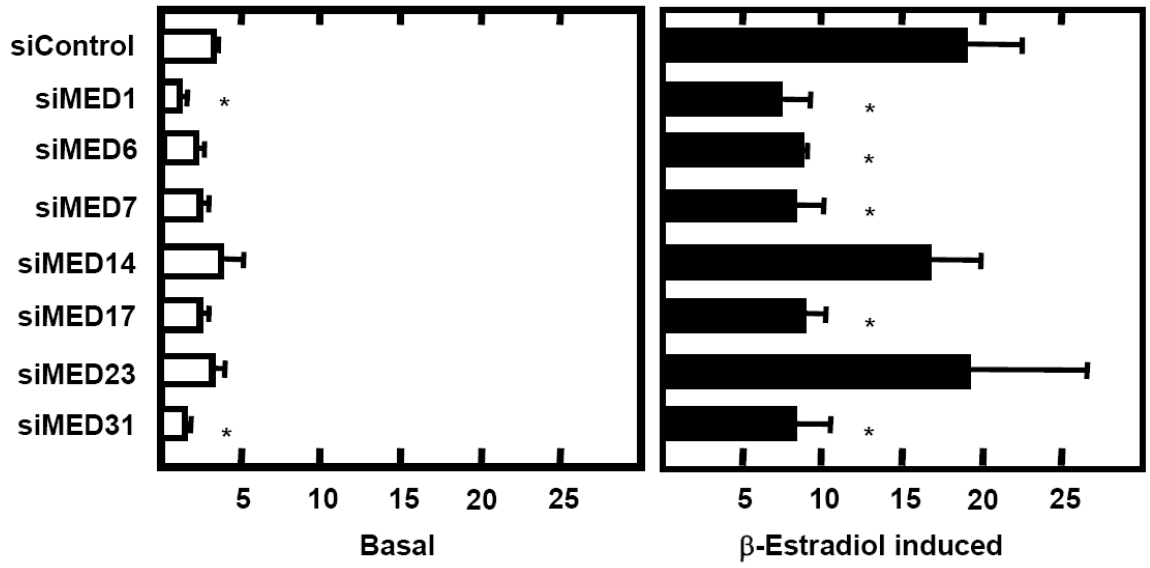

Figure 3-14. Knockdown of specific subunits of the Mediator complex affects both the basal and β-estradiol (E2) induced transcription of the pS2 gene. MCF7 cells were treated with either a control siRNA or a siRNA specific to a subunit of the Mediator complex. After a 48 h siRNA treatment and incubation, cells were incubated in either DMEM or DMEM plus 100 nM β-estradiol for 4 hours, and total RNA was isolated. RT-qPCR for pS2 transcription activity was performed, and the data are graphed as relative values. Each data point represents three samples and the RT-qPCR was performed in duplicate. The data are presented as the average ± sem.

CHAPTER 4
FUNCTION OF THE TRANSCRIPTION FACTORS THAT CONTROL TRANSCRIPTION FROM AARE-CONTAINING GENES

Introduction

Based on data in Chapter 3 of this dissertation and previously published observations cited in the Introduction Chapter (Chapter 1), it has been established that the transcription factors ATF4 and C/EBPβ regulate amino acid starvation-induced gene expression through recruitment and binding to amino acid responsive elements (AAREs). Further experimental data will now be presented to elucidate the function and necessity for each of these transcription factor's actions on AARE elements during amino acid starvation.

Gene Regulation by the ATF4 Protein

Activating transcription factor four (ATF4) is a member of the DNA-binding basic-region leucine zipper (bZIP) proteins which are known to homo- and heterodimerize with other group members (Hai and Curran, 1991). The DNA-binding site for ATF proteins was identified as "TGACGT (C/A) (G/A)," (Ameri and Harris, 2008) in which the first five nucleotides are highly similar to those in the AARE consensus sequence "TGATG (C/A) AA (T/C/A)". Experimental evidence has identified over thirty interaction partners of the ATF4 protein, mostly notably the RPB3 subunit of Pol II (De Angelis et al., 2003). The specificity for the ATF4 protein to function as a transcriptional activator is believed to be influenced by its interaction with different DNA-binding partners. ATF4 is believed to function as a general modulator of cellular stress signaling, given that its protein levels are enhanced by multiple stress conditions such as amino acid deprivation, ER stress, oxidative stress, and hypoxia (Siu et al., 2002; Roybal et al., 2005; Wek and Cavener, 2007; Ameri et al., 2004). All of these stress signaling pathways share the common characteristic of general inhibition of global cellular translation. ATF4 protein levels are rapidly increased during these stress conditions by a translational control mechanism that is

dependent upon ribosome scanning and tRNA charging (Vattem and Wek, 2004; Lu et al., 2004), analogous to the yeast GCN4 mechanism (Figure 1-2). The ATF4 mRNA sequence contains two upstream open reading frame (uORF) sequences 5' relative to the ATF4 translation start site with the second uORF sequence overlapping and out of frame with the ATF4 protein translation start site (Figure 4-1) (Vattem and Wek, 2004). The structure of ATF4 mRNA is highly conserved between vertebrate species with Figure 4-1 depicting both the stucture and its conservation between differet species. When the cellular status is at a basal unstressed state, the ribosome and charged tRNA will initiate at the first uORF and translate this small eight amino acid sequence. After termination of translation through the first uORF, the ribosome reinitiates translation quickly at the second uORF sequence. However, because this second uORF overlaps the ATF4 protein start site, a minimal level of ATF4 protein is produced in the basal condition. When cellular stress occurs, the reinitiation timing of the ribosome and the tRNA complex takes longer, due to lower levels of the eIF2-GTP complex, allowing the ribosome to scan past the second uORF and initiate translation at the ATF4 protein start site. Thus, ATF4 protein can be produced quickly and at high levels during cellular stress conditions by this translational control mechanism.

As a modulator of cellular stress caused by amino acid deprivation specifically, the rapid increase in ATF4 protein levels are associated with its function as a transcriptional activator of AARE-containing genes. ATF4 has been demonstrated to bind the ASNS AARE region as early as thirty minutes after amino acid deprivation occurs (Chen et al., 2004). Though ATF4 activates many genes responsible for alleviation of cellular stress, it also activates a regulatory loop that functions as a feedback signaling pathway responsible for terminating the general inhibition of translation. Phosphorylation of the eIF2 translation complex, specifically the alpha

subunit, is required for inhibition of global cellular translation (Kimball, 2002). GADD34, an eiF2α specific phosphatase, can be transcriptionally induced by ATF4, and ultimately restore global translation (Novoa et al., 2001).

While ATF4 has a defined role in cellular stress signaling, it is also important for both osteogenesis and long-term memory formation (Yang et al., 2004; Costa-Mattioli et al., 2005). In order to determine the function of ATF4 *in vivo*, a mouse model was developed that contains a homozygous deletion of the ATF4 gene by replacing it with a neomycin cassette (Masuoka and Townes, 2002). While most newborn homozygous mice did not survive after one hour of birth, a few where able to survive up to about four weeks. The most severe defect observed was fetal anemia due to defective hematopoiesis in the liver. Defects in lens formation, hair growth, and body size were also observed and were attributed to defects in cellular proliferation. Fibroblasts from these mice were cultured *in vitro* and demonstrated to be unable to induced ER stress responsive genes when compared to wild type fibroblasts (Harding et al., 2003). Wild-type or ATF4 deficient mouse embryonic fibroblasts (MEFs) were generously given to the Kilberg laboratory by Dr. Tim Townes (University of Alabama, Birmingham) and will be used in amino acid deprivation studies featured below to elucidate the role of ATF4 in the AAR pathway.

Gene Regulation by the C/EBPβ Protein

CCAAT/enhancer-binding protein beta (C/EBPβ) is another DNA-binding bZIP protein that can regulate gene expression through partnering with other bZIP proteins. Its consensus binding sequence was originally identified as the palindrome "ATTGCGCAAT," (Johnson, 1993) in which the first five nucleotides are similar to the last five nucleotides of the AARE consensus sequence "TGATG (C/A) AA (T/C/A)". C/EBPβ is an intronless gene whose mRNA sequence contains multiple translation initiation sites. Thus, three isoforms of the C/EBPβ protein, termed liver-enriched activating protein star (LAP*), LAP, and liver-enriched inhibitory

protein (LIP), are produced from a single mRNA sequence by differential translation (Ossipow et al., 1993). The regulated translation of these isoforms is controlled by an upstream uORF, and it has been proposed that differential isoform expression ultimately controls cellular growth or arrest (Calkhoven et al., 2000). Though C/EBPβ was initially characterized by its high expression in liver tissue and regulation of liver specific genes, recent evidence supports a function for C/EBPβ in inflammation, adipogenesis and obesity, diabetes, osteoblast differentiation, and regulation of stress inducible genes (Descombes and Schibler, 1991; Rahman et al., 2007; Naiki et al., 2007; Guo et al., 2001; Hata et al., 2005; Chen et al., 2005). C/EBPβ function in multiple normal and disease states is likely attributed to its opposing roles in cellular growth and repression which may result from its ability to control the cell cycle itself (Grimm et al., 2005). As a result, C/EBPβ protein production and function is also associated with many cancer phenotypes and cellular transformation (Grimm and Rosen, 2003).

C/EBPβ isoforms can act as either enhancers or repressors of transcription, and, as their names imply, the LAP* and LAP isoforms are generally considered as activators while evidence demonstrates the LIP isoform represses transcription by acting as a dominant negative (Descombes and Schibler, 1991). There is evidence, however, illustrating that the LAP protein can also function as a repressor, and it was hypothesized that the ratio of cellular LAP to LIP protein may modulate each isoform's function (Luedde et al., 2004). During cellular amino acid deprivation, C/EBPβ mRNA levels begin to increase after four hours and remain high after 24 hours of cellular stress (Chen et al., 2005). Immunoblotting demonstrates that increased protein levels of the LAP* and LAP isoforms correlates with the timing of induced mRNA levels, but levels of LIP protein do not increase until 18-24 hours of amino acid limitation (Chen et al., 2004). ChIP analysis of the ASNS and CAT-1 genes demonstrated C/EBPβ binding to the

AARE genomic region after amino acid deprivation (Chen et al., 2004; Lopez et al., 2007). My analysis, described in the experiments below, will determine the requirement and function of C/EBPβ during amino acid limitation.

C/EBPβ-deficient mice demonstrate numerous defects related to functionality of the immune system, impairment of adipocyte differentiation, inability to proliferate and differentiate mammary epithelial cells, and no female reproductive capacity (Ramji and Foka, 2002). Given C/EBPβ's regulation of liver-specific genes, it was surprising that livers of C/EBPβ-deficient mice seem to function normally, though they do show proliferation defects after partial hepatectomy (Greenbaum et al., 1998). Another interesting characteristic of C/EBPβ-deficient mice is their resistance to develop skin tumors after treatment with known carcinogenic compounds (Ramji and Foka, 2002). Recent work has demonstrated that administering C/EBPβ-deficient mice a diet lacking methionine and choline results in reduced levels of ER stress induction when compared to their wild type littermates (Rahman et al., 2007). The degree to which C/EBPβ deficiency affects other stress inducible pathways has yet to be determined. MEFs from mice deficient for C/EBPβ protein were generously given to the Kilberg laboratory by Dr. Peter Johnson (National Institutes of Health) and will be used in amino acid deprivation studies featured below to elucidate the role of C/EBPβ in the AAR pathway.

Advantages and Disadvantages of Using Either a Knockout Cell Line or siRNA Knockdown

Until the recent discovery of the small interfering RNA pathway (siRNA), the only method for eliminating gene expression, and subsequent protein production, of any mammalian gene was to create a mouse model in which a marker cassette replaced the gene to be eliminated. The advantage of this method is that since the gene is partially deleted or entirely removed, there is a true one hundred percent knockdown of gene expression in every cell. There are some

disadvantages, however, when selecting this method for gene expression knockout. It can be difficult to make these genomic deletions, especially if the gene is very large, and if the deletion cassette is not placed properly in a large gene, splicing variants of the knockout gene can be produced. Another concern is that any effect seen in a knockout fibroblast line must be compared to a wild type fibroblast line, and even though taken from animals of the same strain, the wild type cell line may well come from a different lineage or tissue than its knockout counterpart. Another considerable disadvantage is that typically only fibroblasts can be isolated and continually cultured *in vitro* from knockout animals, thus restricting experiments in different cell types. A final concern is that the true consequence of specific gene deletion may be masked by the ability of the cell line or animal model to adapt in the absence of gene expression.

With the discovery of the siRNA pathway, a second method of reducing or eliminating gene expression of specific genes in mammalian cells has emerged. This method functions *in vitro* by transiently transfecting a cell line with an 18-22 bp RNA oligonucleotide that has a complementary sequence to the mRNA sequence of a gene whose eliminated expression is desired. The advantages of this method are that it can performed in any mammalian cell line, the effect of knockdown can be easily compared to the same cells treated with a control reagent, and through advances in technology it is easy to get a high efficacy of knockdown in most cell lines. One of the disadvantages of this method is that since it is a knockdown model, rather than a knockout, a hundred percent elimination of gene expression and protein production can never be fully guaranteed. Another disadvantage is that the required use of a small oligonucleotide may cause non-specific knockdown of other genes ("off-target effects"), so multiple experiments with different oligonucleotides may be necessary to illustrate a gene-specific effect. Finally, given that this method involves a transient response, transfection must be performed every time an

assay in the knockdown condition is needed. There are plasmid-siRNA based methods for stable knockdown of gene expression, but this method has drawbacks as well.

While each method, knockout or knockdown of gene expression, has both advantages and disadvantages, the ability to do experiments with each method adds a new level of experimental specificity and reliability. If using both methods produces the same result, it can provide strong evidence that a result is genuine and not biased by the experimental method used. Based on these conclusions, both knockout cell lines and siRNA knockdown were used to assess ATF4 and C/EBPβ function during amino acid limitation

Results

Amino Acid Starvation in an ATF4 KO Cell Line Induces SNAT2 Gene Expression

Based on the nutrient control model of gene expression proposed by Chen et al. (Chen et al., 2004) and the experimental evidence presented in Chapter 3, the transcription factor ATF4 was believed to be required for the amino acid induced gene expression of all AARE containing genes. Given that proposal, Dr. Y. Pan, a post-doctoral fellow in the Kilberg laboratory, measured mRNA levels for amino acid responsive genes in both ATF4 wild type and knockout cell lines before and after amino acid limitation (Figure 4-2). ATF4 mRNA was measured by RT-qPCR as a control to confirm that the two cell lines were in fact wild type or deficient (knockout) for ATF4 mRNA. The ASNS gene demonstrated no induced steady state mRNA levels after eight hours of amino acid limitation in the ATF4 knockout cell line, though it was induced three-fold in the wild type cells. The SNAT2 gene, however, demonstrated the same fold change in amino acid limitation induced mRNA content in both the wild type and knockout cell lines (Figure 4-2). Total levels of SNAT2 mRNA were reduced in the ATF4 knockout cell line, which may be related to the slow growth pattern these cells exhibit. These data demonstrate

that SNAT2 and ASNS may require different DNA binding proteins for amino acid induced gene expression, and that the SNAT2 gene may not require ATF4 binding at all.

Knockdown of ATF4 Protein by siRNA Does Not Block Induction of SNAT2 Transcription

To further determine if ATF4 binding is necessary for induction of SNAT2 gene expression, siRNA against the mRNA sequence of ATF4 was performed in HepG2 cells. Figures 4-3 and 4-4 exhibit control RT-qPCR reactions and immunoblots performed to confirm efficient and specific knockdown of ATF4 mRNA and protein. GAPDH steady state mRNA, which was shown in Chapter 3 to be unaffected by amino acid limitation (Figure 4-2), was measured for each sample to demonstrate that equal levels of mRNA were present in both MEM and MEM + histidinol (HisOH) treated samples. HisOH competitively blocks binding of histidine to its uncharged tRNA and therefore generates induction of the AAR pathway by causing an increased pool of cellular uncharged tRNA (Hansen et al., 1972). The Kilberg laboratory has demonstrated that treating cells with histidinol induces both ASNS transcription and eIF2α phosphorylation to similar levels observed with histidine-deficient medium treatment of cells (Hutson and Kilberg, 1994; Thiaville et al., 2008a). Treating cells with a combination of histidine-deficient medium and histidinol did not provide an additive response in AAR pathway induction also supporting a common mechanism.

Treatment with siRNA also had little to no effect on total gene expression when siRNA treated samples were compared to either non-transfected or mock treated samples. HepG2 cells were also treated with a control siRNA against cyclophilin B (peptidylprolyl isomerase B) to demonstrate the transfection method used was able to efficiently knockdown mRNA expression of a specific gene. RT-qPCR performed with primers specific to cyclophilin b mRNA gave an approximately 90% reduction in cyclophilin b mRNA when compared to all control conditions (Figure 4-3). ATF4 steady state mRNA analyzed by RT-qPCR was reduced by approximately

75% after treatment with a siRNA specific for ATF4. ATF4 mRNA induction by amino acid starvation was also blocked by siRNA treatment (Figure 4-3). An immunoblot of total cellular protein from HepG2 cells treated with either control siRNA or siRNA specific for ATF4 demonstrated almost no production of ATF4 protein in siATF4 treated cells, consistent with the RT-qPCR data (Figure 4-4).

The data presented in Figures 4-3 and 4-4 clearly demonstrate a specific and effective knockdown of ATF4 mRNA and protein by siRNA transfection of HepG2 cells. Given these data, RT-qPCR was used to analyze gene specific transcription activity during amino acid limitation after knockdown of ATF4 protein (Figure 4-5). After four hours of amino acid limitation, ASNS induced transcription activity in siATF4-treated cells was reduced to levels similar to the basal condition in the control treated cells. For SNAT2 transcription activity, however, there was no change in either the basal or induced mRNA levels between the siRNA control and siATF4 conditions. These data, in concert with the data from the ATF4 knockout cell lines, demonstrate that ASNS induced transcription activity is almost entirely dependent on ATF4, while SNAT2 transcription activity can be ATF4 independent.

Effect of ATF4 Protein Knockdown on Recruitment and Increased Binding of C/EBPβ and ATF3 to AARE-containing Genes

The proposed model of gene expression during nutrient stress describes two phases of gene expression. The first phase is characterized by ATF4 recruitment and activation (0-4 hours) whereas the second phase exhibits ATF3 and C/EBPβ recruitment and repression (4-24 hours) (Chen et al., 2004). In order to determine if the second phase can occur without ATF4 binding during the first phase, ChIP analysis was performed on HepG2 cells treated with either control or ATF4-specific siRNA and then induced by amino acid limitation for eight hours. Figure 4-6 depicts transcription factor binding to either the ASNS AARE or coding region after treatment

with siATF4 during amino acid deprivation. ATF3 and C/EBPβ binding to the AARE was clearly increased after starvation in siRNA control conditions, but there was no recruitment of these transcription factors in the siATF4-treated starvation condition. As a control for the siRNA effectiveness, ATF4 was not recruited to the ASNS AARE in siATF4 treatment conditions (Figure 4-6). In corroboration with the ASNS transcription activity data, there was no recruitment of Pol II in the siATF4 starvation condition, indicating that ASNS gene expression cannot be induced by amino acid deprivation when ATF4 protein is not produced. Transcription factor binding to the ASNS coding region is shown as a negative control for background binding.

Given that SNAT2 transcription activity was still induced by amino acid deprivation during knockdown of ATF4 protein, unlike ASNS, ChIP analysis of starvation-induced protein binding to the SNAT2 AARE during siATF4 treatment was performed (Figure 4-7). Though increased ATF4 binding at the SNAT2 AARE was abolished upon siATF4 treatment, Pol II binding still increased approximately three-fold agreeing with the previous data that increased SNAT2 gene expression by amino acid limitation can be ATF4 independent. ChIP analysis also demonstrated that ATF3 and C/EBPβ binding to the SNAT2 AARE increased after ATF4 knockdown even though no ATF4 binding was present. Thus, it seems that if amino acid limitation causes increased gene expression, whether it is ATF4 dependent or independent, the second phase of nutrient regulation (i.e., repression) will occur. In its entirety, the data suggest that different activators, one being ATF4 and the other unknown, may induce ASNS and SNAT2 gene expression during amino acid deprivation.

The AARE Element Requires ATF4 to Function as an Enhancer of Transcription

Previous functional assay data demonstrated that over-expression of ATF4 by transient transfection in HepG2 cells can stimulate both ASNS and SNAT2 AARE-driven transcription in the presence of a wild type, but not a mutated, AARE enhancer element (Siu et al., 2002; Palii et

al., 2006). Given that ChIP can only resolve binding activity to within a range of approximately 500 bp due to sonication of DNA into an average of 500 bp fragments, functional assays were performed to determine if the AARE itself requires ATF4 for transcription activation. HepG2 cells were treated with either control or ATF4-specific siRNA for 24 hours then transiently transfected with luciferase vectors containing either the ASNS or SNAT2 AARE. After 48 hours, cells were transferred to either MEM or MEM plus HisOH for eight hours and luciferase activity was assessed. Figure 4-8 demonstrates that for both the ASNS and SNAT2 AARE-containing constructs, there was little to no activation of luciferase activity (i.e., gene transcription) without ATF4 protein, even in the condition of nutrient deprivation. Though the result seems obvious for the ASNS gene given the previous siRNA and knockout data, it was surprising that the SNAT2 AARE-containing construct was not induced in ATF4-deprived conditions given that the endogenous SNAT2 gene was functional in ATF4 deficient conditions (Figures 4-2, 4-5, and 4-7). One major conclusion from this experiment is that ATF4 protein binding activates the AARE during amino acid limitation. Given that the endogenous SNAT2 gene expression can be induced by starvation in the absence of ATF4 protein, it is possible that a second genomic element may activate SNAT2 transcription through binding of a second transcriptional activator. An attempt was made to find another element in the SNAT2 gene that can induce SNAT2 transcription during starvation by using functional assays. Unfortunately all SNAT2 genomic pieces longer than the –512/+770 bp fragment, even those containing the functional AARE, were not induced by starvation (data not shown). Thus, another method will need to be utilized in the future to determine how SNAT2 gene expression is induced in an ATF4-independent manner.

Knockdown of C/EBPβ by siRNA Does Not Inhibit SNAT2 Transcription

After analyzing the requirement for the ATF4 protein, similar studies were utilized to determine the role of C/EBPβ during amino acid starvation. Previous experimental evidence suggested that C/EBPβ functions as a repressor of AARE-induced gene expression during the second phase (4-24 hour) of amino acid starvation (Chen et al., 2004). Given those results, knockdown of C/EBPβ mRNA and protein in HepG2 cells using a siRNA specific for C/EBPβ was performed. Figure 4-9 illustrates control experiments performed to verify that knockdown of the C/EBPβ protein was efficient and specific. Panel A depicts the amount of steady state C/EBPβ mRNA (normalized to GAPDH mRNA) before and after two or eight hours of amino acid limitation in cells treated with either a control or C/EBPβ-specific siRNA. For both time points, cells treated with siRNA to C/EBPβ demonstrated mRNA levels below or equal to basal conditions, even after the starvation-inducing treatment. In corroboration, immunoblot analysis of protein extracts from cells treated with either siControl or siC/EBPβ RNAs demonstrated little to no protein production when C/EBPβ was knocked down by siRNA (Panel B). No significant induction of C/EBPβ protein was identified after treatment with siC/EBPβ when compared to control siRNA-treated cells. An immunoblot for the protein actin functions as loading control.

Given that Figure 4-9 demonstrates effective and specific knockdown of C/EBPβ mRNA and protein, the transcription activity for the AARE-containing genes ASNS and SNAT2 was measured by RT-qPCR after treatment with the siRNA for C/EBPβ (Figure 4-10). The ASNS gene exhibited similar levels of transcription after two hours of starvation in cells treated with either the control or C/EBPβ specific siRNAs. At there two hours there is little or no increase in C/EBPβ protein synthesis (Figure 4-8) nor was there previously demonstrated to be recruitment to the ASNS gene (Chen et al., 2004). Basal levels of ASNS gene expression were not affected by knockdown of C/EBPβ protein. After eight hours of starvation, a time at which C/EBPβ

protein levels increased (Figure 4-8) and there was increased binding at the ASNS gene (Chen et al., 2004), ASNS expression was induced to a higher level in cells treated with siC/EBPβ. SNAT2 transcription activity, both basal and amino acid starvation-induced levels, were unaffected by knockdown of C/EBPβ protein (Figure 4-10).

AARE Transcription and ATF4 Protein are Induced by Amino Acid Starvation in a C/EBPβ Knockout Cell Line

In order to complement the siRNA data from the C/EBPβ protein knockdown studies, experiments were performed in either wild type or C/EBPβ-deficient MEFs kindly provided by Dr. Peter Johnson. RT-qPCR analysis of both ASNS and SNAT2 transcription activity was performed in wild type and C/EBPβ deficient cells treated with either DMEM or DMEM plus HisOH for a time period of 12 hours (Figure 4-11). Consistent with the siRNA data, ASNS transcription activity was still induced by amino acid deprivation in the C/EBPβ knockout cell line. In fact, ASNS transcription activity was induced to higher levels in the C/EBPβ knockout cell lines, compared to the wild type MEFs, but only at two and four hours after starvation. These data are in contrast to the siRNA data, which showed a difference only after eight hours of starvation. These temporal differences may be attributed to analysis in a knockout cell line versus siRNA knockdown, but they do not alter the basic conclusion that the ASNS gene was induced by amino acid starvation when no C/EBPβ protein was present, and may actually be induced to a greater extent. RT-qPCR analysis of SNAT2 transcription activity in wild type and C/EBPβ knockout MEFs was consistent with the siRNA data demonstrating no change in SNAT2 transcription activity. Given that knockout of C/EBPβ protein yielded a small increase in ASNS transcription activity, which was previously shown to be regulated by ATF4 protein, ATF4 protein levels were measured in whole cell extracts of C/EBPβ knockout cells. As shown

in Figure 4-12, ATF4 induction by amino acid deprivation occurred in the C/EBPβ knockout cell line, and its induction pattern was consistent with previously published data (Chen et al., 2004).

Induced Transcription Factor Binding and Histone Acetylation at the AARE Occurs in C/EBPβ Knockout Cells

To determine if knockout of C/EBPβ protein affected histone acetylation or transcription factor recruitment to an AARE, ChIP analysis was performed in C/EBPβ wild type and knockout MEFs (Figure 4-13). The ASNS AARE was used as a model gene for ChIP analysis of an AARE given that SNAT2-induced transcription in MEFs was barely two fold (see Figure 4-11). In Panel A, ChIP analysis was used to screen for binding of different C/EBP isoforms to the ASNS AARE. C/EBPβ demonstrated both basal and increased binding to the AARE upon amino acid starvation in wild type cells, consistent with ChIP analysis of HepG2 cells (Figure 3-2), but there was no basal or induced binding in C/EBPβ knockout cells as expected. ChIP analysis also established no binding of the proteins C/EBPα or C/EBPδ in either cell type, thus signifying that these proteins do not replace C/EBPβ on the AARE (Figure 4-13). Histone H3 acetylation, Pol II recruitment, and binding of the transcription factors ATF4 and ATF3 to the AARE after amino acid starvation occurred in both wild type and C/EBPβ knockout cell lines (Panel B, Figure 4-13). While ATF4 and Pol II binding occurred at similar levels in the two cell lines, acetylation of histone H3 was generally reduced in the knockout cell line compared to the wild type, although the fold change in HisOH-dependent induction was similar between the two cell lines. Higher levels of ATF3 recruitment to the AARE were demonstrated in the C/EBPβ knockout compared to the wild type. Given that ATF3 and C/EBPβ may work together to repress AARE transcription, higher ATF3 levels may compensate for loss of C/EBPβ protein binding to the AARE.

Conclusions

Previous experimental analysis has demonstrated increased mRNA synthesis and protein production of the transcription factor ATF4 after amino acid deprivation (Siu et al., 2002; Chen et al., 2004). Functional assays demonstrated that ATF4 alone could activate plasmid-based ASNS and SNAT2 transcription through their AARE (Pan et al., 2003), and EMSA analysis confirmed binding of ATF4 to an AARE probe sequence in starved extracts (Siu et al., 2002; Palii et al., 2006). In order to extend these observations, my analysis focused on ASNS and SNAT2 gene expression during amino acid limitation when ATF4 was absent. The data from these studies illustrate that ATF4 is required for ASNS induced gene expression during nutritional stress, and that the recruitment of other transcription factors to the ASNS promoter-AARE region, such as C/EBPβ and ATF3, does not happen in ATF4 deficient conditions. In contrast to ASNS, SNAT2 gene expression was still induced by starvation in ATF4 deficient conditions, and increased Pol II binding to the SNAT2 promoter and C/EBPβ and ATF3 recruitment to the enhancer region also occur. Functional assay data, however, demonstrate that from a plasmid-based reporter gene the AARE requires ATF4 to function as a transcriptional enhancer during nutrient stress. It is interesting to speculate that the SNAT2 gene may contain another amino acid-inducible enhancer element that can function without ATF4 presence. Recently, the System X_c (-) transporter xCT, specific for the transport of cystine and glutamate, was demonstrated to contain two genomic AARE sequences in its promoter region that are separated by nine base pairs (Sato et al., 2004a). One of the AARE elements, AARE-F (5'-3' sequence), is similar to the ASNS AARE, while the other element, AARE-R (5'-3' sequence), is more similar to the SNAT2 AARE when the upper strand of DNA is read 5' to 3'. Even though both of these AAREs are required for maximal induced gene expression of xCT during amino acid limitation, functional assays demonstrated that mutating only one AARE sequence did not

completely abolish starvation induced stimulation (Sato et al., 2004a). For the human C/EBPβ gene, it was demonstrated that though its 3'UTR is required for its induced gene expression during amino acid limitation, no single AARE element able to confer this activity could be identified (Chen et al., 2005). Instead, three genomic regions within a 93 base pair region of the C/EBPβ 3'UTR were all determined to contribute to the C/EBPβ response to amino acid deprivation. It was demonstrated that the IGFBP-1 gene is induced by amino acid starvation through an E-box element, not an AARE (Matsukawa et al., 2001). It was later observed that IGFBP-1 also contains an AARE sequence 6000 bp upstream of the transcription start site that is bound by ATF4 after ER stress (Marchand et al., 2006). Therefore, it is not unlikely that other genomic elements in the SNAT2 gene may be used for amino acid stress induction of the endogenous gene.

Similar to ATF4, C/EBPβ mRNA synthesis and protein levels also increase after amino acid deprivation (Siu et al., 2001; Chen et al., 2004). In contrast to ATF4, however, EMSA and ChIP analysis demonstrated that C/EBPβ binds to the AARE region of ASNS in the basal condition and its binding then increases during amino acid limitation (Siu et al., 2001; Chen et al., 2004). The current experiments have focused on extending those observations to gain additional insight into the function of C/EBPβ during amino acid limitation. Both C/EBPβ knockout MEFs and HepG2 cells treated with siC/EBPβ showed a trend toward increased ASNS activation in the absence of C/EBPβ protein, but not for SNAT2. The ASNS data agrees with previous observations suggesting that C/EBPβ's role during amino acid limitation is to function as a transcriptional repressor, and yet, the lack of an effect on SNAT2 transcription demonstrates that the role of C/EBPβ is not universal for all amino acid-induced genes (Chen et al., 2004). Given that the repressor ATF3 also binds the AARE-containing genes with a timing pattern

similar to that of C/EBPβ, ATF3 alone may be able to modulate AARE transcription without C/EBPβ, thus explaining the modest increase in ASNS gene expression.

Given that the present and previous data demonstrated a significant level of C/EBPβ binding to the AARE in the basal condition, it was surprising that knockout of C/EBPβ protein had no effect on ASNS or SNAT2 basal gene expression. Furthermore, ChIP analysis established no other C/EBP protein replaced C/EBPβ, in its absence, on the AARE. Can Zhong, of the Kilberg laboratory, performed EMSA analysis using nuclear extracts from both C/EBPβ WT and KO MEFs and established that an unknown protein complex can bind the AARE region in the absence of C/EBPβ (Thiaville et al., 2008a). This complex formation was much stronger in the C/EBPβ knockout cells than the wild type, and the entire complex was able to be supershifted with ATF4 specific antibodies, demonstrating it contained ATF4. This result suggests that C/EBPβ may actually antagonize ATF4 binding to the AARE, consistent with the result that ATF4 can still bind the AARE in the absence of C/EBPβ.

After amino acid limitation, by assessing levels of Pol II binding, ChIP analysis demonstrated that the ASNS gene could still be induced in the absence of C/EBPβ. Levels of histone acetylation, though still induced, were diminished at the AARE in the C/EBPβ knockout cell line. C/EBPβ has been demonstrated to interact with a variety of histone and chromatin modulators suggesting that C/EBPβ binding in the basal condition may contribute to the high basal acetylation levels of the AARE (Mink et al., 1997; Kowenz-Leutz and Leutz, 1999; Xu et al., 2007). Absence of C/EBPβ also resulted in higher levels of ATF3 binding to the AARE after amino acid limitation. Increased ATF3 binding may be necessary to modulate the repression phase of amino acid starvation in the absence of C/EBPβ, given the repressive effect it exerts on AARE-containing genes.

All of the data presented demonstrate that the transcription factors ATF4 and C/EBPβ perform specific functions during amino acid limitation. Further detailed analysis of each protein, through determination of post-translational modifications, protein turnover mechanisms, interacting partners, etc., will be necessary to develop a complete model of their function and regulation during nutrient deprivation. By exploring the roles of both a transcriptional activator, ATF4, and a transcriptional repressor, C/EBPβ, during amino acid limitation, the entire mechanism of starvation induced gene expression and its modulation can be further characterized.

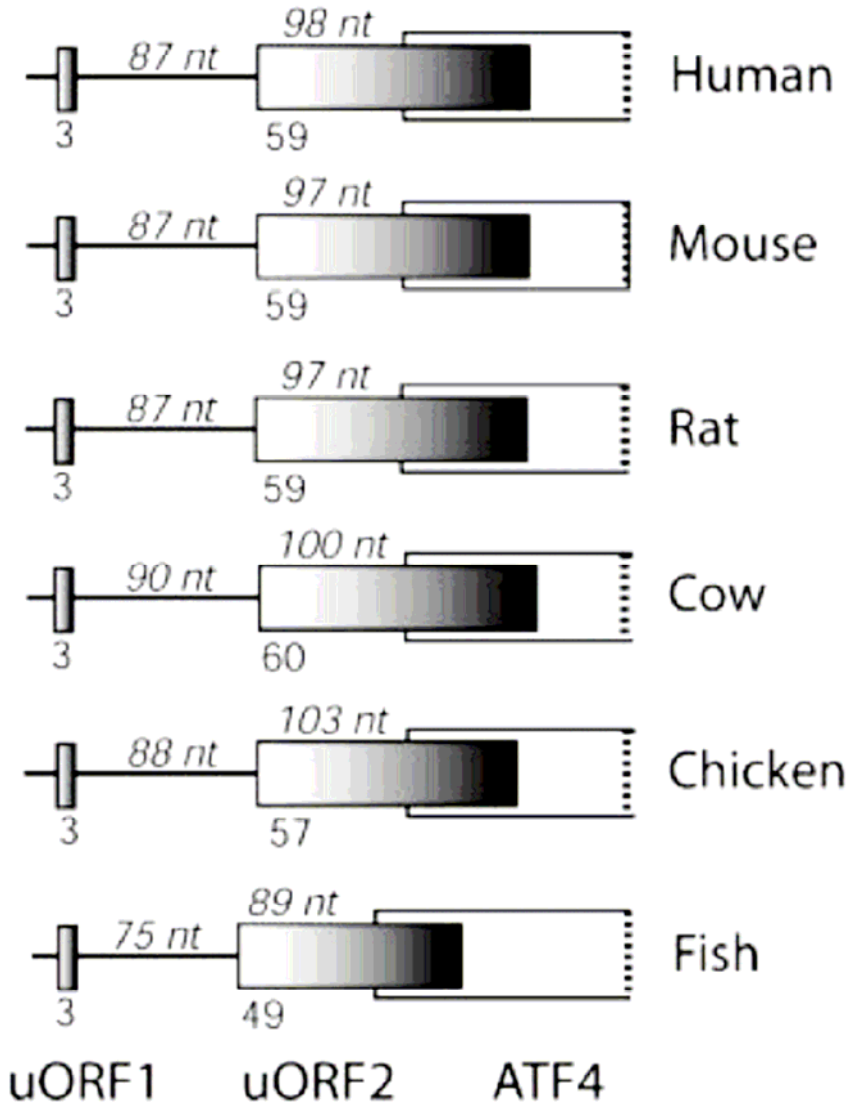

Figure 4-1. The ATF4 mRNA structure and its conservation between different veterbrate species. The ATF4 mRNA structures from human, mouse, rat, cow, chicken, and fish vertebrate species are depicted above. All ATF4 mRNAs contain two uORFs (shaded boxes) with the second uORF overlapping and out-of-frame with the ATG nucleotides used to produce the ATF4 protein (white box). The number of nucleotides between uORF1 and 2 and between uORF2 and the ATG for ATF4 are listed on top. The numbers below represent the number of amino acids encoded by each uORF. This figure was adapted from Vattem et al., PNAS 2004.

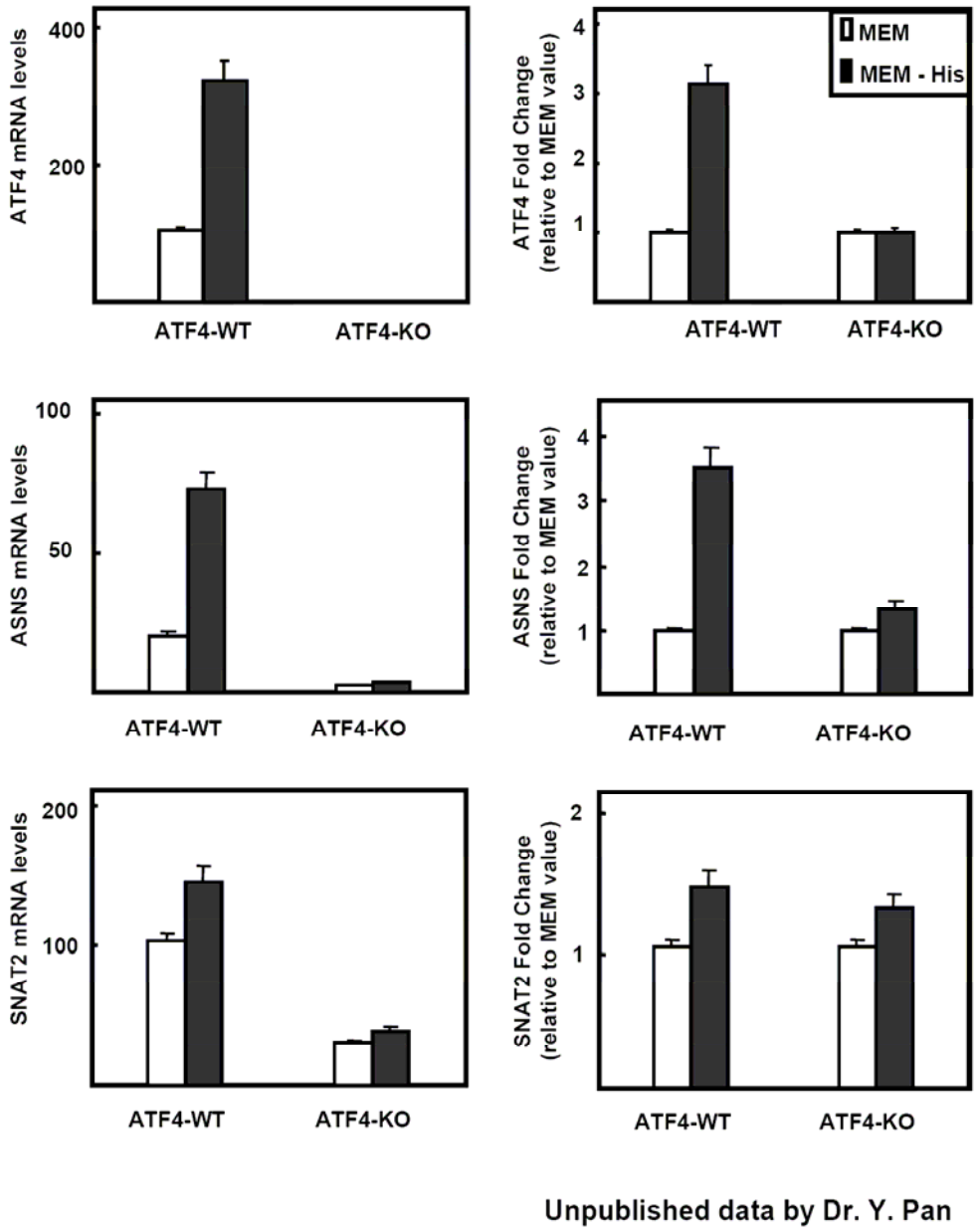

Figure 4-2. SNAT2 gene expression still increases after amino acid deprivation in both ATF4 wild type and knockout MEFs. MEFs were treated with either complete MEM (white box) or MEM lacking histidine (black box) for eight hours, and total RNA was isolated. Specific primers were used to measure ATF4, ASNS, or steady state mRNA by RT-qPCR. RNA for each data point represents three samples and the RT-qPCR for each data point was performed in duplicate, thus, the data on the left side are presented as the average ± the standard error of the means (sem). The data on the right side are adjusted to demonstrate the levels of mRNA induction (Fold Change) by plotting the treatment value divided by the control MEM value. (Data provided by Dr. Y. Pan)

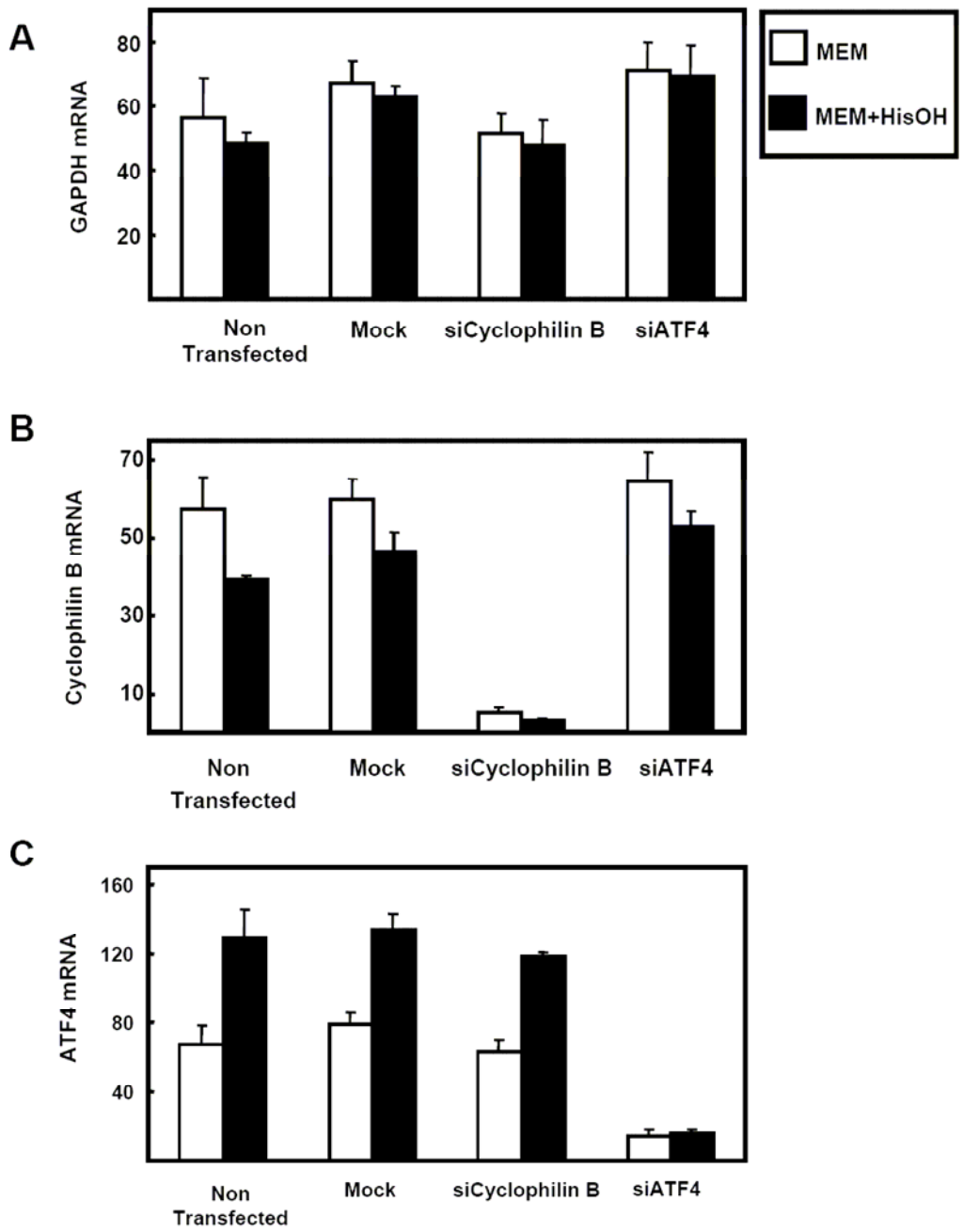

Figure 4-3. Steady state mRNA levels after amino acid deprivation of HepG2 cells treated with siRNA to ATF4. HepG2 cells were transiently transfected with siRNA specific to either cyclophilin B or ATF4 for 24 h, and then given fresh medium and incubated an additional 24 h. These cells were then treated with either MEM (white box) or MEM plus 2 mM HisOH (black box) for 4 h, and total cellular RNA were isolated. Specific primers were used to measure GAPDH, Cyclophilin B, and ATF4 steady state mRNA by RT-q PCR (see Table 2-1 for primer sequences). RNA for each data point represents three samples and the RT-qPCR for each data point was performed in duplicate, thus, the data are presented as the average ± sem.

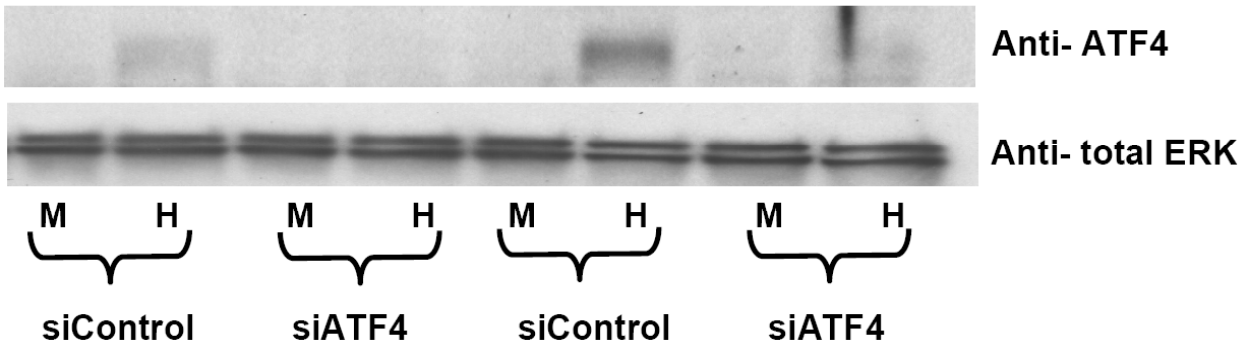

Figure 4-4. Cellular ATF4 protein levels after amino acid deprivation of HepG2 cells treated with siRNA to ATF4. HepG2 cells were transiently transfected with siRNA specific to either cyclophilin B or ATF4 for 24 h, and then given fresh medium and incubated an additional 24 h. These cells were then treated with either MEM (M) or MEM plus 2 mM HisOH (H) for 4 h, and total cellular protein extracts were isolated. Immunoblots for either ATF4 or total extracellular signal-regulated kinase (ERK), as a loading control, were performed with 30 μg of total protein per lane.

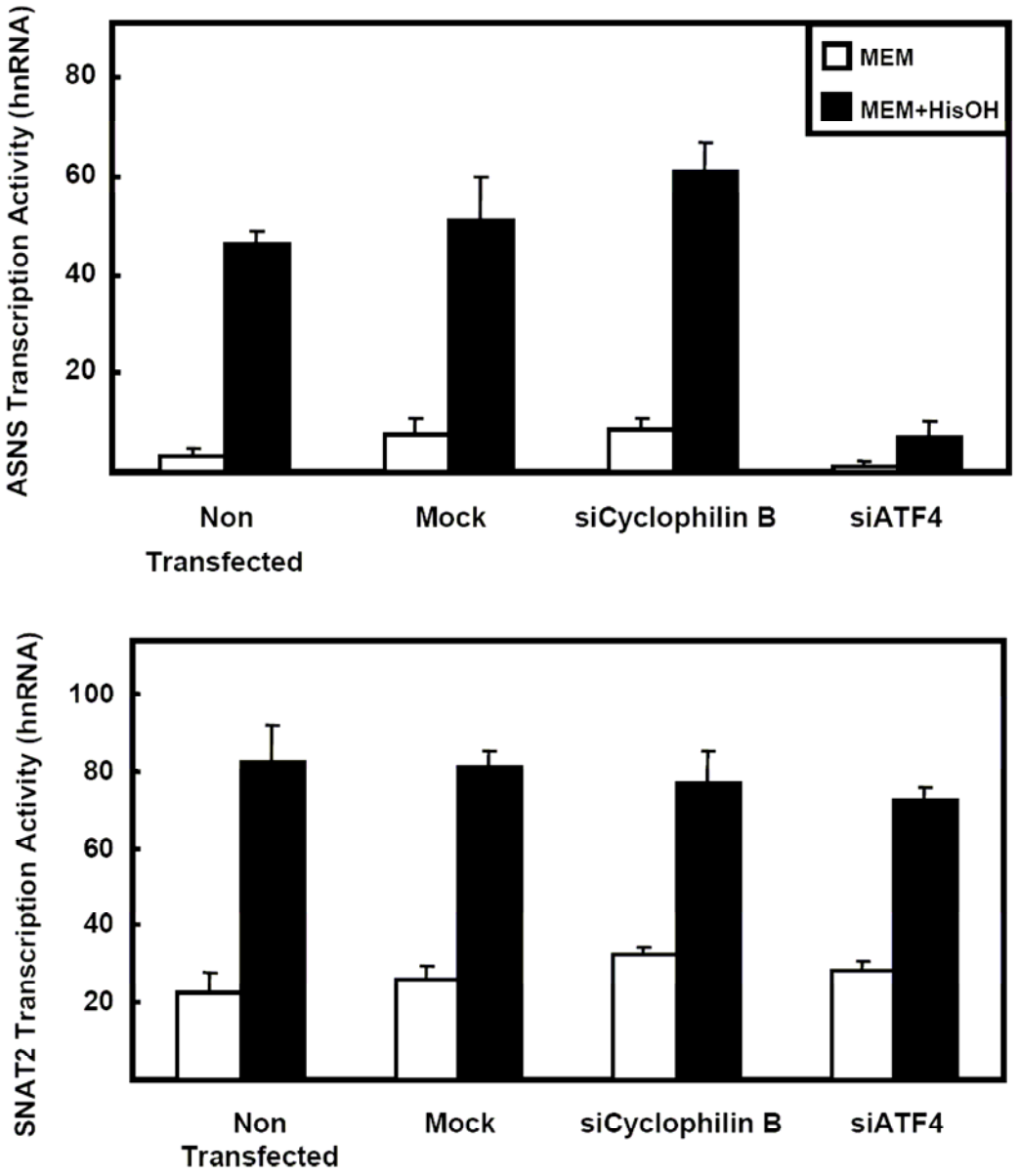

Figure 4-5. SNAT2 transcription activity increases after amino acid deprivation of HepG2 cells treated with siRNA to ATF4. HepG2 cells were transiently transfected with siRNA specific to either cyclophilin B or ATF4 for 24 h, and then given fresh medium and incubated an additional 24 h. These cells were then treated with either MEM (white box) or MEM plus 2 mM HisOH (black box) for 4 h, and total RNA extracts were isolated. Specific primers were used to measure ASNS and SNAT2 transcription activity by RT-qPCR. RNA for each data point represents three samples and the RT-qPCR for each data point was performed in duplicate, thus, the data are presented as the average ± sem.

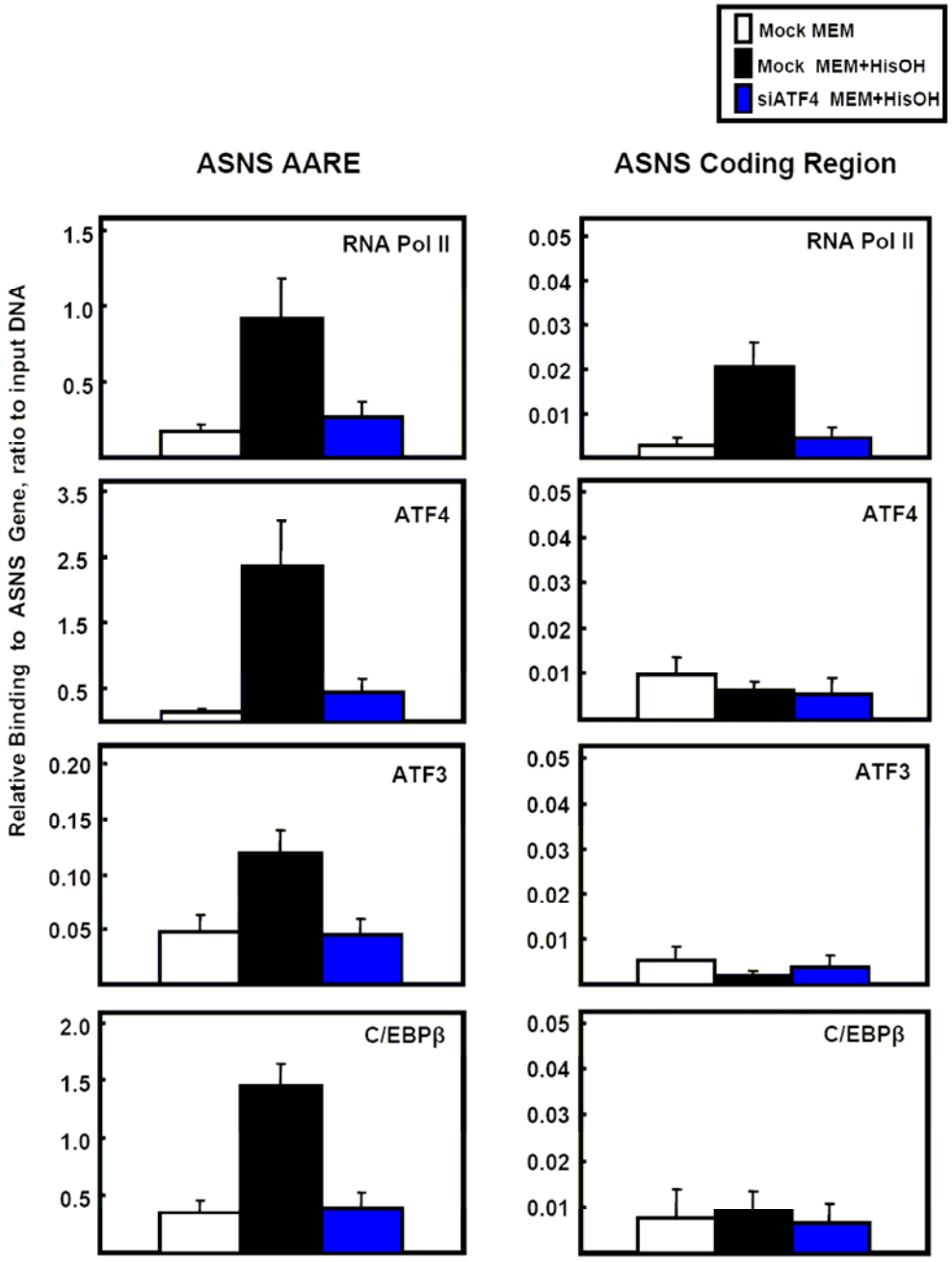

Figure 4-6. ATF4 knockdown prevents transcription factor binding to the ASNS AARE after amino acid deprivation. HepG2 cells were either mock transfected or transiently transfected with an siRNA specific to ATF4 for 24 h, and then given fresh medium and incubated an additional 24 h. These cells were then treated with either MEM or MEM plus 2 mM HisOH for 4 h and used for ChIP analysis. Specific primers were used to measure binding to either the ASNS AARE or the coding region by qPCR (see Figure 2-3 for primer sequences). ChIP analysis was performed in triplicate and qPCR for each data point was performed in duplicate, thus, the data are presented as the average ± sem.

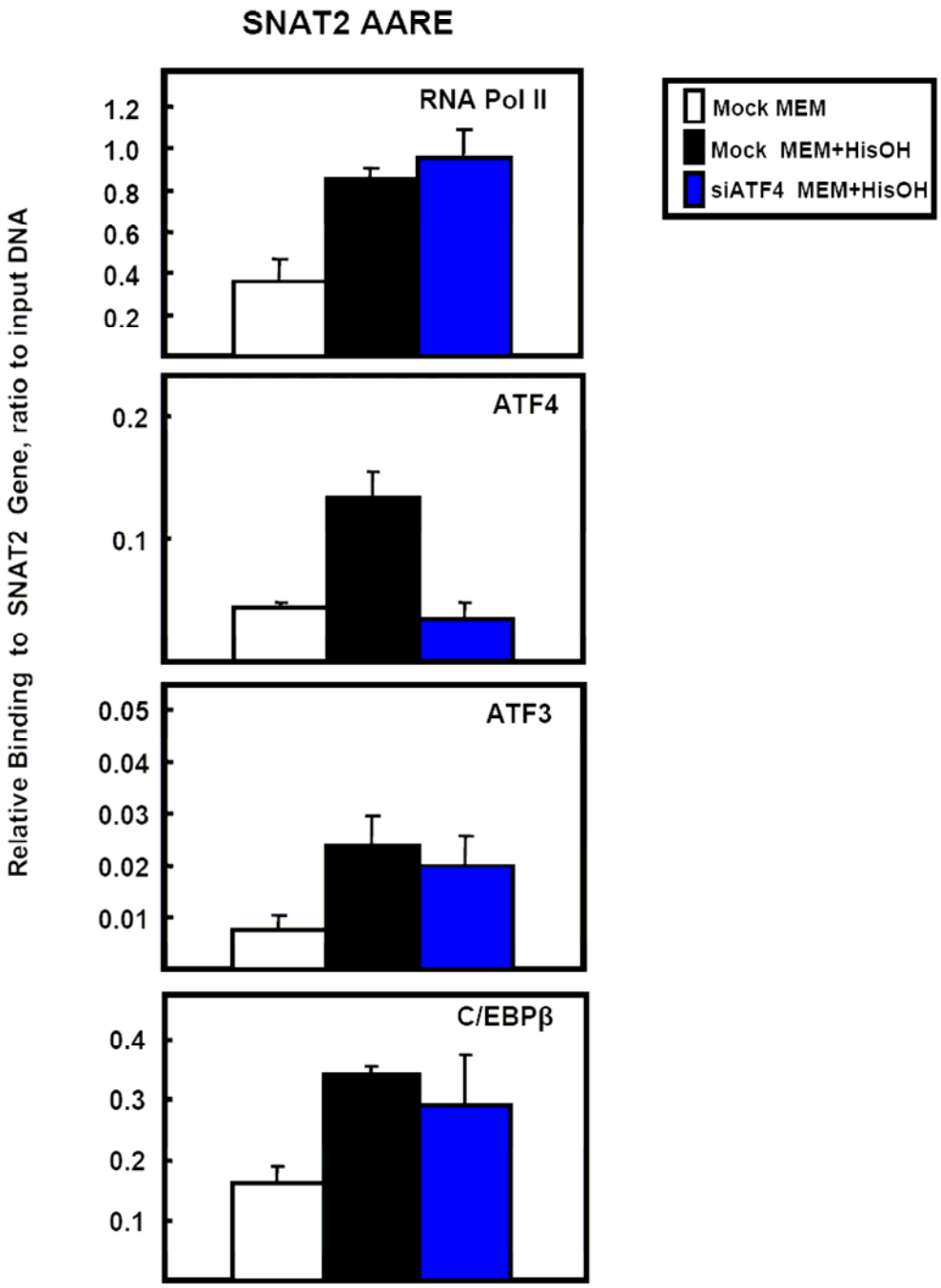

Figure 4-7. Transcription factor binding to the SNAT2 AARE after amino acid deprivation still occurs after ATF4 knockdown. HepG2 cells were transfected with either transfection reagent alone (Mock) or with an siRNA specific to ATF4 for 24 h, and then given fresh medium and incubated an additional 24 h. These cells were then treated with either MEM or MEM plus 2 mM HisOH for 4 h and used for ChIP analysis. Specific primers were used to measure binding to the SNAT2 AARE by qPCR. ChIP analysis was performed in triplicate and qPCR for each data point was performed in duplicate, thus, the data are presented as the average ± sem.

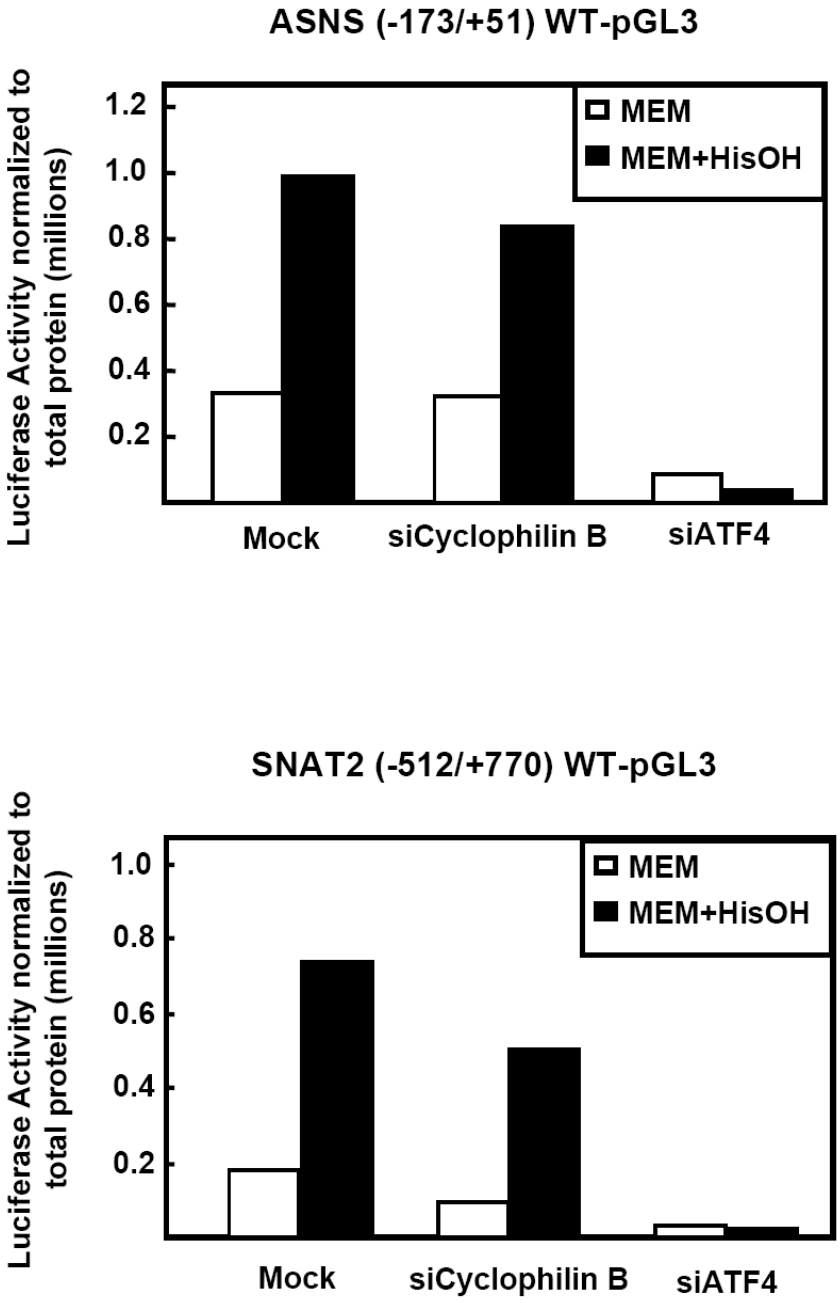

Figure 4-8. AAR-enhanced gene expression through an AARE requires ATF4. HepG2 cells were either Mock (reagent alone) transfected or transiently transfected with siRNA specific to either cyclophilin B or ATF4 for 24 h, and then given fresh medium for 3 to 4 hours. Cells were then transiently transfected for 3 h with plasmids containing the Firefly luciferase gene driven by either the ASNS (-173 / +51) or SNAT2 (-512 / +770) AARE plus promoter before being given fresh medium for additional 18 h. These cells were then treated with either MEM (white boxes) or MEM plus 2 mM HisOH (black boxes) for 48 h and used for luciferase assay analysis. The data are presented as a single experiment performed in duplicate.

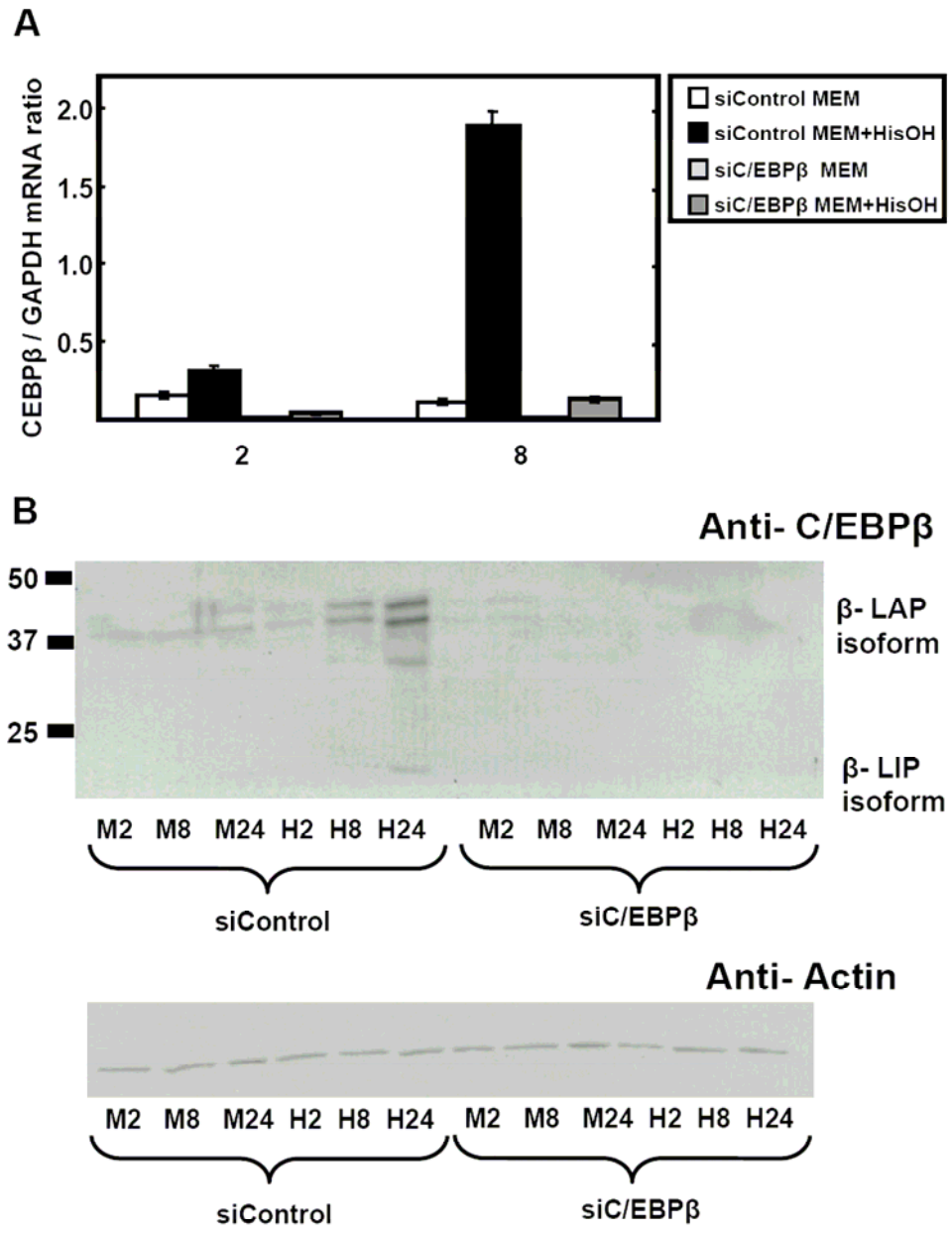

Figure 4-9. Analysis of knockdown efficiency for C/EBPβ in siRNA-treated HepG2 cells. HepG2 cells were transiently transfected with either control siRNA or siRNA specific to C/EBPβ for 24 h, and then given fresh medium and incubated an additional 24 h. These cells were then treated with either MEM or MEM plus 2 mM HisOH for a time period between 2 and 24 hours. A) RNA was isolated from treated cells at 2 or 8 h. Specific primers were used to measure C/EBPβ and GAPDH mRNA by RT-qPCR. RNA for each data point represents three samples and the RT-qPCR for each data point was performed in duplicate. The data are presented as the average ± sem. B) total cellular protein from treated cells was isolated at 2, 8, or 24 h. Immunoblots for either C/EBPβ or actin (protein loading control) were performed with 30 μg of total protein per lane.

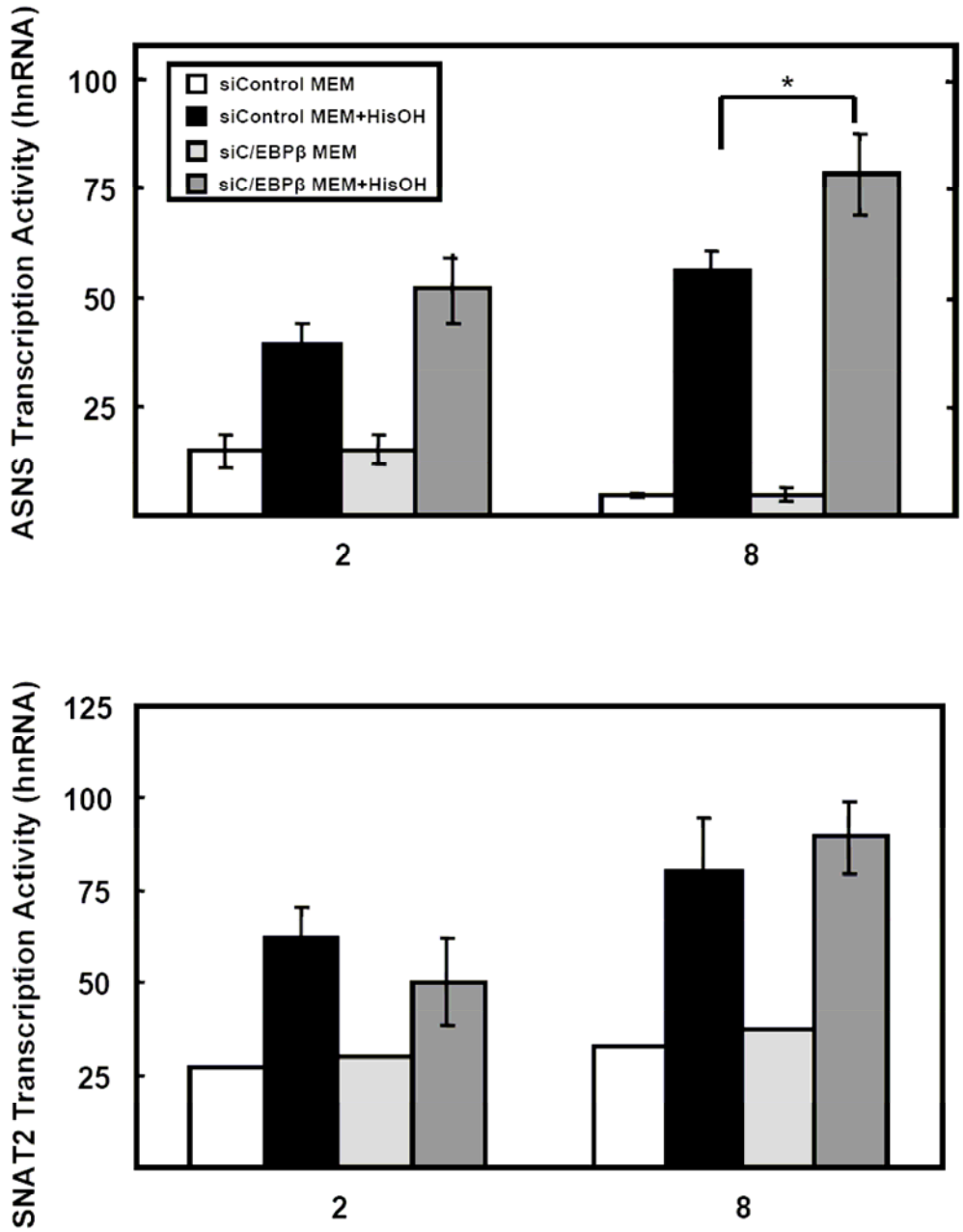

Figure 4-10. Expression of AARE-containing genes increases after amino acid deprivation in the absence of C/EBPβ. HepG2 cells were transiently transfected with either control siRNA or siRNA specific for C/EBPβ for 24 h, and then given fresh medium and incubated an additional 24 h. These cells were then treated with either MEM or MEM plus 2 mM HisOH for 2 or 8 h, and total RNA cellular extracts were isolated. Specific primers were used to measure ASNS and SNAT2 transcription activity by RT-qPCR. RNA for each data point represents three samples and the RT-qPCR for each data point was performed in duplicate. The data are presented as the average ± sem. The data are adapted from Thiaville et al. Biochem J. 2008.

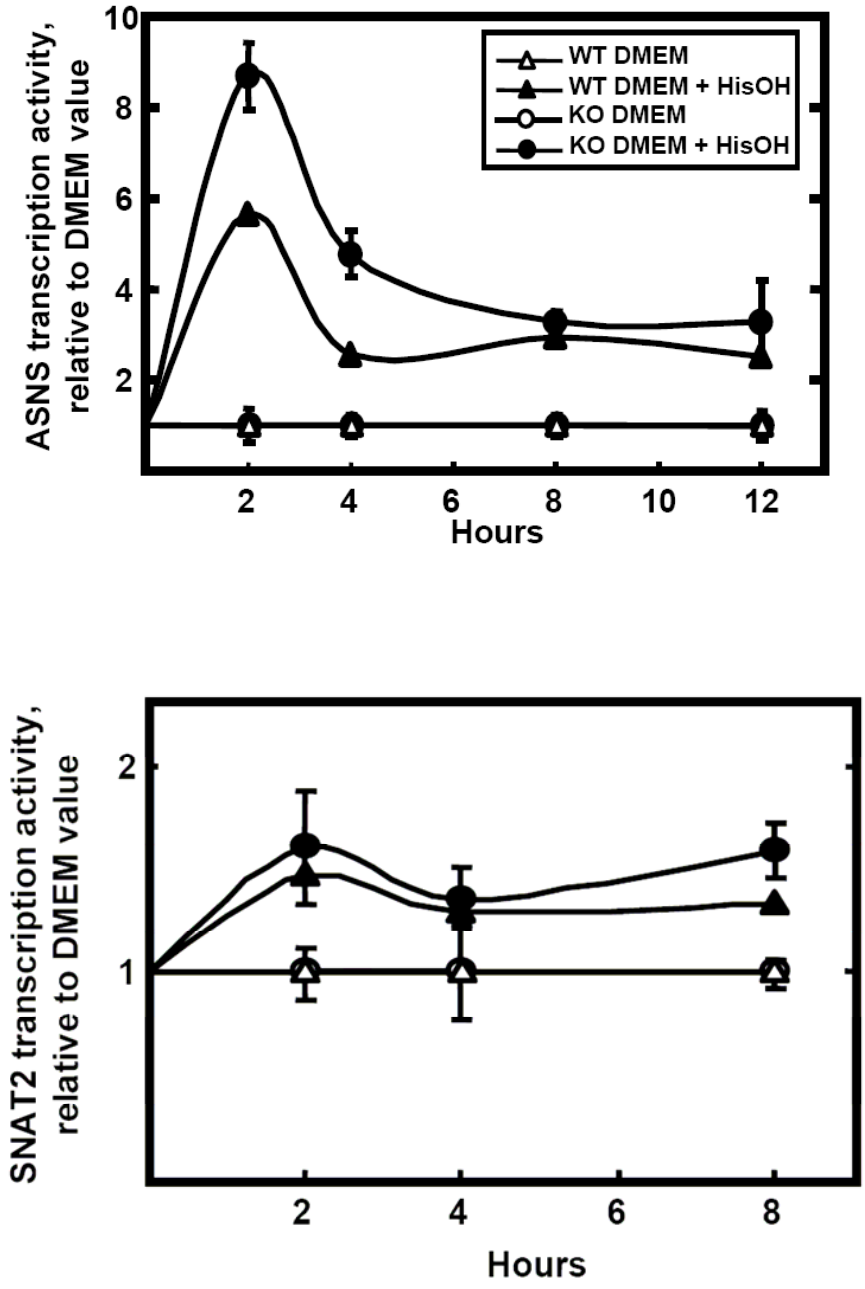

Figure 4-11. Expression of AARE-containing genes increases after amino acid deprivation of C/EBPβ wild type and knockout MEFs. MEFs were treated with either complete DMEM (open circles) or DMEM plus 2 mM HisOH (closed circles) from 0 to 8 hours, and total RNA was extracted. Specific primers were used to amplify ASNS or SNAT2 transcription activity by RT-qPCR. RNA for each data point represents three samples and the RT-qPCR for each data point was performed in duplicate. The data are presented as the average ± sem. The data are adapted from Thiaville et al. Biochem J. 2008.

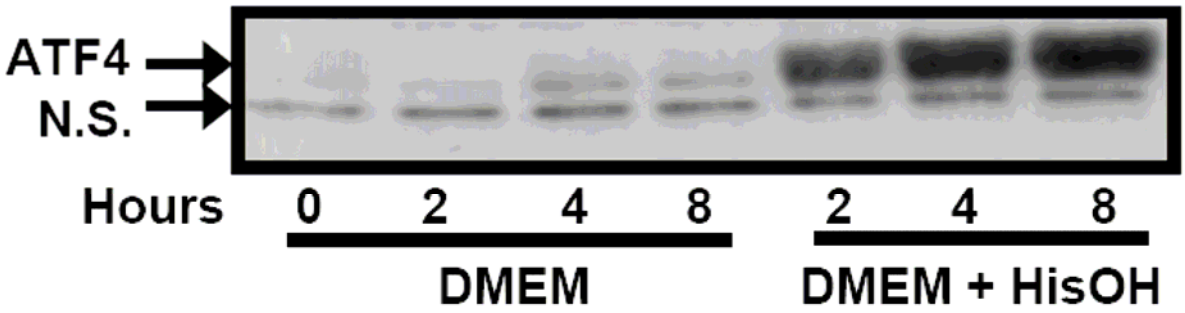

Figure 4-12. ATF4 protein content increases in C/EBPβ knockout cells after amino acid starvation. Wild type and C/EBPβ knockout MEFs were treated with either complete DMEM or DMEM plus 2 mM HisOH from 0 to 8 hours, and total cellular protein was isolated. Immunoblots for ATF4 were performed with 30 μg of total protein per lane. The upper band represents ATF4 protein and the lower band represents a non-specific protein (N.S.). The figure was taken from Thiaville et al. Biochem J. 2008.

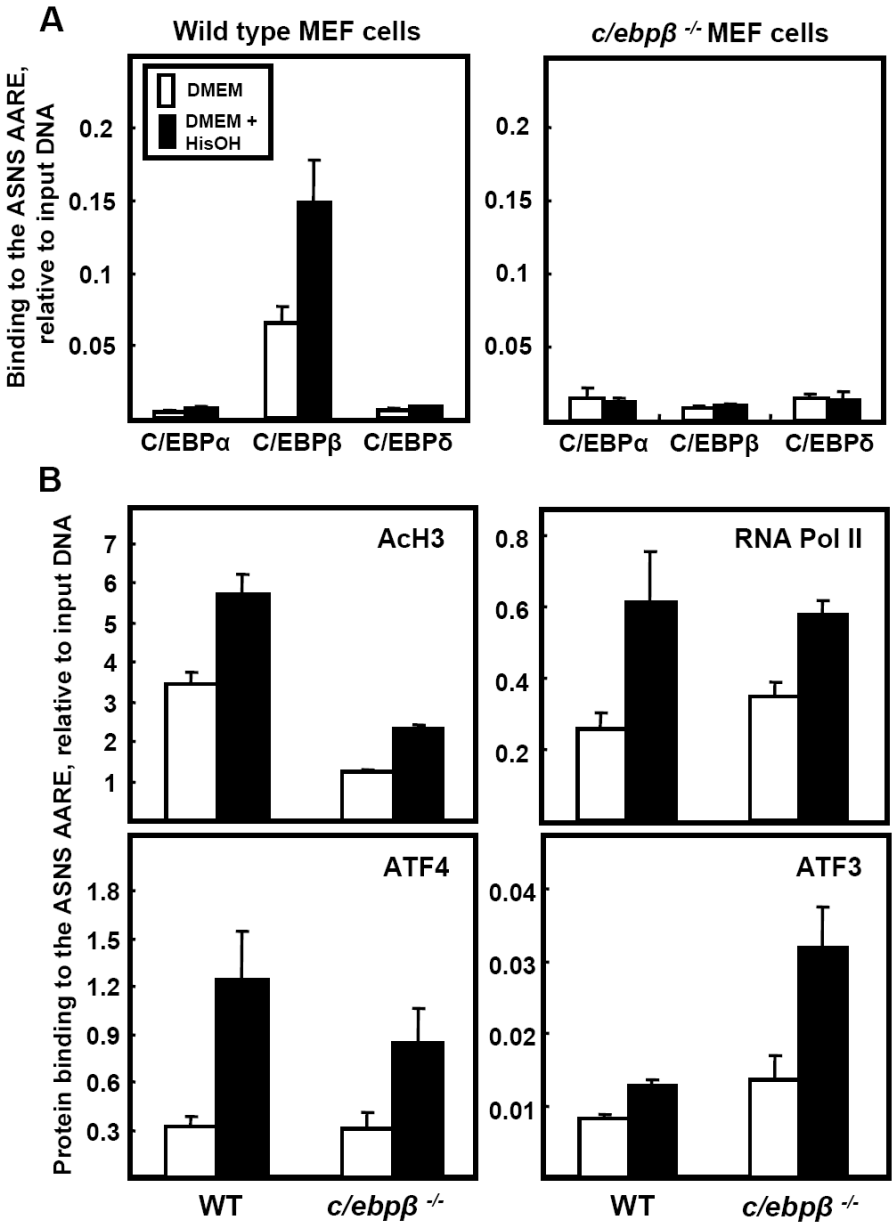

Figure 4-13. Transcription factor recruitment to the AARE increases in both wild type and C/EBPβ knockout MEF cells after amino acid deprivation. MEFs were incubated in either DMEM (white boxes) or DMEM plus 2 mM HisOH (black boxes) for 8 h and used for ChIP analysis. Specific primers were used to measure binding to the ASNS AARE by qPCR. Levels of C/EBPβ binding in C/EBPβ knockout cells were used as a negative control. ChIP analysis was performed in triplicate and qPCR for each data point was performed in duplicate. The data are presented as the average ± sem. A) ChIP analysis of C/EBP family member binding to the ASNS AARE. B) ChIP analysis of AcH3, RNA Pol II, ATF4, and ATF3, binding to the ASNS AARE. The data were taken from Thiaville et al. Biochem J. 2008.

CHAPTER 5
ROLE OF THE MAPK PATHWAY IN REGULATING TRANSCRIPTION OF THE SNAT2 GENE THROUGH THE AAR PATHWAY

Introduction

Cellular regulation by extracellular signaling is often mediated through induction of phosphorylation signaling cascades. The mitogen-activated protein kinase (MAPK) cascade is a three-tiered phospho-signaling cascade in which a cellular stimulus causes phosphorylation of a MAPKKK, whose phosphorylation target is a MAPKK, and ultimately this action leads to phosphorylation of a MAPK protein. It is the final MAPK effector proteins that can modify both cytosolic and nuclear target proteins to elicit a specific cellular response. Four mammalian MAPK signaling cascades have been characterized, ERK (extracellular signal-regulated kinase), p38, JNK (c-Jun N-terminal kinase), and BMK1 (big mitogen-activated protein kinase 1), though other lesser studied pathways exist (Shaul and Seger, 2007). Each of these cascades is named for its MAPK effector protein and consists of three distinct phosphorylation signaling proteins. Stimulation of these pathways, however, can occur simultaneously through overlap between cellular stimuli. For example, all four MAPK pathways can be stimulated by growth factors, though any specific growth factor may cause a more specific pathway activation (Pearson et al., 2001). Given that the majority of the following data will focus on the ERK signaling pathway, it is this pathway that will be described in more detail.

The ERK signaling pathway is activated by various growth factors, mitogens, and g-protein coupled receptors (GPCR) (Dhillon et al., 2007). This stimulation leads to phosphorylation of the MAPKKK, which in the ERK signaling cascade is one of three distinct Raf proteins, a-Raf, b-Raf, or c-Raf (Friday and Adjei, 2008). These different Raf family members are activated, through a complex and poorly understood phosphorylation mechanism, by distinct cellular signals and other kinases thus adding a level of specificity to this generic

signaling cascade. The target proteins of the Raf family are very specific and consist of the MAPKK proteins MEK1 and MEK2. All Raf family members phosphorylate both MEK1 and MEK2 at both serine residues 218 and 222 (Dhillon et al., 2007). The active MEK1/2 kinases have no other known phosphorylation targets besides the MAPK proteins ERK1 and ERK2, which are phosphorylated at the residues Thr202/Tyr204 or Thr185/Tyr187, respectively (Shaul and Seger, 2007). Active ERK1/2 proteins can phosphorylate other cytosolic proteins with kinase activity or traverse to the nucleus to facilitate phosphorylation of specific transcription factors (Pearson et al., 2001). Thus, ERK1/2 are main effector proteins of one of the MAPK signaling pathways that links extracellular signaling with transcriptional regulation.

While active ERK1/2 can phosphorylate transcription factors directly, many of its downstream targets are phosphorylated through an intermediate target, members of the ribosomal S6 kinase (RSK) protein family. ERK1/2 phosphorylates RSK proteins at multiple sites causing their transition to the nucleus and activation of transcription factors (though they also have cytoplasmic targets) (Frodin and Gammeltoft, 1999). Coincidently, recent evidence has demonstrated both ATF4 and C/EBPβ are targets of RSK through ERK signaling. RSK2 phosphorylation of ATF4 on two serine residues near its bZIP domain was demonstrated to be critical for osteoblast biology (Yang et al., 2004). The active ERK signaling pathway can phosphorylate human C/EBPβ at Thr235, and this event was demonstrated to be necessary for further C/EBPβ phosphorylation by other kinases during adipogenesis (Pilipuk et al., 2003; Piwien-Pilipuk et al., 2001; Tang et al., 2005). Other studies demonstrated that RSK itself phosphorylates mouse C/EBPβ at Thr217 after activation by liver injury, though the specific RSK family member was not identified (Buck et al., 2001).

Though the amino acid regulated transcription factors ATF4 and C/EBPβ can be modified by the ERK signaling pathway, there is minimal evidence documenting involvement of MAPK signaling during amino acid deprivation. Initially, Gazzola's laboratory demonstrated that the ERK signaling pathway was required for increased System A amino acid transport activity during amino acid limitation (Franchi-Gazzola et al., 1999). These experiments were performed before the three genes encoding System A transport were cloned, thus it was not known how ERK signaling was mediating its effect. It is now recognized that the SNAT2 gene is responsible for the increased System A transport activity after amino acid starvation (Gazzola et al., 2001; Bain et al., 2002). More recently, the Kilberg laboratory has demonstrated that ERK1/2 phosphorylation is enhanced in HepG2 cells deprived of histidine (Leung-Pineda et al., 2004). Evidence from other groups established that autophagy was induced in a colon cancer cell line after protein deprivation by activation of ERK signaling through c-Raf (Pattingre et al., 2003). In neuronal cells, p-ERK1/2 was demonstrated to co-localize with p-eIF2α in theronine-deprived cells, though no direct interaction was tested (Sharp et al., 2006). Collectively, the evidence suggests a possible role for the ERK signaling pathway during amino acid deprivation. Determining if ERK modulates the AAR pathway or is modulated by this pathway will provide further insight into the transcriptional regulation of AARE-containing genes, given ERK's intimate link with transcription factor regulation (Thiaville et al., 2008b).

Results

Phosphorylated C/EBPβ Associates with the SNAT2 AARE after Amino Acid Limitation

Given that the MEK-ERK signaling pathway is induced by amino acid limitation and that C/EBPβ, known to be phosphorylated at Thr235 by this pathway (Piwien-Pilipuk et al., 2002), binds the SNAT2 AARE, ChIP analysis was performed to assay for binding of p-C/EBPβ on the SNAT2 gene (Figure 5-1). The antibody used for the ChIP analysis specifically recognizes the

phosphorylated threonine 235 residue of the C/EBPβ protein. The data demonstrate that after amino acid deprivation there was a significant increase in phosphorylated C/EBPβ binding to the SNAT2 AARE (Figure 5-1). The increased association of p-C/EBPβ with the AARE was above background binding levels by comparison to the non-specific antibody used at the AARE (n/s IgG) and, as another background measurement, binding to the SNAT2 protein coding region. The p-C/EBPβ binding was specific for the SNAT2 AARE region, given that there was no significant binding at the SNAT2 promoter region. These data coincide with the ChIP analysis for total C/EBPβ, which also demonstrated specific binding at the SNAT2 AARE (see Chapter 3 Figure 3-2 and 3-3). The ChIP data (Figure 5-1) also suggest that the MEK signaling pathway may assert an important functional role of transcription factor modification during amino acid starvation.

Induced SNAT2 Gene Expression by Amino Acid Starvation Requires a Functional MEK Pathway

Given that there are no available antibodies to test the phosphorylation status of other transcription factors known to bind the SNAT2 gene, the effect of blocking the MEK pathway during amino acid deprivation was assessed. Both SNAT2 transcription activity and steady state mRNA was assayed by RT-qPCR during amino acid deprivation and inhibition of the MEK pathway by the inhibitor PD98059 (Figure 5-2). The inhibitor PD98059 is specific for the MEK signaling pathway and functions by binding to the MEK protein itself and preventing MEK phosphorylation by an upstream kinase, in most cases presumably this kinase is Raf (Davies et al., 2000). Though both SNAT2 transcription activity and steady state mRNA increased approximately 3-4 fold after amino acid deprivation, this induction was completely abolished after blocking the MEK pathway. The levels of AAR activated SNAT2 mRNA decreased to approximately basal levels indicating that the MEK pathway may only be required for induced

SNAT2 gene expression (Figure 5-2). These data also demonstrate that the MEK-dependent induction of System A transport activity observed by the Gazzola laboratory actually occurs at the level of gene expression itself and not through translational control of the SNAT2 protein (Franchi-Gazzola et al., 1999).

Increased ATF4 mRNA and Protein Levels Require a Functional MEK Pathway

The transcription factor ATF4 was demonstrated to be necessary for amino acid induction of some AARE-containing genes, such as ASNS (Figure 4-2 and 4-3), and ATF4 expression itself is induced at both the transcriptional and translational level by amino acid deprivation (Siu et al., 2002; Harding et al., 2000). Analysis of ATF4 steady state mRNA and protein content by RT-qPCR and immunoblotting was performed by Dr. Y. Pan in the Kilberg laboratory to determine if the MEK signaling pathway effected ATF4 induction through the AAR pathway (Figure 5-3). The data clearly demonstrate that both ATF4 mRNA (Panel A) and protein content (Panel B) do not increase after amino acid limitation if the MEK signaling pathway is blocked. To determine if the MEK signaling pathway is the only MAPK pathway required for ATF4 induction during amino acid limitation, HepG2 cells were also treated with inhibitors that block either the p38 or JNK signaling pathways. Blocking the p38 pathway had no effect on ATF4 mRNA or protein content (Figure 5-3). Treatment with the JNK inhibitor, however, significantly increased the basal level of ATF4 mRNA and protein, though it had no effect on ATF4 AAR-induced (MEM- histidine) levels. Therefore, the MEK signaling pathway is the only major MAPK pathway required for ATF4 protein induction during amino acid deprivation.

ATF4 Translational Control Requires a Functional MEK Pathway

Amino acid deprivation regulates ATF4 protein content at the translational level through uORF scanning of the steady state mRNA by the ribosome complex (Figure 1-2). Given that inhibiting the MEK pathway blocked the AAR induction of both ATF4 mRNA and protein

levels, the experiments shown in Figure 5–3 do not establish if the MEK pathway is required for ATF4 translational regulation during amino acid deprivation. To directly test ATF4 translational regulation, functional assays were performed with Firefly luciferase constructs containing either the wild-type or mutated ATF4 uORFs, which were previously used by the Wek laboratory to demonstrate ATF4 translational regulation in mammalian cells (Vattem and Wek, 2004). The structure of ATF4 mRNA was previously depicted in Figure 4-1. Firefly luciferase activity of the wild type plasmid was increased after starvation due to increased uORF-regulated translation, but not when the MEK pathway was blocked (Figure 5-4). These data clearly demonstrate that the MEK pathway is required for the translational control of ATF4 protein expression. As a control, functional assays with the mutated ATF4 uORF construct demonstrated that there was no AAR-regulated translation nor was there any effect on translation when the MEK pathway was blocked. The approximately 10-fold higher levels of luciferase activity for the mutated uORF, when compared to the wild type, also signify that translation is now unregulated and thus at a constitutively higher magnitude.

Blocking AARE-Dependent Induction by Inhibiting the MEK Pathway Was Rescued by Over-expression of ATF4

ATF4 was demonstrated to bind the SNAT2 gene and to be required for the induction of SNAT2-driven transcription from a reporter gene through the SNAT2 AARE element. To determine if MEK signaling regulates SNAT2 transcription through ATF4 binding, ATF4 protein was over-expressed in the presence or absence of a functional MEK signaling pathway, and its effect on SNAT2 transcription activity was monitored by examining SNAT2-driven Firefly luciferase expression (Figure 5-5). While inhibiting MEK signaling blocked SNAT2 transcriptional activity (i.e., no increased luciferase activity) in control cells, over-expression of ATF4 caused similar levels of SNAT2 expression in both active and blocked MEK conditions.

These results are consistent with the observation that MEK signaling is necessary for ATF4 synthesis following amino acid limitation. Therefore, MEK signaling regulates SNAT2 gene expression by modulating the availability of its AARE binding transcription factor ATF4.

Phosphorylation of eIF2α during Amino Acid Limitation Requires the MEK Pathway

Translational regulation of ATF4 during AAR signaling requires GCN2-mediated phosphorylation of the translation initiation protein eIF2α on serine 51 (Sood et al., 2000). To determine if eIF2α phosphorylation is effected by MEK signaling, HepG2 cells in either MEM or MEM minus histidine (MEM- His) were incubated with or without the MEK inhibitor PD98059 and total protein was immunoblotted for p-eIF2α levels (Figure 5-6). Phosphorylation of eIF2α occurred after amino acid starvation when the MEK pathway was functional, but blocking the MEK signaling pathway abolished the AAR-induced phosphorylation. Quantification of three immunoblots from three separate experiments is also shown to demonstrate the reproducibility of this effect. Immunoblotting for total eIF2α demonstrates that the block in phosphorylation was not due to decreased eIF2α protein (Figure 5-6). To demonstrate the effectiveness of treatment with the MEK inhibitor, immunoblots for both p-ERK and total ERK were performed, and as expected, phosphorylation of ERK was induced by histidine starvation and this response was blocked by PD98059 treatment.

Though the inhibitor PD98059 was demonstrated to specifically block MEK phosphorylation, another specific inhibitor of MEK signaling has been identified. This inhibitor, U0126, is also a non-competitive inhibitor of MEK believed to bind MEK at a site similar or identical to the PD98059 binding site (Favata et al., 1998). Data provided by A. Gjimisksa in the Kilberg laboratory demonstrated that using the inhibitor U0126 during amino acid deprivation also blocked the increase in ATF4 protein and phosphorylation of eIF2α. Given that similar

results were obtained using two different inhibitors to MEK signaling, it is clear that the MEK signaling pathway is necessary for the AAR-dependent increase in ATF4 synthesis.

Over-expression of Active MEK Does Not Induce eIF2α Phosphorylation Nor Does Over-expression of eIF2α Induce MEK Phosphorylation

GCN2 is believed to be the only kinase responsible for eIF2α phosphorylation at serine 51 during amino acid limitation. In order to determine if MEK or its downstream target ERK can phosphorylate eIF2α directly at serine 51, a constitutively active MEK construct was transiently transfected into 293T cells (Figure 5-8). 293T cells were used, instead of HepG2 cells, due to the high levels (approximately 70%) of transfection efficiency that are achieved with this cell line. Though over-expression of an active MEK construct was able to phosphorylate its downstream target, p-ERK, as demonstrated by immunoblotting, it was not able to phosphorylate eIF2α at residue serine 51. Though no antibodies are available to test MEK phosphorylation of eIF2α on other residues, computer analysis of the eIF2α protein sequence did not reveal any MAPK consensus phosphorylation sites. As a control, both eIF2α wild type and mutant constructs were also over-expressed. Over-expression of eIF2α, with or without serine phosphorylation, did not cause phosphorylation of ERK (Figure 5-8).

Knockdown of the Phosphatase GADD34 Does Not Restore EIF2α Phosphorylation or SNAT2 Gene Expression when the MEK Pathway is Blocked

Though the AAR causes GCN2 to phosphorylate eIF2α, it also induces a feedback mechanism that upregulates the eIF2α phosphatase GADD34 (Novoa et al., 2001). Given that the MEK pathway does not seem to phosphorylate eIF2a directly, it is possible that it may function to inhibit the eIF2α phosphatase, GADD34, thus allowing for increased p-eIF2α. To test this possibility, HepG2 cells were treated with either control siRNA or siRNA specific for GADD34 (Figure 5-9). Panel A demonstrates the effectiveness of GADD34 knockdown by RT-qPCR of steady state GADD34 mRNA levels. GADD34 mRNA increases after amino acid

limitation, but knockdown of GADD34 by siRNA treatment reduces its mRNA to basal levels. There is no antibody available to measure endogenous GADD34 protein levels, but previous studies have demonstrated that most GADD34 protein is produced by de *novo synthesis* of induced mRNA (Novoa et al., 2003). Given the effectiveness of siRNA treatment, immunoblots of HepG2 cells were performed to analyze phosphorylation of eIF2α. Knockdown of GADD34 did not restore eIF2α phosphorylation during the AAR when the MEK pathway was blocked (Figure 5-9, Panel B). ATF4 protein was also monitored by immunoblot after knockdown of GADD34, and its induction was also blocked in the MEK-inactive and GADD34-depleted conditions. As a final test, SNAT2 transcription activity and steady state mRNA were measured in GADD34 knockdown and control conditions (Figure 5-9, Panel C). Inhibiting MEK blocks the AAR-induced SNAT2 gene expression, and knockdown of GADD34 did not restore SNAT2 gene expression. The data collectively demonstrate that MEK signaling does not block the phosphatase GADD34 and thereby allow for increased phosphorylation of eIF2α. Consequently, it appears that the action of MEK is to permit or promote the phosphorylation of eIF2α by GCN2.

Amino Acid Starvation Does Not Affect ERK1/2 Gene Expression

There is no commercial antibody available to monitor phosphorylation of GCN2, so it could not be determined if the MEK signaling pathway affects the kinase that phosphorylates eIF2α. Therefore, attention was turned to determining the mechanism by which the amino acid deprivation pathway activates the MEK signaling pathway. To determine if ERK1 or ERK2 mRNA expression is regulated by amino acid starvation, RT-qPCR analysis was performed with HepG2 cells treated with either MEM or MEM minus histidine (Figure 5-10). No induced mRNA expression for either ERK1 or ERK2 was detected after amino acid limitation. These experiments complement those of Figure 5-6 showing that the total amount of ERK protein is not

increased by amino acid limitation or PD98059 treatment. Consequently, treating with the MEK inhibitor PD98059, which blocks the induction of amino acid responsive genes, has no effect on ERK1/2 gene expression. Therefore, the AAR pathway does not affect the MEK pathway at the level of ERK transcription or translation.

Phosphorylation of ERK by Amino Acid Limitation Was Abolished in a GCN2 Knockout Cell Line and an eIF2α Mutant Knock-in Cell Line

Self-activation of the GCN2 kinase following binding of uncharged tRNA is believed to be the cellular sensing mechanism for amino acid limitation (Kimball, 2005). Activation of the MEK pathway, assessed by phosphorylation of ERK1/2, was assayed by immunoblot analysis in a GCN2 wild type or knockout cell line after 2, 4, or 8 hours of histidine limitation to determine if AAR-induced MEK activation is dependent upon GCN2 activation (Figure 5-11). Starvation-dependent expression of ATF4 protein, known to be GCN2-dependent, was monitored as a positive control. In the GCN2 wild type cells, amino acid limitation clearly induces ERK phosphorylation and ATF4 protein levels. In the GCN2 knockout cell line, however, there was no increase in either ERK phosphorylation or ATF4 protein levels after amino acid limitation. Quantification of three individual immunoblots demonstrates that this result is reproducible (Figure 5-11). GCN2 activation, therefore, is required for MEK activation during the AAR.

MEK activation by amino acid limitation was also assessed in an eIF2α knock-in cell line, in which the serine 51 residue of eIF2α has been mutated to alanine (eIF2α A/A) thus blocking the translationally regulated increase in ATF4 synthesis (Figure 5-12). Immunoblots of histidine-deprived cells displayed no activation of ERK phosphorylation in the eIF2α A/A cells. In the control cell line, eIF2α S/S, both ERK phosphorylation and ATF4 protein synthesis increased after amino acid limitation as expected. However, both the GCN2 knockout and eIF2α A/A cell lines did display higher p-ERK basal levels than their wild type counterparts. That

observation, while not fully understood with regard to the mechanism, is taken as further evidence for interplay between the AAR and MEK-ERK pathways.

Conclusions

The data presented in this chapter are the first demonstration of the requirement for the MEK signaling pathway as a component for complete activation of the AAR pathway. Phosphorylation of eIF2α and increased ATF4 synthesis after amino acid limitation requires an active MEK signaling pathway, and thus, increased expression of AAR-containing genes after amino acid limitation is also dependent on MEK signaling. Conversely, activation of the MEK signaling pathway during amino acid deprivation requires both GCN2 and phosphorylation of eIF2α. Clearly, there is interdependence between these two critical stress-induced pathways that is required for increased gene expression after amino acid deprivation.

Unfortunately, the direct effect of MEK signaling of p-GCN2 could not be tested due to the lack of available antibody reagents. Amino acid sequence analysis of GCN2 determined it does contain MAPK phosphorylation consensus sequences at both serine and theronine residues. Thus, it is possible that GCN2 may be activated by MEK signaling which then further enhances GCN2 phosphorylation of eIF2α. Recent analysis of phosphorylation sites on GCN2 in the yeast *Saccharomyces cerevisiae* from the Hinnebusch laboratory demonstrated that it does contain phosphorylated serine and theronine residues *in vivo* (Garcia-Barrio et al., 2002). Their data demonstrated that phosphorylation of GCN2 on serine 577 inhibited GCN2-dependent phosphorylation of eIF2α. Their data also established that other GCN2 serine and theronine sites were phosphorylated *in vivo*, but unfortunately they were unable to resolve the specific sites by mass spectroscopy.

Though GCN2 is the only known kinase in yeast to phosphorylate eIF2α, several different kinases in mammalian cells are capable of phosphorylating eIF2α depending upon the specific

cellular stress pathway that is activated. This observation has led to the characterization of a mammalian "Integrated Stress Response" in which many cellular stress signaling pathways converge at the point of p-eIF2α to ATF4 synthesis (Harding et al., 2002). Though the present data focuses on the AAR stress response, it is likely that other stress signaling pathways may also be interdependent on MAPK signaling pathways. It has recently been demonstrated that PKR phosphorylation of eIF2α by the stress UV-irradiation also requires the ERK signaling pathway but not the p38 pathway (Zykova et al., 2007). In contrast, Liang et al demonstrated that PERK activation, by disruption of calcium homeostasis, also activates the JNK and p38 signaling pathways (Liang et al., 2006). Thus, activation and repression of different MAPK signaling pathways may provide specificity during different cellular stress responses.

Though the MEK pathway was the only MAPK pathway demonstrated to be required for increased ATF4 synthesis after amino acid deprivation, an interesting observation is that treatment with inhibitors to JNK actually induced ATF4 synthesis. Recently, aging related studies have showed decreased p-eIF2α and ATF4 protein in older rats compared to younger ones (Hussain and Ramaiah, 2007). The researchers correlated this decrease to increased levels of p-JNK and CHOP, an inducer of the eIF2α phosphatase GADD34, in the older rats. Thus, it is possible that the JNK pathway actively represses the AAR by inhibiting eIF2α phosphorylation. Unfortunately, levels of p-ERK were not measured in the Hussain and Ramaiah study, but presumably they should decrease with increased aging.

All of the data presented demonstrate a requirement for the active regulation of MAPK signaling pathways during amino acid limitation. Further detailed analysis of each individual protein component of the MEK signaling cascade will be necessary to develop a complete model of its activation and AAR pathway interaction during nutrient deprivation. Furthermore,

discovery of other transcription factors modified by the MEK signaling pathway during amino acid limitation will provide further mechanistic insight of amino acid regulated gene expression.

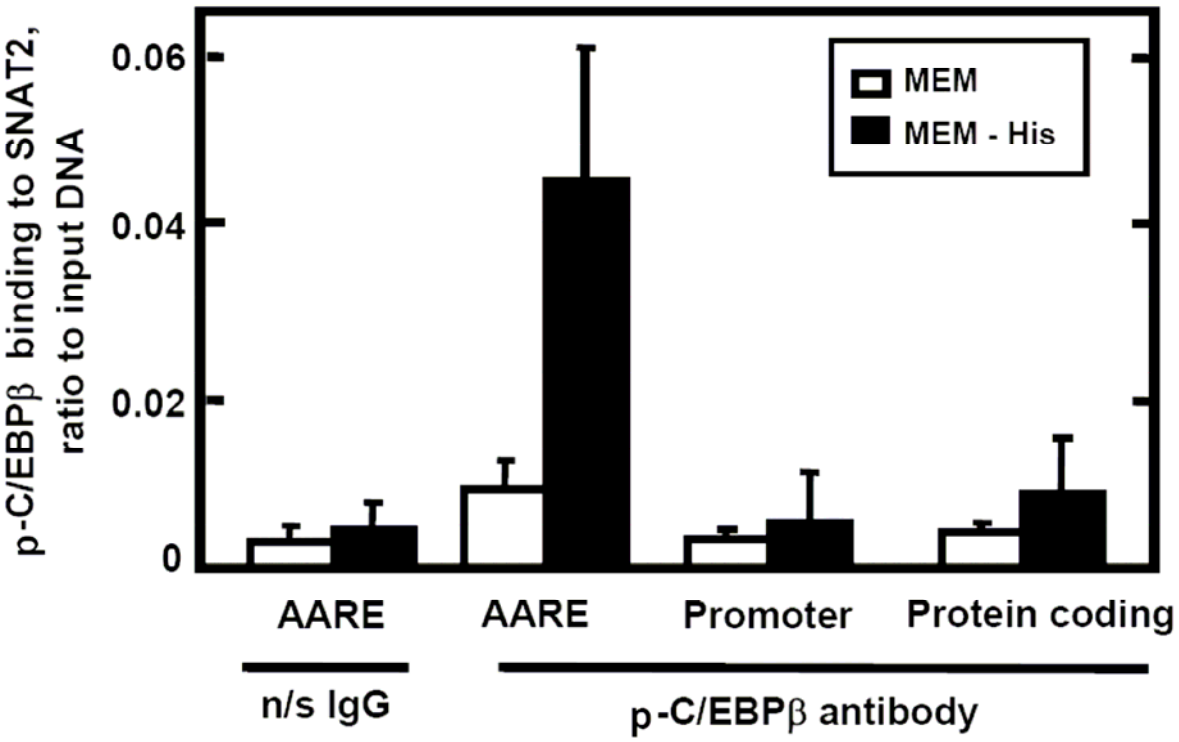

Figure 5-1. p-C/EBPβ binds specifically to the SNAT2 AARE. HepG2 cells were treated with either complete MEM (white boxes) or MEM lacking histidine (black boxes) for 8 hours and ChIP analysis was performed. The antibodies used were either specific for Thr235 phosphorylation of C/EBPβ or a non-specific IgG control (n/s IgG). Primers were designed against the indicated regions of the SNAT2 gene for analysis by qPCR, and the primer sequences are listed in Table 2-3. Data are presented as the ratio of the qPCR signal for immunoprecipitated DNA to the qPCR signal for total DNA (input) for three independent experiments. Figure taken from Thiaville et al. Biochem J. 2008.

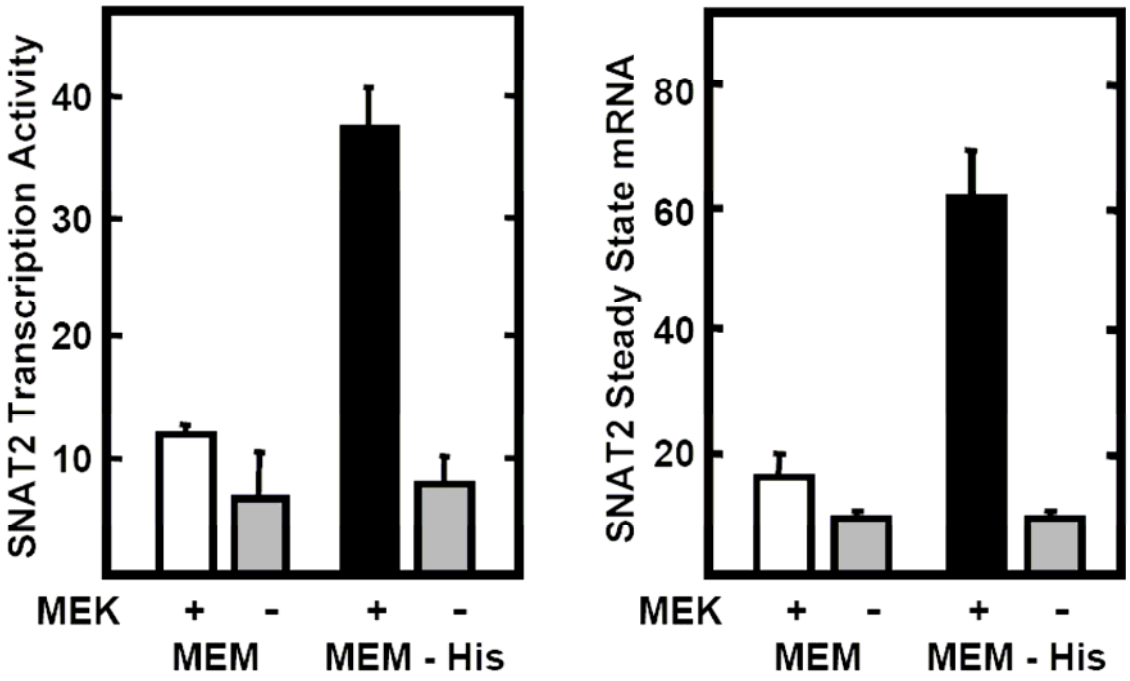

Figure 5-2. AAR induction of SNAT2 gene expression requires an active MEK signaling pathway. HepG2 cells were pre-treated with either DMSO (0.5 %) or 50 μM PD98059 (MEK inhibitor) for 1 h and then transferred to either MEM or MEM minus histidine in the absence (+ MEK) or presence (- MEK) of PD98059 for 8 h. Total RNA was isolated and RT-qPCR analysis of SNAT2 transcription activity and steady state mRNA was performed. The RNA for each data point represents three samples and the qPCR was performed in duplicate. The data are presented as the averages ± sem. Figure taken from Thiaville et al. JBC 2008.

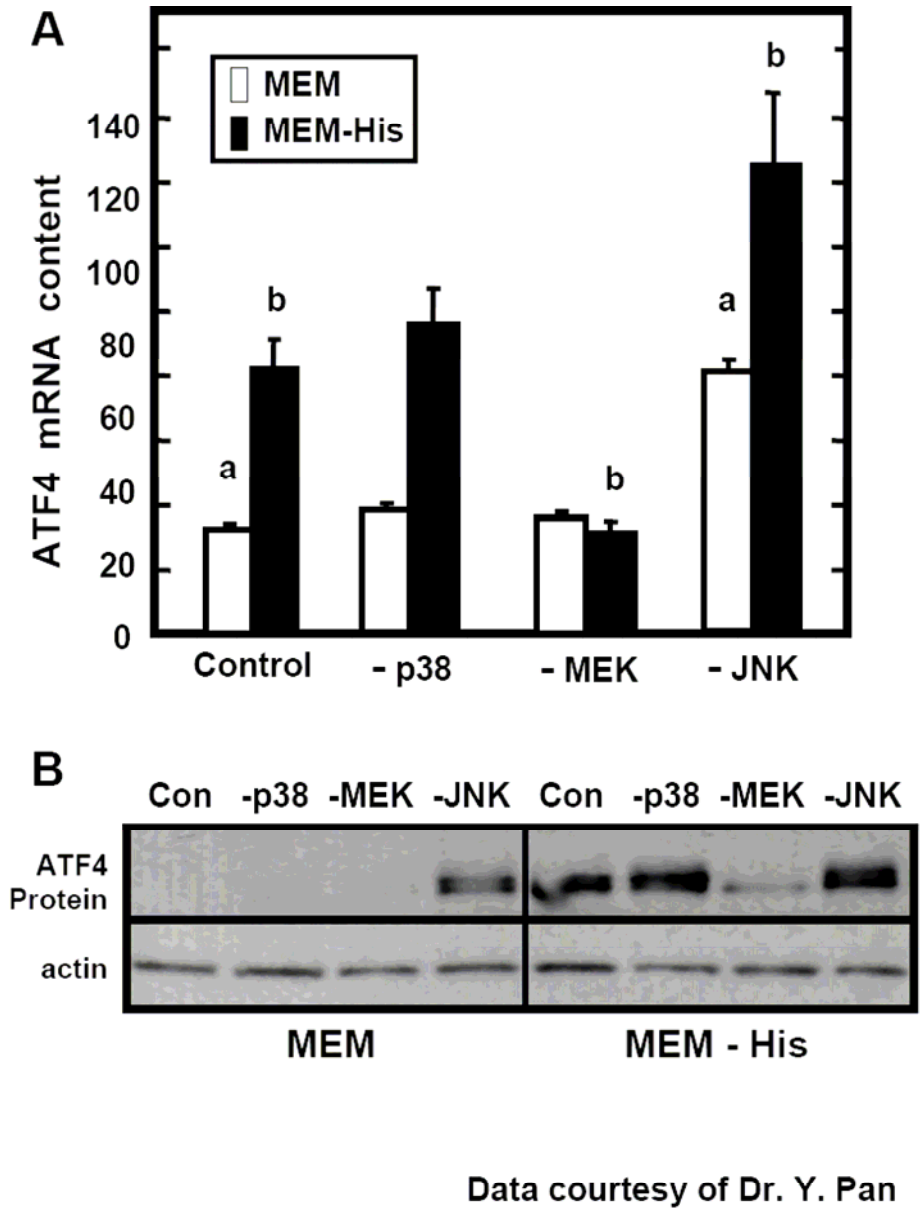

Figure 5-3. AAR induction of ATF4 mRNA and protein synthesis requires the MEK pathway, but neither the p38 nor JNK pathway. HepG2 cells were incubated in either MEM or MEM minus histidine in the absence (+p38, +MEK, or +JNK) or presence (-p38, -MEK, or -JNK) of specific MAPK inhibitors for 8 h. The inhibitors used were 10 μM SB203580 (p38), 50 μM PD98059 (MEK), and 20 μM SP600125 (JNK). A) Total RNA was isolated after treatment and RT-qPCR analysis of ATF4 steady state mRNA was performed. Letter marked values (a = MEM and b = MEM-His) are statistically different from their letter marked control. B) Total protein was isolated after treatment and immunoblotted for either ATF4 protein or actin (as a loading control). The figure was taken from Thiaville et al. JBC 2008. The data are courtesy of Dr. Y. Pan.

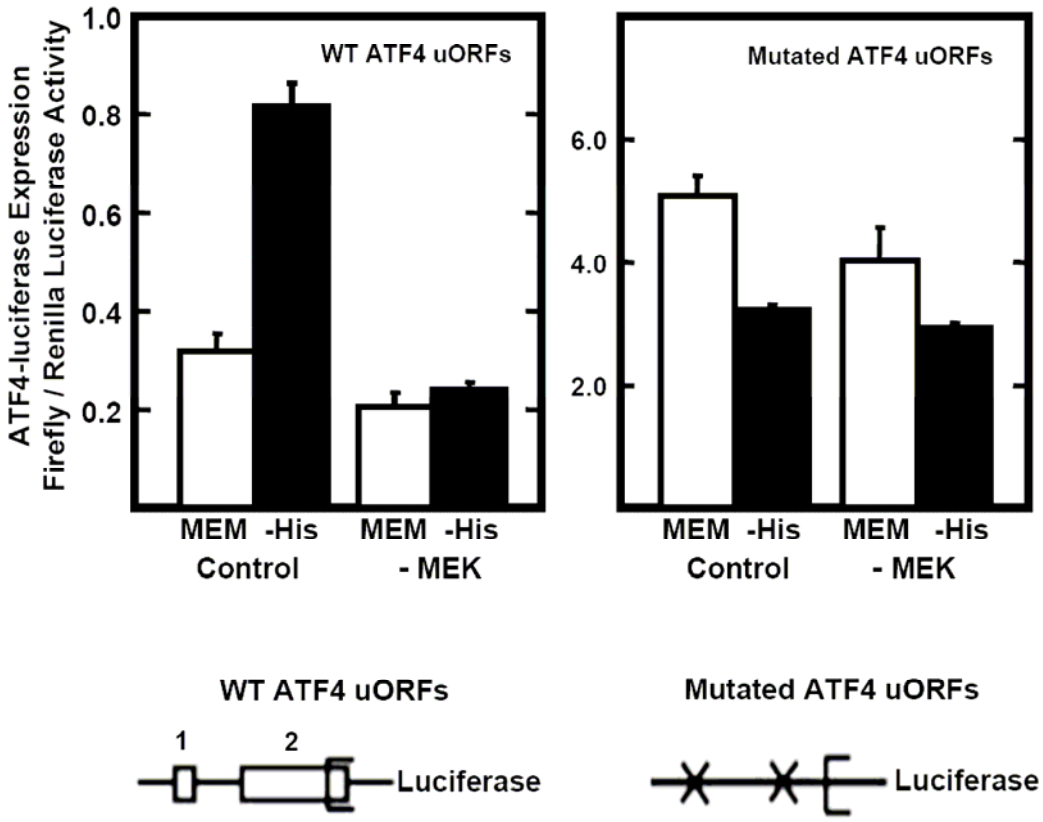

Figure 5-4. ATF4 translational regulation by amino acid limitation requires the MEK pathway. HepG2 cells were transiently co-transfected with a plasmid containing either the wild type or mutated ATF4 uORFs upstream of the Firefly luciferase protein coding sequence and a plasmid containing a Renilla luciferase gene driven by the SV40 promoter. After 18 h transfection, cells were incubated for 4 h in either MEM (white boxes) or MEM minus histidine (black boxes) in the absence (+ MEK) or presence (- MEK) of the MEK inhibitor PD98059. Total cell lysate was collected and luciferase activity was measured. Data are expressed as the ratio of Firefly to Renilla luciferase activity, and represent the average ± standard deviation from one of two independent assays. The figure was taken from Thiaville et al. JBC 2008.

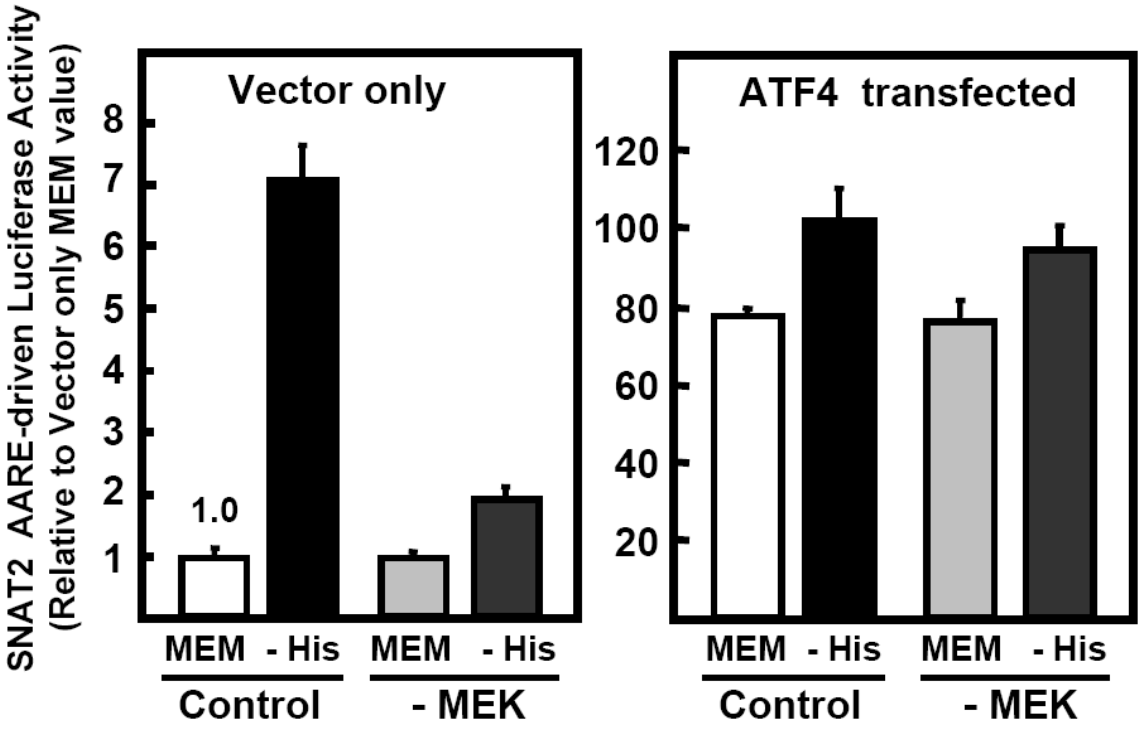

Figure 5-5. Induction of SNAT2 through the AARE element can be induced in the presence of MEK inhibition, if exogenous ATF4 protein is over-expressed. HepG2 cells were transiently transfected with a vector containing the SNAT2 promoter and AARE (nts -512 to +770) genomic sequences upstream of the Firefly luciferase protein coding sequence. In addition, ATF4 transfected cells were also given 10 ng of a vector expressing full-length ATF4 protein while "Vector only" cells received 10 ng of empty plasmid. After 18 h transfection, cells were incubated for 12 h in either MEM or MEM minus histidine in the absence (+ MEK) or presence (-MEK) of the MEK inhibitor PD98059. Total cell lysate was collected and Firefly luciferase activity was measured. The Lowry assay was used to quantify the total protein content for each sample, and Firefly luciferase activity was normalized to total protein. The data in both panels are expressed relative to the "Vector only" "Control" sample treated in complete MEM without PD98059, for which the value has been set to 1.0. The figure was taken from Thiaville et al. JBC 2008.

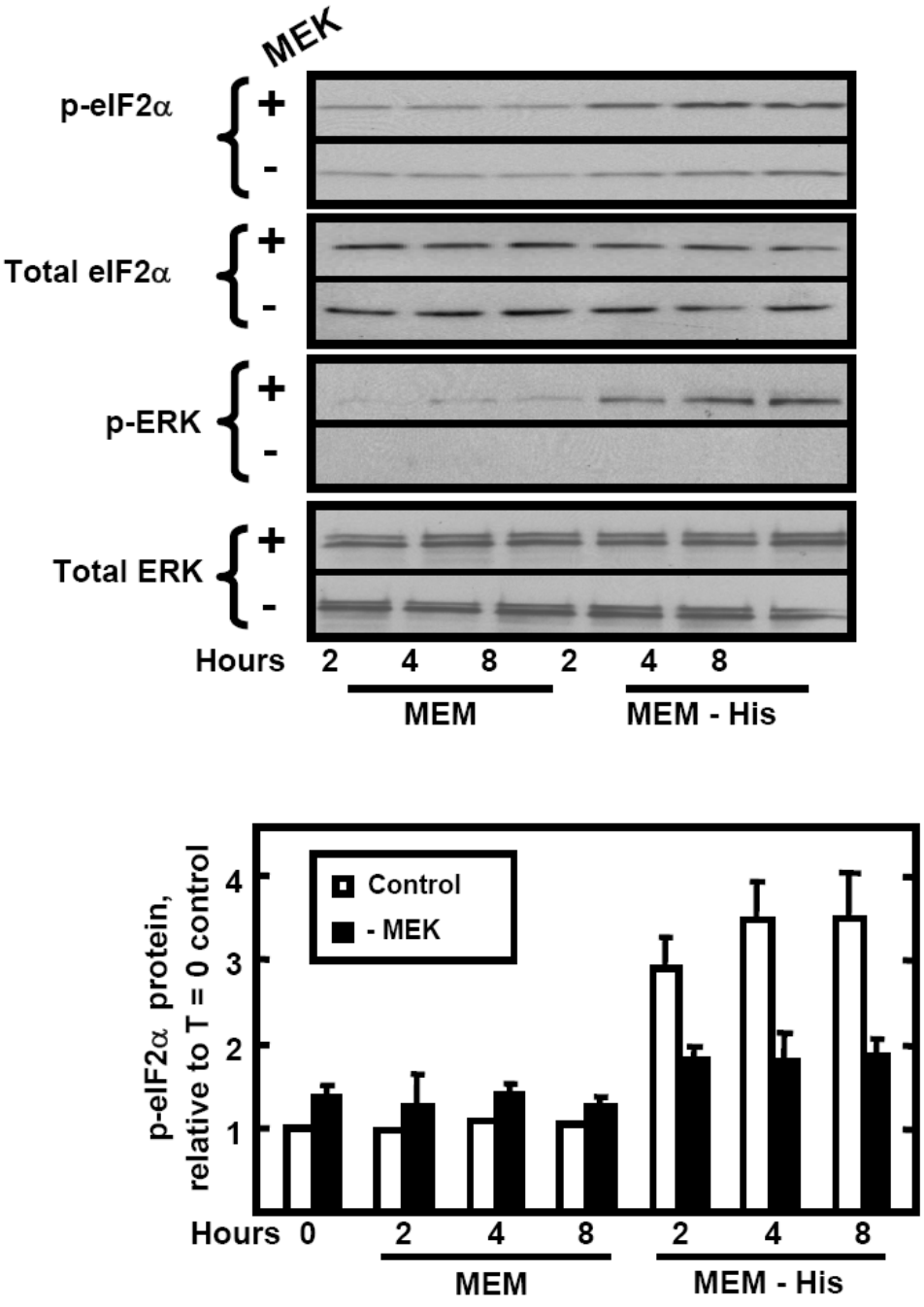

Figure 5-6. MEK activation is required for phosphorylation of eIF2α by the AAR pathway. HepG2 cells were incubated for 2, 4, or 8 h in either MEM or MEM minus histidine in the absence (+MEK) or presence (-MEK) of the inhibitor PD98059. Total protein extracts were isolated and immunoblotted for either p-eIF2α, total eIF2α, p-ERK, or total ERK. For p-eIF2α, the data from three experiments was quantified by densitometry and are expressed as a bar graph with all values expressed relative to the value for time zero in the control condition (MEM without inhibitor). The figure was taken from Thiaville et al. JBC 2008.

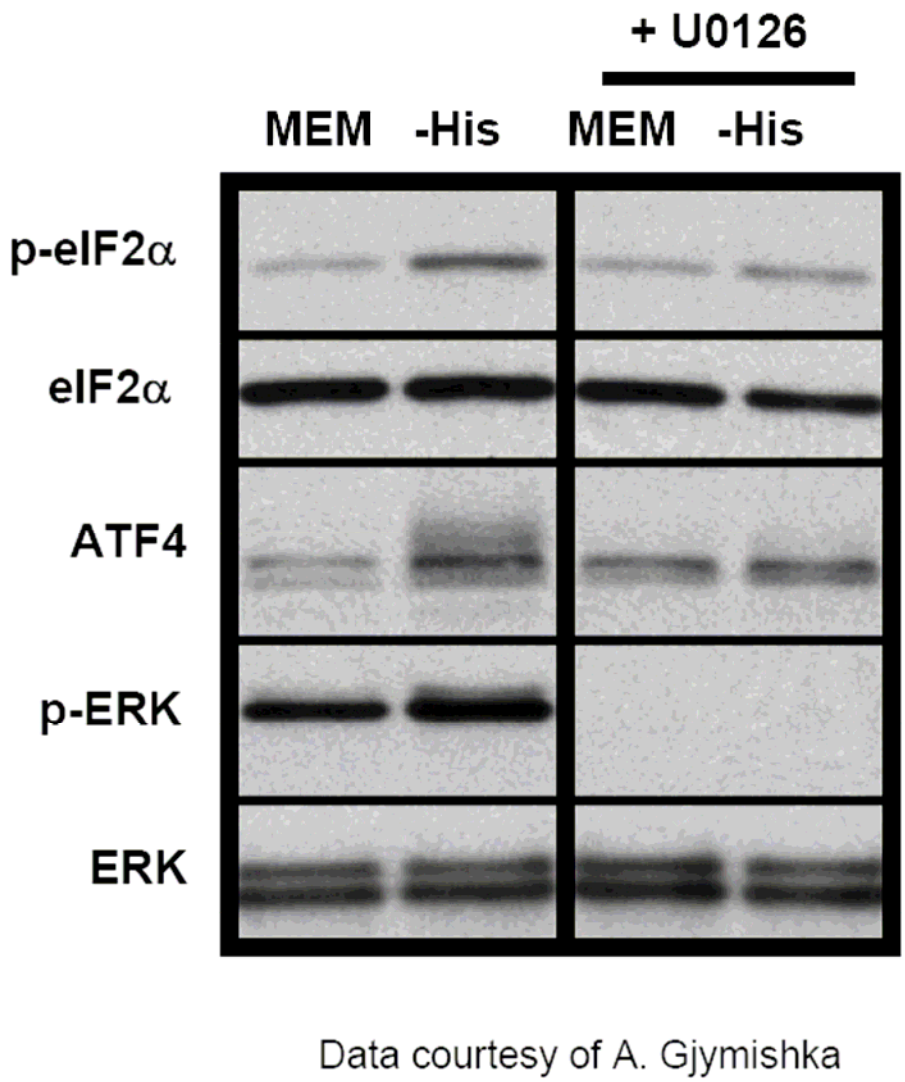

Figure 5-7. An inhibitor of the MEK pathway other than PD98059, which is U0126, also blocks the AAR pathway. HepG2 cells were incubated for 4 h in either MEM or MEM minus histidine in the absence (+MEK) or presence (-MEK) of the inhibitor U0126 (10 μM). Total protein extracts were isolated and immunoblotted for either p-eIF2α, total eIF2α, ATF4, p-ERK, or total ERK. The figure was taken from Thiaville et al. JBC 2008. Data are courtesy of A. Gymishka.

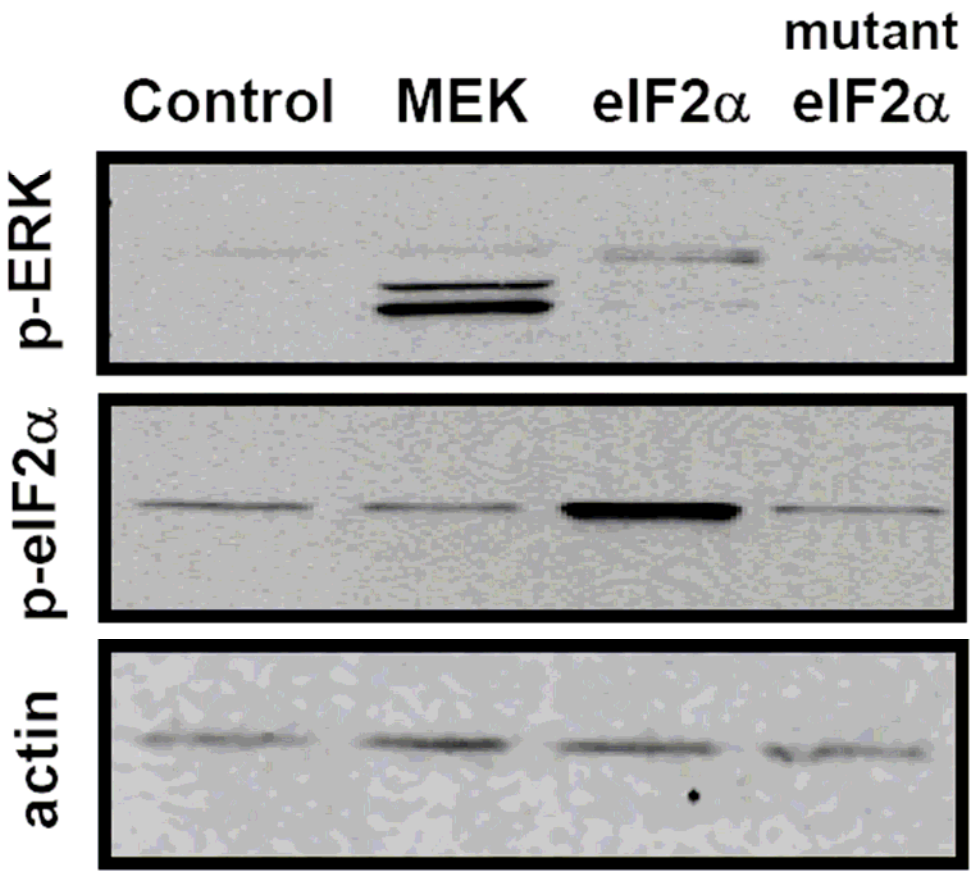

Figure 5-8. The MEK pathway does not directly phosphorylate ser51 of eIF2α. 293T cells were transiently transfected using a calcium-phosphate protocol with vectors containing either a constitutively active MEK, wild type eIF2α, or mutant eIF2α in which serine 51 has been mutated to alanine. After 36 h, whole cell lysates were collected and immunoblotted for either p-ERK, p-eIF2α, or actin. The figure was taken from Thiaville et al. JBC 2008.

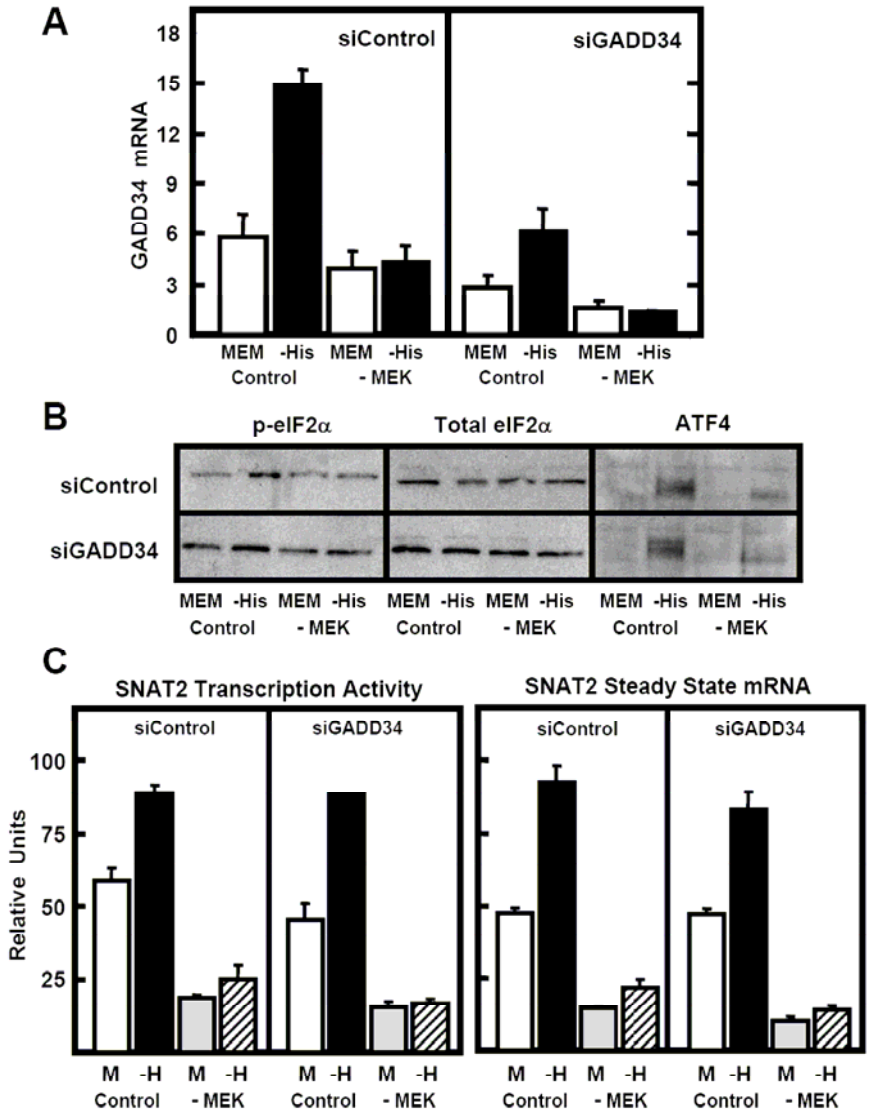

Figure 5-9. Knockdown of the eIF2α phosphatase, GADD34, does not restore eIF2α phosphorylation, ATF4 synthesis, or SNAT2 gene expression when the MEK pathway is inhibited. HepG2 cells were transfected with either a control siRNA or a siRNA specific for GADD34. After 48 h, transfected cells were incubated for 4 h in either MEM or MEM minus histidine (-His or -H) in the absence (+MEK) or presence (-MEK) of the inhibitor PD98059 and either RNA or total protein was collected. A) RT-qPCR analysis of RNA samples was performed to measure the steady state mRNA of GADD34. B) Protein samples were immunoblotted for either p-eIF2α, total eIF2α, or ATF4. C) RT-qPCR analysis of RNA samples was performed to measure the transcription activity and steady state mRNA of the SNAT2 gene. For all RT-qPCR experiments, the RNA for each data point represents three samples and the qPCR was performed in duplicate. The data are presented as the averages ± sem. The figure was taken from Thiaville et al. JBC 2008.

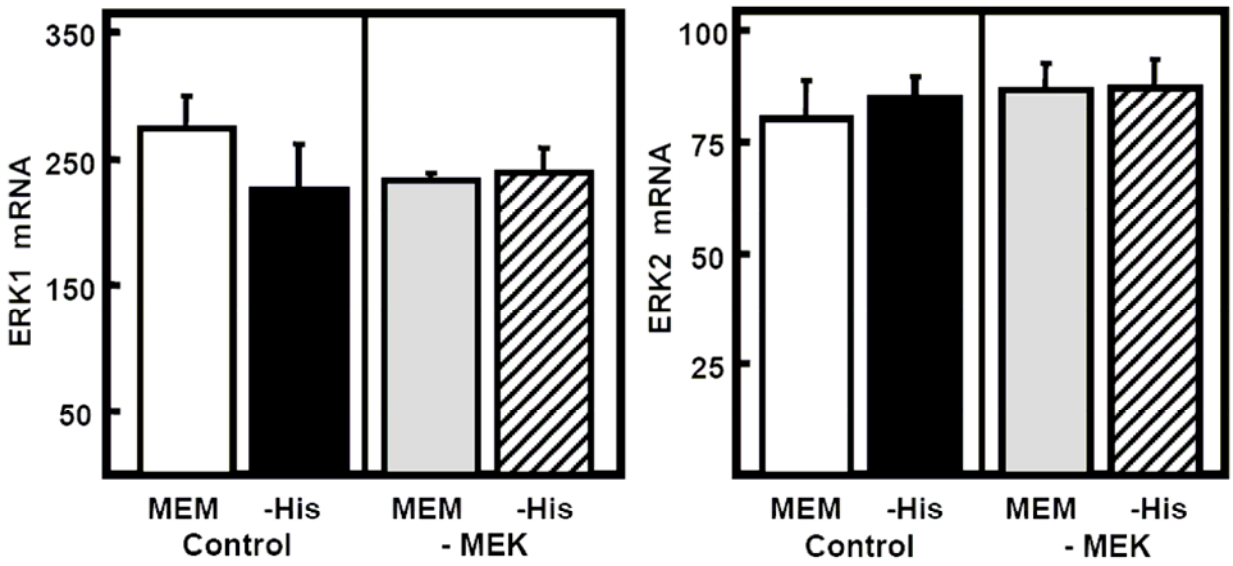

Figure 5-10. Neither ERK1 nor ERK2 mRNA levels are affected by histidine starvation or PD98059 treatment. HepG2 cells were incubated in either MEM or MEM minus histidine in the absence (+MEK) or presence (-MEK) of the inhibitor PD98059 for 8 h and total RNA was isolated. RT-qPCR analysis of RNA samples was performed to measure the steady state mRNA of either ERK1 or ERK2. The RNA for each data point represents three samples and the qPCR was performed in duplicate. The data are presented as the averages ± sem. The figure was taken from Thiaville et al. JBC 2008.

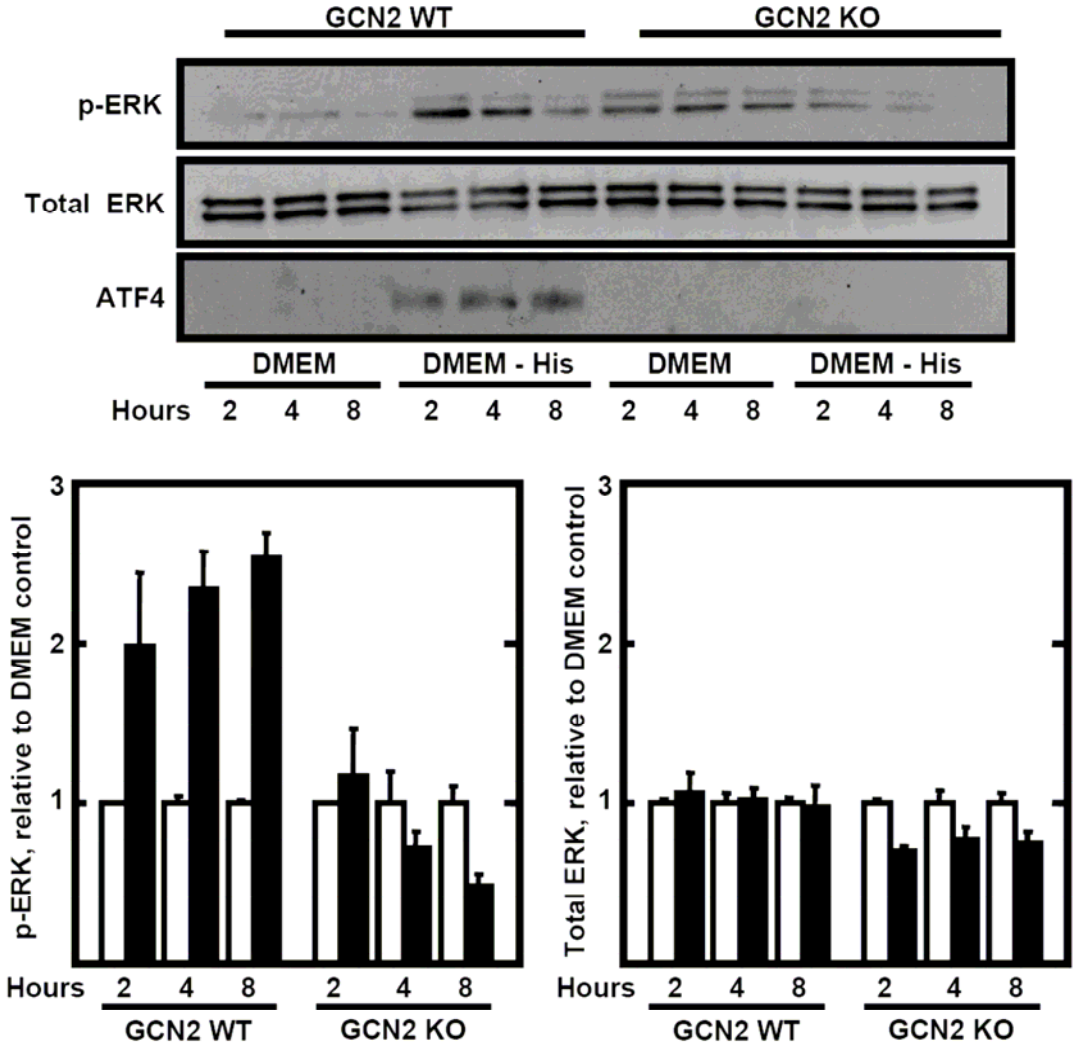

Figure 5-11. MEK activation during amino acid limitation requires active GCN2. GCN2 wild type and knockout MEF cells were incubated 2, 4, or 8 h in either DMEM or DMEM minus histidine in the absence (+MEK) or presence (-MEK) of the inhibitor PD98059 for. Total protein extracts were isolated and immunoblotted for either p-ERK, total ERK, or ATF4. For p-ERK and total ERK, the immunoblots from three experiments were quantified by densitometry and are expressed as a bar graph with all values expressed relative to their individual DMEM controls. The figure was taken from Thiaville et al. JBC 2008.

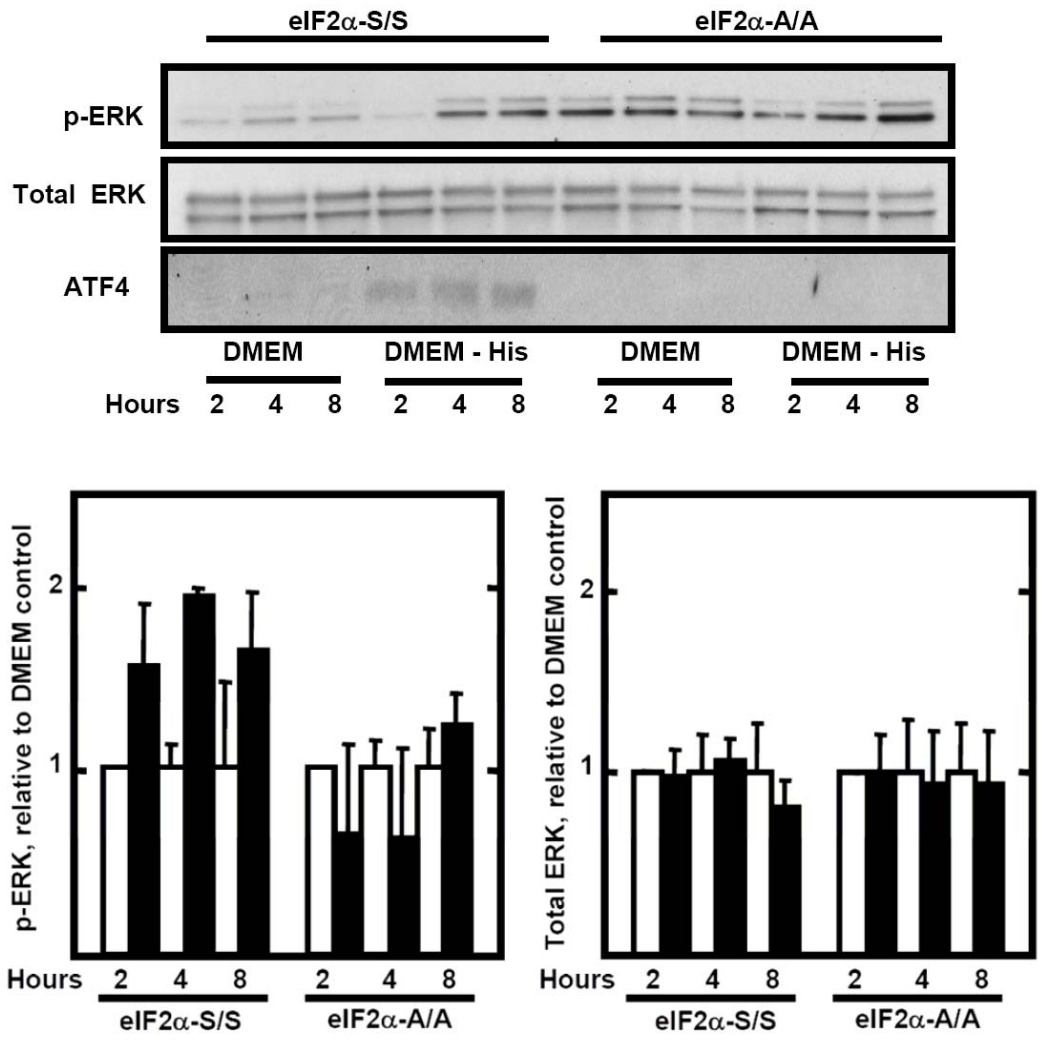

Figure 5-12. MEK activation during amino acid limitation requires eIF2α phosphorylation. eIF2α wild type (S/S) and mutant (A/A) MEF cells were incubated 2, 4, or 8 h in either DMEM or DMEM minus histidine in the absence (+MEK) or presence (-MEK) of the inhibitor PD98059 for. Total protein extracts were isolated and immunoblotted for either p-ERK, total ERK, or ATF4. For p-ERK and total ERK, the immunoblots from three experiments were quantified by densitometry and are expressed as a bar graph with all values expressed relative to their individual DMEM controls.

CHAPTER 6
CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Given that transport of amino acids by the System A transporter is regulated by hormones and amino acids at the transcriptional level, cloning of the genes responsible for this activity has allowed for characterization of the genomic elements responsible for this regulation. Previous experiments identified a region in intron 1 of the SNAT2 gene that is responsible for increased gene expression by amino acid limitation (Palii et al., 2004). The region contains three 9 bp regions termed the CAAT box, AARE, and PuR box, though only the AARE region was demonstrated to be required for induction by starvation (Palii et al., 2004). Though the SNAT2 AARE is similar to the ASNS AARE, and identical to the CHOP AARE, unlike those genes, the SNAT2 gene does not respond to glucose starvation, because it lacks an element that responds to the unfolded protein response (Bain et al., 2002). Characterization of ASNS gene regulation established a two-phased model to describe both its activation and subsequent repression by amino acid limitation (Chen et al., 2004). ATF4 binding to the ASNS AARE along with histone acetylation and Pol II recruitment characterized the activation phase, while ATF3 and C/EBPβ AARE binding established the repression phase (Chen et al., 2004). This model of ASNS activation and repression by amino acid limitation was the basis for investigating the regulation of other AARE-containing genes, such as SNAT2.

My study demonstrates that the transcription factors ATF4, ATF3, and C/EBPβ bind the SNAT2 AARE element in a temporal coordinated manner similar to the model of binding established for the ASNS gene. In fact, when four other AARE-containing genes (VEGF, CHOP, ATF3, C/EBPβ) were analyzed by ChIP analysis after amino acid limitation, transcription factor binding to their AARE regions also correlated with this model. Thus, the

model of ASNS gene regulation first proposed by Chen et al. has held true so far for all tested genes containing a functional AARE element (Chen et al., 2004). Further analysis of the SNAT2 gene established that transcription factor binding was either promoter or AARE region specific, as no direct interaction between the GTFs and the ATF/C/EBP proteins on the gene could be established. This disparity caused me to develop the idea that the general co-activator complex Mediator may be present to bridge these two regions, 700 bp apart, given that other investigators had suggested Mediator is present at all Pol II transcribed promoters (Kornberg, 2007). ChIP analysis of the SNAT2 gene demonstrated no enhanced recruitment of the Mediator complex after amino acid limitation, and knockdown of individual subunits by siRNA also had no effect on induced SNAT2 gene expression. In contrast, a control gene induced by β-estradiol, pS2, displayed required Mediator recruitment to its promoter for enhanced gene expression. The data suggest that enhanced recruitment of the Mediator complex is not required for all activator-bound enhancers to interact with their promoter region. As a consequence, the precise mechanism of interaction between the SNAT2 promoter and the distal AARE enhancer element remains to be defined.

The modeled activation and repression phases of AARE-containing gene transcription during amino acid limitation were further studied by analyzing the roles of ATF4 and C/EBPβ during these phases. Knockdown of ATF4 by siRNA demonstrated that ASNS gene regulation by amino acid limitation requires ATF4 protein, whereas SNAT2 gene regulation can function without ATF4 protein. Though the regulation of SNAT2 through its AARE is ATF4-dependent, when tested in a transient transfection study, it seems likely that *in vivo* an ATF4-independent pathway of regulation also exists. Given that IGFBP-1 gene expression can be induced during amino acid limitation by both USF binding to an E-box element and ATF4 binding to an AARE,

the SNAT2 ATF4-independent pathway likely consists of a second uncharacterized functional element with different transcription factor requirements (Matsukawa et al., 2001; Marchand et al., 2006). Analysis of the repression phase of amino acid limitation by knockdown of the C/EBPβ protein demonstrated that both ASNS and SNAT2 gene expression was slightly elevated in the absence of C/EBPβ. While C/EBPβ does exert a modest repressive effect on AARE-containing gene transcription during amino acid limitation, it does not seem to function alone as a major repressor. Given that both C/EBPβ and ATF3 bind to the AARE during this repressive phase, either ATF3 alone or a combination of C/EBPβ and ATF3 binding may be required for a maximal repressive effect. In vitro, when either ATF3 and/or C/EBPβ were overexpressed with a constant amount of ATF4, transcription from a reporter construct containing the ASNS AARE was repressed in ATF3-C/EBPβ conditions, but the contribution by ATF3 was demonstrated to be more important than the contribution from C/EBPβ (Chen et al., 2004).

The AAR pathway activated by amino acid limitation is one of several main signaling cascades responsible for cellular response to nutrient stress. The activation of other signaling pathways, such as MAPK pathways, by amino acid limitation has been documented but not well characterized. Previously published data demonstrated an importance for either ERK activity or phosphorylation during amino acid limitation in fibroblast, liver, and muscle cell lines (Franchi-Gazzola et al., 1999; Leung-Pineda et al., 2004; Kashiwagi et al., 2008). The current analysis establishes an absolute requirement of the MEK-ERK signaling cascade for activation of the AAR cascade during amino acid limitation in HepG2 cells. The MEK-ERK pathway was required for phosphorylation of eIF2α, increased synthesis of ATF4, and increased AARE gene transcription during amino acid limitation, and conversely, GCN2 and eIF2α phosphorylation were also required for the induction of the MEK pathway itself during amino acid limitation.

Although the data do not establish the exact mechanism by which these two pathways interact, it does demonstrate a required interdependence between the two during amino acid limitation. This interdependence between the MEK and AAR pathways, however, may be liver-tissue specific or even hepatoma specific. Aubel et al have reported that Hela cells, a cervical adenocarcinoma cell line, deprived for a single amino acid demonstrate upregulation of the JNK pathway and actually show a slight downregulation of the ERK pathway (Aubel et al., 2001). In L6 cells, a muscle tissue cell line, amino acid limitation induced both the JNK and ERK phosphorylation cascades (Hyde et al., 2007). Clearly, the link between the AAR and ERK pathway established for amino acid limitation of HepG2 cells is not applicable to all cell types, but is potentially beneficial for studying liver and/or hepatoma specific phenomena.

Future Directions

Initial analysis of the SNAT2 AARE by ChIP has identified a specific set of transcription factors whose binding increases after amino acid limitation. Undoubtedly, there are other unknown transcription factors that bind this region *in vivo*. As more AAR regulated transcription factors are identified, their possible presence at AARE regions should be investigated by ChIP. Likewise, as antibodies become available to phosphorylation, acetylation, and other post-translational modification sites on transcription factors, specifically ATF4, ATF3, C/EBPα, and C/EBPβ, binding of those modified proteins to the AARE can also be tested by ChIP analysis. These experiments will be especially interesting for C/EBPα, given that it binds to the AARE, but the absolute amount bound is unchanged by amino acid limitation. It is possible that the amount of total protein at the AARE does not change, but the amount of modified (i.e., phosphorylated) protein does. Evidence for this type of regulation was demonstrated for the AARE of the amino acid responsive gene CHOP. During amino acid limitation, the binding of total ATF2 to the CHOP AARE remains constant while the binding of

p-ATF2 increases (Averous et al., 2004). ChIP analysis for ATF2 at the SNAT2 AARE demonstrated no binding, thus indicating potential diversity in transcription regulation by the AAR pathway for specific genes may exist. ATF2 is also a histone acetyltransferase that acetylates histones H4 and H2B and was demonstrated to be necessary for the acetylation of these histones at the CHOP AARE (Bruhat et al., 2007). Thus, although my studies have documented an increase in general acetylation of H3 on the SNAT2 gene, ChIP analysis of the SNAT2 gene with antibodies for specific singular site histone modifications may provide clues about the other transcription factors associated with the AARE region.

The finding that the Mediator complex does not display inducible binding at the SNAT2 gene after amino acid limitation is one of the most significant findings from this research. This result is important not only for the field of nutrient regulation of gene transcription, but also for the more global study of basic gene transcription. Though it has been proposed that Mediator interacts with all inducible activator-bound promoters, this observation may need to be reconsidered given the present data for the SNAT2 gene. In order to test Mediator's necessity further, a knockout of all non-lethal Mediator subunits in yeast would ensure a complete complex could not be formed while testing whether inducible gene transcription can still take place. Since the yeast and human Mediator complexes may also have different specific functions, this experiment could be under taken in human cells as well, though it would be much harder to perform.

The data presented also begin further functional characterization of two transcription factors that actually bind the SNAT2 AARE region, these are the transcription factors ATF4 and C/EBPβ. The data suggest that SNAT2, unlike most other known AARE-containing genes, can be transcriptionally induced after amino acid limitation by an ATF4-independent pathway.

Recently, it has been demonstrated that other genes can be induced by amino acid limitation through E-boxes, multiple AARE elements in close proximity, or a combination of yet undefined elements (Matsukawa et al., 2001; Sato et al., 2004a; Chen et al., 2005). Though I performed a few experiments to look for a second ATF4-independent element, none were successful. Future studies of this ATF4-independent pathway could use functional assays paired with species alignment to test for conserved potential AAR elements in an ATF4 KO cell line or after siATF4 treatment. Given that several AARE-containing genes have been described, transcription activation of these genes by amino acid limitation could be tested in both ATF4 replete and depleted conditions to provide a more complete picture of the ATF4-independent pathway.

To further determine the role of C/EBPβ function on the AARE, studies have already begun in the Kilberg laboratory to use newly available C/EBPβ isoform specific antibodies for ChIP analysis. These antibodies will distinguish LAP* binding alone and the contribution of LAP*/LAP binding to the AARE without interference from the LIP protein. Given that the entire LIP protein coding sequence is contained within the LAP protein, there are no specific antibodies for LIP. It is also possible that the maximal repressive function C/EBPβ exerts on the AARE may be through partnered binding with the ATF3 protein at the AARE itself. Knockdown of both C/EBPβ and ATF3 may be required to see a significant enhancement of AARE gene expression. Co-immunoprecipitation studies with antibodies to C/EBPβ and ATF3 to detail the interaction between these proteins may also provide insight into the mechanism of repression of AARE gene expression during the latter phase of amino acid limitation.

Another significant finding from this research is the interdependence between the MEK and AAR signaling pathways in the HepG2 cell line. This observation has been documented in Chapter 5 of this work, but clearly the data raised as many questions as it answered. How is the

MEK signaling cascade actually activated? Is Ras, an upstream activator of MEK, required for amino acid deprivation through MEK signaling? Do any of these proteins directly interact with GCN2? Given that no known plasma membrane sensor protein has been proven to mediate an AAR signal, activation of Ras, known to localize to the plasma membrane, by AAR signaling may provide some evidence for activation of transmembrane receptors. Likewise, questions about the downstream effectors of MEK signaling can also be asked. What downstream proteins of ERK are specifically phosphorylated by either ERK itself or its downstream target RSK? Are these phosphorylated proteins transcription factors and do they interact with AARE containing gene? Given that RSK is a known target of ERK and has been demonstrated to phosphorylate both ATF4 and C/EBPβ in other cellular contexts (Yang et al., 2004; Buck et al., 2001), the next step would be to establish if RSK is phosphorylated by amino acid deprivation. Constitutively active RSK constructs could then be used to check modulation of ATF4 and C/EBPβ by mutating suspected phosphorylation sites. RSK knockout cell lines or RSK-specific siRNA treated cells could also be probed for induction of AAR proteins and genes.

Recent studies have also demonstrated that tribbles homolog 3 (TRIB3) protein levels are increased after amino acid deprivation in a phosphoinositide-3-kinase (PI3K) dependent manner (Schwarzer et al., 2006). Though PI3K's established target is AKT, recent evidence has demonstrated that PI3K signaling can indirectly either inhibit or enhance MEK activation (Sebolt-Leopold and Herrera, 2004; Merighi et al., 2006). TRIB3 over-expression studies established that TRIB3 can modulate MEK activity and inhibit gene transcription from an AP-1 site (Kiss-Toth et al., 2004). Thus, a feedback mechanism may also exist in which the AAR pathway first induces the MEK signaling cascade and secondly, induces TRIB3 protein levels, such that TRIB3 can now modulate MEK activity. This scenario is not unlikely given that the

phosphorylation of eIF2α during amino acid limitation is creates its own feedback inhibition by later activation of the GADD34-PP1 phosphatase complex (Brush et al., 2003). Clearly, the interdependence between the MEK and AAR signaling pathways has only begun to be characterized and understood, and there are many opportunities for additional mechanistic studies.

APPENDIX A
CO-ACTIVATOR BINDING TO THE ASNS AND SNAT2 AARE

Protein/Activity	**ASNS AARE- Present**	**SNAT2 AARE- Present**
GCN5 (HAT)	NO	NO
PCAF (HAT)	NO	NO
CBP (HAT)	NO	NO
p300 (HAT)	NO	NO
TIP60 (HAT)	NO	NO
SRC1 (HAT)	NO	NO
MLL	NO	NO
SET9	NO	NO
p-ATF2	NO	NO
CREB1	NO	NO
p-H3 Ser10	NO	NO
NFκB p65	NO	NO
Histone deacetylase (HDAC) 1	NO	Not tested
Histone deacetylase (HDAC) 3	NO	Not tested
MSin3A	NO	Not tested
YY1	NO	Not tested
Cyclin D1	NO	Not tested
XBP-1	NO	Not tested
c-fos	NO	Not tested
SP3	NO	Not tested
NRF-2	Not tested	NO
BRG1	Not tested	NO
c-Jun	Not tested	NO
ATF1	Not tested	NO
ATF5	Not tested	NO

Figure A-1. ChIP analysis of specific protein binding to the ASNS or SNAT2 gene. HepG2 cells were incubated for 8 h in either MEM, MEM minus histidine, or MEM plus 2 mM histidinol and subjected to ChIP analysis with antibodies for different proteins. The figure depicts a compilation of the ChIP result for all negative protein binding analysis to the ASNS or SNAT2 gene. All data analyzed for the ASNS gene only was provided by Dr. H. Chen. These proteins were determined not to interact with the ASNS and/or SNAT2 genes during amino acid limitation though some of these proteins were demonstrated to interact with ATF4 and/or C/EBPβ by other investigators.

APPENDIX B
RT-qPCR ANALYSIS OF NORMAL HUMAN HEPATOCYTES INDUCED BY AAR AND UPR PATHWAYS

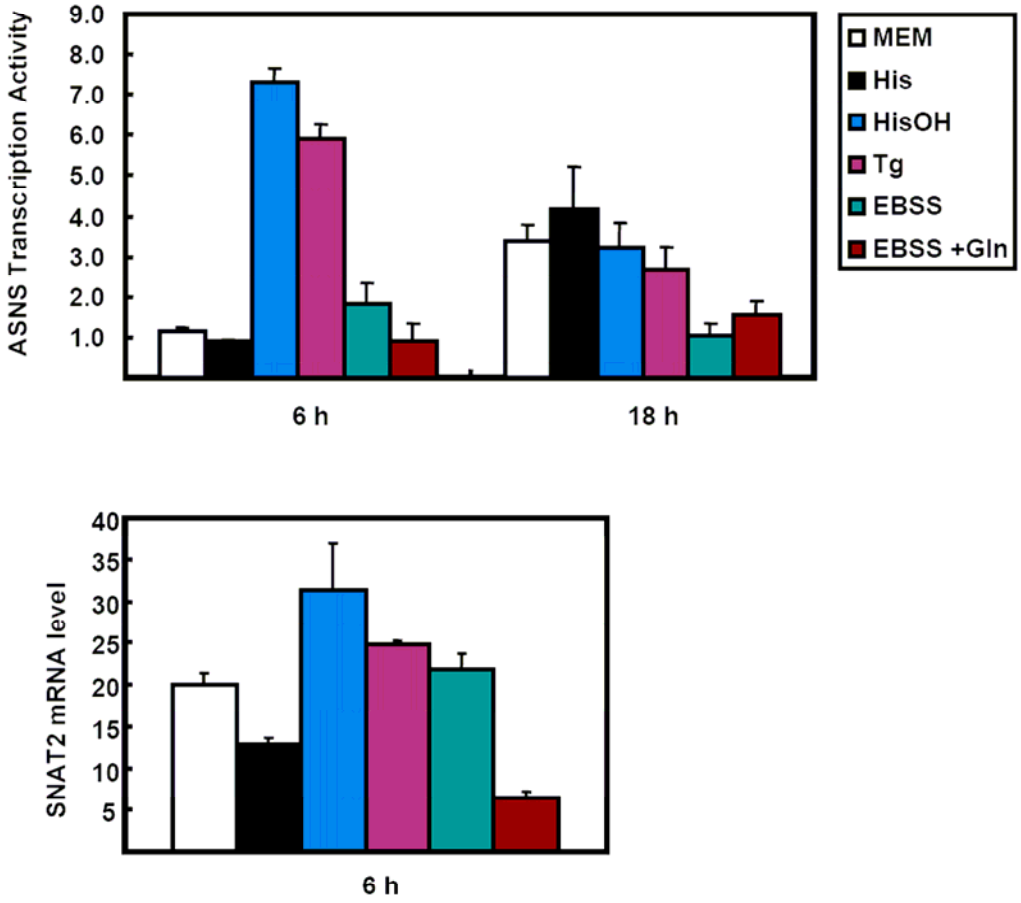

Figure B-1. SNAT2 and ASNS induction was monitored during the AAR and UPR in a primary culture of normal human hepatocytes. Freshly prepared normal human hepatocytes were plated in collagen-coated 6-well plates at a density of approximately 0.5 x 10^6 cells per well. After 24 h, the cells were incubated in either complete culture media (MEM- white), MEM minus the amino acid histidine (His- black), MEM plus 2 mM histidinol (HisOH- blue), MEM plus 300 nM thapsigargin (Tg- purple), Earle's Balances Salt Solution (EBSS- green), or EBSS plus the amino acid glutamine (EBSS+Gln- red). Cells were cultured in these conditions for either 6 or 18 h, and total RNA was isolated at each of these time points. For each time point, RNA was isolated independently from 3 different wells per condition, and RT-qPCR was performed for each independent sample in duplicate with primers (shown in Table 2-1) specific to either the human ASNS or SNAT2 gene. The data are presented as the average ± the standard deviation. After 6 h of drug treatment to induce the AAR, ASNS transcription activity and SNAT2 mRNA increased, while only ASNS transcription activity was induced by 6 h of drug treatment to induce the UPR.

APPENDIX C
CELL GROWTH AND AMINO ACID DEPRIVATION STUDIES IN THE SV40 TEMPERATURE SENSITIVE RAT FETAL HEPATOCYTE CELL LINE RLA209

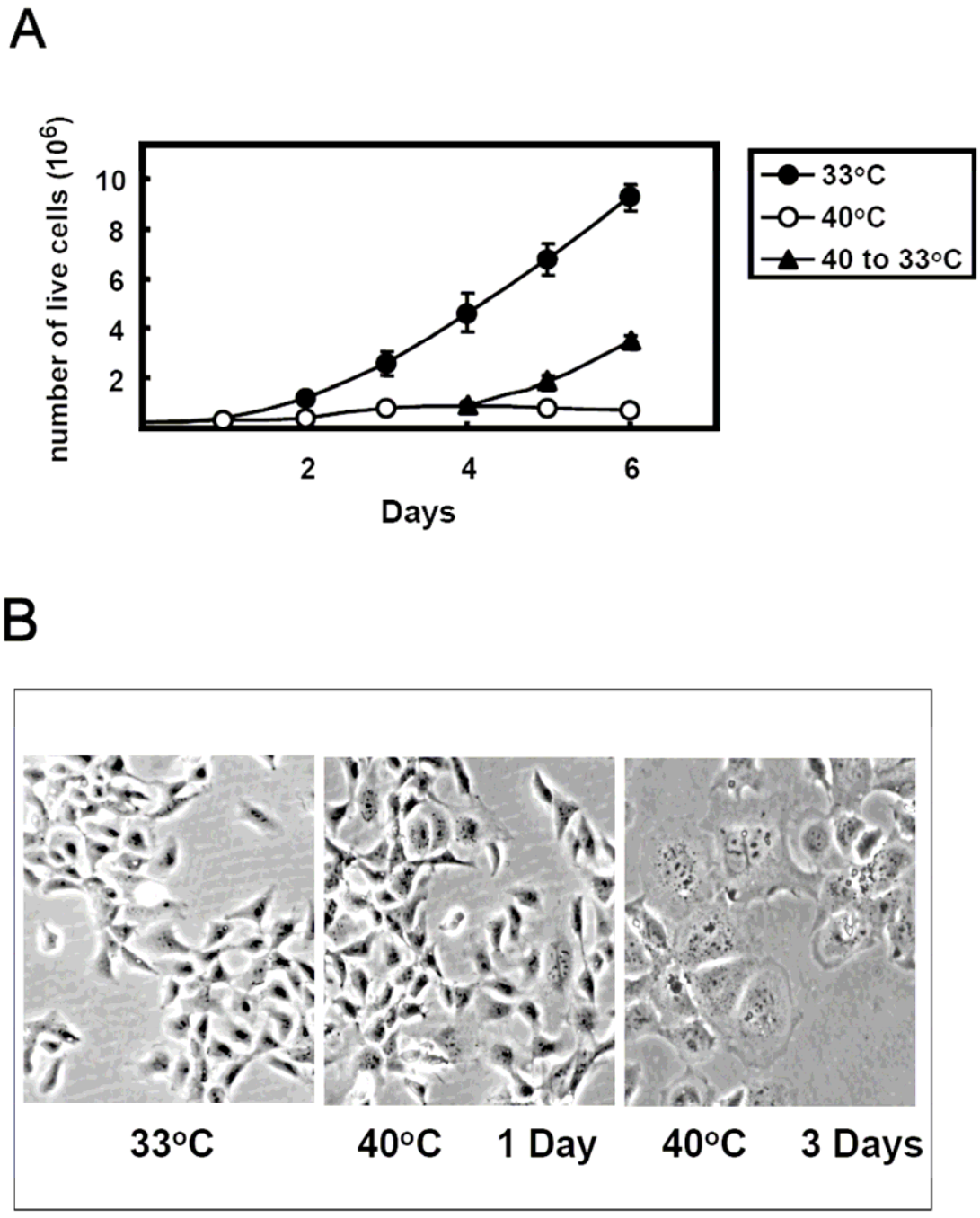

Figure C-1. Growth conditions and cell morphology of RLA209-15 cells incubated at either 33°C or 40°C. A) RLA209-15 cells were plated in multiple 60 mm dishes at a density of 0.3 x 10^6 cells per plate and incubated at either 33°C (closed circles) or 40°C (open circles) for 6 days. On day 4, a subset of the cells cultured at 40°C were moved to

33^{o}C (closed triangles). Every day for 6 days, one 60 mm dish of cells at each incubation temperature was counted using the Trypan blue exclusion method. This experiment was repeated independently three times, and the data are graphed as the number of live cells (per one million cells) ± sem. B) RLA209-15 cells were plated in 60 mm dishes at a density of 0.3 x 10^{6} cells per plate and incubated at 33^{o}C for 1 day. Cells were then transferred to 40^{o}C and cultured for 3 days. Pictures of cellular morphology were obtained using light microscopy after either 1 day at 33^{o}C, 1 day at 40^{o}C, or 3 days at 40^{o}C. Cell growth and morphology was monitored to ensure cellular response was the same as previously published observations for this cell line.

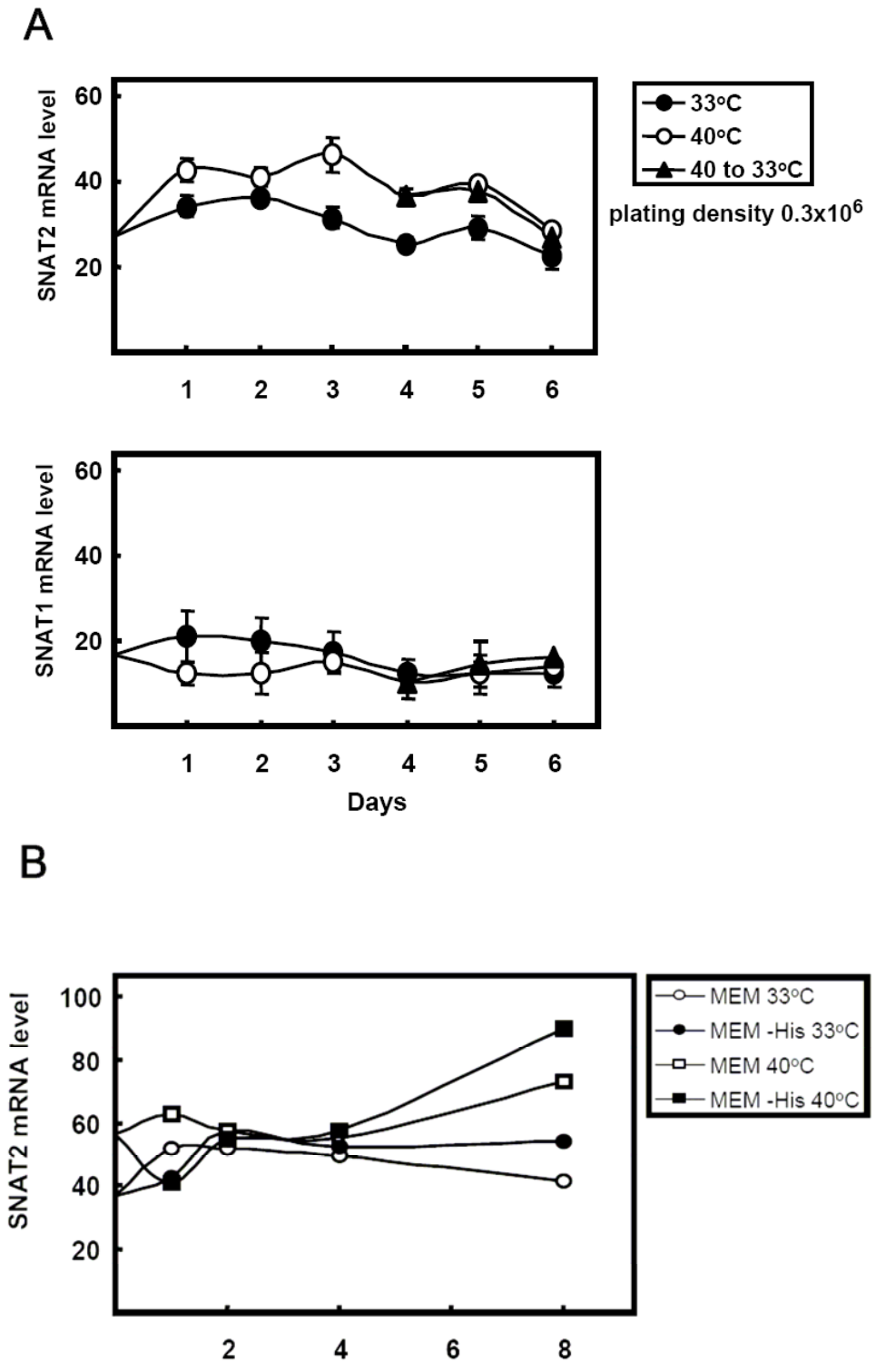

Figure C-2. SNAT1 and SNAT2 steady state mRNA levels in RLA209-15 cells cultured at either 33°C or 40°C. A) RLA209-15 cells were plated in 60 mm dishes at a density of 0.3 x 10^6 cells per plate and incubated at either 33°C (closed circles) or 40°C (open circles) for 6 days. On day 4, a subset of the cells cultured at 40°C were moved to

33°C (closed triangles). Every day for 6 days, total RNA was isolated from one 60 mm dish of cells at each incubation temperature. RT-qPCR analysis of RNA samples was performed with primers specific for either rat SNAT1 or SNAT2 steady state mRNA levels. Though primers to amplify SNAT4 mRNA were created and validated to work, no detectable levels of SNAT4 mRNA were produced by RT-qPCR. RNA for each data point represents three samples and the RT-qPCR for each data point was performed in duplicate. The data are presented as the average ± sem. B) RLA209-15 cells were plated in 60 mm dishes at a density of 0.3 x 10^6 cells per plate and cultured at either 33°C (circles) or 40°C (squares). After 12-16 h, cells were incubated in either MEM (open shape) or MEM minus histidine (closed shape) for 0 to 8 h. Total RNA was isolated and RT-qPCR analysis was performed with primers specific to rat SNAT2 mRNA. The data shown are from a single experiment. SNAT mRNA levels and starvation response did not vary between cells incubated at different temperatures, though System A transport activity was previously demonstrated to increase when cells were incubated at 40°C as compared with cells incubated at 33°C.

APPENDIX D
EFFECT OF CD98 KNOCKDOWN ON THE AAR PATHWAY

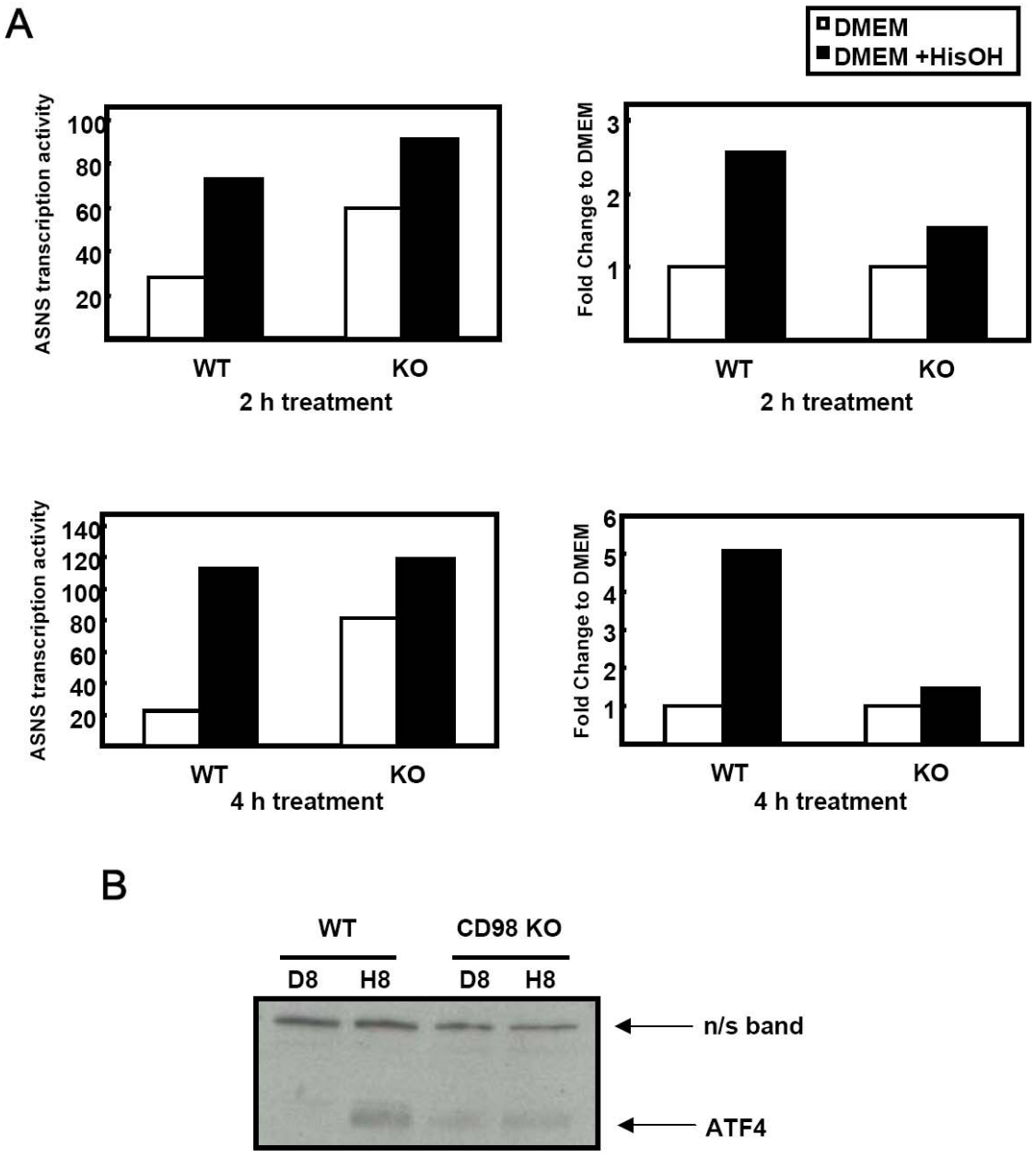

Figure D-1. ASNS transcription activity and ATF4 protein concentration in CD98 (amino acid transporter subunit 4F2hc) wild type and knockout mouse ES cells. A) CD98 wild type and knockout embryonic stem (ES) cells were plated in 60 mm dishes at a density of 1.0 x 10^6 cells per dish. The next day, cells were incubated in either complete DMEM (white box) or DMEM plus 2 mM histidinol (black boxes) for 2 or 4 h. RNA was isolated at each time point, and RT-qPCR analysis was performed

with primers (listed in Table 2-1) specific for mouse ASNS transcription activity. The data are graphed as either relative activity levels or fold change relative to DMEM for a single experiment. B) CD98 wild type and knockout ES cells were plated in 60 mm dishes at a density of 1.0 x 10^6 cells per dish. The next day, cells were incubated in either complete DMEM (white box) or DMEM plus 2 mM histidinol (black boxes) for 8 h and total protein was isolated. Immunoblots were performed with antibody specific to ATF4. Basal levels of ASNS transcription activity and ATF4 protein were increased in a CD98 knockout cell line alluding to CD98 involvement in amino acid deprivation sensing through its known intramembrane interaction with amino acid transporter proteins.

APPENDIX E
C-MAF BINDING TO THE SNAT2 PROMOTER DOES NOT INCREASE DURING AMINO ACID DEPRIVATION

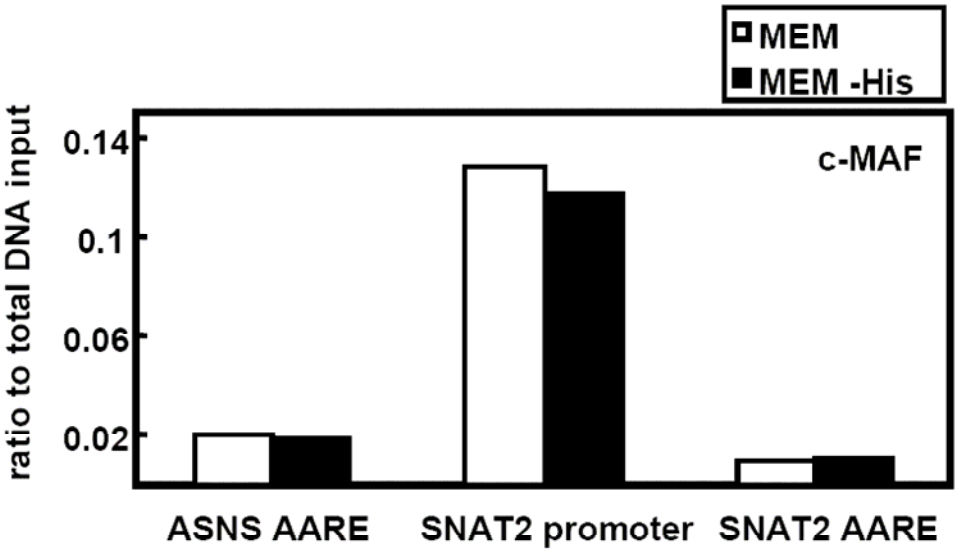

Figure E-1. c-Maf binds specifically to the SNAT2 proximal promoter region. HepG2 cells were treated with either complete MEM (white box) or MEM minus histidine (black box) for 8 h, and then ChIP analysis was performed with antibodies specific for the c-Maf protein. Specific primers were used to measure levels of c-Maf binding to either the ASNS AARE, SNAT2 promoter, or SNAT2 AARE by qPCR. The data are graphed as the relative immunoprecipitated value for c-Maf to total DNA (input) for a single experiment. Though its binding is not elevated after starvation, c-Maf is constitutively present at the SNAT2 promoter, consistent with its role in gene interaction with other bZIP and transcription co-activator proteins.

LIST OF REFERENCES

Abcouwer,S.F., Marjon,P.L., Loper,R.K., and Vander Jagt,D.L. (2002). Response of VEGF expression to amino acid deprivation and inducers of endoplasmic reticulum stress. Invest Ophthalmol. Vis. Sci. *43*, 2791-2798.

Ameri,K. and Harris,A.L. (2008). Activating transcription factor 4. Int. J. Biochem. Cell Biol. *40*, 14-21.

Ameri,K., Lewis,C.E., Raida,M., Sowter,H., Hai,T., and Harris,A.L. (2004). Anoxic induction of ATF-4 through HIF-1-independent pathways of protein stabilization in human cancer cells. Blood *103*, 1876-1882.

Arai,M., Kondoh,N., Imazeki,N., Hada,A., Hatsuse,K., Kimura,F., Matsubara,O., Mori,K., Wakatsuki,T., and Yamamoto,M. (2006). Transformation-associated gene regulation by ATF6alpha during hepatocarcinogenesis. FEBS Lett. *580*, 184-190.

Armstrong,J.A. (2007). Negotiating the nucleosome: factors that allow RNA polymerase II to elongate through chromatin. Biochem. Cell Biol. *85*, 426-434.

Aubel,C., Dehez,S., Chabanon,H., Seva,C., Ferrara,M., and Brachet,P. (2001). Activation of c-Jun N-terminal kinase 1 (JNK-1) after amino acid deficiency in HeLa cells. Cell Signal. *13*, 417-423.

Auberger,P., Samson,M., and LeCam,A. (1983). Effects of growth factors on hormonal stimulation of amino acid transport in primary cultures of rat hepatocytes. Biochem. J. *210*, 361-366.

Averous,J., Bruhat,A., Jousse,C., Carraro,V., Thiel,G., and Fafournoux,P. (2004). Induction of CHOP Expression by Amino Acid Limitation Requires Both ATF4 Expression and ATF2 Phosphorylation. J. Biol. Chem. *279*, 5288-5297.

Bain,P.J., LeBlanc-Chaffin,R., Chen,H., Palii,S.S., Leach,K.M., and Kilberg,M.S. (2002). The mechanism for transcriptional activation of the human ATA2 transporter gene by amino acid deprivation is different than that for asparagine synthetase. J. Nutr. *132*, 3023-3029.

Balciunas,D. and Ronne,H. (1995). Three subunits of the RNA polymerase II mediator complex are involved in glucose repression. Nucleic Acids Res. *23*, 4421-4425.

Barbosa-Tessmann,I.P., Pineda,V.L., Nick,H.S., Schuster,S.M., and Kilberg,M.S. (1999). Transcriptional regulation of the human asparagine synthetase gene by carbohydrate availability. Biochem. J. *339*, 151-158.

Bhattacharyya,S.N., Habermacher,R., Martine,U., Closs,E.I., and Filipowicz,W. (2006). Relief of microRNA-Mediated Translational Repression in Human Cells Subjected to Stress. Cell *125*, 1111-1124.

Bjorklund,S. and Kim,Y.J. (1996). Mediator of transcriptional regulation. Trends Biochem. Sci. *21*, 335-337.

Borghetti,A.F., Piedimonte,G., Tramacere,M., Severini,A., Ghiringhelli,P., and Guidotti,G.G. (1980). Cell density and amino acid transport in 3T3, SV3T3, and SV3T3 revertant cells. J. Cell Physiol *105*, 39-49.

Boube,M., Joulia,L., Cribbs,D.L., and Bourbon,H.M. (2002). Evidence for a mediator of RNA polymerase II transcriptional regulation conserved from yeast to man. Cell *110*, 143-151.

Bourbon,H.M., Aguilera,A., Ansari,A.Z., Asturias,F.J., Berk,A.J., Bjorklund,S., Blackwell,T.K., Borggrefe,T., Carey,M., Carlson,M., Conaway,J.W., Conaway,R.C., Emmons,S.W., Fondell,J.D., Freedman,L.P., Fukasawa,T., Gustafsson,C.M., Han,M., He,X., Herman,P.K., Hinnebusch,A.G., Holmberg,S., Holstege,F.C., Jaehning,J.A., Kim,Y.J., Kuras,L., Leutz,A., Lis,J.T., Meisterernest,M., Naar,A.M., Nasmyth,K., Parvin,J.D., Ptashne,M., Reinberg,D., Ronne,H., Sadowski,I., Sakurai,H., Sipiczki,M., Sternberg,P.W., Stillman,D.J., Strich,R., Struhl,K., Svejstrup,J.Q., Tuck,S., Winston,F., Roeder,R.G., and Kornberg,R.D. (2004). A unified nomenclature for protein subunits of mediator complexes linking transcriptional regulators to RNA polymerase II. Mol. Cell *14*, 553-557.

Boyd,K.E. and Farnham,P.J. (1999). Coexamination of site-specific transcription factor binding and promoter activity in living cells. Mol. Cell Biol. *19*, 8393-8399.

Bruhat,A., Averous,J., Carraro,V., Zhong,C., Reimold,A.M., Kilberg,M.S., and 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*, 48107-48114.

Bruhat,A., Cherasse,Y., Maurin,A.C., Breitwieser,W., Parry,L., Deval,C., Jones,N., Jousse,C., and Fafournoux,P. (2007). ATF2 is required for amino acid-regulated transcription by orchestrating specific histone acetylation. Nucleic Acids Res. *35*, 1312-1321.

Bruhat,A., Jousse,C., Carraro,V., Reimold,A.M., Ferrara,M., and Fafournoux,P. (2000). Amino acids control mammalian gene transcription: activating transcription factor 2 is essential for the amino acid responsiveness of the CHOP promoter. Mol. Cell Biol. *20*, 7192-7204.

Bruhat,A., Jousse,C., Wang,X.-Z., Ron,D., Ferrara,M., and Fafournoux,P. (1997). Amino acid limitation induces expression of CHOP, a CCAAT/enhancer binding protein-related gene, at both transcriptional and post-transcriptional levels. J. Biol. Chem. *272*, 17588-17593.

Brush,M.H., Weiser,D.C., and 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*, 1292-1303.

Buck,M., Poli,V., Hunter,T., and Chojkier,M. (2001). C/EBPbeta phosphorylation by RSK creates a functional XEXD caspase inhibitory box critical for cell survival. Mol. Cell *8*, 807-816.

Burakov,D., Crofts,L.A., Chang,C.P., and Freedman,L.P. (2002). Reciprocal recruitment of DRIP/mediator and p160 coactivator complexes in vivo by estrogen receptor. J. Biol. Chem. *277*, 14359-14362.

Calkhoven,C.F., Muller,C., and Leutz,A. (2000). Translational control of C/EBPalpha and C/EBPbeta isoform expression. Genes Dev. *14*, 1920-1932.

Chen,C., Dudenhausen,E., Chen,H., Pan,Y.X., Gjymishka,A., and Kilberg,M.S. (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*, 649-658.

Chen,H. and Kilberg,M.S. (2006). Alignment of the transcription start site coincides with increased transcriptional activity from the human asparagine synthetase gene following amino acid deprivation of HepG2 cells. J. Nutr. *136*, 2463-2467.

Chen,H., Pan,Y.X., Dudenhausen,E.E., and Kilberg,M.S. (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 bZIP transcription factors as well as localized histone acetylation. J. Biol. Chem. *279*, 50829-50839.

Conaway,J.W., Shilatifard,A., Dvir,A., and Conaway,R.C. (2000). Control of elongation by RNA polymerase II. Trends Biochem. Sci. *25*, 375-380.

Costa-Mattioli,M., Gobert,D., Harding,H., Herdy,B., Azzi,M., Bruno,M., Bidinosti,M., Ben,M.C., Marcinkiewicz,E., Yoshida,M., Imataka,H., Cuello,A.C., Seidah,N., Sossin,W., Lacaille,J.C., Ron,D., Nader,K., and Sonenberg,N. (2005). Translational control of hippocampal synaptic plasticity and memory by the eIF2alpha kinase GCN2. Nature *436*, 1166-1173.

Davies,S.P., Reddy,H., Caivano,M., and Cohen,P. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem. J. *351*, 95-105.

De Angelis,R., Iezzi,S., Bruno,T., Corbi,N., Di Padova,M., Floridi,A., Fanciulli,M., and Passananti,C. (2003). Functional interaction of the subunit 3 of RNA polymerase II (RPB3) with transcription factor-4 (ATF4). FEBS Lett. *547*, 15-19.

Descombes,P. and Schibler,U. (1991). A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. Cell *67*, 569-579.

Dhillon,A.S., Hagan,S., Rath,O., and Kolch,W. (2007). MAP kinase signalling pathways in cancer. Oncogene *26*, 3279-3290.

Endo,Y., Fu,Z., Abe,K., Arai,S., and Kato,H. (2002). Dietary protein quantity and quality affect rat hepatic gene expression. J. Nutr. *132*, 3632-3637.

Fan,X., Chou,D.M., and Struhl,K. (2006). Activator-specific recruitment of Mediator in vivo. Nat. Struct. Mol. Biol. *13*, 117-120.

Farnham,P.J. (2002). In vivo assays to examine transcription factor localization and target gene specificity. Methods *26*, 1-2.

Favata,M.F., Horiuchi,K.Y., Manos,E.J., Daulerio,A.J., Stradley,D.A., Feeser,W.S., Van Dyk,D.E., Pitts,W.J., Earl,R.A., Hobbs,F., Copeland,R.A., Magolda,R.L., Scherle,P.A., and Trzaskos,J.M. (1998). Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J. Biol. Chem. *273*, 18623-18632.

Fawcett,T.W., Martindale,J.L., Guyton,K.Z., Hai,T., and Holbrook,N.J. (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*, 135-141.

Fernandez,J., Lopez,A.B., Wang,C., Mishra,R., Zhou,L., Yaman,I., Snider,M.D., and Hatzolgou,M. (2003). Transcriptional control of the arginine/lysine transporter, cat-1, by physiological stress. J. Biol. Chem. *278*, 50000-50009.

Fong,A.D., Handlogten,M.E., and Kilberg,M.S. (1990). Substrate-dependent adaptive regulation and trans-inhibition of Systems A-mediated amino acid transport. Studies using rat hepatoma plasma membrane vesicles. Biochim. Biophys. Acta *1022*, 325-332.

Foster,D.O. and Pardee,A.B. (1969). Transport of amino acids by confluent and nonconfluent 3T3 and polyoma virus-transformed 3T3 cells growing on glass cover slips. J. Biol. Chem. *244*, 2675-2681.

Franchi-Gazzola,R., Visigalli,R., Bussolati,O., Dall'Asta,V., and Gazzola,G.C. (1999). Adaptive increase of amino acid transport System A requires ERK1/2 activation. J. Biol. Chem. *274*, 28922-28928.

Freeman,T.L., Ngo,H.Q., and Mailliard,M.E. (1999). Inhibition of system A amino acid transport and hepatocyte proliferation following partial hepatectomy in the rat. Hepatology *30*, 437-444.

Friday,B.B. and Adjei,A.A. (2008). Advances in Targeting the Ras/Raf/MEK/Erk Mitogen-Activated Protein Kinase Cascade with MEK Inhibitors for Cancer Therapy. Clin. Cancer Res. *14*, 342-346.

Frodin,M. and Gammeltoft,S. (1999). Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction. Mol. Cell Endocrinol. *151*, 65-77.

Garcia-Barrio,M., Dong,J., Cherkasova,V.A., Zhang,X., Zhang,F., Ufano,S., Lai,R., Qin,J., and Hinnebusch,A.G. (2002). Serine 577 is phosphorylated and negatively affects the tRNA binding and eIF2alpha kinase activities of GCN2. J. Biol. Chem. *277*, 30675-30683.

Gazzola,R.F., Sala,R., Bussolati,O., Visigalli,R., Dall'Asta,V., Ganapathy,V., and Gazzola,G.C. (2001). The adaptive regulation of amino acid transport system A is associated to changes in ATA2 expression. FEBS Lett. *490*, 11-14.

Gietzen,D.W., Hao,S., and Anthony,T.G. (2007). Mechanisms of food intake repression in indispensable amino acid deficiency. Annu. Rev. Nutr. *27*, 63-78.

Greenbaum,L.E., Li,W., Cressman,D.E., Peng,Y., Ciliberto,G., Poli,V., and Taub,R. (1998). CCAAT enhancer-binding protein β is required for normal hepatocyte proliferation in mice after partial hepatectomy. J. Clin. Invest *102*, 996-1007.

Grimm,S.L., Contreras,A., Barcellos-Hoff,M.H., and Rosen,J.M. (2005). Cell cycle defects contribute to a block in hormone-induced mammary gland proliferation in CCAAT/enhancer-binding protein (C/EBPbeta)-null mice. J. Biol. Chem. *280*, 36301-36309.

Grimm,S.L. and Rosen,J.M. (2003). The role of C/EBPbeta in mammary gland development and breast cancer. J. Mammary. Gland. Biol. Neoplasia. *8*, 191-204.

Guerrini,L., Gong,S.S., Mangasarian,K., and Basilico,C. (1993). *Cis*- and *trans*-acting elements involved in amino acid regulation of asparagine synthetase gene expression. Mol. Cell. Biol. *13*, 3202-3212.

Guo,S., Cichy,S.B., He,X., Yang,Q., Ragland,M., Ghosh,A.K., Johnson,P.F., and Unterman,T.G. (2001). Insulin suppresses transactivation by CAAT/enhancer-binding proteins beta (C/EBPbeta). Signaling to p300/CREB-binding protein by protein kinase B disrupts interaction with the major activation domain of C/EBPbeta. J. Biol. Chem. *276*, 8516-8523.

Hai,T. and 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*, 3720-3724.

Hampsey,M. (1998). Molecular genetics of the RNA polymerase II general transcriptional machinery. Microbiol. Mol. Biol. Rev. *62*, 465-503.

Handlogten,M.E. and Kilberg,M.S. (1988). Growth-dependent regulation of System A in SV40-transformed fetal rat hepatocytes. Am. J. Physiol. *255*, C261-C270.

Hansen,B.S., Vaughan,M.H., and Wang,L.-J. (1972). Reversible inhibition by histidinol of protein synthesis in human cells at the activation of histidine. J. Biol. Chem. *247*, 3854-3857.

Hao,S., Sharp,J.W., Ross-Inta,C.M., McDaniel,B.J., Anthony,T.G., Wek,R.C., Cavener,D.R., McGrath,B.C., Rudell,J.B., Koehnle,T.J., and Gietzen,D.W. (2005). Uncharged tRNA and sensing of amino acid deficiency in mammalian piriform cortex. Science *307*, 1776-1778.

Harding,H.P., Calfon,M., Urano,F., Novoa,I., and Ron,D. (2002). Transcriptional and translational control in the Mammalian unfolded protein response. Annu. Rev. Cell Dev. Biol. *18*, 575-599.

Harding,H.P., Novoa,I., Zhang,Y., Zeng,H., Wek,R., Schapira,M., and Ron,D. (2000). Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol. Cell *6*, 1099-1108.

Harding,H.P., Zhang,Y., Zeng,H., Novoa,I., Lu,P.D., Calfon,M., Sadri,N., Yun,C., Popko,B., Paules,R., Stojdl,D.F., Bell,J.C., Hettmann,T., Leiden,J.M., and Ron,D. (2003). An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. Mol. Cell *11*, 619-633.

Hata,K., Nishimura,R., Ueda,M., Ikeda,F., Matsubara,T., Ichida,F., Hisada,K., Nokubi,T., Yamaguchi,A., and Yoneda,T. (2005). A CCAAT/enhancer binding protein beta isoform, liver-enriched inhibitory protein, regulates commitment of osteoblasts and adipocytes. Mol. Cell Biol. *25*, 1971-1979.

He,C.H., Gong,P., Hu,B., Stewart,D., Choi,M.E., Choi,A.M., and 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*, 20858-20865.

Hinnebusch,A.G. (2005). Translational regulation of GCN4 and the general amino acid control of yeast. Annu. Rev. Microbiol. *59*, 407-450.

Hinnebusch,A.G. (1997). Translational regulation of yeast GCN4. J. Biol. Chem. *272*, 21661-21664.

Hope,I.A. and Struhl,K. (1985). GCN4 protein, synthesized in vitro, binds HIS3 regulatory sequences: Implications for general control of amino acid biosynthetic genes in yeast. Cell *43*, 177-188.

Hope,I.A. and Struhl,K. (1987). GCN4, a eukaryotic transcriptional activator protein, binds as a dimer to target DNA. EMBO J. *6*, 2781-2784.

Hussain,S.G. and Ramaiah,K.V. (2007). Reduced eIF2alpha phosphorylation and increased proapoptotic proteins in aging. Biochem. Biophys. Res. Commun. *355*, 365-370.

Hutson,R.G. and Kilberg,M.S. (1994). Cloning of rat asparagine synthetase and specificity of the amino acid-dependent control of its mRNA content. Biochem. J. *303*, 745-750.

Hyde,R., Cwiklinski,E.L., Macaulay,K., Taylor,P.M., and Hundal,H.S. (2007). Distinct Sensor Pathways in the Hierarchical Control of SNAT2, a Putative Amino Acid Transceptor, by Amino Acid Availability. J. Biol. Chem. *282*, 19788-19798.

Hyde,R., Hajduch,E., Powell,D.J., Taylor,P.M., and Hundal,H.S. (2005). Ceramide down-regulates System A amino acid transport and protein synthesis in rat skeletal muscle cells. FASEB J. *19*, 461-463.

Hyde,R., Peyrollier,K., and Hundal,H.S. (2002). Insulin promotes the cell surface recruitment of the SAT2/ATA2 system A amino acid transporter from an endosomal compartment in skeletal muscle cells. J. Biol. Chem. *277*, 13628-13634.

Jager,P.L., Vaalburg,W., Pruim,J., de Vries,E.G., Langen,K.J., and Piers,D.A. (2001). Radiolabeled amino acids: basic aspects and clinical applications in oncology. J. Nucl. Med. *42*, 432-445.

Jessen,W.J., Hoose,S.A., Kilgore,J.A., and Kladde,M.P. (2006). Active PHO5 chromatin encompasses variable numbers of nucleosomes at individual promoters. Nat. Struct. Mol. Biol. *13*, 256-263.

Johnson,P.F. (1993). Identification of C/EBP basic region residues involved in DNA sequence recognition and half-site spacing preference. Mol. Cell Biol. *13*, 6919-6930.

Kashiwagi,H., Yamazaki,K., Takekuma,Y., Ganapathy,V., and Sugawara,M. (2008). Regulatory mechanisms of SNAT2, an amino acid transporter, in L6 rat skeletal muscle cells by insulin, osmotic shock and amino acid deprivation. Amino. Acids *In Press*.

Kilberg,M.S. (1986). System A-mediated amino acid transport: Metabolic control at the plasma membrane. Trends Biochem. Sci. *11*, 183-186.

Kilberg,M.S., Barber,E.F., and Handlogten,M.E. (1985). Characteristics and hormonal regulation of amino acid transport system A in isolated rat hepatocytes. Topics in Cell. Regulation *25*, 133-163.

Kilberg,M.S., Handlogten,M.E., and Christensen,H.N. (1980). Characteristics of an amino acid transport system in rat liver for glutamine, asparagine, histidine, and closely related analogs. J. Biol. Chem. *255*, 4011-4019.

Kilberg,M.S. and Neuhaus,O.W. (1977). Hormonal regulation of hepatic amino acid transport. J. Supramolecular Struct. *6*, 191-204.

Kilberg,M.S., Pan,Y.X., Chen,H., and Leung-Pineda,V. (2005). Nutritional Control of Gene Expression: How Mammalian Cells Respond to Amino Acid Limitation. Annu. Rev. Nutr. *25*, 59-85.

Kim,T.H., Barrera,L.O., Zheng,M., Qu,C., Singer,M.A., Richmond,T.A., Wu,Y., Green,R.D., and Ren,B. (2005). A high-resolution map of active promoters in the human genome. Nature *436*, 876-880.

Kim,Y.J., Bjorklund,S., Li,Y., Sayre,M.H., and Kornberg,R.D. (1994). A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. Cell *77*, 599-608.

Kimball,S.R. (2002). Regulation of global and specific mRNA translation by amino acids. J. Nutr. *132*, 883-886.

Kimball, S. R. Anthony T. G. Cavener D. R. and Jefferson L. S. Nutrient signaling through mammalian GCN2. [7], 113-129. 2005. Springer-Verlag. Topics in Current Genetics. Winderickx, J. and Taylor, P. M.
Ref Type: Serial (Book,Monograph)

Kiss-Toth,E., Bagstaff,S.M., Sung,H.Y., Jozsa,V., Dempsey,C., Caunt,J.C., Oxley,K.M., Wyllie,D.H., Polgar,T., Harte,M., O'neill,L.A., Qwarnstrom,E.E., and Dower,S.K. (2004).

Human tribbles, a protein family controlling mitogen-activated protein kinase cascades. J. Biol. Chem. *279*, 42703-42708.

Koehnle,T.J., Russell,M.C., Morin,A.S., Erecius,L.F., and Gietzen,D.W. (2004). Diets deficient in indispensable amino acids rapidly decrease the concentration of the limiting amino acid in the anterior piriform cortex of rats. J. Nutr. *134*, 2365-2371.

Kornberg,R.D. (1996). RNA polymerase II transcription control. Trends Biochem. Sci. *21*, 325-326.

Kornberg,R.D. (1999). Eukaryotic transcriptional control. Trends Cell Biol. *9*, M46-M49.

Kornberg,R.D. (2007). The molecular basis of eukaryotic transcription. Proc. Natl. Acad. Sci. U. S. A *104*, 12955-12961.

Kowenz-Leutz,E. and Leutz,A. (1999). A C/EBP beta isoform recruits the SWI/SNF complex to activate myeloid genes. Mol. Cell *4*, 735-743.

Lagrange,T., Kapanidis,A.N., Tang,H., Reinberg,D., and Ebright,R.H. (1998). New core promoter element in RNA polymerase II-dependent transcription: sequence-specific DNA binding by transcription factor IIB. Genes Dev. *12*, 34-44.

Lee,D. and Lis,J.T. (1998). Transcriptional activation independent of TFIIH kinase and the RNA polymerase II mediator in vivo. Nature *393*, 389-392.

Lee,S.J. and Kim,S.G. (2006). Role of p90 ribosomal S6-kinase-1 in oltipraz-induced specific phosphorylation of CCAAT/enhancer binding protein-beta for GSTA2 gene transactivation. Mol. Pharmacol. *69*, 385-396.

Lee,Y.C. and Kim,Y.J. (1998). Requirement for a functional interaction between mediator components Med6 and Srb4 in RNA polymerase II transcription. Mol. Cell Biol. *18*, 5364-5370.

Leung-Pineda,V., Pan,Y., Chen,H., and Kilberg,M.S. (2004). Induction of p21 and p27 Expression By Amino Acid Deprivation of HepG2 Human Hepatoma Cells Involves mRNA Stabilization. Biochem. J. *379*, 79-88.

Lewis,B.A. and Reinberg,D. (2006). Promoter activation when the ChIPs are down. Nat. Struct. Mol. Biol. *13*, 96-97.

Li,A.G., Piluso,L.G., Cai,X., Gadd,B.J., Ladurner,A.G., and Liu,X. (2007a). An acetylation switch in p53 mediates holo-TFIID recruitment. Mol. Cell *28*, 408-421.

Li,B., Carey,M., and Workman,J.L. (2007b). The role of chromatin during transcription. Cell *128*, 707-719.

Liang,S.H., Zhang,W., McGrath,B.C., Zhang,P., and Cavener,D.R. (2006). PERK (eIF2alpha kinase) is required to activate the stress-activated MAPKs and induce the expression of immediate-early genes upon disruption of ER calcium homoeostasis. Biochem. J. *393*, 201-209.

Linder,T., Zhu,X., Baraznenok,V., and Gustafsson,C.M. (2006). The classical srb4-138 mutant allele causes dissociation of yeast Mediator. Biochem. Biophys. Res. Commun. *349*, 948-953.

Ling,R., Bridges,C.C., Sugawara,M., Fujita,T., Leibach,F.H., Prasad,P.D., and Ganapathy,V. (2001). Involvement of transporter recruitment as well as gene expression in the substrate-induced adaptive regulation of amino acid transport system A. Biochim. Biophys. Acta *1512*, 15-21.

Lipson,K.E. and Baserga,R. (1989). Transcriptional activity of the human thymidine kinase gene determined by a method using the polymerase chain reaction and an intron-specific probe. Proc. Natl. Acad. Sci. U. S. A *86*, 9774-9777.

Lopez,A.B., Wang,C., Huang,C.C., Yaman,I., Li,Y., Chakravarty,K., Johnson,P.F., Chiang,C.M., Snider,M.D., Wek,R.C., and Hatzoglou,M. (2007). A feedback transcriptional mechanism controls the level of the arginine/lysine transporter cat-1 during amino acid starvation. Biochem. J. *402*, 163-173.

Lu,P.D., Harding,H.P., and Ron,D. (2004). Translation reinitiation at alternative open reading frames regulates gene expression in an integrated stress response. J. Cell Biol. *167*, 27-33.

Luedde,T., Duderstadt,M., Streetz,K.L., Tacke,F., Kubicka,S., Manns,M.P., and Trautwein,C. (2004). C/EBP beta isoforms LIP and LAP modulate progression of the cell cycle in the regenerating mouse liver. Hepatology *40*, 356-365.

Luger,K. (2006). Dynamic nucleosomes. Chromosome. Res. *14*, 5-16.

Mackenzie,B. and Erickson,J.D. (2004). Sodium-coupled neutral amino acid (System N/A) transporters of the SLC38 gene family. Pflugers Arch. *447*, 784-795.

Marchand,A., Tomkiewicz,C., Magne,L., Barouki,R., and Garlatti,M. (2006). Endoplasmic reticulum stress induction of insulin-like growth factor-binding protein-1 involves ATF4. J. Biol. Chem. *281*, 19124-19133.

Markwell,M.A.K., Haas,S.M., Bieber,L.L., and Tolbert,N.E. (1978). A Modification of the Lowry Procedure to Simplify Protein Determination in Membrane and Lipoprotein Samples. Anal. Biochem. *87*, 206-210.

Masuoka,H.C. and Townes,T.M. (2002). Targeted disruption of the activating transcription factor 4 gene results in severe fetal anemia in mice. Blood *99*, 736-745.

Matsukawa,T., Inoue,Y., Oishi,Y., Kato,H., and Noguchi,T. (2001). Up-regulation of upstream stimulatory factors by protein malnutrition and its possible role in regulation of the IGF-binding protein-1 gene. Endocrinology *142*, 4643-4651.

Merighi,S., Benini,A., Mirandola,P., Gessi,S., Varani,K., Leung,E., Maclennan,S., Baraldi,P.G., and Borea,P.A. (2006). Modulation of the Akt/Ras/Raf/MEK/ERK pathway by A(3) adenosine receptor. Purinergic. Signal. *2*, 627-632.

Mink,S., Haenig,B., and Klempnauer,K.H. (1997). Interaction and functional collaboration of p300 and C/EBPbeta. Mol. Cell Biol. *17*, 6609-6617.

Mizuno,Y., Sotomaru,Y., Katsuzawa,Y., Kono,T., Meguro,M., Oshimura,M., Kawai,J., Tomaru,Y., Kiyosawa,H., Nikaido,I., Amanuma,H., Hayashizaki,Y., and Okazaki,Y. (2002). Asb4, Ata3, and Dcn are novel imprinted genes identified by high-throughput screening using RIKEN cDNA microarray. Biochem. Biophys. Res. Commun. *290*, 1499-1505.

Naiki,T., Saijou,E., Miyaoka,Y., Sekine,K., and Miyajima,A. (2007). TRB2, a mouse Tribbles ortholog, suppresses adipocyte differentiation by inhibiting AKT and C/EBPbeta. J. Biol. Chem. *282*, 24075-24082.

Natarajan,K., Meyer,M.R., Jackson,B.M., Slade,D., Roberts,C., Hinnebusch,A.G., and Marton,M.J. (2001). Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast. Mol. Cell Biol. *21*, 4347-4368.

Novoa,I., Zeng,H., Harding,H.P., and Ron,D. (2001). Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2α. J. Cell Biol *153*, 1011-1021.

Novoa,I., Zhang,Y., Zeng,H., Jungreis,R., Harding,H.P., and Ron,D. (2003). Stress-induced gene expression requires programmed recovery from translational repression. EMBO J. *22*, 1180-1187.

Ossipow,V., Descombes,P., and Schibler,U. (1993). CCAAT/enhancer-binding protein mRNA is translated into multiple proteins with different transcription activation potentials. Proc. Natl. Acad. Sci. U. S. A *90*, 8219-8223.

Oxender,D.L. and Christensen,H.N. (1963). Distinct Mediating Systems for the Transport of Neutral Amino Acids by the Ehrlich Cell. J. Biol. Chem. *238*, 3686-3699.

Palii,S.S., Chen,H., and Kilberg,M.S. (2004). Transcriptional control of the human sodium-coupled neutral amino acid transporter system A gene by amino acid availability is mediated by an intronic element. J. Biol. Chem. *279*, 3463-3471.

Palii,S.S., Thiaville,M.M., Pan,Y.X., Zhong,C., and Kilberg,M.S. (2006). Characterization of the amino acid response element within the human SNAT2 system A transporter gene. Biochem. J. *395*, 517-527.

Pan,Y.-X., Chen,H., Siu,F., and Kilberg,M.S. (2003). Amino acid deprivation and endoplasmic reticulum stress induce expression of multiple ATF3 mRNA species which, when overexpressed in HepG2 cells, modulate transcription by the human asparagine synthetase promoter. J. Biol. Chem. *278*, 38402-38412.

Pan,Y.X., Chen,H., Thiaville,M.M., and Kilberg,M.S. (2007). Activation of the ATF3 gene through a co-ordinated amino acid-sensing response programme that controls transcriptional regulation of responsive genes following amino acid limitation. Biochem. J. *401*, 299-307.

Park,J.M., Kim,H.S., Han,S.J., Hwang,M.S., Lee,Y.C., and Kim,Y.J. (2000). In vivo requirement of activator-specific binding targets of mediator. Mol. Cell Biol. *20*, 8709-8719.

Pattingre,S., Bauvy,C., and Codogno,P. (2003). Amino acids interfere with the ERK1/2-dependent control of macroautophagy by controlling the activation of Raf-1 in human colon cancer HT-29 cells. J. Biol. Chem. *278*, 16667-16674.

Pearson,G., Robinson,F., Beers,G.T., Xu,B.E., Karandikar,M., Berman,K., and Cobb,M.H. (2001). Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr. Rev. *22*, 153-183.

Pedersen,T.A., Kowenz-Leutz,E., Leutz,A., and Nerlov,C. (2001). Cooperation between C/EBPalpha TBP/TFIIB and SWI/SNF recruiting domains is required for adipocyte differentiation. Genes Dev. *15*, 3208-3216.

Peterson,C.L. and Laniel,M.A. (2004). Histones and histone modifications. Curr. Biol. *14*, R546-R551.

Pilipuk,G.P., Galigniana,M.D., and Schwartz,J. (2003). Subnuclear Localization of C/EBP{beta} Is Regulated by Growth Hormone and Dependent on MAPK. J. Biol. Chem. *278*, 35668-35677.

Piwien-Pilipuk,G., Van Mater,D., Ross,S.E., MacDougald,O.A., and Schwartz,J. (2001). Growth Hormone Regulates Phosphorylation and Function of CCAAT/Enhancer-binding Protein beta by Modulating Akt and Glycogen Synthase Kinase-3. J. Biol. Chem. *276*, 19664-19671.

Piwien-Pilipuk,G., MacDougald,O., and 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*, 44557-44565.

Rahman,S.M., Schroeder-Gloeckler,J.M., Janssen,R.C., Jiang,H., Qadri,I., Maclean,K.N., and Friedman,J.E. (2007). CCAAT/enhancing binding protein beta deletion in mice attenuates inflammation, endoplasmic reticulum stress, and lipid accumulation in diet-induced nonalcoholic steatohepatitis. Hepatology *45*, 1108-1117.

Ramji,D.P. and Foka,P. (2002). CCAAT/enhancer-binding proteins: structure, function and regulation. Biochem. J. *365*, 561-575.

Richards,N.G. and Kilberg,M.S. (2006). Asparagine synthetase chemotherapy. Annu. Rev. Biochem. *75*, 629-654.

Roeder,R.G. (1996). The role of general initiation factors in transcription by RNA polymerase II. Trends Biochem. Sci. *21*, 327-335.

Roybal,C.N., Hunsaker,L.A., Barbash,O., Jagt,D.L., and Abcouwer,S.F. (2005). The Oxidative Stressor Arsenite Activates Vascular Endothelial Growth Factor mRNA Transcription by an ATF4-dependent Mechanism. J. Biol. Chem. *280*, 20331-20339.

Sato,H., Nomura,S., Maebara,K., Sato,K., Tamba,M., and Bannai,S. (2004a). Transcriptional control of cystine/glutamate transporter gene by amino acid deprivation. Biochem. Biophys. Res. Commun. *325*, 109-116.

Sato,S., Tomomori-Sato,C., Parmely,T.J., Florens,L., Zybailov,B., Swanson,S.K., Banks,C.A., Jin,J., Cai,Y., Washburn,M.P., Conaway,J.W., and Conaway,R.C. (2004b). A set of consensus mammalian mediator subunits identified by multidimensional protein identification technology. Mol. Cell *14*, 685-691.

Schwarzer,R., Dames,S., Tondera,D., Klippel,A., and Kaufmann,J. (2006). TRB3 is a PI 3-kinase dependent indicator for nutrient starvation. Cell Signal. *18*, 899-909.

Sebolt-Leopold,J.S. and Herrera,R. (2004). Targeting the mitogen-activated protein kinase cascade to treat cancer. Nat. Rev. Cancer *4*, 937-947.

Sharp,J.W., Ross-Inta,C.M., Hao,S., Rudell,J.B., and Gietzen,D.W. (2006). Co-localization of phosphorylated extracellular signal-regulated protein kinases 1/2 (ERK1/2) and phosphorylated eukaryotic initiation factor 2alpha (eIF2alpha) in response to a threonine-devoid diet. J. Comp Neurol. *494*, 485-494.

Shaul,Y.D. and Seger,R. (2007). The MEK/ERK cascade: from signaling specificity to diverse functions. Biochim. Biophys. Acta *1773*, 1213-1226.

Siu,F., Bain,P.J., LeBlanc-Chaffin,R., Chen,H., and Kilberg,M.S. (2002). ATF4 Is a Mediator of the Nutrient-sensing Response Pathway That Activates the Human Asparagine Synthetase Gene. J. Biol. Chem. *277*, 24120-24127.

Siu,F.Y., Chen,C., Zhong,C., and Kilberg,M.S. (2001). CCAAT/enhancer-binding protein beta (C/EBPb) is a mediator of the nutrient sensing response pathway that activates the human asparagine synthetase gene. J. Biol. Chem. *276*, 48100-48107.

Smith,R.J., Dean,W., Konfortova,G., and Kelsey,G. (2003). Identification of novel imprinted genes in a genome-wide screen for maternal methylation. Genome Res. *13*, 558-569.

Sood,R., Porter,A.C., Olsen,D.A., Cavener,D.R., and Wek,R.C. (2000). A mammalian homologue of GCN2 protein kinase important for translational control by phosphorylation of eukaryotic initiation factor-2α. Genetics *154*, 787-801.

Sugawara,M., Nakanishi,T., Fei,Y.J., Huang,W., Ganapathy,M.E., Leibach,F.H., and Ganapathy,V. (2000). Cloning of an amino acid transporter with functional characteristics and tissue expression pattern identical to that of system A. J. Biol. Chem. *275*, 16473-16477.

Sutinen,E., Jyrkkio,S., Alanen,K., Nagren,K., and Minn,H. (2003). Uptake of [N-methyl-11C]alpha-methylaminoisobutyric acid in untreated head and neck cancer studied by PET. Eur. J. Nucl. Med. Mol. Imaging *30*, 72-77.

Sutinen,E., Jyrkkio,S., Gronroos,T., Haaparanta,M., Lehikoinen,P., and Nagren,K. (2001). Biodistribution of [11C] methylaminoisobutyric acid, a tracer for PET studies on system A amino acid transport in vivo. Eur. J. Nucl. Med. *28*, 847-854.

Takagi,Y. and Kornberg,R.D. (2006). Mediator as a general transcription factor. J. Biol. Chem. *281*, 80-89.

Tang,Q.Q., Gronborg,M., Huang,H., Kim,J.W., Otto,T.C., Pandey,A., and Lane,M.D. (2005). Sequential phosphorylation of CCAAT enhancer-binding protein beta by MAPK and glycogen synthase kinase 3beta is required for adipogenesis. Proc. Natl. Acad. Sci. U. S. A *102*, 9766-9771.

Thiaville,M.M., Dudenhausen,E.E., Zhong,C., Pan,Y.X., and Kilberg,M.S. (2008a). Deprivation of protein or amino acid induces C/EBPbeta synthesis and binding to amino acid response elements, but its action is not an absolute requirement for enhanced transcription. Biochem. J. *410*, 473-484.

Thiaville,M.M., Pan,Y.X., Gjymishka,A., Zhong,C., Kaufman,R.J., and Kilberg,M.S. (2008b). MEK Signaling Is Required for Phosphorylation of eIF2{alpha} following Amino Acid Limitation of HepG2 Human Hepatoma Cells. J. Biol. Chem. *283*, 10848-10857.

Trinklein,N.D., Aldred,S.J., Saldanha,A.J., and Myers,R.M. (2003). Identification and functional analysis of human transcriptional promoters. Genome Res. *13*, 308-312.

Uehara,H., Miyagawa,T., Tjuvajev,J., Joshi,R., Beattie,B., Oku,T., Finn,R., and Blasberg,R. (1997). Imaging experimental brain tumors with 1-aminocyclopentane carboxylic acid and alpha-aminoisobutyric acid: comparison to fluorodeoxyglucose and diethylenetriaminepentaacetic acid in morphologically defined tumor regions. J. Cereb. Blood Flow Metab *17*, 1239-1253.

Vattem,K.M. and Wek,R.C. (2004). Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. Proc. Natl. Acad. Sci. U. S. A *101*, 11269-11274.

Walker,P.R. and Whitfield,J.F. (1978). Inhibition by colchicine of changes in amino acid transport and initiation of DNA synthesis in regenerating rat liver. Proc. Natl. Acad. Sci. U. S. A *75*, 1394-1398.

Wek,R.C. and Cavener,D.R. (2007). Translational control and the unfolded protein response. Antioxid. Redox. Signal. *9*, 2357-2371.

Wolfgang,C.D., Chen,B.P., Martindale,J.L., Holbrook,N.J., and Hai,T. (1997). gadd153/Chop10, a potential target gene of the transcriptional repressor ATF3. Mol. Cell Biol. *17*, 6700-6707.

Xu,R., Spencer,V.A., and Bissell,M.J. (2007). Extracellular matrix-regulated gene expression requires cooperation of SWI/SNF and transcription factors. J. Biol. Chem. *282*, 14992-14999.

Yang,X., Matsuda,K., Bialek,P., Jacquot,S., Masuoka,H.C., Schinke,T., Li,L., Brancorsini,S., Sassone-Corsi,P., Townes,T.M., Hanauer,A., and Karsenty,G. (2004). ATF4 is a substrate of

RSK2 and an essential regulator of osteoblast biology; implication for Coffin-Lowry Syndrome. Cell *117*, 387-398.

Yao,D., Mackenzie,B., Ming,H., Varoqui,H., Zhu,H., Hediger,M.A., and Erickson,J.D. (2000). A novel system A isoform mediating Na+/neutral amino acid cotransport. J. Biol. Chem. *275*, 22790-22797.

Yu,S., Jiang,Y., Galson,D.L., Luo,M., Lai,Y., Lu,Y., Ouyang,H.J., Zhang,J., and Xiao,G. (2008). General transcription factor IIA-gamma increases osteoblast-specific osteocalcin gene expression via activating transcription factor 4 and runt-related transcription factor 2. J. Biol. Chem. *283*, 5542-5553.

Zhang,F., Sumibcay,L., Hinnebusch,A.G., and Swanson,M.J. (2004). A triad of subunits from the Gal11/tail domain of Srb mediator is an in vivo target of transcriptional activator Gcn4p. Mol. Cell Biol. *24*, 6871-6886.

Zhang,X., Krutchinsky,A., Fukuda,A., Chen,W., Yamamura,S., Chait,B.T., and Roeder,R.G. (2005). MED1/TRAP220 exists predominantly in a TRAP/ Mediator subpopulation enriched in RNA polymerase II and is required for ER-mediated transcription. Mol. Cell *19*, 89-100.

Zykova,T., Zhu,F., Zhang,Y., Bode,A.M., and Dong,Z. (2007). Involvement of ERKs, RSK2, and PKR in UVA-induced signal transduction toward phosphorylation of eIF2{alpha} (Ser51). Carcinogenesis *28*, 1543-1551.

BIOGRAPHICAL SKETCH

Michelle M. Magee was born in 1981 in the city of Baton Rouge, Louisiana, as the first child to parents Ray and Diane Magee. In 1999, she graduated as the salutatorian of her high school class, and enrolled as an undergraduate student at Centenary College of Louisiana in Shreveport. She graduated with a Bachelor of Science degree in 2003 under the distinction of magna cum laude after completion of a major in biochemistry (with honors) and a minor in English. In August of 2003, she enrolled as a graduate student in the interdisciplinary program in biomedical sciences at the University of Florida. Later that December, she married Patrick Thiaville to become Mrs. Michelle M. Thiaville. She joined the laboratory of Dr. Michael Kilberg in May 2004 and became an official PhD candidate in October of 2005. She received her Ph.D. in medical sciences-biochemistry and molecular biology from the University of Florida in 2008.