CELLULAR AND ANIMAL METABOLIC RESPONSES TO INDISPENSABLE AMINO ACID LIMITATION

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Angelos Sikalidis

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CELLULAR AND ANIMAL METABOLIC RESPONSES TO INDISPENSABLE AMINO ACID LIMITATION

Angelos Sikalidis, Ph.D.

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The current thesis was guided by three principal research objectives. The first objective was to identify target genes responsive to indispensable amino acid deprivation with the goal of identifying potential components of the Integrated Stress Response as well as possibly identifying some genes that are differentially expressed in response to amino acid deprivation but not other stress conditions. The second objective was to determine whether the previously identified targets and eIF2α-kinase-mediated responses would be induced in animals fed a diet that was marginal in essential amino acid composition. The third objective was to elucidate the role of elevated 4E-BP1 expression in regulating translation initiation during nutrient deficiency.

To assess the role of amino acid deprivation on eIF2α phosphorylation and downstream transcriptional responses, we conducted additional microarray studies for leucine-depleted HepG2/C3A cells and compared these with results for cysteine-depleted cells. The comparison of cells exposed to deficiencies of two different amino acids was done to facilitate selection of genes whose expression is more likely to be altered specifically in response to GCN2-induced eIF2α phosphorylation. In addition, we assessed the effects of both cysteine and leucine deprivation on the phosphorylation of eIF2α and on the protein expression patterns of ATF4 and other ISR-related proteins. We have compiled a list of 120 genes whose expression was

differentially expressed in HepG2/C3A cells cultured in either cysteine- or leucine-deficient medium, and this list contains many of the genes known to respond to eIF2α kinase activation and to be components of the ISR. Clearly, amino acid deficiency, including cysteine deficiency in the absence of oxidative stress, induces eIF2α phosphorylation, presumably by activation of GCN2, leading to changes in expression of ATF4 and stress-related target genes.

To answer whether eIF2α-kinase–mediated responses would be induced in animals fed a sulfur amino acid (SAA) deficient diet not as imbalanced as one in which a single essential amino acid is totally absent, we fed rats soy protein-based diets that were either adequate or limiting in SAA. Rats fed a SAA-deficient diet grew more slowly than rats fed the control diet. Analysis of liver from rats fed these diets for 7 days showed that the SAA-deficient rats had higher levels of eIF2α phosphorylation and higher levels of activating transcription factor (ATF) 4, ATF3, asparagine synthetase, solute carrier 7A11, cysteinyl-tRNA synthetase, and cystathionine γ-lyase. On the other hand, components of the integrated stress response (ISR) known to promote apoptosis or translational recovery were not induced. These results indicate that rats fed the SAA-deficient diet had a prolonged activation of an eIF2α kinase that leads to upregulation of adaptive components of the ISR.

To assess the role of elevated 4E-BP1 expression in regulating translation initiation during nutrient deficiency, we fed rats various protein/amino acid-deficient diets with a varying degree of deficiency and achieved amino acid deficiency and reduction of growth. In these experiments we consistently observed a significant induction of total 4E-BP1 protein levels that did not parallel a reduction in 4E-BP1 phosphorylation and was independent of energy restriction or feed intake. Further, the induction of total 4E-BP1 appeared independent of eIF2α phosphorylation.

BIOGRAPHICAL SKETCH

Angelos was born on December 13th, 1979 in Thessaloniki, Greece, to Konstantinos a chemist and Eirini a chemist and a medical doctor. A son of two educators, he spent a significant amount of time reading books on a variety of non-science topics, mostly from the fields of literature, ethics, history, politics and humanities in general. He initially planned to become a lawyer but, since in life things take longer than expected and rarely go as planned, he chose to enter the physical sciences track while a lyceum student as he felt that this kind of training fitted his personality and way of thinking better. As an undergraduate, Angelos joined the School of Agriculture of the Aristotle University of Thessaloniki and graduated after attending a 5-year program with a degree in Food Science and Technology in 2003. During his undergraduate tenure Angelos became increasingly more interested in Nutrition and decided to pursue graduate studies in this field thinking that "…if the societal request is for food industries to contribute to public health in addition to the elimination of hunger, food scientists should become nutritionists and vice-versa", a view thought by many to be rather romantic and naive. At the University of California, Berkeley as a Master's student, Angelos worked on the mechanisms by which obesity contributes to the risk of colonic cancer in mouse models and graduated with a Master of Science in 2006. He decided to continue with a Ph.D. as a means to broaden his knowledge and understanding in metabolism and with the aspiration of becoming able to contribute to the growth of the Nutritional Sciences academic field back in his homeland where this field is in a more preliminary state. However, as things in life take longer than expected and rarely go as planned whether this path will be walked or not remains to be seen. Regardless of what the future holds, he is glad to have had his Ph.D. experience as it has been a remarkably educational one at various levels, as well as very self-revealing.

I would like to dedicate this piece of work to my family and all my teachers, mentors and friends who have helped me develop as a person and a scientist.

Αγαπητοί μου γονείς, ελπίζω και εύχομαι να μπορέσω να δώσω στα παιδιά μου ό,τι εσείς δώσατε σε μένα...

"My beloved parents, I hope and wish to be able to provide to my children what you have given to me…"

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I would also like to thank my parents Konstantinos and Eirini and my brother Alexandros for their continued support and faith in me throughout my efforts in graduate school all these years that I have been studying in the USA.

Other individuals who have helped me maintain perspective of things and a healthy and positive outlook of life even at difficult times during my graduate school experience, whom I'd like to thank from the bottom of my heart, are:

The "older brother" I never had, Prof. Dr. Peter Diamessis (to friends, "Pete"). His wise and thoughtful observations and experience sharing regarding life and grad school in particular, always constituted a beacon of optimism even in the darkest seas and skies of my grad school years.

The legendary Olin Café manager Mr. Gregory Halkiopoulos (to friends, "Mr. Greg"), who with his amazing and unparalleled sense of humor, with the necessary dose of sarcasm, kept reminding me that there was/is a whole wide other world waiting for me outside of the "Grad school bubble".

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keeping in touch and always encouraging me to do more and reach higher while treasuring a loyal and honest friendship towards me. A great “thank you” to each and every-one of you for accompanying me in this journey, each in his own unique way. I wouldn’t have made it without you!

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LIST OF ABBREVIATIONS

AARE: Amino Acid Response Element

ASNS: Asparagine synthetase

ATF4: Activating Transcription Factor 4

CARS: Cysteinyl-tRNA synthetase

CBX4: Chromobox homolog 4

C/EBP: CCAAT-enhancer binding protein

CHOP: C/EBP-homologous protein

CTH: Cystathionase gamma-lyase

eIF2: eukaryotic Initiation Factor 2 heterotrimeric complex

GADD34: Growth Arrest and DNA Damage inducible protein 34 complex

GCN2: General Control Nonderepressible kinase 4, also known as mammalian eIF2α kinase 4

HSP5A: Heat Shock Protein 5A

ISR: Integrated Stress Response

mTOR: mammalian Target Of Rapamycing

OPA: o-Phthalaldehyde

ORF: Open Reading Frame

SAA: Sulfur Amino Acid

SARS: Seryl-tRNA synthetase

SDS-PAGE: Sodium dodecylsulfate-polyacrylamide gel electrophoresis

SLC: Solute carrier

TRIB3: Tribbles homolog 3

xCT: Transporter subunit of the cystine-glutamate exchanger X_{c-}

4E-BP1: eIF4E binding protein 1

CHAPTER 1

AN INTRODUCTION

Cellular growth repression can mediate positive health outcomes and ameliorate the manifestation, while decelerating the progression, of chronic diseases. Protein synthesis and growth regulation are known to be regulated by essential amino acids through a variety of pathways. The lack of amino acids leads to suppression of global protein synthesis. There are two major systems that sense and respond to amino acid limitation by reducing protein synthesis, the GCN2 system and the mTOR system.

Amino acid availability is sensed via GCN2 (General Control Nonderepressible kinase 2, also known as eukaryotic Initiation Factor 2α kinase 4), which functions by sensing the increase of non-aminoacylated tRNAs that occurs when essential amino acids are limiting. Sensing of "uncharged" tRNAs activates GCN2 which phosphorylates eIF2α which in turn leads to reduction of global translation due to inhibition of ternary complex formation and subsequent suppression of translation initiation. Another way translation initiation is regulated is through the action of eukaryotic Initiation Factor 4E – Binding Protein 1 (4E-BP1). When amino acids, glucose or energy substrates are limiting, there is less phosphorylation of 4E-BP1, rendering the protein available to competitively bind to eIF4E displacing eIF4G and subsequently suppressing translation initiation.

In addition to reduction of global translation, the eIF2α phosphorylation response in particular regulates expression of a variety of genes that are involved in cellular repair and defense. For this reason, the response is considered beneficial. Other stresses can activate other eIF2α kinases that in turn phosphorylate eIF2α

leading to outcomes /responses that are similar to those induced by amino acid deprivation (Figure 1.1). The downstream response of eIF2α phosphorylation is termed the Integrated Stress Response (ISR) and is associated with cell survival and stress resistance since it leads to the selective upregulation of genes that promote adaptation to stress and survival.

Thus, a modest restriction of protein or essential amino acid intake may, via the ISR pathway activation, extend a variety of beneficial physiological effects, such as slowing the aging process and/or preventing age-related diseases (e.g., coronary heart disease, cancer, and type II diabetes mellitus) (Salminen and Kaarniranta, 2010). Other beneficial effects could include the promotion of hormesis prior to a known stress (e.g., treatment with a toxic drug); inhibition of osteoclast formation and activity and thus suppression of bone resorption (osteoporosis); and suppression of hypertrophic/hyperplastic conditions (e.g., cancer) (Calabrese et al., 2010; Rattan SI, 2008). Consequently, understanding the relationship between amino acid intake and the regulation of the ISR could reveal health strategies for manipulating ISR in a way that would improve health outcomes pertinent to the aforementioned health conditions.

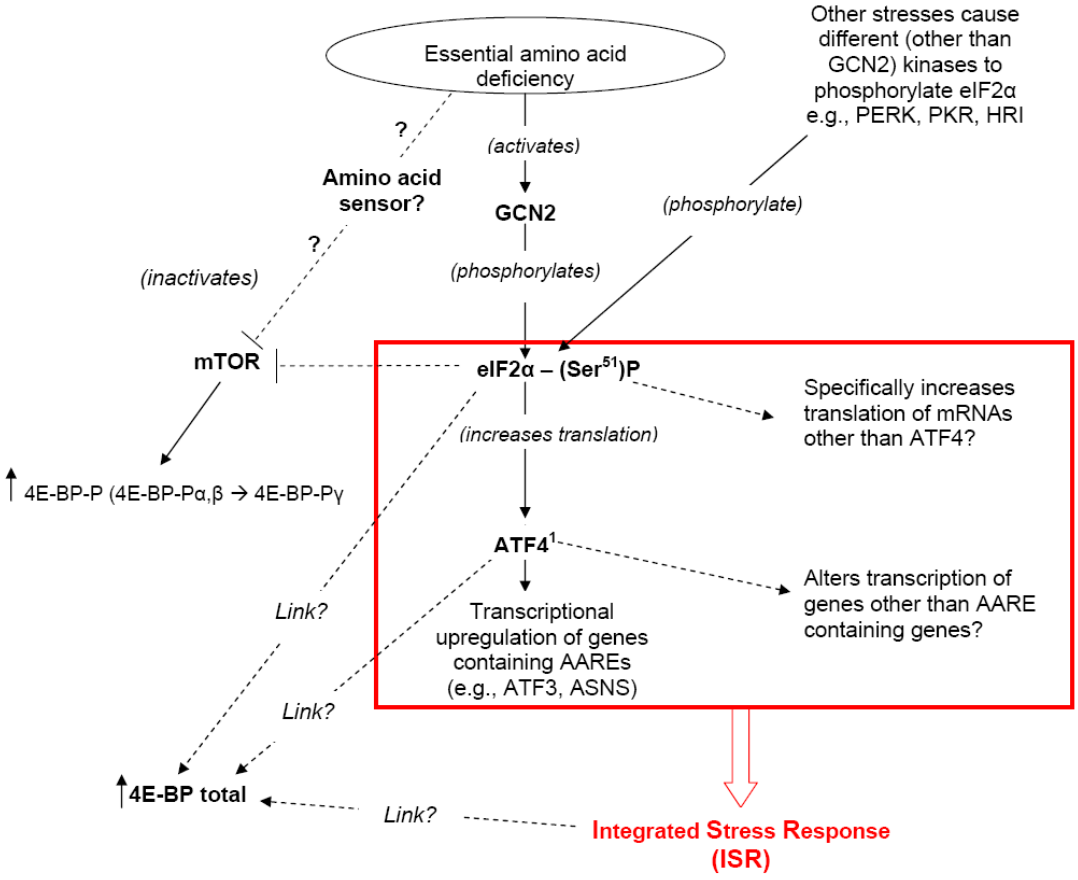

Figure 1.1. The diagram shows a potential link between eIF2α phosphorylation and the levels of 4E-BP. A potential link between Activating Transcription Factor 4 (ATF4) and the levels of 4E-BP is also illustrated. 4E-BP mRNA levels have been shown increased when eIF2α is phosphorylated (Lu et al., 2004) suggesting a potential link between the ISR and the induction of 4E-BP. Another potential link depicted in the diagram is the one between eIF2α phosphorylation and the mammalian Target of Rapamycin (mTOR) pathway. Finally, as seen in the diagram, the ISR is eIF2α phosphorylation-dependent but not all components of the ISR are necessarily ATF4 or Amino Acid Response Elements (AARE)-dependent.

[1] Phosphoryation of eIF2α leads to the global reduction of translation of most mRNAs. However, the translation of a few specific mRNAs with inhibiting uORFs or other structural features is actually increased. ATF4 is the best-understood example.

Overall, exposure to stress (as long as severe cell injury does not occur) has been considered a beneficial strategy in building resistance and tolerance towards more severe stressful conditions that may arise in the future (preconditioning) (Lu et al., 2004; Harding et al., 2000). Pathways, such as the ISR, that couple diverse stressful conditions to a common signaling event (such as eIF2α phosphorylation) leading to a common downstream program are likely candidates for playing a role in preconditioning. This postulation holding a key role for eIF2α and its phosphorylation has been supported by a variety of *in vitro* (Lu et al., 2004; Harding et al., 2000) and *in vivo* (Raffaghello et al., 2008) studies. The evidence that subjecting an organism to a mild stress facilitates the organism's ability to subsequently withstand a second more severe or different stress has important clinical implications. For example, short-term starvation has been associated with protection of mice against a high dose of the chemotherapy drug/pro-oxidant cyclophosphamide (Raffaghello et al., 2008). Induction of the ISR may be particularly useful in circumstances where acute tissue injury by Reactive Oxygen Species (ROS) can be anticipated, such as in organ procurement for transplantation and vascular surgery, and in circumstances where potentially cytotoxic drugs are administered.

Caloric restriction (or dietary restriction), defined as reduced dietary intake without malnutrition, has been proposed to be a mild stress with hormetic effects (Masoro EJ, 2007; Masoro EJ, 1998). Dietary restriction has been long shown to drastically improve health parameters and increase longevity in a variety of species including worms (*Caenorhabditis elegans*), flies (*Drosophila melanogaster*), yeast (*Saccharomyces cerevisiae*), rats (McCay et al., 1939; McCay et al., 1935) and mice (McCarty et al., 2008; Martin et al., 2007). Although the data for humans are not direct it is likely that the basic biological mechanisms via which caloric restriction exerts its beneficial effects are conserved in humans. Even though the exact

mechanism via which caloric restriction induces health benefits is not clear it appears that in general, when caloric restriction is applied, cells turn to a maintenance/repair phase as opposed to a growth one. Whether the benefit of caloric restriction should be attributed to the restriction of energy (calories) per se or to the restriction of protein, which often occurs when caloric restriction regiments are applied, is not fully resolved. Some studies have tried to disentangle these two arms and it seems that at least part of the beneficial response of caloric restriction is due to protein restriction (Lee et al., 2008; Yamaguchi et al., 2008; Sanz et al., 2006; Zimmerman et al., 2003; Ooka et al., 1988; De Marte and Enesco, 1986). The evidence for a role of amino acid or protein deficiency in extending lifespan in mice and fruit flies suggest that GCN2 plays a role in this process by mediating eIF2α phosphorylation, leading both to depression of global protein synthesis/growth and to activation of the downstream ISR.

The long-term overall objective of this work was to increase understanding of the protein and essential amino acid contribution to the regulation of protein translation and the induction of the ISR by investigating cellular and animal responses to indispensable amino acid deficiency. In this context we aimed to assess the effect of dietary protein and essential amino acid adequacy on induction of the ISR and the expression/regulation of proteins involved in translation inhibition in the liver. In addition, we investigated whether dietary restriction has a similar or different effect than protein/essential amino acid restriction on the induction of the ISR and the expression/regulation of proteins involved in translation inhibition in the liver of rats. Additionally, we attempted to determine whether eIF2α phosphorylation by GCN2 is essential for the induction of 4E-BP1. Further, we investigated the time-course for the induction of the ISR and the expression/regulation of proteins involved in translation

inhibition in the liver of rats when they are switched from an adequate diet to one limiting in protein and/or essential amino acids.

CHAPTER 2

LITERATURE REVIEW

Significance of indispensable amino acids in optimizing health and survival

A question fundamental in biology is how cells sense and respond to changes in their nutrient supply. The ability to synthesize protein and other nitrogenous compounds is essential for growth, maintenance, healing, and survival. Of the 20 common amino acids required for protein synthesis, nine are considered indispensable (Leu, Ile, Val, Lys, Thr, Trp, Phe, Met and His) for humans and rats, while cysteine and tyrosine are considered semi-indispensable because they can be synthesized if intake of their indispensable amino acid precursors (Met and Phe respectively) is adequate, via transulfuration and transamination reactions, respectively. A continuous supply of all amino acids is necessary for protein synthesis to proceed at a maximal rate. Insufficient intake of protein or of indispensable amino acids may occur in individuals with restricted dietary intakes due to choice, illness, or low caloric expenditure/intake as well as in individuals consuming diets consisting of foods that provide low amounts of protein or a protein mixture deficient in one or more indispensable amino acid. Because excess amino acids are not stored in the body, tissues tend to respond quickly to amino acid limitation by reducing their amino acid/protein catabolism, slowing rates of protein synthesis, and upregulating amino acid transporters. In general, scientific advisory committees (e.g., Institute of Medicine/National Academies; USDA) have recommended the intake of diets that provide protein and essential amino acids at levels well above the average requirement in order to provide a safety allowance for all age groups except infants for whom human milk is used as the standard. On the other hand, intake of largely plant-based

diets has been associated with health in industrialized and industrializing populations, and these plant-based diets typically provide lower intakes of protein and essential amino acids (Milward and Jackson, 2003). Although not fully explored, it is possible that some health benefits of plant-based diets may relate to the content and composition of proteins in these diets. The consequences of marginal intakes of protein on overall health have not yet been fully assessed, but these could relate both to lower rates of protein synthesis and growth and to generation of a physiological state of increased stress-resistance.

How mammalian cells respond to changes in the availability of essential amino acids has been a major interest of research in the past decade, particularly in the context of the regulation of nutrient sensing by the mammalian target of rapamycin (mTOR) pathway and of cellular sensing of essential amino acid levels by GCN2 [general control nonderepressible 2, or eukaryotic initiation factor (eIF) 2 alpha kinase 4]. Amino acids and energy are necessary for synthesis of proteins, which are needed for cells to grow and proliferate. When fuels or amino acids become limiting, protein production needs to be carefully regulated, both in terms of total protein synthesis as well as which specific proteins are synthesized, so that cells can optimally utilize their limited resources to survive.

A greater capacity of an organism to adapt to and cope with stress in order to defend homeostasis constitutes a critical advantage and can determine survival over death. In this context, the study of stress response mechanisms is of great interest in terms of possible applications to health promotion and disease prevention or treatment. Nutrient deprivation and other situations of stress require a rapid response as an early line of defense against the stressful environment that ultimately allows organisms to adapt to their changing environment by controlling cell growth, proliferation, and optimize survival.

How amino acid availability regulates translation initiation

The regulation of protein synthesis in mammalian tissues has been demonstrated to occur largely at the translation initiation phase (i.e., at steps involved in the formation of a ribosome binding site/translation start site, rather than in the elongation phase). Regulation occurs at two parts of the translation initiation process: (1) formation of a functional eIF4F complex at the 5′-cap and (2) formation of ternary complex (Met-$tRNA_i^{Met}$ · eIF2 – GTP) needed to form the 43S pre-initiation complex containing the 40S ribosomal subunit (Wek et al., 2006; Tettweiler et al., 2005; Hinnebusch, 2000).

The eIF4F complex comprises the 5′-cap-binding protein eIF4E that binds the m^7GTP cap present at the 5′-end (cap structure) of the mature mRNA, the RNA helicase eIF4A, and the scaffolding protein eIF4G (Gabauer, 2004; Prévôt et al., 2003). This eIF4F complex recruits the 43S pre-initiation complex. The 43S pre-initiation complex is the result of the association of the ternary complex (Met-$tRNA_i^{Met}$ · eIF2 – GTP) with the 40S ribosomal subunit and a variety of other initiator factors (eIF1A, eIF3). The recruitment of the 43S pre-initiation complex to eIF4F yields a translation-competent 48S complex, which begins to scan for the AUG start codon to initiate translation. Stable binding of the 48S complex to the AUG codon triggers the joining of the 60S ribosomal subunit, which, along with the hydrolysis of eIF2-bound GTP and subsequent release of eIF2-GDP, forms the 80S complex (Figure 2.1). To begin another round of initiation, eIF2-GDP is recycled back to eIF2-GTP (eIF2-GTP regeneration) via a reaction catalyzed by eIF2B, the guanine nucleotide exchange factor for eIF2 (Kubica et al., 2006). The ability of eIF4E to bind eIF4G to form a functional eIF4F complex has been shown to be regulated by the mTOR pathway (Avruch et al., 2009; Schmelzle, 2000), while the regeneration of eIF2-GTP

for the formation of ternary complex has been shown to be regulated by stress-activated eIF2α kinases, including GCN2 (Wek et al., 2006; Holcik et al., 2005).

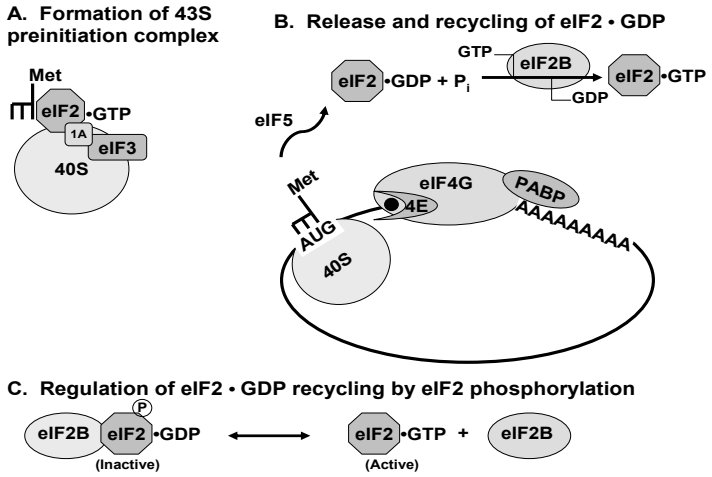

Figure 2.1. Formation of the 43S pre-initiation complex and its regulation by phosphorylation of eIF2's alpha subunit by an eIF2α kinase (e.g., GCN2).

The mammalian target of rapamycin (mTOR) pathway regulates translation initiation through phosphorylation of eIF4E binding protein (4E-BP). eIF4E-binding proteins (4E-BPs) are a family of three small (10-12 kDa) acidic proteins that act as repressors of translation initiation (Gingras et al., 1999). The three members of the 4E-BP family, 4E-BP1, 4E-BP2, and 4E-BP3, interact with eIF4E, the cap-binding component of the eIF4F complex, which recruits mRNA to the polysomes (Poulin et al., 1998). eIF4E is present in rate-limiting amounts, so the binding of eIF4E by 4E-BPs inhibits complex assembly and represses cap-dependent translation (Mader et al., 1995). The binding of eIF4E is reversible and is dependent on the phosphorylation state of 4E-BP. Hypophosphorylated 4E-BP interacts with eIF4E whereas hyperphosphorylated 4E-BP does not (Gingras et al., 1999). The three members of the 4E-BP family are homologous to one another, with most striking homology found in the middle region of the protein which contains the eIF4E binding motif and the

residues that are phosphorylated. Such redundancy in translational control mechanism emphasizes the importance of eIF4E binding hence its need for regulation (Poulin et al., 1998) (Figure 2.2).

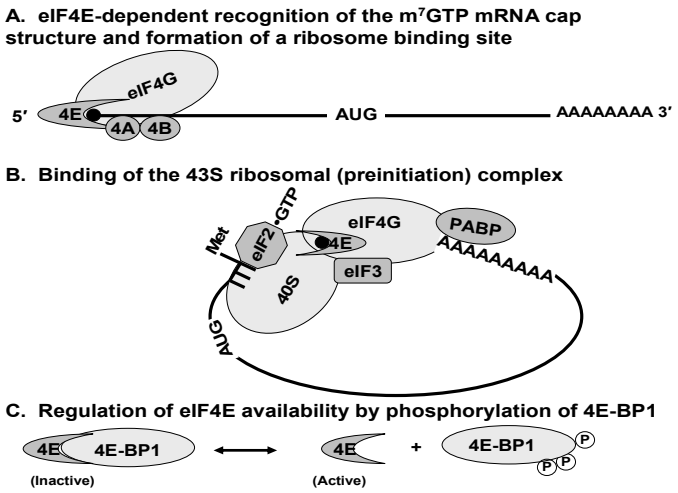

Figure 2.2. Formation of the 5′-cap binding complex eIF4F (eIF4E, 4G, 4A complex) and its regulation by association of eIF4E with 4E-BP.

Phosphorylation of 4E-BP1 by mTOR complex 1 (mTORC1) is positively regulated by insulin and other growth factors, availability of fuels, and the presence of amino acids, especially leucine (Choo and Blenis, 2009; Schmelzle and Hall, 2000). Mechanisms for insulin and growth factor signaling and sensing of cellular energy status have been at least partially elucidated but the mechanism for amino acid or leucine sensing is still unknown (Avruch et al., 2009). The hyperphosphorylation of 4E-BP by mTORC1 blocks the binding protein's ability to bind eIF4E and allows eIF4E to associate with eIF4G to promote formation of functional eIF4F complex. In contrast, low energy or low amino acid status in cells or a lack of insulin or IGF-1 can reduce the activation state of mTORC1. Inactive mTORC1 is unable to hyperphosphorylate its downstream target 4E-BP, making 4E-BP available to bind to

eIF4E and displace eIF4G. With the eIF4F complex unable to be assembled, recruitment of the 43S complex and subsequent translation initiation are inhibited (Tettweiler et al., 2005; Gingras et al., 1999; Haghighat et al., 1995; Mader et al., 1995).

The Integrated Stress Response (ISR) pathway

The eIF2α kinases regulate eIF2-GTP availability for ternary complex formation, and hence 43S pre-initiation complex formation, by phosphorylation of the Ser^{51} residue of the alpha subunit of the eIF2 heterotrimeric complex (i.e., eIF2α). Although there are four mammalian eIF2α kinases, with each responding to different types of cellular stress, it is only GCN2 that specifically responds to a lack of amino acids (Figure 2.3). In contrast to the mTORC1 pathway, which appears to show specificity for leucine and secondarily other branched-chain amino acids in terms of its activation by the presence of amino acids, GCN2 responds to deprivation of any essential amino acid (Avruch et al., 2009) (Figure 2.3). An increase in the abundance of non-aminoacylated tRNAs ("uncharged" tRNAs) is sensed by a histidyl-tRNA synthetase homologous domain of GCN2 (Wek et al., 2006; Padyana et al., 2005; Hao et al., 2005; Hinnebusch, 2000; Wek et al., 1995). Binding of uncharged tRNAs to this domain activates the juxtaposed protein kinase (PK) domain of GCN2 rendering the PK domain component of GCN2 active and leading to subsequent phosphorylation of eIF2α (Padyana et al., 2005; Hao et al., 2005; Wek et al., 1995). The phosphorylation of eIF2α reduces the dissociation rate of the eIF2 complex from eIF2B, thereby sequestering eIF2B (in fact, eIF2α-P binds eIF2B very tightly) and inhibiting its GDP-GTP exchange activity. This results in less eIF2-GTP and consequently less formation of the ternary complex needed for 43S pre-initiation complex formation/translation initiation and ultimately suppression of global protein

synthesis (Guo and Cavener, 2007; Padyana et al., 2005; Hao et al., 2005; Gabauer and Hentze, 2004; Harding et al., 2000; Hinnebusch, 2000; Wek et al., 1995; Kimball et al., 1991; Wek et al., 1989) (Figure 2.3).

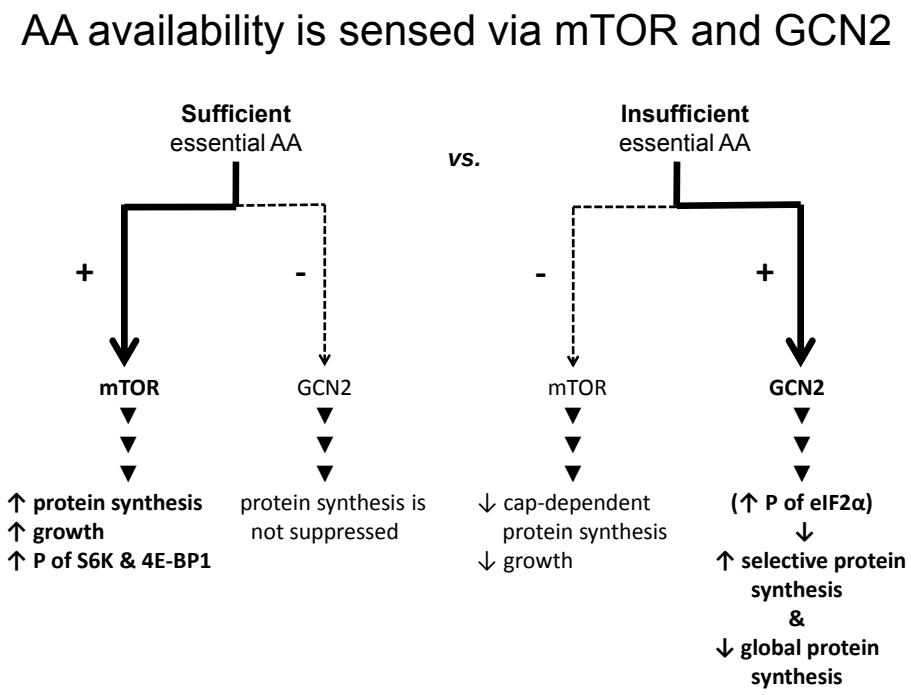

Figure 2.3. Conceptual scheme presenting the sensing mechanisms for amino acids and their responses to amino acid availability and limitation. Dashed arrows indicate lower vs. solid arrows higher, activation of the pathway.

Although both mechanisms of regulation of translation initiation regulate the rate of translation initiation (for general/global translation), eIF2α phosphorylation also, somewhat paradoxically, increases the translation of a subset of mRNAs, most notably of activating transcription factor 4 (ATF4). The presence of inhibitory up-stream open reading frame (ORF) that overlaps the ATF4 ORF, promotes read-through of the ATF4 translational start codon, thus preventing ATF4 translation under normal conditions in which ternary complex is abundant (Wek et al., 2006; Lu et al., 2004a). In amino acid deprivation or other stress conditions in which an eIF2α kinase

is activated and ternary complex formation is reduced, delayed initiation or re-initiation (i.e., ribosomal scanning through the inhibitory up-stream ORF start codon) is favored so that initiation occurs at the ATF4 ORF start codon, therefore allowing ATF4 to be translated (Figures 2.4 & 2.5).

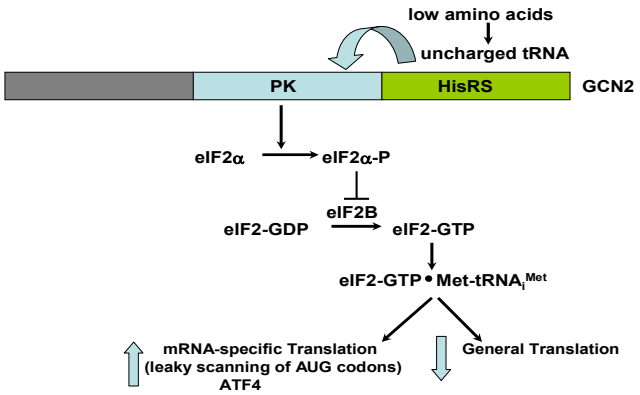

Figure 2.4. Conceptual scheme of the amino acid deprivation response which leads to reduction of global protein synthesis and the selective upregulation of the *ATF4* gene and its downstream targets.

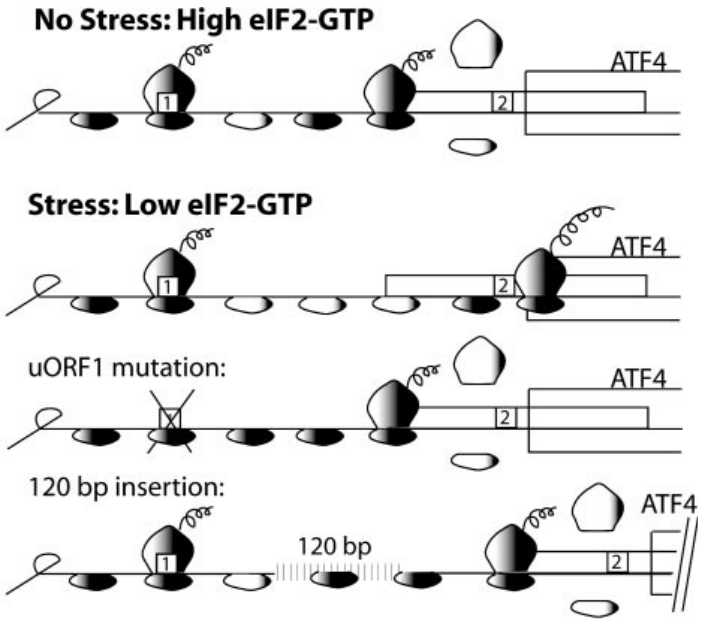

Figure 2.5. Model for ATF4 translational control by its leader sequences (Vattem and Wek, 2004). The *ATF4* mRNA is illustrated as a straight line that has uORFs 1 and 2 which are presented as boxes. The shading of the small ribosomal subunit indicates its association with eIF2-GTP bound Met-tRNA$_i^{Met}$. After translation of the positive-acting uORF1, ribosomes retain the capacity to resume translation initiation at a downstream ORF. The basis for this re-initiation capacity is currently not clear. In the related *GCN4* translation mechanism, in yeast, the termination context of the analogous uORF1 is thought to facilitate the retention of the small ribosomal subunit with the *GCN4* mRNA (Hinnebusch 2000, Hinnebusch 1997, Grant and Hinnebusch 1994). After translation of the *ATF4* uORF1, the 40S ribosomal subunits are proposed to resume scanning in a 5′ to 3′ direction along the *ATF4* transcript. When eIF2-GTP bound Met-tRNA$_i^{Met}$ is plentiful during nonstressed conditions, the small ribosomal subunits quickly acquire the eIF2 ternary complex and, coupled with the 60S ribosome, re-initiate translation at uORF2. After translation of this inhibitory uORF2, ribosomes dissociate from the *ATF4* mRNA, thereby reducing expression of the *ATF4*-coding region. When cells are subjected to ER stress or to nutrient deprivation, the levels of eIF2 phosphorylation are enhanced leading to reduced eIF2-GTP levels. After translation of uORF1, there is an increased time required for reacquisition of eIF2-GTP coupled Met-tRNA$_i^{Met}$ that allows a portion of the scanning 40S ribosomal subunits to scan through the negative-acting uORF2. While scanning the mRNA-leader region from beginning of uORF2 to the initiation codon of the *ATF4*-coding region, ribosomes reacquire the eIF2 ternary complex, facilitating translational expression of *ATF4*. If uORF1 is mutated, ribosomes scanning from the 5′-end of the *ATF4* mRNA will initiate translation at uORF2. After translation of the inhibitory

Figure 2.5 (Continued) uORF2, ribosomes dissociate from the *ATF4* mRNA, thus lowering translation of the *ATF4*-coding region even when eIF2-GTP levels are reduced in response to cellular stress. When the distance between uORF1 and uORF2 is increased compared to WT, most ribosomes are competent for re-initiation before reaching uORF2, thereby reducing *ATF4* translation independent of eIF2-GTP availability. (Figure and legend from Vattem and Wek, 2004.)

ATF4 is a member of the ATF subfamily of the basic leucine zipper (bZIP) transcription factor super-family. ATF4 is involved in upregulation of expression of a subset of genes, including the *ATF4* gene, at the transcriptional level. ATF family members heterodimerize within the ATF family as well as with members of another bZIP subgroup, the CCAAT/enhancer-binding protein (C/EBP) family. Many of the genes known to be transcriptionally upregulated by amino acid deprivation or the eIF2α-P/ATF4-mediated stress response pathway, contain C/EBP-ATF composite sites that are also known as amino acid response elements (AAREs) or CCAAT-enhancer binding protein-activating transcription factor response elements (CAREs) (Kilberg et al., 2009; Shan et al., 2009; Harding et al., 2000). The ATF4 half-site within these composite sites is highly conserved, which is consistent with the central role of ATF4 in the transcriptional program that follows activation of an eIF2α kinase. The AARE sites have a 9bp core element (NTTNCATCA) but differ by several nucleotides among genes (Harding et al., 2003). Genes that have been clearly established to contain a functional AARE and to be transcriptionally induced by the eIF2α-P/ATF4 pathway include those encoding several transcription factors [ATF4, ATF3, C/EBPβ, and CHOP (C/EBP-homologous protein)]; several members of the solute carrier (SLC) family including amino acid transporters [SNAT2 (sodium-coupled neutral amino acid transporter 2 or SLC38A2); CAT1 (high affinity cationic amino acid transporter 1 or SLC7A1); and xCT (the transporter subunit of the cystine-glutamate exchanger X_C^- or SLC7A11)]; an enzyme for amino acid biosynthesis

[ASNS (asparagine synthetase)]; and a putative protein kinase [TRIB3 (tribbles homolog 3)].

GCN2 is the only known eIF2α kinase in yeast. Activation of GCN2 in yeast results in increased translation of GCN4, the functional counterpart to mammalian ATF4, and GCN4 acts to induce expression of an array of genes that encode amino acid biosynthetic enzymes (Hinnebusch, 2005). Unlike yeast, mammalian cells are unable to synthesize a subset of amino acids, the so-termed essential or indispensable amino acids, and must obtain them from the environment. Not surprisingly, in mammalian cells, ATF4 induces the expression of a somewhat different array of genes, which includes other transcriptional regulators, solute carrier family transporters, and genes involved in cell cycle and DNA repair as well as amino acid uptake and amino-acyl tRNA synthetases (Lee et al., 2008; Sato et al., 2004; Lu et al., 2004b; Harding et al., 2003, 2000). This selection of upregulated genes assists the cell in coping with the amino acid limitation by increasing the efficiency of the uptake and utilization of the available amino acids, while minimizing the requirement. The downstream response of eIF2α phosphorylation in mammalian cells has been termed the integrated stress response (ISR) and shown to be associated with cell survival and stress resistance. The ISR was so-named because various kinases, activated by different types of stress situations, phosphorylate eIF2α to activate the ISR, hence eIF2α can be viewed as a molecule that integrates stress-related signaling and initiates a response to the induced stress. Other kinases that phosphorylate eIF2α, besides GCN2, are the heme-regulated inhibitor kinase (HRI), which is stimulated by heme depletion; protein kinase activated by double-stranded RNA (PKR), which is stimulated by viral infection; and PKR-like endoplasmic reticulum kinase (PERK), which is activated under endoplasmic reticulum (ER) stress (Lu et al., 2004a; Harding et al., 2003, 2000) (Figure 2.6).

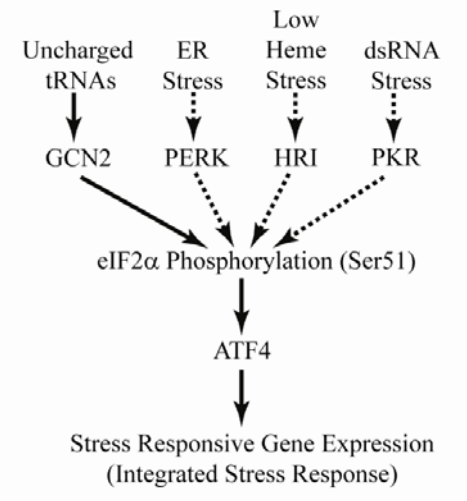

Figure 2.6. In mammals there are four eIF2α kinases that are capable of promoting eIF2α phosphorylation and the consequent downstream effects.

Although many of the responses to different stresses are similar, the responses also differ due to eIF2α phosphorylation-independent induction of other transcription factors or signaling pathways by the specific stress condition. These auxiliary pathways are best understood for ER stress responses in which PERK and ATF6 are also key factors. So even though amino acid deprivation and other stresses that activate mammalian eIF2α kinases commonly trigger increased ATF4 synthesis, a somewhat distinct subset of genes is activated in response to different types of cellular stress, perhaps due to eIF2α kinase-independent signaling, which, among other things, may lead to induction of different C/EBP family members (Dang et al., 2009; Shan et al., 2009; Wek et al., 1995). It is also likely that some of the longer-term responses to sustained induction of eIF2α may simply be consequences of the global repression of translation and/or of stress granule formation.

It has been shown that mammalian cells respond to a deficiency of one or more essential amino acids by induction of the eIF2α-P/ATF4-mediated ISR (Kilberg et al., 2009; Sato et al., 2004; Harding et al., 2000; Wek et al., 1995). Studies in GCN2 knockout mice and murine cells have shown that the GCN2 pathway is the major

signaling pathway involved in the up- and down-regulation of gene expression in response to amino acid starvation. Studies in murine embryonic stem cells and immortalized embryonic fibroblasts showed that eIF2α phosphorylation was not activated in $GCN2^{-/-}$ cells cultured in leucine-devoid medium (Deval et al., 2009; Zhang et al., 2002). Zhang et al. (2002) further showed that perfused livers of $GCN2^{-/-}$ mice did not display either induction of eIF2α-P or repression of eIF2B activity in response to histidine deprivation as occurred in wild-type mice. Anthony et al. (2004) reported that $GCN2^{-/-}$ mice fed a leucine-free diet for 1 h or 6 days did not show increased phosphorylation of hepatic eIF2α or restriction of liver protein synthesis, whereas increased phosphorylation of eIF2α and concomitant decreased protein synthesis were observed in liver of wild-type mice. Also the $GCN2^{-/-}$ mice maintained their liver mass but lost muscle mass and lost more body mass than the wild-type mice. Similarly, asparaginase treatment of $GCN2^{-/-}$ mice to lower asparagine levels did not lead to increased phosphorylation of eIF2α, whereas asparaginase treatment induced hepatic eIF2α-P levels in wild-type mice and in mice with a liver-specific eIF2α kinase 3 (PERK) deletion (Bunpo et al., 2009).

Although the mTOR pathway clearly plays a substantial role in regulation of protein synthesis and growth in response to nutrient availability, the eIF2α phosphorylation pathway may be better suited to respond to diets limiting in specific indispensable amino acids. Normal diets or food proteins are rarely, if ever, limiting in leucine but are commonly limiting in sulfur-containing amino acids (SAAs), lysine, threonine or tryptophan (Milward and Jackson, 2003).

Suppression of general protein synthesis and induction of the ISR by SAA restriction may lead to stress-resistance and/or increased lifespan

The evidence that protein or amino acid restriction can play a critical role in extending lifespan in model organisms, as well as the possible relation of this to induction of a stress-resistant physiological state, has wide-ranging implications for human nutrition and health. Zimmerman et al. (2003) reported that lowering the content of SAAs in the diet by removing cysteine and restricting the concentration of methionine extended all measured survival parameters in various rat strains, and pair-feeding of control rats with a SAA-supplemented diet demonstrated that the increase in survival was not simply due to caloric restriction. Although methionine restriction led to a decrease in levels of hepatic glutathione and cysteine, glutathione levels in most extrahepatic tissues did not decline while cysteine levels did (Richie et al., 2004). Methionine restriction was further shown to decrease mitochondrial oxygen radical production, as well as to decrease oxidative damage to mitochondrial DNA and cellular proteins (Sanz et al., 2006).

A number of studies have shown an association of eIF2α phosphorylation with induction of a state of stress resistance in cells. Studies in murine hippocampal (HT22) cells involving stable genetic mutations that resulted in reduced activity of the eIF2 complex (Tan et al., 2001) and subsequent studies employing a genetic system for inducing rapid reversible phosphorylation of eIF2α independent of cell stress (Lu et al., 2004b) demonstrated that reduced translational activity of the eIF2 complex induced resistance to subsequent oxidative glutamate toxicity in HT22 cells. Lu et al. (2004b) showed that eIF2α phosphorylation, or loss of functional eIF2 complex, resulted in translational inhibition, reduced incorporation of [^{35}S]-methionine into newly synthesized protein, and increased mRNA expression of many genes encoding enzymes involved in amino acid uptake and utilization, thiol-metabolism and

sufficiency, as well as resistance to oxidative and other cellular stresses. Knockdown of GADD34 (i.e., a decrease in eIF2α phosphatase activity) enhanced eIF2α phosphorylation, activated the ISR, and strongly protected mammalian cells against oxidative stress, peroxynitrite stress, and more modestly against ER stress induced by thapsigargin (Jousse et al., 2007). In addition, Ranganathan et al. (2008) reported that the activation of eIF2α kinase signaling in highly malignant squamous T-HEp3 cells or SW620 colorectal carcinoma cells induced both survival and tumor growth suppression, which was associated with a loss of tumorgenicity. The activation of eIF2α kinase inhibited T-HEp3 and SW620 carcinoma growth *in vivo*, suggesting that the growth-inhibitory function of eIF2α phosphorylation dominated in the tumor cells. This hypothesis was further supported by the observation that reduction of eIF2α kinase activity in a spontaneously arising *in vivo* quiescent variant of HEp3 cells, which displays strong basal PERK (eIF2α kinase 3) activation, led to reversion of the quiescent phenotype and restored tumor growth. These observations suggested a key and protective, pro-survival role for eIF2α-P. With our work we aimed to explore whether a mild, nutritional stress can lead to increased eIF2α-P and the associated events both *in vitro* and *in vivo*.

CHAPTER 3

GENE EXPRESSION AND INTEGRATED STRESS RESPONSE IN HepG2/C3A CELLS CULTURED IN AMINO ACID DEFICIENT MEDIUM

INTRODUCTION

A fundamental question in biology is how cells sense and respond to changes in their nutrient supply. How mammalian cells respond to changes in the availability of essential amino acids has been a major research focus in recent years due to evidence for various roles of essential amino acids in the regulation of protein translation and growth as well as regulation of stress response pathways.

In our studies of cysteine metabolism in mammalian cells, we discovered that cysteine deprivation does not necessarily induce an oxidative state in unstressed cells (Lee et al., 2008) but, rather, the data suggested that expression of genes for cyst(e)ine uptake and GSH synthesis may be induced as part of the cell's normal response to amino acid deficiency. In particular, we observed highly significant upregulation of expression of a group of genes (ASNS, ATF3, C/EBPβ, SLC7A11, TRIB3, ATF4, and SLC7A1) that are known to contain an amino acid response element (AARE) and to respond to amino acid deprivation via the binding of activating transcription factor 4 (ATF4).

ATF4 is translationally, as well as transcriptionally, upregulated in response to activation of stress response eIF2α kinases which then phosphorylate Ser^{51} of the alpha subunit of eukaryotic initiation factor 2 (eIF2). One of the four mammalian eIF2α kinases is GCN2 (protein kinase general control nonderepressible 2, or eIF2α kinase 4) which is activated specifically in response to amino acid deprivation (Wek et al., 2006). The GCN2 kinase senses an increase in the abundance of non-

aminoacylated tRNAs via its histidyl-tRNA synthetase homologous domain (Wek et al. 1995; Padyana et al. 2005; Hao et al. 2005). Binding of uncharged tRNAs to this domain activates the juxtaposed protein kinase domain of GCN2, rendering the kinase active and leading to phosphorylation of Ser^{51} of eIF2α. As shown in Figure 3.1A, the phosphorylation of eIF2 subsequently leads to inhibition of the GTP-GDP exchange activity of eIF2B, and this in turn leads to diminished formation of the ternary complex (Met-$tRNA_i^{Met}$ · eIF2 – GTP) that is needed for all cytoplasmic translation initiation events (Gabauer and Hentze 2004; Hinnebusch 2000; Kubica et al., 2006; Kimball et al., 1991). In addition to globally reducing translation initiation events and hence the overall rate of protein synthesis, reduced ternary complex formation paradoxically results in increases in the translation of some specific mRNAs, most notably that for ATF4 (Dang Do et al., 2009), as illustrated in Figure 3.1B. The presence of an inhibitory upstream open reading frame (ORF) that overlaps the ATF4 ORF promotes read-through of the ATF4 translational start codon, preventing ATF4 translation under normal conditions in which ternary complex is abundant (Lu et al., 2004a, b; Wek et al., 2006). In amino acid deprivation, in which eIF2B is inhibited and ternary complex formation is reduced, ribosomal scanning through the inhibitory upstream ORF start codon is favored so that initiation occurs at the downstream ATF4 ORF start codon, allowing an increase in ATF4 mRNA translation.

This translationally mediated increase in ATF4 leads to a transcriptional response that involves the upregulation of a number of genes including that for ATF4 itself (Lu et al., 2004a, b; Harding et al., 2000, 2003). GCN2 is the only eIF2α kinase in yeast, and activation of GCN2 in yeast results in increased translation of GCN4, the functional counterpart to mammalian ATF4, and GCN4 acts to induce expression of an array of genes that encode amino acid biosynthetic enzymes (Hinnebusch 2005). Unlike yeast, mammalian cells express several different eIF2α kinases, which are

activated by various kinds of stress including accumulation of unfolded proteins, heme deficiency, and viral infection, in addition to amino acid deprivation (Wek et al., 2006). Because mammalian cells, unlike yeast, are unable to synthesize a subset of amino acids, the so-called essential or indispensable amino acids, they must obtain them from the environment. Not surprisingly, the phospho-eIF2α/ATF4 signaling cascade induces the expression of a somewhat different array of genes in mammalian cells compared with those induced by the GCN2/GCN4 pathway in yeast. The genes induced in mammalian cells include those encoding transcriptional regulators, solute carrier family transporters, aminoacyl-tRNA synthetases, and proteins involved in cell cycle and DNA repair (Harding et al., 2000, 2003; Lee et al., 2008; Lu et al., 2004b; Sato et al., 2004). Because the mammalian response to eIF2α phosphorylation is induced by kinases that respond to a variety of stressors, the downstream translational/transcriptional response is often termed the integrated stress response (ISR).

Although amino acid deprivation and other stresses that activate mammalian eIF2α kinases commonly trigger increased ATF4 synthesis, a somewhat distinct subset of genes is activated in response to different types of cellular stress, perhaps due to eIF2α kinase-independent signaling, which, among other things, may lead to induction of different C/EBP family members (Wek et al., 1995; Dang Do et al., 2009; Shan et al., 2009). Gene expression surveys have been conducted with cells lacking ATF4 (Harding et al., 2003), in cells with ER-stress induced by tunicamycin (Harding et al., 2003; Lu et al., 2004b), with drug-induced activity of an artificial eIF2α kinase, Fv2E-PERK (Lu et al., 2004b), and in cells lacking GCN2 (Deval et al., 2009). All of these studies have been done using immortalized murine fibroblasts. To our knowledge, no large gene expression surveys have been reported for mammalian cells exposed to amino acid deprivation other than our studies in cysteine-deficient HepG2/C3A cells

(Lee et al., 2008). Results could vary with both the type of stress and with the type of cell that is used for the gene expression studies.

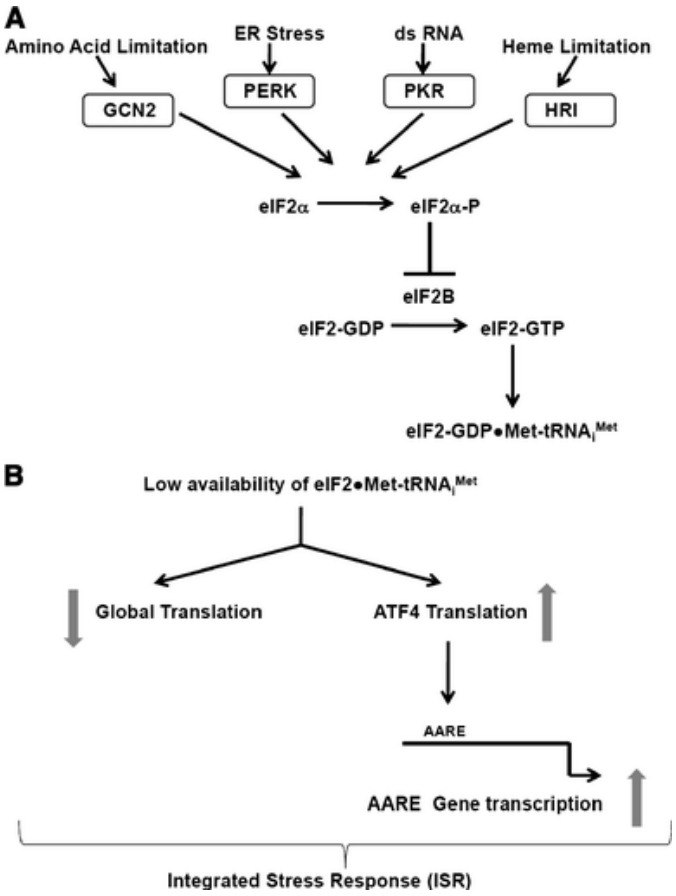

Figure 3.1. Mechanisms by which eIF2α kinases downregulate formation of ternary complex ($Met\text{-}tRNA_i^{Met} \cdot eIF2 - GTP$) formation (A) and by which the reduced availability of ternary complex suppresses global protein translation while selectively increasing ATF4 mRNA translation (B). The increase in ATF4 translation leads to induction of genes containing amino acid response elements (AARE). The collective consequences of eIF2α phosphorylation are termed the integrated stress response (ISR).

Thus, to further assess the role of amino acid deprivation on eIF2α phosphorylation and downstream transcriptional responses, we conducted additional microarray studies for leucine-depleted HepG2/C3A cells and compared these with

results for cysteine-depleted cells. The comparison of cells exposed to deficiencies of two different amino acids was done to facilitate selection of genes whose expression is more likely to be altered specifically in response to GCN2-induced eIF2α phosphorylation. In addition, we assessed the effects of both cysteine and leucine deprivation on the phosphorylation of eIF2α and on the protein expression patterns of ATF4 and other ISR-related proteins. The results of this study add to our understanding of how gene expression changes in response to amino acid deprivation in a human-derived cell line. Amino acid deprivation frequently induces a milder degree of stress than the agents used to induce ER stress (e.g., tunicamycin, thapsigargin), and the human HepG2/C3A cell line represents a more-differentiated cell line than the immortalized murine embryonic fibroblasts used in the studies mentioned above. The HepG2/C3A cell line is a derivative of the human hepatocarcinoma HepG2 cell line, with the C3A derivative being selected for its more liver-specific phenotype. HepG2 cells have been used in other studies of the amino acid deprivation response (Jousse et al., 2000; Thiaville et al., 2008; Palii et al., 2009).

MATERIALS AND METHODS

Experimental treatment of HepG2/C3A cells

HepG2/C3A cells (ATCC CRL-10741) were cultured in a humidified incubator at 37^{o}C and 5% CO_2 in complete medium prepared using sulfur amino acid (SAA)- and leucine-free Dulbecco's modified Eagle's medium (DMEM; from Gibco/ Invitrogen) supplemented with 0.8 mM L-leucine, 0.2 mM L-methionine, 0.2 mM L-cysteine, 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1×MEM nonessential amino acid solution and 0.05 mM bathocuproine disulfonate.

All cells were plated in complete medium at a density of 1×10^6 cells per 100 mm diameter culture dish. After 24 h of culture in complete medium to allow cells to reach 50-60% confluence, the medium was replaced with experimental medium, which was the same complete medium (+Leu, +Cys) or medium prepared without leucine (-Leu) or without cysteine (-Cys). Cells were cultured with a medium change at 18 h and harvested at the indicated time-points.

RNA extraction, microarray analysis, and selection of differentially expressed genes

HepG2/C3A cells cultured in complete or Leu-free treatment medium for 36 h as described above were washed twice with ice-cold PBS and then directly lysed into denaturation solution. Total RNA was extracted from three separate plates of cells grown under each treatment with an RNeasy Micro kit (Qiagen). Microarray analysis was performed on each of the three samples for each treatment using Affymetrix GeneChip Human plus 2.0 Arrays, a GeneChip Scanner 3000, and Affymetrix GCOS software, as described previously (Lee et al., 2008). To look for differential gene expression between the two treatment conditions, the Student's t test was applied on the normalized signals for each probe set. To select the differential gene set, a significance level cutoff was empirically established at $P < 0.0001$, based on the number of genes passing the cutoff, the magnitude of fold changes, and the relationship between fold changes and *P* values. The complete set of microarray data has been deposited in the NCBI Gene Expression Omnibus (accession no. GSE13142, on-line: http://www.ncbi.nlm.nih.gov/geo/).

Comparison of gene expression array results for HepG2/C3A cells cultured in leucine-free medium versus complete medium with those for HepG2/C3A cells cultured in cysteine-free medium versus complete medium

Using a dataset generated earlier for HepG2/C3A cells cultured in cysteine-free (-Cys) medium (SAA-free DMEM supplemented with 0.1 mM L-methionine) versus cells cultured in cysteine-supplemented (+Cys) medium (SAA-free DMEM supplemented with 0.1 mM L-methionine plus 0.1 mM L-cysteine) (GEO, accession no. GSE9517), we similarly selected differentially expressed genes ($P \leq 0.0001$). The two sets of selected genes were then compared to each other to select those that were differentially expressed ($P \leq 0.0001$) in both Cys-deficient and Leu-deficient HepG2/C3A cells. It should be noted that HepG2/C3A cells are readily depleted of cysteine, even when cultured in methionine-containing medium, due to the lack of the high K_m isoform of methionine adenosyltransferase (Lee et al., 2008; Lu and Huang 1994).

Western blot analysis of protein levels in HepG2/C3A cells cultured in -Leu, -Cys, or complete medium

Dishes of HepG2/C3A cells were cultured in complete, -Leu, or -Cys medium as described earlier. After 6, 12, 24, or 30 hours of culture, treatment medium was aspirated and cells were washed with ice-cold PBS containing 10 mM NaF. Monolayers were then harvested into TNESV lysis solution (50 mM Tris, pH 7.5, 1% (v/v) Nonidet P-40, 2 mM EDTA, 150 mM NaCl, and 10 mM sodium ortho-vanadate) supplemented with 1×PhosSTOP phosphatase inhibitor cocktail (Roche Applied Science) and 1×Complete Protease Inhibitor Cocktail (Roche). Cell lysates were centrifuged at 17,000×g for 30 min, and the protein concentration of the supernatants was determined using the bicinchonic acid assay (Pierce). For western blotting, 60 μg

of total supernatant protein from each sample were separated by one-dimensional SDS-PAGE (12% w/v acrylamide) and electroblotted overnight onto 0.45 μm (pore size) Immobilon-P PVDF membranes (Millipore). Membranes were immunoblotted for proteins of interest using the following antibodies: anti-pS^{51}-eIF2α and anti-eIF2α (total) from Cell Signaling Technology; anti-ATF4 (gift from M.S. Kilberg, University of Florida, Gainesville, FL); antiATF3 (C-19), anti-ASNS (C-14), anti-CHOP, and antiHSP5A from Santa Cruz Biotechnology; and anti-GCLC from Neomarkers (Freemont, CA). Bands were visualized using horseradish peroxidase-coupled secondary antibodies and chemiluminescent substrates (West Dura, Pierce) and autoradiography. The cell culture studies were repeated three or more times for designated time-points to assure the repeatability of the results.

RESULTS

Effect of amino acid deficiency on gene expression in HepG2/C3A cells

A total of 670 genes were identified as differentially expressed with a $P \leq 0.0001$ in cells cultured in leucine deficient (-Leu) versus complete (+Leu) medium, with 301 being upregulated and 369 being downregulated (Tables S1 and S2 in supplemental materials, see appendix 5). Similarly, 807 genes were identified as differentially expressed with a $P \leq 0.0001$ in cells cultured in cysteine deficient (-Cys) versus complete (+Cys) medium (GSE13142 data set; Tables S3 and S4 in supplemental materials, see appendix 5); 407 genes were upregulated and 400 were downregulated. To obtain a list of genes that are potential targets of regulated expression in response to essential amino acid deprivation, genes that met the following conditions were selected: (1) the gene was differentially expressed at $P \leq 0.0001$ in both the -Leu and the -Cys conditions; (2) the differential expression of the

gene was at least twofold; and (3) the differential expression of the gene was detected with the same probe set in both series of studies. Application of these criteria yielded the list of 120 genes that are listed in Table 3.1. Of these genes that were differentially expressed in response to both leucine and cysteine deprivation, 67 were downregulated. This downregulated group includes many genes involved in cell cycle/cell division, fatty acid and sterol metabolism/synthesis, and nucleotide metabolism/synthesis, as shown in Table 3.1. The remaining 53 genes were upregulated, and this upregulated group of genes includes a number of genes involved in amino acid uptake, aminoacyl-tRNA synthesis, transcriptional regulation, and growth inhibition. Most notably, many of the genes known to contain AAREs and to be induced in response to eIF2α (Ser^{51}) phosphorylation and ATF4 heterodimer binding (i.e., ATF3, C/EBPβ, SLC7A1, SLC7A11, and TRIB3), as well as others shown to be induced downstream of eIF2α (Ser^{51}) phosphorylation (C/EBPγ, CARS, SARS, CLCN3, CBX4, and PPP1R15A, which is also known as GADD34, were among these 53 genes (Lee et al., 2008; Lu et al., 2004b) (see Table 3.1 for names of gene products). Expression of ASNS, another AARE-dependent gene, met all criteria except one in that the fold increase for expression in the -Cys medium was only 1.6. Two other well-characterized AARE-containing genes, ATF4 and (also known as DDIT3 or GADD153) were also identified as significantly differentially expressed in cells cultured in both -Leu and -Cys medium using a lower statistical significance cutoff of $P \leq 0.001$.

Confirmation that the amino acid deprivation response was actually initiated in HepG2/C3A cells cultured in -Leu or -Cys medium was obtained by western blot analysis of total and phosphorylated eIF2α, ATF4, activating transcription factor 3 (ATF3), and asparagine synthase (ASNS) proteins in the cells. Glutamate-cysteine ligase catalytic subunit (GCLC) and heat shock 70-kDa protein 5 (HSPA5) were also

assayed to confirm that proteins that are not part of the amino acid deprivation response were not upregulated. As shown in Figure 3.2, the amount of phosphorylated eIF2α increased between 6 and 24 hours of culture in cells cultured in -Leu or -Cys, as well as in control medium. However, the increases were greater in cells cultured in -Leu or -Cys medium than in cells cultured in control medium, such that the amount of phosphorylated eIF2α in cells cultured for 24 to 30 hours in amino-acid-deficient medium was double that in cells cultured in control medium (2.0-times for -Leu and 2.2times for -Cys). The amount of total eIF2α did not change significantly across time or culture conditions. A significant increase in total ATF4 levels in the cells was noted only at the 24 h time-point at which time ATF4 in cells cultured in the -Leu medium was 1.3-times and that in cells cultured in -Cys medium was 1.2-times that in control cells. Expression of ATF3 and ASNS, whose genes are well-established targets for upregulation in response to amino acid deprivation or eIF2α phosphorylation, paralleled the increases in eIF2α phosphorylation. ATF3 levels in cells cultured in amino acid-deficient medium for 24 h/30 h were 8.0-and 9.8-times for -Leu and -Cys cells, respectively, and ASNS levels were 2.7- and 1.9-times, respectively, those in cells cultured in complete medium. CHOP was strongly induced in cells cultured in -Leu medium but only weakly detected in cells cultured in -Cys medium at the longest time-point (30 h). The amount of GCLC, which served as a loading control, did not change with time or treatment. As a control on the specificity of the amino acid deprivation/GCN2-mediated response, a chaperone protein that is strongly induced in response to activation of a different eIF2α kinase (i.e., PERK, or eIF2α kinase 3) by a different type of stress (endoplasmic reticulum stress, or ER stress) was measured. The ER chaperone HSPA5 was not increased over time or with amino acid deprivation of the medium and, in fact, was consistently lower in cells cultured in -Leu medium than in those cultured in control medium. Similar results were obtained for

Table 3.1. Genes differentially upregulated and downregulated in amino acid-deficient G2/C3A cells.

GENES UPREGULATED IN AMINO ACID DEFICIENT CELLS				
Probe ID	**Gene Symbol**	**Gene Title**	**Fold Difference**	
			-Cys/ +Cys	**-Leu/ +Leu**
Genes with known functional AAREs				
202672_s_at	ATF3	activating transcription factor 3	10.3	4.0
212501_at	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	4.6	3.3
209921_at	SLC7A11	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	13.8	4.2
217678_at			9.9	3.2
1555788_a_at	TRIB3	tribbles homolog 3 (Drosophila)	5.8	4.0
218145_at			4.0	3.2
212295_s_at	SLC7A1	solute carrier family 7 (cationic amino acid transporter, y+ system), member 1	3.3	2.6
Amino Acid Metabolism				
212971_at	CARS	cysteinyl-tRNA synthetase	2.7	2.3
206085_s_at	CTH	cystathionase (cystathionine gamma-lyase)	2.9	3.1
202014_at	PPP1R15A (GADD34)	protein phosphatase 1, regulatory (inhibitor) subunit 15A	8.6	2.2
37028_at			6.2	2.9
231894_at	SARS	seryl-tRNA synthetase	4.4	3.5
200629_at	WARS	tryptophanyl-tRNA synthetase	2.6	4.5

Table 3.1 (Continued)

GENES UPREGULATED IN AMINO ACID DEFICIENT CELLS				
Probe ID	**Gene Symbol**	**Gene Title**	**Fold Difference**	
			-Cys/ +Cys	**-Leu/ +Leu**
Endosomal/lysosomal system				
201734_at	CLCN3	chloride channel 3	2.1	2.4
201735_s_at			2.0	2.4
205569_at	LAMP3	lysosomal-associated membrane protein 3	3.0	5.9
208786_s_at	MAP1LC3 B	microtubule-associated protein 1 light chain 3 beta	3.7	2.9
209822_s_at	VLDLR	Very low density lipoprotein receptor	3.5	3.0
GAPs and GEFs				
209435_s_at	ARHGEF2	rho/rac guanine nucleotide exchange factor (GEF) 2	5.5	4.4
224822_at	DLC1	deleted in liver cancer 1	2.6	2.0
Growth regulation				
209409_at	GRB10	growth factor receptor-bound protein 10	3.1	2.8
210587_at	INHBE	inhibin, beta E	6.8	4.6
202393_s_at	KLF10	kruppel-like factor 10	3.5	3.1
226275_at	MXD1	MAX dimerization protein 1	4.4	2.1
228846_at			4.2	3.3
203373_at	SOCS2	suppressor of cytokine signaling 2	3.5	2.4

Table 3.1 (Continued)

GENES UPREGULATED IN AMINO ACID DEFICIENT CELLS				
Probe ID	**Gene Symbol**	**Gene Title**	**Fold Difference**	
			-Cys/ +Cys	**-Leu/ +Leu**
Cytoskeleton				
226084_at	MAP1B	microtubule-associated protein 1B	3.5	2.9
226342_at	SPTBN1	spectrin, beta, non-erythrocytic 1	3.5	4.6
Other				
209993_at	ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	2.3	2.0
210517_s_at	AKAP12	A kinase (PRKA) anchor protein (gravin) 12	6.1	3.0
220088_at	C5R1	complement component 5 receptor 1 (C5a ligand)	9.4	2.1
201925_s_at	DAF	decay accelerating factor for complement (CD55, Cromer blood group system)	4.2	5.5
235296_at	EIF5A2	eukaryotic translation initiation factor 5A2	3.5	2.3
209098_s_at	JAG1	jagged 1 (Alagille syndrome)	5.9	2.1
201010_s_at	TXNIP	thioredoxin interacting protein	3.1	3.2
Transcriptional regulators				
227558_at	CBX4	chromobox homolog 4 (Pc class homolog, Drosophila)	2.5	2.8
204203_at 225527_at	CEBPG	CCAAT/enhancer binding protein (C/EBP), gamma	2.6 3.3	2.2 2.4
223287_s_at	FOXP1	forkhead box P1	3.5	2.2

Table 3.1 (Continued)

GENES UPREGULATED IN AMINO ACID DEFICIENT CELLS				
Probe ID	**Gene Symbol**	**Gene Title**	**Fold Difference**	
			-Cys/ +Cys	**-Leu/ +Leu**
Unknown functions				
225283_at	ARRDC4	arrestin domain containing 4	3.8	5.3
226398_s_at	C10orf4	chromosome 10 open reading frame 4	2.4	2.2
227443_at	C9orf150	chromosome 9 open reading frame 150	5.0	2.3
201919_at	FLJ10618	Hypothetical protein FLJ10618	2.7	2.2
222953_at	GPR83	G protein-coupled receptor 83	10.0	6.4
202147_s_at	IFRD1	interferon-related developmental regulator 1	5.5	2.7
236565_s_at	LARP6	La ribonucleoprotein domain family, member 6	3.5	3.4
209205_s_at	LMO4	LIM domain only 4	3.5	2.1
221501_x_at	LOC339047	hypothetical protein LOC339047	3.0	2.27
220437_at	LOC55908	hepatocellular carcinoma-associated gene TD26	2.4	5.26
236285_at	MGC16635	hypothetical protein BC009980	11.3	7.04
204538_x_at	NPIP /// LOC339047 /// LOC440341	nuclear pore complex interacting protein /// hypothetical protein LOC339047 /// similar to hypothetical protein LOC339047	2.8	2.38
215707_s_at	PRNP	prion protein (p27-30) (Creutzfeld-Jakob disease, Gerstmann-Strausler-Scheinker syndrome, fatal familial insomnia)	2.1	2.04

Table 3.1 (Continued)

GENES UPREGULATED IN AMINO ACID DEFICIENT CELLS				
Probe ID	**Gene Symbol**	**Gene Title**	**Fold Difference**	
			-Cys/ +Cys	**-Leu/ +Leu**
209568_s_at	RGL1	ral guanine nucleotide dissociation stimulator-like 1	2.2	2.52
202130_at	RIOK3	RIO kinase 3 (yeast) /// RIO kinase 3 (yeast)	2.8	2.04
202241_at	TRIB1	tribbles homolog 1 (Drosophila)	2.4	2.08
222408_s_at	YPEL5	yippee-like 5 (Drosophila)	2.9	2.13
223506_at	ZC3H8	Zinc finger CCCH-type containing 8	2.2	3.23
225522_at		similar to BMP2 inducible kinase	2.6	3.13
227755_at		cDNA clone IMAGE:4077090, partial cds	5.9	5.26
Cell Cycle/Cell Division				
222608_s_at	ANLN	anillin, actin binding protein (scraps homolog, Drosophila)	0.33	0.32
209642_at	BUB1	BUB1 budding uninhibited by benzimidazoles 1 homolog (yeast)	0.36	0.33
203418_at	CCNA2	cyclin A2	0.27	0.27
213226_at			0.31	0.34
214710_s_at	CCNB1	cyclin B1	0.35	0.35
228729_at			0.33	0.35

Table 3.1 (Cotinued)

GENES UPREGULATED IN AMINO ACID DEFICIENT CELLS				
Probe ID	**Gene Symbol**	**Gene Title**	**Fold Difference**	
			-Cys/ +Cys	**-Leu/ +Leu**
203214_x_at	CDC2	cell division cycle 2, G1 to S and G2 to M	0.32	0.30
210559_s_at			0.27	0.29
203967_at	CDC6	CDC6 cell division cycle 6 homolog (S. cerevisiae)	0.19	0.27
203968_s_at			0.27	0.30
204159_at	CDKN2C	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	0.20	0.27
209714_s_at	CDKN3	cyclin-dependent kinase inhibitor 3 (CDK2-associated dual specificity phosphatase)	0.28	0.34
204962_s_at	CENPA	centromere protein A, 17kDa	0.36	0.38
209194_at	CETN2	centrin, EF-hand protein, 2	0.41	0.38
205394_at	CHEK1	CHK1 checkpoint homolog (S. pombe)	0.23	0.42
210416_s_at	CHEK2	CHK2 checkpoint homolog (S. pombe)	0.45	0.38
203764_at	DLG7	discs, large homolog 7 (Drosophila)	0.31	0.37
223556_at	HELLS	helicase, lymphoid-specific	0.29	0.35
201555_at	MCM3	MCM3 minichromosome maintenance deficient 3 (S. cerevisiae)	0.19	0.39

Table 3.1 (Cotinued)

GENES UPREGULATED IN AMINO ACID DEFICIENT CELLS				
Probe ID	**Gene Symbol**	**Gene Title**	**Fold Difference**	
			-Cys/ +Cys	**-Leu/ +Leu**
219978_s_at	NUSAP1	nucleolar and spindle associated protein 1	0.34	0.37
219148_at	PBK	PDZ binding kinase	0.21	0.26
221521_s_at	Pfs2	DNA replication complex GINS protein PSF2	0.14	0.23
205909_at	POLE2	polymerase (DNA directed), epsilon 2 (p59 subunit)	0.29	0.34
203554_x_at	PTTG1	pituitary tumor-transforming 1	0.26	0.40
203696_s_at	RFC2	replication factor C (activator 1) 2, 40kDa	0.34	0.45
204127_at	RFC3	replication factor C (activator 1) 3, 38kDa	0.21	0.33
204128_s_at			0.19	0.26
204023_at	RFC4	replication factor C (activator 1) 4, 37kDa	0.25	0.37
209891_at	SPBC25	spindle pole body component 25 homolog (S. cerevisiae)	0.24	0.23
204026_s_at	ZWINT	ZW10 interactor	0.17	0.34
Calcium signaling				
1554483_at	TMEM37	transmembrane protein 37	0.22	0.34
1554485_s_at			0.20	0.36
203797_at	VSNL1	visinin-like 1	0.17	0.33

Table 3.1 (Cotinued)

GENES UPREGULATED IN AMINO ACID DEFICIENT CELLS				
Probe ID	**Gene Symbol**	**Gene Title**	**Fold Difference**	
			-Cys/ +Cys	**-Leu/ +Leu**
Cytoskeleton				
212320_at	TUBB	tubulin, beta polypeptide	0.34	0.46
Fatty acid and sterol metabolism				
209608_s_at	ACAT2	acetyl-Coenzyme A acetyltransferase 2 (acetoacetyl Coenzyme A thiolase)	0.23	0.27
209389_x_at	DBI	diazepam binding inhibitor (GABA receptor modulator, acyl-Coenzyme A binding protein)	0.21	0.25
202735 _at	EBP	emopamil binding protein (sterol isomerase)	0.38	0.32
213787_s_at			0.31	0.29
208962_s_at	FADS1	fatty acid desaturase 1	0.39	0.29
208964_s_at			0.42	0.33
210950_s_at	FDFT1	farnesyl-diphosphate farnesyltransferase 1	0.24	0.30
201036_s_at	HADH SC	L-3-hydroxyacyl-Coenzyme A dehydrogenase, short chain	0.22	0.43
204615_x_at	IDI1	isopentenyl-diphosphate delta isomerase 1	0.32	0.24
202245_at	LSS	lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)	0.36	0.25

Table 3.1 (Cotinued)

GENES UPREGULATED IN AMINO ACID DEFICIENT CELLS				
Probe ID	**Gene Symbol**	**Gene Title**	**Fold Difference**	
209146_at	SC4MOL	sterol-C4-methyl oxidase-like	0.31	0.28
Nucleotide synthesis/metabolism				
202534_x_at	DHFR	dihydrofolate reductase	0.30	0.43
48808_at			0.29	0.36
1553984_s_at	DTYMK	deoxythymidylate kinase (thymidylate kinase)	0.24	0.43
203270_at			0.30	0.39
209932_s_at	DUT	dUTP pyrophosphatase	0.35	0.40
201476_s_at	RRM1	ribonucleotide reductase M1 polypeptide	0.33	0.49
209773_s_at	RRM2	ribonucleotide reductase M2 polypeptide	0.10	0.28
209980_s_at	SHMT1	serine hydroxymethyltransferase 1 (soluble)	0.26	0.38
1554408_a_at	TK1	thymidine kinase 1, soluble	0.27	0.21
202338_at			0.23	0.24
202330_s_at	UNG	uracil-DNA glycosylase	0.19	0.42

Table 3.1 (Cotinued)

GENES UPREGULATED IN AMINO ACID DEFICIENT CELLS				
Probe ID	**Gene Symbol**	**Gene Title**	**Fold Difference**	
			-Cys/ +Cys	**-Leu/ +Leu**
Other				
208951_at	ALDH7A1	aldehyde dehydrogenase 7 family, member A1	0.18	0.47
206651_s_at	CPB2	Carboxypeptidase B2 (plasma, carboxypeptidase U)	0.07	0.30
226980_at	DEPDC1B	DEP domain containing 1B	0.28	0.36
228033_at	E2F7	E2F transcription factor 7	0.41	0.45
219990_at	E2F8	E2F transcription factor 8	0.26	0.36
201341_at	ENC1	ectodermal-neural cortex (with BTB-like domain)	0.41	0.49
203564_at	FANCG	Fanconi anemia, complementation group G	0.35	0.45
202503_s_at	KIAA0101	KIAA0101	0.14	0.39
221952_x_at	KIAA1393	KIAA1393	0.38	0.48
218755_at	KIF20A	kinesin family member 20A	0.35	0.29
207256_at	MBL2	mannose-binding lectin (protein C) 2, soluble (opsonic defect)	0.43	0.46
213599_at	OIP5	Opa interacting protein 5	0.26	0.31
235113_at	PPIL5	peptidylprolyl isomerase (cyclophilin)-like 5	0.34	0.37

Table 3.1 (Cotinued)

GENES UPREGULATED IN AMINO ACID DEFICIENT CELLS				
Probe ID	**Gene Symbol**	**Gene Title**	**Fold Difference**	
			-Cys/ +Cys	**-Leu/ +Leu**
203824_at	TSPAN8	tetraspanin 8	0.17	0.34
223229_at	UBE2T	ubiquitin-conjugating enzyme E2T (putative)	0.33	0.27
Unknown				
225687_at	C20orf129	chromosome 20 open reading frame 129	0.33	0.37
212279_at	MAC30	hypothetical protein MAC30	0.17	0.31
212282_at			0.13	0.40
226456_at	MGC24665	hypothetical protein MGC24665	0.21	0.27

HepG2/C3A cells cultured in medium deficient in L-methionine (but containing 0.2 mM L-cysteine) (data not shown).

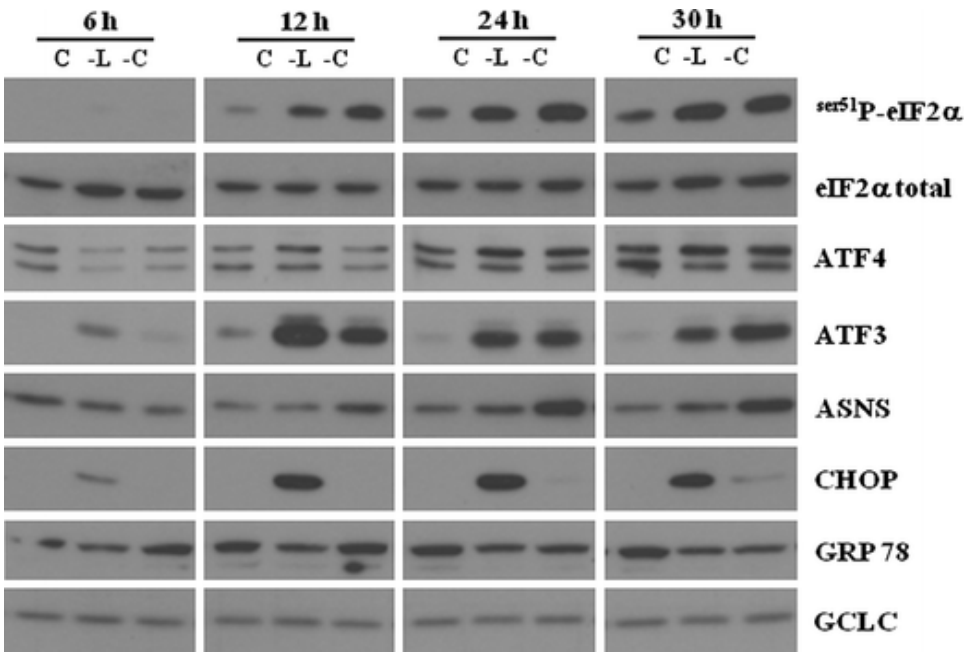

Figure 3.2. Western blot of protein levels in cells cultured for 6, 12, 24 or 30 hours in complete control medium (C), leucine-free (-L) or cysteine-free (-C). For each sample, 60 μg of total soluble protein was loaded in each lane.

Comparison of results for genes induced in response to amino acid deficiency with lists of upregulated genes previously generated for eIF2a phosphorylation/ATF4-mediated changes in gene expression

To further explore the list of 53 upregulated genes that met stringent criteria for designation as differentially expressed ($P \leq 0.0001$) in HepG2/C3A cells cultured in amino acid deficient medium, this list of genes was compared with lists of upregulated genes previously generated for eIF2α phosphorylation/ATF4-mediated changes in gene expression (Figure 3.3). These comparison lists include (1) that for murine fibroblasts that was generated by Harding et al. (2003) by selecting genes whose expression was at least twofold lower in tunicamycin-treated $ATF4^{-/-}$ cells compared with tunicamycin-treated wild-type cells (i.e., genes upregulated by tunicamycin in an ATF4-dependent fashion) and (2) that for murine fibroblasts that

was generated by Lu et al. (2004b) by selecting genes whose expression was induced at least twofold in both drug-induced Fv2E-PERK cells and 1.5-fold in tunicamycin-treated wild-type cells (i.e., genes upregulated by both drug-induced eIF2α phosphorylation and by induction of ER stress). Interestingly, only two genes appear on all three lists: C/EBPβ and C/EBPγ (CCAAT/enhancer binding protein, β and γ, respectively). These two genes represent the only overlap with the selected list of Harding et al. (2003). Comparison of our list with that of Lu et al. (2004b) yields seven additional overlapping genes: ATF3, SARS (seryl-tRNA synthetase), CARS (cysteinyl-tRNA synthetase), CBX4 (chromobox homolog 4), CLCN3 (chloride channel 3), LMO4 (LIM domain only 4), and TXNIP (thioredoxin interacting protein).

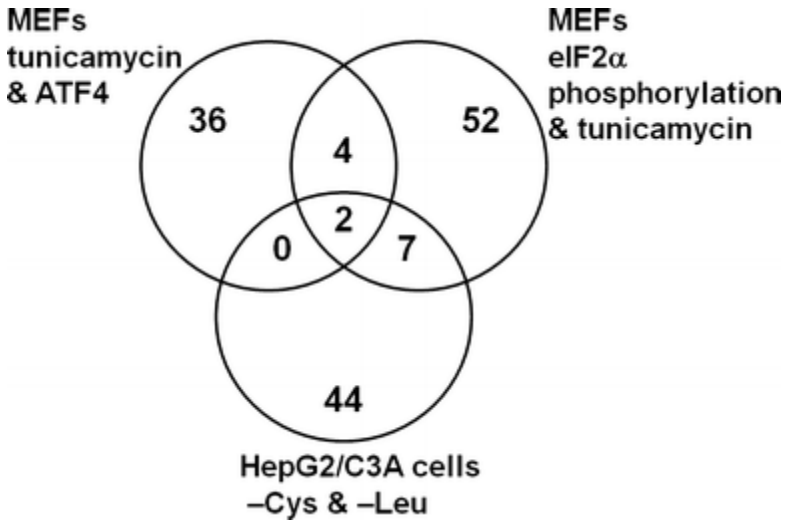

Figure 3.3. Comparison of genes proposed to be induced in response to eIF2α phosphorylation and/or ATF4 induction. Lists of upregulated genes generated by Harding et al. (2003) by selecting genes whose expression was at least two-fold lower than tunicamycin-treated ATF4$^{-/-}$ murine fibroblasts compared to tunicamycin-treated wild-type cells; Lu et al. (2004b) by selecting genes whose expression was induced at least two-fold in both drug-induced Fv2E-PERK murine fibroblasts and 1.5-fold in tunicamycin-treated wild-type cells; and in our work by selecting genes that were induced at least twofold in HepG2/C3A cells when cultured in either cysteine-deficient medium or leucine-deficient medium.

ASNS (asparagine synthetase), SLC1A7 (glutamate/neutral amino acid transporter), NARS (asparaginyl-tRNA synthetase), and ERO1L (endoplasmic reticulum 1-like oxidoreductase) made the lists of both Harding et al. (2003) and Lu et al. (2004a, b) but not our list of genes induced by amino acid deprivation in HepG2/C3A cells.

DISCUSSION

Amino acid deprivation leads to downregulation of genes involved in amino acid uptake, aminoacyl-tRNA synthesis, transcriptional regulation, and growth inhibition

The 120 genes that met stringent criteria for designation as differentially expressed ($P \leq 0.0001$) in HepG2/C3A cells cultured in amino acid deficient medium should all be considered potential targets for regulation by amino acids in HepG2/C3A cells.

The upregulated group of 53 genes includes a number of genes involved in amino acid uptake, aminoacyl-tRNA synthesis, transcriptional regulation, and growth inhibition; comparison of this group of upregulated genes with the groups of upregulated genes identified by other investigators as potential downstream targets of eIF2α phosphorylation and ATF4 induction, amino acyl-tRNA synthetases and amino acid transporters stand out as consistently upregulated although the specific ones varied among studies. The cystine-glutamate exchanger, SLC7A11, was the most highly induced amino acid transporter gene in HepG2/C3A cells in both -Cys and -Leu medium. Several amino acyl-tRNA synthetases, CARS, SARS, and WARS, were induced under conditions of both Cys and Leu deficiency.

Another striking observation from our comparison of various studies to identify common genes induced by various stresses that induce eIF2α phosphorylation and ATF4 expression is that a number of genes that are known to contain AAREs and

to be induced by ATF4 binding were identified, as would be anticipated. These include C/EBPβ, ATF3, and ASNS. Other genes induced in the amino acid deficient HepG2/C3A cells that are known to contain AAREs and to be induced by ATF4 binding include SLC7A1, SLC7A11, and TRIB3. The identification of these AARE-containing genes is consistent with transcriptional responses to amino acid deprivation being mediated by ATF4 binding to AARE and with phosphoeIF2α/ATF4-dependent gene expression being a key component of the ISR.

Further support for the induction of the ISR in HepG2/ C3A cells cultured in amino acid deficient medium was obtained from the eIF2α phosphorylation and protein expression patterns in the HepG2/C3A cells. Increases in eIF2α phosphorylation were apparent by 12 h after the removal of leucine or cysteine from the medium as were increases in ATF3 protein levels, and increases in ATF4 and ASNS protein levels were apparent in both cysteine-and leucine-depleted cells by 24 h. Whereas ATF3 protein was strongly induced by either leucine deprivation or cysteine deprivation, ASNS protein was consistently more strongly induced in the cysteine-depleted cells. On the other hand, CHOP protein was strongly induced in the leucine-depleted cells whereas induction was not evident in the cysteine-depleted cells until the 30-h time-point. The stronger induction of CHOP by leucine depletion is consistent with our microarray results that showed a 6.7-fold induction (significant at $P \leq 0.0001$) of CHOP mRNA in -Leu-treated cells but only a 4.1-fold, less significant ($P \leq 0.001$) induction in -Cys-treated cells. Jousse et al. (2000) previously reported a similar finding for both HeLa and HepG2 cells, finding that CHOP protein induction was greater in cells incubated for 16 h in -Leu medium than in cells incubated in -Cys medium. Thus, a robust upregulation of components of the amino acid deprivation pathway or ISR was observed, although not all downstream responses were highly correlated with the degree of eIF2α phosphorylation or with cellular ATF4 levels.

There are several possible reasons for the lack of apparent correlation of all upstream responses with activation or expression of early components of the ISR pathway or of mRNA and protein expression levels for individual genes. First of all, the time-course for changes in levels of various mRNAs and proteins is highly dependent upon the turnover rate of the individual mRNAs and proteins. Second, the levels of ATF4 in the nucleus may not parallel the amount of total ATF4 in the cell. Third, other transcriptional regulators including ATF4's heterodimerization partners may also play an important role in gene activation. ATF4 forms heterodimers with members of another bZIP family, which consists of C/EBP α, β, γ, δ, ε, and CHOP. ATF4-C/EBP complexes have been detected at the C/EBP-ATF composite sites (functional AAREs) on promoters of genes induced by amino acid deprivation. ATF4 activity is subjected to regulation (activation or repression) by its various binding partners, with some of these dimerization partners (e.g., ATF3, TRIB3, C/EBPβ, and CHOP), as well as the ATF4 gene itself, being transcriptionally induced by ATF4 heterodimers (Shan et al., 2009; Mungrue et al., 2009; Pan et al., 2007). Fourth, some of the effects of amino acid deprivation are undoubtedly mediated by GCN2-independent pathways and these may vary for different amino acids (Jousse et al., 2000; Su et al., 2009; Deval et al., 2009; Thiaville et al., 2008; Palii et al., 2009). Finally, regulation of gene expression and/or protein level may involve the integration of a number of regulators and points of regulation, with these additional signaling pathways either enhancing or suppressing the GCN2/ phospho-eIF2α/ATF4 signaling pathway (Shan et al., 2009; Ishikawa et al., 2009; Vesely et al., 2009).

Amino acid deprivation leads to downregulation of genes involved in the cell cycle, fatty acid desaturation, sterol synthesis, and nucleotide synthesis

The group of genes that were downregulated in cells cultured in either -Leu or -Cys medium included a number of genes involved in cell cycle/cell division, fatty acid and sterol metabolism/synthesis, and nucleotide metabolism/synthesis (Table 3.1). Genes that are downregulated in response to eIF2α phosphorylation/ATF4 induction have generally received much less attention, although one would expect that DNA synthesis and cell proliferation and growth would be restricted under conditions of cell stress so that resources can be diverted to deal with the imposed stress. Downregulation of the expression of genes involved in cell division and nucleotide synthesis, along with the eIF2α phospho-mediated reduction in protein synthesis, would seem to be synergistic in facilitating the cell's survival during times of nutrient deprivation or other stress. We observed downregulation of expression of a cluster of genes in the folate/deoxythymidine nucleotide synthetic pathway as well as downregulation of genes involved in all phases of the cell cycle.

Guo and Cavener (2007) speculated that GCN2 may act as a master regulator of metabolic adaptation to nutrient deprivation, because an essential amino acid limitation in nature would almost always be correlated with the deprivation of other nutrients as well. Using $GCN2^{-/-}$ and wild-type mice, they found that triglyceride synthesis was repressed in the livers of wild-type mice that were fed a leucine-devoid diet for 7 or 17 days but continued unabated in the livers of $GCN2^{-/-}$ mice. In the $GCN2^{-/-}$ mice, consumption of a leucine-deficient diet was accompanied by accumulation of hepatic triglyceride as well as impaired lipolysis of adipose stores. Guo and Cavener (2007), using quantitative RT-PCR and western blotting, showed that the -Leu diet resulted in diminished fatty acid synthase (FAS) expression in wild-type but not $GCN2^{-/-}$ mice. Similarly, hepatic mRNA levels for stearoyl-CoA

desaturase (SCD) and other enzymes involved in fatty acid synthesis were diminished in GCN2$^{-/-}$ mice fed the –Leu diet but remained the same or even increased in wild-type mice. Furthermore, they showed that the mRNA levels for enzymes involved in β-oxidation (e.g., fatty acyl-CoA oxidase, long-and medium-chain acyl-CoA dehydrogenases) were not affected by leucine deprivation in wild-type mice, whereas the expression of these genes was markedly increased in GCN2$^{-/-}$ mice fed a leucine-devoid diet. Our gene expression studies in HepG2/C3A cells cultured in medium deficient in either Cys or Leu also showed the consistent downregulation of a group of genes involved in fatty acid and sterol metabolism, although the particular genes showing high fold induction in our microarray study were somewhat different than those observed by Guo and Cavener. The genes whose mRNA levels were significantly reduced ($P \leq 0.0001$, ≤0.5-fold) in our analysis included genes involved in fatty acid desaturation (FADS1 and SCD), in isoprenoid and sterol synthesis (FDFT1, IDI1, LSS, EBP, SQLE, and SC4MOL), and in cholesterol ester synthesis (ACAT2). The only gene in this repressed group that was also included in the more-limited screen done by Guo and Cavener (2007) is SCD, and they found that SCD mRNA level was reduced to < 20% of control in liver of wild-type mice fed the leucine-devoid diet. Furthermore, Guo and Cavener (2007) showed that this reduction was strictly dependent on GCN2 because SCD mRNA levels were not different in GCN2$^{-/-}$ mice suggesting that regulation of SREBP-1c may underlie some of the other transcriptional changes in genes involved in fatty acid synthesis. They did not, however, pursue the role of SREBP-2 in the regulation of sterol synthesis in their study which focused on regulation of triglyceride synthesis. Nevertheless, our findings support their hypothesis that amino acid deficiency has effects on overall macronutrient metabolism and, in particular, leads to a restriction of lipid synthesis.

Deprivation of a single amino acid likely results in amino acid-specific changes in gene expression in addition to those mediated by the ISR

In the previously reported analysis of -Cys/+Cys microarray results, we selected genes that were differentially expressed under conditions of both 6-and 42-h culture in treatment medium. Of the 24 genes significantly differentially expressed ($P \leq 0.0001$) after both 6-and 42-h culture in -Cys/+Cys medium (Table 2 of Lee et al., 2008, see appendix 3), 16 remained on the list of genes differentially expressed in both the -Leu/+Leu and -Cys/+Cys conditions reported here. All of the genes known to contain functional AAREs that were differentially expressed in response to both short- and long-term culture in -Cys medium (ATF3, C/EBPβ, SLC7A11, SLC7A1, and TRIB3), along with CARS, MAP1LC3B, VLDLR, ARHGEF2, INHBE, DAF, CBX4. C/EBPc, FLJ10618, MGC16635, and NPIP///LOC339047// LOC440341, were also significantly upregulated in response to leucine deprivation in this study. ASNS, FOXO3A, GADD45A, GCLM, LNK, STC2, TUBE1, TXNRD1, and ZFAND1, which were significantly upregulated in response to both 6- and 42-h culture in -Cys medium were not detected as significantly upregulated in response to -Leu. These genes might be more responsive to cysteine depletion than to depletion of leucine or possibly other amino acids. GLCM and TXNRD1 contain known EpREs and may have been responding to glutathione depletion caused by cysteine deprivation.

Identification of transcriptionally regulated targets of the phospho-eIF2a/ATF4 pathway

Comparison of the genes identified in this study with those previously identified in different studies also aimed at identification of genes that respond to eIF2α phosphorylation and ATF4 induction yielded a limited number of genes that

were identified in more than one study (Figure 3.3). These include C/EBPβ, C/EBPγ, ATF3, SARS, CARS, CBX4, CLCN3, LMO4, and TXNIP, which were identified in this study, as well as ASNS, SLC1A7, NARS, and ERO1L, which were identified in two studies of murine fibroblasts but not in our study with HepG2/C3A cells. It is quite interesting that only three of these 13 genes (C/EBPβ, ATF3, and ASNS) have been extensively studied as ISR target genes. C/EBPγ, CBX4, and LMO4 may also be important transcriptional regulators of the ISR. Amino acyl-tRNA synthetases, including CARS and SARS, are likely targets and would serve to preserve synthesis of critical stress-response proteins in the face of amino acid deprivation. The roles of induction of CLCN3 and TXNIP expression are less clear, and confirmation that levels of these proteins increase is still needed. TXNIP binds reduced thioredoxin and inhibits the ability of thioredoxin to reduce other proteins, thus increasing redox stress, inhibiting growth and hypertrophy, and causing increased apoptosis (Patwari et al., 2006). CLCN3 has been localized to late endosomes/lysosomes where it apparently is involved in organelle acidification (Li et al., 2002; Okamoto et al., 2008), and this would be consistent with the increase in macroautophagy observed in nutrient-depleted cells (Tallóczy et al., 2002; Kuma et al., 2004; Komatsu et al., 2005).

SUMMARY

The ISR is generally viewed to be adaptive and to promote cell survival in the face of stress. The downregulation of expression of genes involved in cell growth and division, as well as the global reduction in protein synthesis that is a consequence of eIF2α phosphorylation, is consistent with cell survival under conditions of nutrient limitation. At the same time, the induction of expression of genes involved in amino acid uptake and aminoacyl-tRNA synthesis and reduced expression of lipogenic genes suggest that eukaryotic cells may attempt to adapt to an amino acid-or nutrient-limited

environment by facilitating more efficient utilization of the limited resources (i.e., amino acids, other nutrients) and upregulating stress response genes, while at the same time suppressing overall protein synthesis, cell proliferation, and growth. All of these responses can be seen as consistent with cell survival under conditions of nutrient limitation. We have compiled a list of 120 genes whose expression was differentially expressed in HepG2/C3A cells cultured in either cysteine-or leucine-deficient medium, and this list contains many of the genes known to respond to eIF2α kinase activation and to be components of the ISR. Clearly, amino acid deficiency, including cysteine deficiency in the absence of oxidative stress, induces eIF2α phosphorylation, presumably by activation of GCN2, leading to changes in expression of ATF4 and stress-related target genes. This list of target genes should be useful, as an initial step, in identifying potential components of the ISR as well as possibly identifying some genes that are differentially expressed in response to amino acid deprivation but not other stress conditions. Comparison of genes that were highly upregulated in HepG2/C3A cells cultured in -Cys and also in -Leu medium with lists generated by other investigators for genes induced in murine fibroblasts in response to eIF2α phosphorylation yielded a small list of only nine common genes (C/EBPβ, C/EBPγ, ATF3, SARS, CARS, CBX4, CLCN3, LMO4, and TXNIP). These nine genes are likely to be robust targets of the phospho-eIF2α/ATF4-mediated pathway of transcriptional regulation, although only two of them (C/EBPβ and ATF3) have so far been well characterized as target genes.

CHAPTER 4

GROWING RATS RESPOND TO A SULFUR AMINO ACID-DEFICIENT DIET BY PHOSPHORYLATION OF THE ALPHA SUBUNIT OF EUKARYOTIC INITIATION FACTOR 2 HETEROTRIMERIC COMPLEX AND INDUCTION OF ADAPTIVE COMPONENTS OF THE INTEGRATED STRESS RESPONSE

INTRODUCTION

The ability of an organism to adapt to and cope with stress constitutes a crucial survival advantage, and the study of stress response mechanisms is of great interest in terms of possible applications to health promotion and disease prevention or treatment. Mammalian cells respond to various kinds of stress, including nutritional stress, via pathways that are initiated by phosphorylation of the α subunit of the eukaryotic initiation factor 2 heterotrimeric complex (eIF2α). Mammalian eIF2α kinase 4, which is usually called general control of amino acid biosynthesis, nonderepressing 2 (GCN2) after its yeast ortholog, is responsible for the cellular response to a lack of essential amino acids (Wek et al., 1995). Phosphorylation of eIF2α leads to simultaneous control of global and gene-specific translation, a response that is collectively referred to as the integrated stress response (ISR) (Harding et al., 2003; Harding et al., 2000; Kimball et al., 1991).

A key component of the ISR is an increase in translation of activating transcription factor (ATF) 4 (Kilberg et al., 2009; Shan et al., 2009; Wek et al., 2006; Lu et al., 2004a; Harding et al., 2000). The presence of inhibitory upstream open reading frames (ORF) promotes read-through of the ATF4 translational start codon, preventing ATF4 translation under normal conditions in which ternary complex is

abundant. Under stress conditions in which eIF2B is inhibited by eIF2α-P and ternary complex formation is reduced, ribosomal scanning through the inhibitory upstream ORF start codon is favored so that initiation occurs at the downstream ATF4 ORF start codon, allowing ATF4 to be translated. ATF4 then is involved in upregulation of expression of a subset of genes, including the ATF4 gene at the transcriptional level.

Many of the genes known to be transcriptionally upregulated by the eIF2α-P/ATF4-mediated stress response pathway contain C/EBP-ATF composite sites that are also known as amino acid response elements (AARE) or CCAAT-enhancer binding protein activation transcription factor response elements (Kilberg et al., 2009; Shan et al., 2009; Harding et al., 2000). Genes that have been clearly established to contain a functional AARE and to be transcriptionally induced by the eIF2α-P/ATF4 pathway include those encoding several transcription factors [ATF4, ATF3, C/EBPβ, and C/EBP-homologous protein (CHOP)]; several members of the solute carrier (SLC) family amino acid transporters (sodium-coupled neutral amino acid transporter 20 or SLC38A2); high affinity cationic amino acid transporter 1 (CATl) or SLC7A1; and the transporter subunit of the cystineglutamate exchanger X_{c-} (xCT) or SLC7A11; an enzyme for amino acid biosynthesis [asparagine synthetase (ASNS)]; and a putative protein kinase [nibbles homolog 3 (TRIB3)] (Shang et al., 2010; Kilberg et al., 2009; Shan et al., 2009; Pan et al., 2007; Palii et al., 2006; Ohoca et al., 2005; Sato et al., 2004; Harding et al., 2000).

Studies in GCN2 knockout mice and murine cells have shown that the GCN2 pathway is the major signaling pathway involved in the up- and down-regulation of gene expression in response to amino acid starvation. Studies in murine embryonic stem cells and immortalized embryonic fibroblasts showed that eIF2α phosphorylation was not activated in $GCN2^{-/-}$ cells exposed to leucine-devoid medium (Deval et al., 2009; Zhang et al., 2002). Zhang et al. (2002), further showed that perfused livers of

GCN2$^{-/-}$ mice did not display induction of eIF2α phosphorylation in response to histidine deprivation as occurred in wild-type mice. Anthony et al. (2004) reported that GCN2$^{-/-}$ mice fed a leucine-free diet for 1 h or 6 days did not show increased phosphorylation of hepatic eIF2α or restriction of liver protein synthesis, whereas increased phosphorylation of eIF2α and concomitant decreased protein synthesis were observed in liver of wild-type mice. Similarly, asparaginase treatment of GCN2$^{-/-}$ mice lowered asparagine levels but did not lead to increased phosphorylation of eIF2α, whereas hepatic eIF2α-P levels were increased in wild-type mice and in mice with a liver-specific deletion of a different eIF2α kinase (i.e. eIF2α kinase 3, also known as PERK) (Bunpo et al., 2009).

Because the models used to study eIF2α-kinase-mediated responses to amino acid deficiency have commonly used media or diets devoid of one or more essential amino acids, we asked whether eIF2α-kinase-mediated responses would be induced in animals fed a more typical diet that was marginal in essential amino acid composition but not as imbalanced as one in which a single essential amino acid is totally absent. To answer this question, we fed rats soy protein-based diets that were either adequate (+SAA) or limiting in sulfur-containing amino acids (-SAA) and evaluated eIF2α phosphorylation and protein expression levels for ATF4 and other ISR-target genes in the livers of these rats.

MATERIALS AND METHODS

Diets

Rats were fed a semipurified diet that contained 100 g of soy protein isolate plus 3.4 g L-methionine/kg diet (+SAA) or that contained 100 g of soy protein-isolate but no supplemental SAA (-SAA) (Table 4.1). L-Threonine and L-lysine were added to both diets to ensure that the -SAA diet would be limiting only in SAA. These experimental diets were mixed with an equal volume of a hot 0.003% (wt:v) agar solution, cooled, refrigerated, and cut into cubes for feeding. The -SAA diet provided 1.1 g of methionine plus 1.1 g of cyst(e)ine, or a total of 2.46 g of methionine equivalents (1.24 g cystine = 1.0 g methionine)/kg diet. With the addition of L-methionine, the +SAA diet provided a total of 5.86 methionine equivalents.

Table 4.1. Composition of experimental diets

Ingredient	+SAA	-SAA
	g/kg diet	
Soy protein isolate (86% purity)	100	100
L-Methionine	3.40	0
L-Threonine	1.82	1.82
L-Lysine	0.58	0.58
Cornstarch	497.5	497.5
Dextrinized cornstarch	165	165
Sucrose	94.2	97.2
Cellulose	50	50
Soybean oil	40	40
AIN-93-M vitamin mix	35	35
AIN-93-M mineral mix	10	10
Choline bitartrate	2.5	2.5
Tetra-butylhydroquinone	0.008	0.008

Rats, treatments, and sample collection

Male Sprague-Dawley rats (N = 24) that were ~5 weeks old and weighed ~120 g were purchased from Harlan Sprague Dawley and housed in individual polycarbonate cages with corncob bedding at 20°C and 60-70% humidity with light from 2100 to 0900 h. Rats were allowed free access to diet and water at all times. All rats were fed the +SAA diet for 1 week prior to experimental group assignment. At the end of the acclimation week, the rats were distributed among 4 treatment groups using a random block design to ensure an even distribution of larger and smaller rats. Rats were fed the assigned experimental diets, with fresh diet given at the beginning of each dark cycle each day. Feed intake and body weight were measured at intervals. On day 8, 1 group of rats fed each diet was killed between 0800 and 0900 h (feed-deprived) and the other group fed each diet was killed between 1400 to 1500 h (fed). Rats were anesthetized with CO_2 and quickly decapitated. The liver was rapidly removed, rinsed with ice-cold saline, blotted, weighed, and quickly frozen in liquid nitrogen. Frozen tissues were stored at -80°C until analysis. Rats were killed and samples were collected in the order of assigned blocks, but rats within each block were killed randomly. All animal procedures were approved by the Cornell University Institutional Animal Care and Use Committee.

Analysis of protein expression levels

Rat liver was homogenized in TNESV lysis buffer supplemented with protease and phosphatase inhibitors [50 mmol/L Tris, pH 7.5, 1% (v:v) Nonidet P-40, 2 mmol/L EDTA, 150 mmol/L NaCl, and 10 mmol/L sodium orthovanadate] supplemented with 1 × PhosSTOP phosphatase inhibitor cocktail (Roche Applied Science) and 1 × Complete Protease Inhibitor Cocktail (Roche), to form 20% (wt:v) homogenates. Homogenates were centrifuged at 4°C at 18,000×g for 20 min to obtain

the soluble fraction. Nuclear extracts of liver were obtained by the method of Sha et al. (Sha et al., 2009). Protein concentration was determined using the bicinchonic acid assay (Pierce) and equal amounts of total protein were loaded for western blotting. For western blotting, 50 μg of total supernatant protein from each sample was separated by one-dimensional SDS-PAGE (12% wt:v acrylamide) and electroblotted overnight onto 0.45-μm (pore size) Immobilon-P PVDF membranes (Millipore). Membranes were immunoblotted for proteins of interest using the following antibodies: anti-pS^{51}-eIF2α, anti-eIF2α (total), and anti-β-actin from Cell Signaling Technology; antiATF4 (gift from M.S. Kilberg, University of Florida, Gainesville, FL); anti-ATF3 (C-1 9), anti-ASNS (C-14), anti-CHOP, anti-heat shock protein 5A (HSP5A), anti-TRIB3, and anti-growth arrest and DNAdamage-inducible protein 34 (GADD34) (H-1 93) from Santa Cruz Biotechnology; anti-SLC7All (xCT) and anti-cysteinyl-tRNA synthetase (CARS) from Abcam Inc; and anti-cystathionine γ-lyase (CTH) from Abnova. Bands were visualized using horseradish peroxidase-coupled secondary antibodies and chemiluminescent substrates (West Dura, Pierce) and autoradiography. Visualization, normalization, and analysis of the bands were done as described by Dominy et al. (2007).

Measurement of hepatic glutathione and cysteine levels

Total intracellular cyst(e)ine levels were measured by a modified acid ninhydrin method of Gaitonde (1967) as described by Dominy et al. (2007). Total glutathione levels were measured by the method of Cereser et al. (2001) (for more detailed methodological procedures see appendix 1).

Statistical analysis

Results for the 2 dietary treatment groups and 2 time points were compared by 2-way ANOVA. Liver GSH values were transformed to $\log_{10}$ prior to ANOVA. Results for all variables except nuclear CHOP levels were similar for both time points. Thus, except for the nuclear CHOP results, data for both time-points were collapsed. Results for the 2 dietary treatments were analyzed by Student's t test. Significance was accepted at $P \leq 0.05$.

RESULTS

Upon switching from the +SAA diet to the -SAA diet at the beginning of the experimental period, feed intake of the -SAA group over the first 4 days was less ($P \leq 0.01$) than that of rats that continued to consume the +SAA diet and weight gain over the same 4 days was only 10% that for +SAA rats (Figure 4.1A). Rats appeared to adapt to the -SAA diet, however, as both feed intake and weight gain of the -SAA rats were much greater during days 5-7 of the diet treatment period. In fact, the feed intake of the -SAA rats for the last 3 days of the diet treatment period did not differ from that of +SAA for control rats and mean weight gain was 66% of that for the +SAA rats. Although the -SAA rats were smaller than the +SAA rats (152 ± 3 vs. 181 ± 2 g after 7 days of treatment), the differences in feed intake and weight gain remained when data were expressed relative to body weight (Figure 4.1B).

Hepatic thiol levels, measured at the end of the 7-day dietary treatment period, were markedly lower in the -SAA rats than in the +SAA rats. Total cysteine in the -SAA rats was 49% of that in +SAA rats, whereas total glutathione was only 17% of the level in +SAA rats (Figure 4.2).

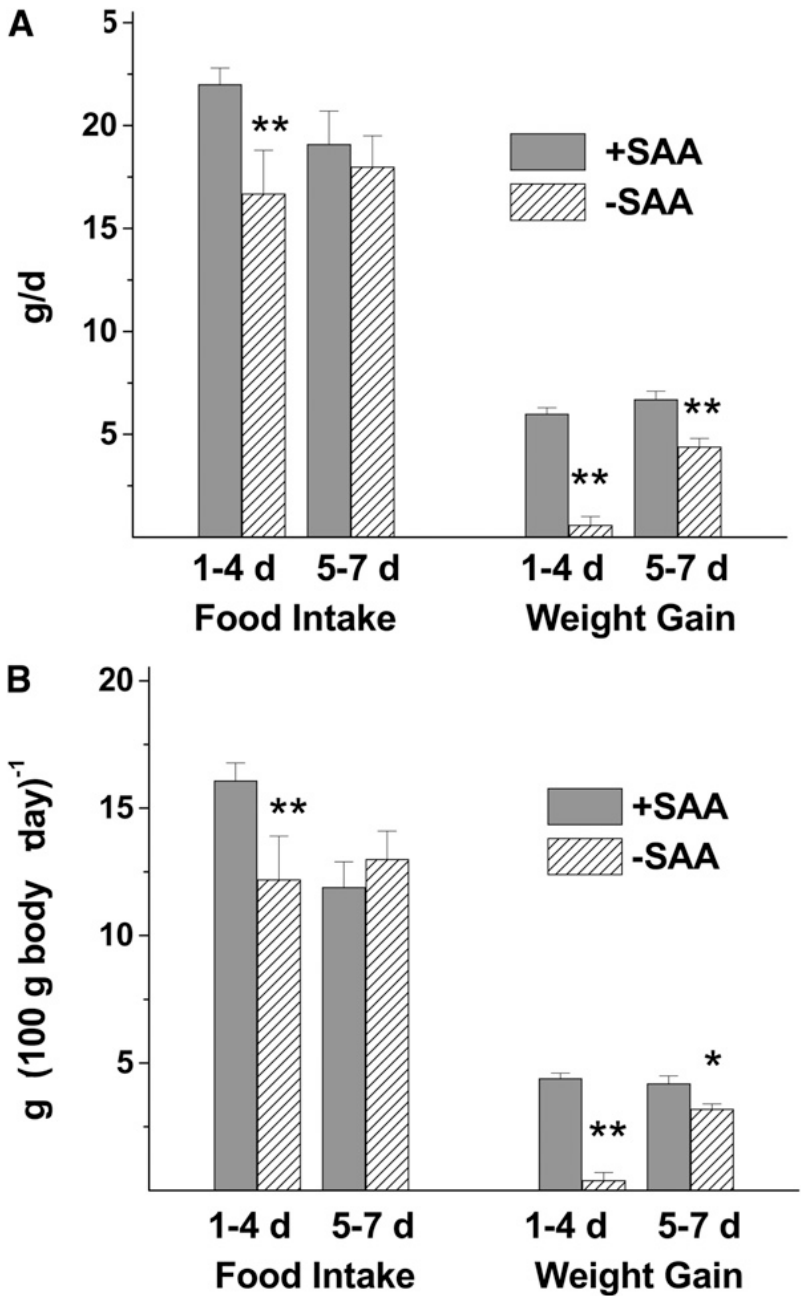

Figure 4.1. Absolute (A) and relative (B) daily weight gain and daily feed intake of rats fed the -SAA and +SAA diets for 7 days. Gains and intakes were determined over 2 periods, days: 1–4 (mean of 4 days) and days: 5–7 (mean of 3 days). In B, data are expressed relative to body weight at days 0 or 4, as appropriate. Values are means ± SEM, N=12. Asterisks indicate different from +SAA: $^{**}P \leq 0.001$, $^{*}P \leq 0.05$.

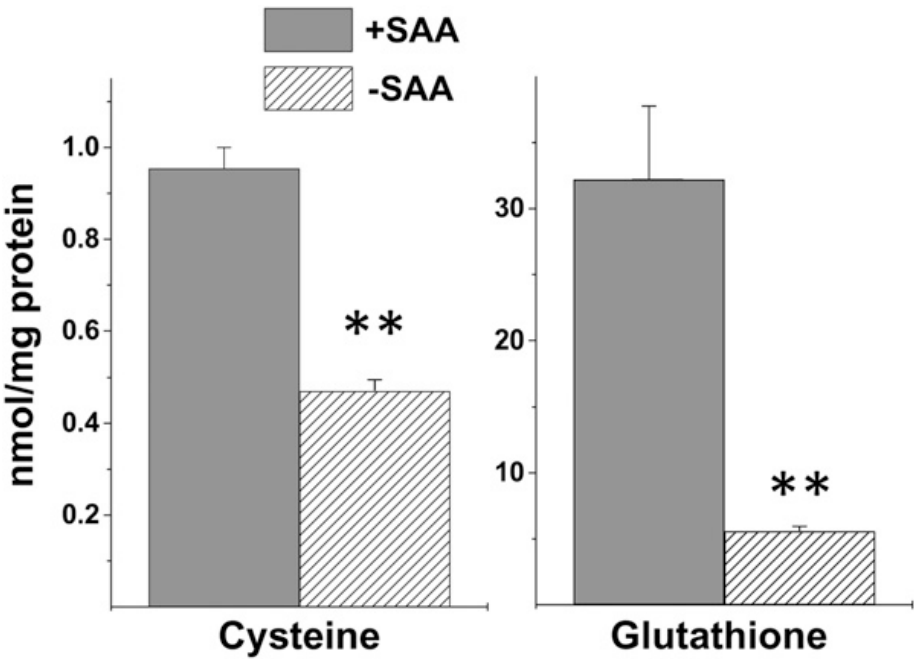

Figure 4.2. Total cysteine and total glutathione levels in liver of rats fed the -SAA and +SAA diets for 7 days. Values are means ± SEM, N=12. Asterisks indicate different from +SAA: $^{**}P \leq 0.001$, $^{*}P \leq 0.01$.

Phosphorylation of eIF2α (Ser^{51}) was induced in rats fed the -SAA diet (Figure 4.3A & B). Values for eIF2α-P and total eIF2α did not differ for rats killed at the 2 different time-points (feed-deprived vs. fed). The amount of eIF2α-P in liver of the -SAA rats was 1.6 times that of the +SAA rats, whereas total eIF2α levels did not differ for livers of the -SAA and +SAA rats.

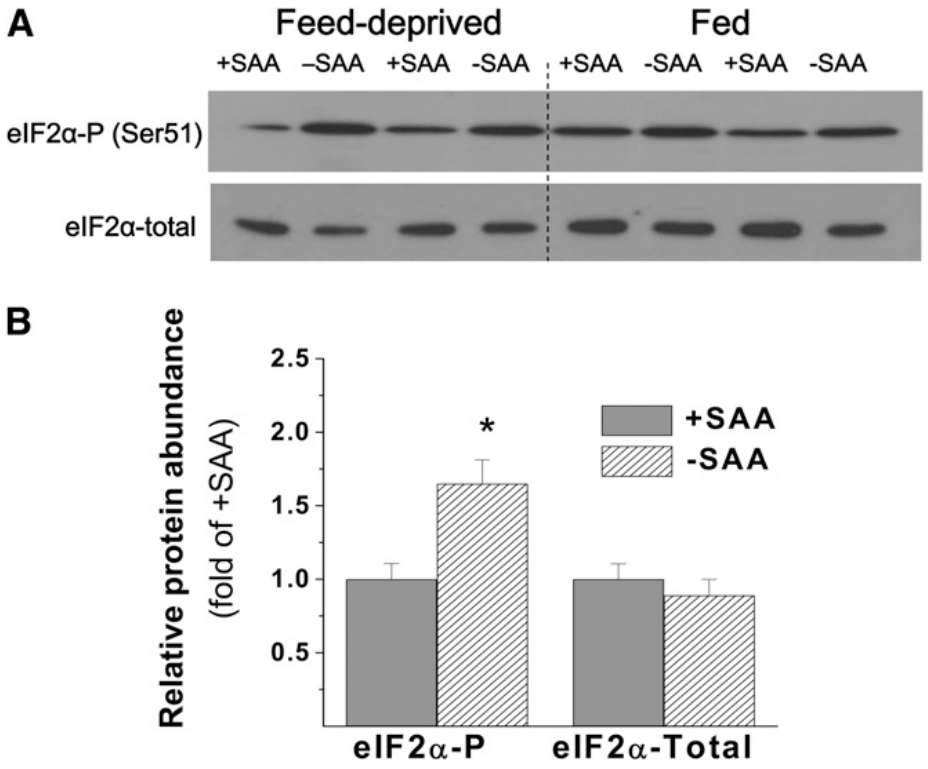

Figure 4.3. Western blots (A) and mean fold values (B) for phosphorylation of Ser^{51} of eIF2α in liver of rats fed the -SAA vs. +SAA diet. (A) Examples of western blots for rats killed at either the end of the light period (feed-deprived) or at the mid-point of the dark period (fed). Equal amounts of total protein were loaded per lane. (B) Mean fold values for phospho-eIF2α and total eIF2α relative to the mean density for the +SAA rats. Values are means ± SEM, N=12. Asterisks indicate different from +SAA: $*P \leq 0.05$.

Protein levels for ATF4 in liver of the -SAA rats was 4.5 times that in liver of the +SAA rats (Figure 4.4). Expression of several other proteins whose genes contain a functional AARE and are induced by ATF4 was also higher in the -SAA rats than in the +SAA rats. ATF3, ASNS and SLC7A11 protein levels in liver of the -SAA rats were 2.4, 1 .6, and 2.5 times, respectively, those for +SAA rats. Similarly, the level of CARS and CTH, 2 proteins involved in SAA metabolism whose gene expression

(mRNA) levels were upregulated (>2-fold difference; $P < 0.0001$) in our previous studies of cysteine and leucine deprivation in HepG2 cells (Lee et al., 2008; A.K. Sikalidis, J-I. Lee, and M.H. Stipanuk, unpublished data), were also induced in liver of -SAA rats to 1.6 and 2.1 times, respectively, the levels in liver of +SAA rats.

On the other hand, expression of CHOP was not detectable in liver homogenates from either group of rats and protein expression levels for TRIB3 (a direct ATF4 target) and growth arrest and DNA damage protein 34 [GADD34; also known as PPP1R15A for protein phosphatase 1, regulatory (inhibitor) subunit 15A, which is a downstream target], were not increased. Because CHOP was not detectable in the whole tissue preparations, we also assessed CHOP expression in nuclear extracts. Only basal low levels of CHOP were measured in the nuclear extracts and these levels did not differ for the 2 dietary groups, although they did differ for the fed and feed-deprived states in both diet groups (Figure 4.4A), with expression levels being lower ($P \leq 0.05$) in feed-deprived rats than in fed rats.

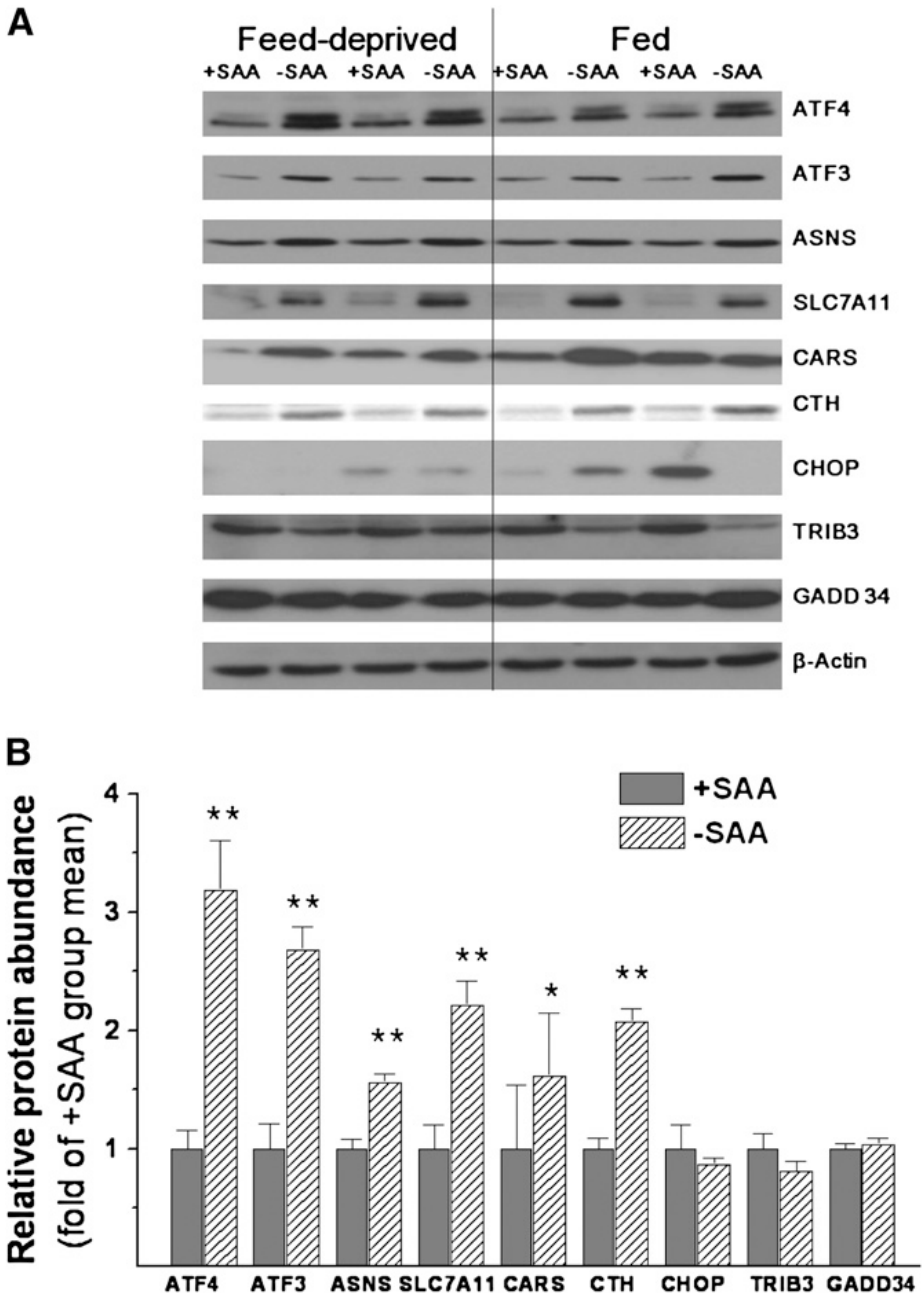

Figure 4.4. Western blots (A) and mean fold values (B) for protein expression levels of ATF4 and its transcriptional targets in liver of rats fed the -SAA and +SAA diets. (A) See Figure 3A legend for details. (B) Mean fold values for protein levels relative to the mean density for the +SAA rats. Values are means ± SEM, N=12, except for values for CHOP. For CHOP, values are reported only for rats killed in the fed state (N=6), because values were different, $P < 0.05$, for rats killed in the feed-deprived state and values are for nuclear extracts instead of total soluble protein. Nuclear CHOP in -SAA rats was 0.44-fold that of the +SAA rats. Asterisks indicate different from +SAA: $^{**}P \leq 0.001$, $^{*}P \leq 0.01$.

DISCUSSION

Rats fed the -SAA diet displayed reduced growth and low cysteine and glutathione levels. Rats in this study were fed a -SAA diet that provided ~70% of the mean SAA requirement for growing rats but was adequate in all other amino acids (Bella et al., 1999; Bella et al., 1996; Stockland et al., 1973). The diminished levels of hepatic cysteine and glutathione in rats fed the -SAA diet demonstrated that the rats were indeed deficient in SAA. Hepatic glutathione levels were reduced to a greater extent (17% of control levels) than hepatic cysteine levels (49% of control), suggesting that body cysteine levels were sustained at the expense of the glutathione pools and most likely that SAA were used preferentially for protein synthesis compared with glutathione synthesis under conditions of dietary SAA limitation. This is consistent with previous reports that glutathione levels become depleted at SAA intakes that are marginal but adequate for sustaining protein synthesis and growth (Lee et al., 2004; Stipanuk et al., 1992).

Consistent with the inadequate level of dietary SAA and with the well-established effects of essential amino acid-deficient diets on growth of rats, rats fed the -SAA diet grew more slowly throughout the 7-day experimental period. Over the first 4 days of the dietary treatment period, -SAA rats gained only 10% as much weight as did rats fed the +SAA diet. This difference was reduced markedly over the subsequent 3 days of treatment, however, with rats fed the -SAA diet gaining 66% as much weight as did rats fed the +SAA diet. During the first 4 days of the experimental period, the -SAA rats consumed only 76% as much diet as the rats that continued to be fed the +SAA diet and the reduced food intake undoubtedly contributed to their low weight gain over this period. However, food intake for the subsequent 3 days did not differ between rats fed the -SAA and +SAA diets. Although food intake of the -SAA rats increased by only 1.3 g/d (7.8%) between the first and second halves of the

experimental period, mean daily weight gain increased by 3.8 g (633%). Thus, the increase in food intake cannot explain the much better growth of rats during the second part of the dietary treatment period. This suggests that the more adequate rate of growth during the latter time-period may have mainly been due to metabolic adaptations of the rats to allow growth when consuming the nutrient-limited diet. Such a metabolic adaptation could relate to a suppression of SAA degradation and/or to a more efficient utilization of the limited amounts of SAA available. Nevertheless, despite an apparent adaptation to the diet, the mean daily weight gain during the period from day 4 to 7 (expressed relative to day 4 weight) remained less ($P \leq 0.05$) for -SAA rats (3.2 ± 0.3 g/100 g body weight) than for +SAA control rats (4.3 ± 0.2 g/100 g body weight), which is consistent with the fact that animals cannot completely adapt to a diet lacking in an essential nutrient that they are not able to synthesize.

We did not include a pair-fed control group in this study, because we did not anticipate a difference in food intake between the 2 groups. In previous studies in which rats were fed a SAA-deficient diet that contained 100 g casein/kg, food intake was the same or slightly greater than that of rats fed an adequate diet that contained either 100 g casein/kg diet supplemented with methionine or 200 g casein/kg without SAA supplementation (Bella et al., 1999; Bella et al., 1996). Nevertheless, because we collected samples on day 8, several days after food intake of the -SAA rats had returned to levels not significantly different from those for +SAA rats, a major effect of food intake on our results seems unlikely. Some of the consequences of the -SAA dietary treatment in this study could have been due to the modest early restriction of food intake rather than to the lack of SAA and a pair-fed group or a longer feeding period would be appropriate for any future studies using the -SAA diet prepared with 100 g soy protein isolate/kg.

Rats fed the -SAA diet displayed increased phosphorylation of eIF2α

Phosphorylation of eIF2α was clearly induced in liver of intact rats fed the -SAA diet for 7 days, which is consistent with the activation of a stress-induced eIF2α kinase, presumably GCN2, in response to SAA deprivation. In contrast to previous studies of the role of GCN2 and/or eIF2α phosphorylation in the response of intact animals to amino acid deprivation (Guo et al., 2007; Anthony et al., 2004; Anthony et al., 2001), the diet used in this study was limiting in, but not devoid of, an essential amino acid. Thus, our results extend the observations in mice to the situation in which the diet is only partially deficient in an essential amino acid (i.e. SAA), to an amino acid pattern that is more representative of that in diets consumed by humans and animals, and to a diet that has been shown to support both growth and longevity (Zimmerman et al., 2003; Orentreich et al., 1993; Hosokawa et al., 1988).

One of the consequences of eIF2α phosphorylation is a reduction in the rate of protein synthesis. Both absolute liver weight and relative liver weight were lower in rats fed the -SAA diet than in rats fed the +SAA diet, with the relative liver weights being 3.5 ± 0.3 and 4.6 ± 0.5 g/100 g body weight for the -SAA and +SAA groups, respectively. The lower relative liver weight in -SAA rats is consistent with diminished protein synthesis in liver in response to eIF2α phosphorylation, although, of course, we cannot exclude an effect of protein turnover, because we did not measure synthesis rates directly in this study. Suppression of hepatic protein synthesis might facilitate increased utilization of the limiting amino acid for muscle protein synthesis and growth and this interpretation would be consistent with the observation of no difference in the relative weights of the gastrocnemius and planteris muscles and epididymal adipose depot between rats fed the -SAA and +SAA diets (data not shown). This interpretation of our data is supported by the earlier work of Anthony et al. (2004) with GCN2 knockout mice. Wild-type control mice fed a leucine-free diet

for 6 days lost liver mass while preserving muscle mass, whereas GCN2$^{-/-}$ mice fed the same leucine-free diet maintained their liver mass but lost muscle mass and lost more body mass than the wild-type mice. Furthermore, the rate of hepatic protein translation was reduced in wild-type but not in GCN2$^{-/-}$ mice fed the leucine-free diet, suggesting that translational repression in the liver of rats fed diets limiting in essential amino acids may play an important role in conserving muscle and body mass.

Rats fed the -SAA diet displayed increased expression of proadaptive proteins that are known components of the ISR

Our protein expression data indicate that the eIF2α-P/ATF4-mediated ISR was induced in liver of the -SAA rats compared with the +SAA rats. In particular, we saw large increases in the relative abundance (amount per g total soluble protein) of ATF4, ATF3, SLC7A11, and CTH, along with smaller fold-increases in ASNS and CARS. ATF4 and ATF3 are important transcription factors that initiate and regulate the ISR, whereas the other proteins are involved in amino acid transport and metabolism and have been reported to be induced under stress conditions that initiate eIF2α phosphorylation (Lee et al., 2008; Lu et al., 2004a; Lu et al., 2004b; Harding et al., 2003) (A.K. Sikalidis, J-I. Lee, and M.H. Stipanuk, unpublished data). Induction of genes involved in amino acid uptake and aminoacyl-tRNA synthesis may play an important role in the adaptation of rats to diets limiting in essential amino acids. In the case of SAA deprivation, the strong induction of SLC7A11 (xCT), which is the transporter subunit of the cystineglutamate exchanger X_c^-, of CARS, the cysteinyl-tRNA synthetase, and of CTH, which is involved in cysteine synthesis from homocysteine and serine as well as in desulfhydration of cysteine, suggests that cysteine utilization is highly regulated under conditions of SAA deficiency.

Rats fed the -SAA diet did not display increased expression of pro-apoptotic proteins that are known components of the ISR

Despite the clear induction of eIF2α phosphorylation and increased expression of ATF4, ATF3, ASNS, SLC7A11, CARS, and CTH in liver of the -SAA rats, several other downstream targets of the eIF2α-P/ATF4 signaling pathway were not induced. Expression of CHOP, which has a well-established role in the induction of apoptosis (Su and Kilberg, 2008; Tamaki et al., 2008; Marciniak et al., 2004; Oyadomari et al., 2004), was clearly not induced in liver of -SAA rats, being nearly undetectable, even in nuclear extracts. Consistent with the lack of upregulation of CHOP in liver of rats fed the -SAA diet, levels of TRIB3 and GADD34 proteins, whose transcriptional induction is dependent upon CHOP (Shang et al., 2010; Ohoka et al., 2005), were not elevated. The lack of increased expression of CHOP, TRIB3, or GADD34 proteins suggests that activation of the GCN2/eIF2α-P/ATF4 signaling pathway does not necessarily result in induction of all downstream target genes and is consistent with the variable induction of mRNA and/or protein levels for ISR genes reported previously for HepG2 cells (Palii et al., 2006; Zimmerman et al., 2003) (A. K. Sikalidis, J-I. Lee, and M. H. Stipanuk, unpublished data). Induction of pro-survival and pro-apoptotic components of the ISR might be anticipated to vary with the degree or type of stress and with whether the ultimate response is cell survival or apoptotic death. In the case of this study, although the stress of a modest SAA deficiency was sustained, rats appeared to effectively adapt and showed no obvious adverse effects other than a slightly lower rate of growth.

Induction of the ISR in response to a marginal intake of essential amino acids could play a role in the beneficial effects of amino acid restriction on longevity in animal models

A number of studies have shown an association of eIF2α phosphorylation with induction of a state of stress resistance in cells (Orentreich et al., 1993; Hosokawa et al., 1988). This study now shows that modest SAA restriction can induce the ISR in liver of intact growing rats. Others have shown that lowering the SAA level in the diet can extend lifespan in rats. Zimmerman et al. (2003) reported that lowering the content of SAA in the diet by removing cysteine and restricting the concentration of methionine extended all measured survival variables in various rat strains, and pair-feeding of control rats with a SAA-supplemented diet demonstrated that the increase in survival was not simply due to caloric restriction. Additionally, methionine restriction was shown to decrease mitochondrial oxygen radical production and oxidative damage to mitochondrial DNA and cellular proteins (Sanz et al., 2006). The postulate that eIF2α phosphorylation in response to modest levels of cellular stress can play a key role in building resistance and tolerance toward subsequently imposed more severe stressful conditions has been supported by a variety of *in vitro* studies (Raffaghello et al., 2008; Lu et al., 2004b; Harding et al., 2003; Jousse et al., 2003). Additional studies, however, will be required to assess whether mild essential amino acid limitation can result in a more stress-resistant animal and whether enhanced phosphorylation of eIF2α and its downstream consequences contribute to the favorable effects of SAA restriction on longevity.

CHAPTER 5

TOTAL 4E-BP1 LEVELS ARE ELEVATED IN LIVERS OF RATS FED DIETS LOW IN PROTEIN OR SULFUR AMINO ACIDS

INTRODUCTION

Adaptation to environmental stresses is important for survival. In this context adaptation mechanisms for stressful conditions such as amino acid limitation are widely seen in nature. Because mammals lack a mechanism to store amino acids, a constant supply of them is essential (Wek et al., 2006; Tettweiler et al., 2005). Amino acid availability needs to be ensured so that protein synthesis can occur and support survival, growth and healing processes. Optimization of amino acid uptake and utilization constitutes an important adaptation strategy during amino acid limitation. Such optimization can be achieved via reduction of protein synthesis, decreased growth and cell division, and increased synthesis of particular proteins including amino acid transporters and amino acyl-tRNA synthetases.

Under normal circumstances, translation initiation requires the formation of two main complexes, the 43S pre-initiation complex and the eukaryotic translation initiation factor 4F (eIF4F) complex (Yamaguchi et al., 2008). The formation of the 43S pre-initiation complex begins with the formation of a ternary complex composed of Met-tRNA$_i^{Met}$, eukaryotic initiation factor 2 (eIF2), and GTP (Holcik et al., 2005, Gabauer et al., 2004). Once the ternary complex is formed it then associates with the 40S pre-initiation complex and other initiation factors to generate the 43S pre-initiation complex. On the other hand, the eIF4F complex is comprised of three subunits: eIF4E, which is the cap-binding protein; eIF4A, which is an RNA helicase; and eIF4G, which serves as a scaffolding protein (Gabauer et al., 2004). The eIF4F

complex associates with the 5′-cap structure of the mRNA through the binding of the eIF4E subunit to the m^7GTP (m^7GpppN) cap at the 5′-end of the mature mRNA. The eIF4G subunit recruits the 43S pre-initiation complex to the mRNA strand through the binding of the 43S pre-initiation complex to eIF3. The 43S pre-initiation complex and the eIF4 proteins thus form a translation competent 48S complex, which begins to scan for the AUG (start codon) to begin translation. Stable binding of the 43S pre-initiation complex to the AUG codon triggers the joining of the large (60S) ribosomal subunit, associated with the hydrolysis of eIF2-bound GTP and release of eIF2-GDP, to form the 80S complex. The 80S complex can catalyze the formation of the first peptide bond in the generation of the polypeptide chain (Gabauer et al., 2004). To begin another round of initiation, eIF2-GDP is recycled to eIF2-GTP by a reaction catalyzed by eIF2B, the guanine nucleotide exchange factor for eukaryotic initiation factor 2 (Gabauer et al., 2004). The 43S pre-initiation complex is needed for both cap-dependent and cap-independent initiation, whereas the eIF4F complex is needed only for cap-dependent initiation.

In situations of nutrient deprivation or other stresses, translation initiation is regulated to allow for immediate and rapid response to changes in the environment. Two mechanisms have been found to prevent the assembly of the aforementioned complexes, one involving the regulation of the ability of eIF4E-binding protein (4E-BP) to bind to eIF4E and block the formation of the eIF4F complex and another involving the regulation of the formation of the ternary complex which in turn affects the rate of formation of the 43S pre-initiation complex (Holcik et al., 2005).

The 4E-BPs constitute a family of three small (10-12 kDa) acidic proteins that act as repressors of translation initiation (Gingras et al., 1999). The three members of the 4E-BP family, 4E-BP1, 4E-BP2, and 4E-BP3, interact with eIF4E, the cap-binding component of the eIF4F complex, which recruits mRNA to the polysomes (Poulin et

al., 1998). eIF4E is present in rate-limiting amounts so the binding of eIF4E by 4E-BP inhibits complex assembly and thus represses cap-dependent translation (Mader et al., 1995). The binding of eIF4E is reversible and is dependent on the phosphorylation state of 4E-BP. Hypophosphorylated 4E-BP interacts with eIF4E whereas hyperphosphorylated 4E-BP does not (Gingras et al., 1999). The three members of the 4E-BP family are homologous to one another, with most striking homology in the middle region of the protein that contains the eIF4E binding motif and the residues that are phosphorylated. The redundancy in genes encoding4E-BP emphasizes the importance of eIF4E binding (Poulin et al., 1998).

The mammalian target of rapamycin (mTOR) pathway is responsible for regulating the phosphorylation state and eIF4E binding affinity of 4E-BP in response to insulin and other growth factors, amino acids, and cellular energy status. mTOR signaling is positively regulated by growth factors such as insulin, ATP/AMP ratio, and amino acids, especially leucine (Schmelzle, 2000). Under conditions of nutrient availability, active mTOR phosphorylates 4E-BP, rendering it inactive (Schmelzle, 2000). Under these conditions, eIF4E is able to bind to eIF4G, promoting the formation of the eIF4F complex and the initiation of cap-dependent mRNA translation. However, low energy or low amino acid status in cells or a lack of insulin or IGF-1 can reduce the activation state of mTOR. Inactive mTOR is unable to phosphorylate its downstream target 4E-BP, making 4E-BP available to bind to eIF4E and to displace eIF4G. With the eIF4F complex unable to be assembled, cap-dependent translation initiation is inhibited (Haghighat et al., 1995).

The second known mechanism for regulation of translation initiation in response to nutrient deprivation or other stresses acts via the regulation of a group of eIF2α kinases. One of these eIF2α kinases is General Control Nonderepressible kinase 2 (GCN2), which is activated in cells that are deprived of essential amino acids

(Harding et al., 2000). The histidyl-tRNA synthetase (HisRS)-like domain of GCN2 senses the non-aminoacylated tRNAs that are increased during an amino acid deprivation state. With the binding of tRNA, the HisRS domain undergoes a conformational change that propagates the juxtaposed protein kinase domain, eliciting kinase activation (Padyana, 2005). Consequently, GCN2 kinase phosphorylates the Ser^{51} residue of the alpha subunit of eukaryotic initiation factor 2 (eIF2α). The phosphorylation of eIF2α reduces the dissociation rate of eIF2 from eIF2B, thereby sequestering eIF2B. As a result, GDP-GTP exchange is inhibited (Gabauer et al., 2004). The lack of eIF2-GTP reduces the formation of the ternary complex and hence the 43S pre-initiation complex, and global mRNA translation in suppressed (Gabauer et al., 2004; Harding et al., 2000).

Somewhat paradoxically, the phosphorylation of eIF2α also increases the translation of a few specific existing mRNAs. Upon eIF2α phosphorylation, upstream open reading frames (uORFs) allow for the upregulated translation of certain proteins, with activating transcription factor 4 (ATF4) being the best characterized. ATF4 induces the expression of other transcriptional regulators, solute carrier family acid transporters, genes involved in amino acid biosynthesis, and genes involved in cell cycle and DNA repair (Harding et al., 2000). The downstream response of eIF2α phosphorylation has been termed the integrated stress response (ISR) and is associated with cell survival and stress resistance. The ISR is so-named because other kinases in addition to GCN2 also phosphorylate eIF2α to activate the ISR. These other kinases include the heme-regulated inhibitor kinase (HRI), which is stimulated by heme depletion; protein kinase activated by double-stranded RNA (PKR), which is stimulated by viral infection; and PKR-like endoplasmic reticulum kinase (PERK), which is activated under endoplasmic reticulum (ER) stress (Gabauer et al., 2004).

In a previous animal study (Sikalidis and Stipanuk, 2010), we noticed a marked increase in levels of total 4E-BP1 in rats fed a sulfur amino acid-deficient diet that was not accompanied with a change in the proportion of 4E-BP1 in the hyperphosphorylated state or in the phosphorylation of p70S6 kinase. As these results suggested that mTOR signaling was not suppressed in the livers of rats fed the sulfur amino acid deficient diet, we decided to investigate further how amino acid/protein deficiency under various dietary regimens affect 4E-BP1 levels, mTOR signaling, and eIF2α phosphorylation.

In this study, we exposed rats to a variety of diets that were variably deficient in protein, energy, or both, and then examined changes in both 4E-BP total abundance and its phosphorylation state (target of mTOR), ribosomal protein S6 (rpS6 a target of p70S6 kinase which is a direct target of mTOR) phosphorylation state, and eIF2α phosphorylation state (target of GCN2). Thus we aimed to investigate the possibility of an increase in 4E-BP1 expression as a third mechanism in regulating translation initiation and whether this increased 4E-BP1 expression was associated with eIF2α phosphorylation and/or independent of mTOR activation state.

We hypothesized that a deficiency of either protein or energy at the level of the liver would lead to a decrease in hyperphosphorylation of 4E-BP1, as predicted by our present understanding of signaling through the mTOR pathway. In addition, we hypothesized that total 4E-BP1 would also be upregulated in response to a deficiency in protein or essential amino acids but not in response to energy or feed intake per se. Additionally, we conducted a time-course study with one of the deficient diets to determine how rapidly the changes in 4E-BP1 occur.

MATERIALS AND METHODS

Diets. All experimental diets were prepared by Dyets Inc. (Bethlehem, PA) in powdered form. The composition of the experimental diets is shown in Table 5.1. Diets varied in amount of soy protein or in the amount of L-methionine (M) or L-cystine (C). For diet preparation, the powdered diets were mixed with an equal volume (1 L/kg diet) of hot agar solution (3 g/L), and the mixture was cooled at room temperature, refrigerated and cut into cubes for feeding. Three of these diets supported maximal or near-maximal rates of growth and were considered control group for the various deficient diets.

Study 1

Animals. Male Sprague-Dawley rats that weighed approximately 110 g were purchased from Harlan Sprague Dawley (Indianapolis, IN) and were housed in individual polycarbonate cages containing corncob bedding, in a holding room maintained at 20°C and 60–70% humidity with light from 21:00 to 09:00 h. Rats were allowed free access to diet and water at all times (except for the pair-fed animals).

All rats were fed the 20% soy protein diet, which provided 4.9 Met equivalents/kg of diet, for 1 week for acclimation purposes prior to experimental group assignment. At the end of the adaptation week, rats were grouped by weight. Rats within each block were randomly distributed among the following dietary treatment groups: 20% soy protein diet, 10% soy protein diet, 10% soy protein supplemented with 0.34% methionine diet, protein free diet, 20% soy protein diet pair-fed to intake of the protein-free group, 0.23% methionine/0.35% cysteine amino acid diet, 0.11% methionine/0.35% cysteine amino acid diet, 0.23% methionine amino acid diet, and 0.11% methionine amino acid diet.

Table 5.1. Composition of experimental diets

Ingredient	Control 10% soy + 0.34% M	10% soy	Control 20% soy	Protein free	Control 0.23%M / 0.35%C	0.11%M / 0.35%C	0.11% M	0.23% M
	g/kg							
Soy protein isolate (86% purity)	100	100	200	0	0	0	0	0
L-AA mix*	0	0	0	0	172	172	172	172
L-Methionine	3.40	0	0	0	2.3	1.1	1.1	2.3
L-Threonine	1.82	1.82	0	0	0	0	0	0
L-Lysine	0.58	0.58	0	0	0	0	0	0
L-Cystine	0	0	0	0	3.5	3.5	0	0
Cornstarch	497.5	497.5	377.5	471.9	389.9	389.9	389.9	389.9
Dextrinized cornstarch	165	165	155	193.8	155	155	155	155
Sucrose	94.2	97.6	100	125	102.4	103.6	107.1	105.9
Cellulose	50	50	50	62.5	50	50	50	50
Soybean oil	40	40	70	87.5	70	70	70	70
TBHQ	0.008	0.008	0.014	0.018	0.01	0.01	0.01	0.01

Table 5.1 (Continued)

Ingredient	Control		Control		Control			
	10% soy + 0.34% M	10% soy	20% soy	Protein free	0.23%M / 0.35%C	0.11%M / 0.35%C	0.11% M	0.23% M
Mineral mix	35	35	35	43.8	35	35	35	35
Vitamin mix	10	10	10	12.5	10	10	10	10
Choline bitartarate	2.5	2.5	2.5	3.1	2.5	2.5	2.5	2.5
Sodium bicarbonate	0	0	0	0	7.4	7.4	7.4	7.4
Methionine equivalents	5.9	2.5	4.9	0	6.6	5.4	1.1	2.3

* L-AA mix (g/kg): L-Arginine 6.3, L-Histidine 4.5, L-Tyrosine 9.2, L-Phenylalanine 8.7, L-Leucine 15.3, L-Isoleucine 8.4, L-Valine 9.9, Glycine 3.1, L-Proline 20.4, L-Glutamic acid 36.2, L-Alanine 4.5, L-Aspartic acid 11.3, L-Serine 9.4, L-Lysine-HCl 16.1, L-Threonine 6.6, L-Tryptophan 2.1.
- M= L-Methionine ; C= L-Cystine

Experimental diets were provided to rats for one week, with fresh diet being given daily at the beginning of each dark cycle. Feed intake and body weights were measured daily over the experimental period. On each day the pair-fed group was given control diet (20% soy diet) at the amount the protein free group had consumed the previous day.

At the midpoint (between 12:50 and 14:20 h) of the dark cycle (feeding cycle) on day 7, rats were anesthetized and killed for the collection of samples. Previous experiments (Sikalidis and Stipanuk, 2010) revealed no difference in the choice of the termination time-point (fed vs. overnight fast) on phosphorylation status of eIF2α, 4E-BP1, or S6K. Liver and muscle (gastrocnemius) tissues were rapidly removed, rinsed with ice-cold saline, blotted, weighed and frozen in liquid nitrogen. Frozen tissues were stored at -80°C until analyses were performed. Rats were euthanized and samples were collected in the order of assigned weight blocks, but rats within each block were killed randomly. All animal procedures were approved by the Cornell University Institutional Animal Care and Use Committee.

Study 2

Animals. Male Sprague-Dawley rats that weighed approximately 115 g were housed with light from 6:00 to 18:00 h. Rats were allowed free access to food (with the exception of the pair-fed group) at all times. All rats were fed an amino acid-based control diet that contained 0.23% methionine/ 0.35% cysteine for 1 week prior to experimental group assignment. At the end of the acclimation week rats were blocked and rats within each block were assigned to 9 treatment groups (3 diets × 3 time-points, 4 rats per group). Rats were fed the control 0.23% methionine/0.35% cysteine diet, the 0.11% methionine diet or were pair-fed with control diet at the intake of

0.11% methionine group. Rats were fed the assigned experimental diets, with fresh diet given at the beginning of the dark cycle each day. Each day the pair-fed group was given control diet (0.23% methionine/0.35% cysteine) at the amount the 0.11% methionine group had consumed the previous day. Feed intake and body weight were measured daily. Rats were killed at the beginning of the light cycle on days 1, 3 and 5 (4 rats from each of the 3 dietary treatments). For termination, rats were quickly decapitated so as to avoid anesthetics that might alter the plasma insulin and glucose measurements. The liver was rapidly removed, quickly rinsed with ice-cold saline, blotted, weighed and quickly frozen in liquid nitrogen. Frozen tissues were stored at -80°C until analysis.

Blood parameters. Blood glucose was assessed immediately after decapitation with a commercial glucometer (Contour, Bayer GmbH). Plasma insulin and IGF-1 measurements were done at the Hormone Assay & Analytical Services Core, Vanderbilt Diabetes Research and Training Center, supported by NIH grant DK20593. Plasma insulin was assayed via a radioimmunoassay (RIA), using a double antibody procedure by the Hormone Assay and Analytical Services Core, Vanderbilt Diabetes Research and Training Center. Plasma IGF-1 was measured by a multi-plexed assay using X-map technology via the Luminex100 system by the Hormone Assay and Analytical Services Core, Vanderbilt Diabetes Research and Training Center.

Analysis of protein expression levels in liver homogenates. Rat livers were homogenized to form a 20% (w/v) homogenate [1 part of liver (mg) plus 4 parts of buffer (μL)] in TNES lysis buffer with 1× mammalian protease inhibitor cocktail (Sigma-Aldrich) and 1× PhosStop phosphatase inhibitor cocktail (Roche Applied Science) as well as 50mM of NaF. The same procedure was followed for the muscle samples. Homogenates were centrifuged for 20 min at 4^{o}C at 18,000×g to obtain the soluble protein fraction. The BCA assay was used to determine total protein

concentration of the soluble fraction. Samples containing equal amounts of protein (50 μg) were loaded onto a 12% w/v acrylamide gel and run using a large-vertical electrophoresis apparatus at a constant I=25 mA per gel. The gel was electroblotted overnight onto a 0.45 μm Immobilin-P PVDF membrane (Millipore, Medford, MA). Membranes were immunoblotted using anti-4E-BP1, anti-eIF2α, and anti-eIF2αP, anti-S6 ribosomal total and S6 ribosomal-P, anti-PKD/PKCμ (Ser^{916}), anti-PKD/PKCμ ($Ser^{744/748}$), anti-PKC(pan) (βII Ser^{660}), anti-PKCα/β II ($Thr^{638/641}$), anti-PKCδ (Thr^{505}), anti-PKCδ/μ ($Ser^{643/676}$), anti-PKCμ (Thr^{538}) and anti-PKCζ/λ ($Thr^{410/403}$), anti-REDD1 (Cell Signaling, Danvers, MA). For study 1, visualization of bands was accomplished using horseradish peroxidase-coupled (HRP) secondary antibodies (1:25,000 dilution in 5% (w/v) dry fat free milk in 1×TBST) and chemiluminescent substrates (West Dura, Pierce) with exposure to autoradiography film. Film images were digitized and analyzed using NIH Image 1.63 software. Band intensities were normalized against corresponding bands for β-actin loading and transfer controls. For study 2, after incubation with respective primary antibodies, membranes were incubated in dark with IRDye®-labeled secondary antibody (1:15,000) in Odyssey blocking buffer plus 0.1%Tween-20 and 0.01%SDS. Then, membranes were washed with PBS and scanned on an Odyssey scanner by Li-Cor Biosciences, Lincoln NE, on 680 nm (for β-actin) and 800 nm (for the protein of interest) channels, using intensity values of 4.0 and 8.5 respectively. Quantification of bands and normalization by β-actin was done using the Li-Cor Odyssey V3.0 software.

Measurement of hepatic glutathione and cysteine levels. Total intracellular cyst(e)ine levels were measured by a modified acid ninhydrin method of Gaitonde (1967), as described by Dominy et al. (2007). Total glutathione levels were measured by the method of Cereser et al. (2001).

Statistical analysis. For comparisons among dietary studies involving more than two groups (i.e., 20% soy, protein-free and pair-fed; 0.23%M/0.35%C, 0.11%M and pair-fed groups; and 0.23%M/0.35%C, 0.11%M/0.35%C, 0.23%M, and 0.11%M groups), the numerical data were analyzed by one-way ANOVA. Differences were accepted as significantly different at $P \leq 0.05$ by Dunnett's mean comparison procedure, for which the 20% soy and the 0.23%M/0.35%C groups were used as control groups. For comparisons between two groups (i.e., 10% soy +0.34%M and 10% soy groups), results were evaluated by Student's t-test, and significance was accepted at $P \leq 0.05$.

RESULTS

Study 1

Body weight and feed intake

Overall, at the end of the 7-day experimental period, rats switched from a 20% soy diet to a protein-free diet and rats switched from a complete amino acid diet (0.23%M/0.35%C) to either of three amino acid deficient diets (0.11%M, 0.23%M or 0.11%M/0.35%C) exhibited lower feed intake (Table 5.2) as well as lower body weight (Table 5.3) compared to their corresponding controls. The group on the protein-free diet exhibited a weight loss at an average daily rate of -4.3±1.3g, whereas the group pair-fed to the protein-free group grew marginally at a rate of only 10% that of the 20% group (control). The feed intake of the protein-free and pair-fed groups was ~63% of the intake of the 20% soy group. Rats fed diets containing 0.11%M, 0.23%M, and 0.11%M/0.35%C consumed 62%, 53% and 60%, respectively, as much diet as control (0.23%M/0.35%C) rats. The rats on these deficient diets lost weight at an average daily rate of -4.3±1.8g, -2.9±2.2g and -1.1±1.4g, respectively, over 7 days as shown in Table 5.2. Rats fed a 10% soy protein diet did not differ significantly in

their feed intake, but the 10% soy protein group gained only 40% as much weight over 7 days as did the control group that was fed the 10% soy + 0.34%M diet.

Table 5.2. Animal average daily weight change over the experimental periods

Diet	10% soy + 0.34 %M	10% soy	0.23% M/ 0.35%C	0.11%M / 0.35%C	0.23% M	0.11% M	20% soy	Pair-fed	Protein free
Mean weight change $(rat \times day)^{-1}$ (g) ± SD, N=6	6.3 ±0.4	2.5 ±2.0	6.9 ±0.4	-1.1 ±1.4	-2.9 ±2.2	-4.3 ±1.8	6.6 ±0.5	0.6 ±0.8	-4.3 ±1.3

Hepatic cysteine and GSH

As shown in Figure 5.1, hepatic thiol levels, measured at the end of the 7-day dietary treatment period, were markedly lower for rats fed the 10% soy, protein-free, pair-fed (to protein-free) and the amino acid deficient diets (0.11%M, 0.23%M, and 0.11%M/0.35%C) than in their respective controls (i.e., 10% soy+0.34%M, 20% soy and 0.23%M/0.35%C). Total cysteine in the 10% soy rats was 49% that in the 10% soy + 0.34% M control group. Total cysteine in the protein-free and pair-fed rats was 41% and 49%, respectively, that of control 20% soy-fed rats. Further, hepatic cysteine levels of the rats on deficient amino acid-defined diets (0.11%M/0.35%C, 0.23%M

Table 5.3. Animal average daily feed intake over the experimental periods

Diet	10% soy + 0.34%M	10% soy	0.23%M/ 0.35%C	0.11%M/ 0.35%C	0.23%M	0.11%M	20% soy	Pair-fed	Protein free
Mean feed intake $(rat \times day)^{-1}$ (g) ± SD, N=6	19.8 ±0.4[a*]	17.3 ±0.7[a*]	19.3 ±0.5[a]	11.6 ±0.6[c]	10.2 ±1.2[c]	11.9 ±0.7[c]	19.2 ±0.5[a]	12.0 ±0.3[c]	12.0 ±0.3[c]
Met-equivalents consumed $(rat \times day)^{-1}$ (g) ± SD	0.117 ±0.002	0.043 ±0.002	0.127 ±0.003	0.063 ±0.003	0.023 ±0.003	0.013 ±0.001	0.094 ±0.002	0.059 ±0.002	0
Met-equivalents consumed as % of the 20% soy group	124	46	135	67	24	14	100	63	0

*Cumulative feed intake at the end of the 7-day experimental period was not different between the two groups (N=12).

and 0.11%M) were 71%, 52%, and 38% of control, respectively. The reductions of hepatic total cysteine levels reflect the sulfur amino acid deficiency induced by diet in the animals.

Hepatic total glutathione levels tended to parallel cysteine levels, as shown in Figure 5.1. Total hepatic glutathione for the 10% soy fed rats was only 17% of the level in 10% soy +0.34%M rats. Total hepatic glutathione was 48% and 36% of *ad libitum* control (20% soy) for the protein-free and pair-fed-to-protein-free groups, respectively. Additionally, levels of glutathione in the rats fed the 0.11%M/0.35%C, 0.23%M and 0.11%M diets were 69%, 25% and 18% of control, respectively.

mTOR and eIF2α kinase activation

Total levels of 4E-BP1 protein were significantly increased in the livers of rats fed deficient diets compared to their respective controls, as shown in Figure 5.2. More specifically, rats on a 10% soy diet exhibited 2.4-times as much ($P<0.001$) 4E-BP1 as the methionine-supplemented control animals. Furthermore, hepatic levels of 4E-BP1 in rats fed a 0.23%M or 0.11%M diet were nearly double ($P<0.05$) that of rats fed either the control 0.23%M/0.35%C diet or even the 0.11%M/0.35%C diet (that had the same average feed intake as the more deficient groups). Rats fed a protein-free diet demonstrated a nearly 60% increase ($P<0.05$) in hepatic 4E-BP1 levels compared to either control or their pair-fed counterparts. These results thus show that 4E-BP1 protein levels increased in rats fed diets deficient in protein or sulfur amino acids but not in rats with restricted intakes of diets adequate in protein or sulfur amino acids.

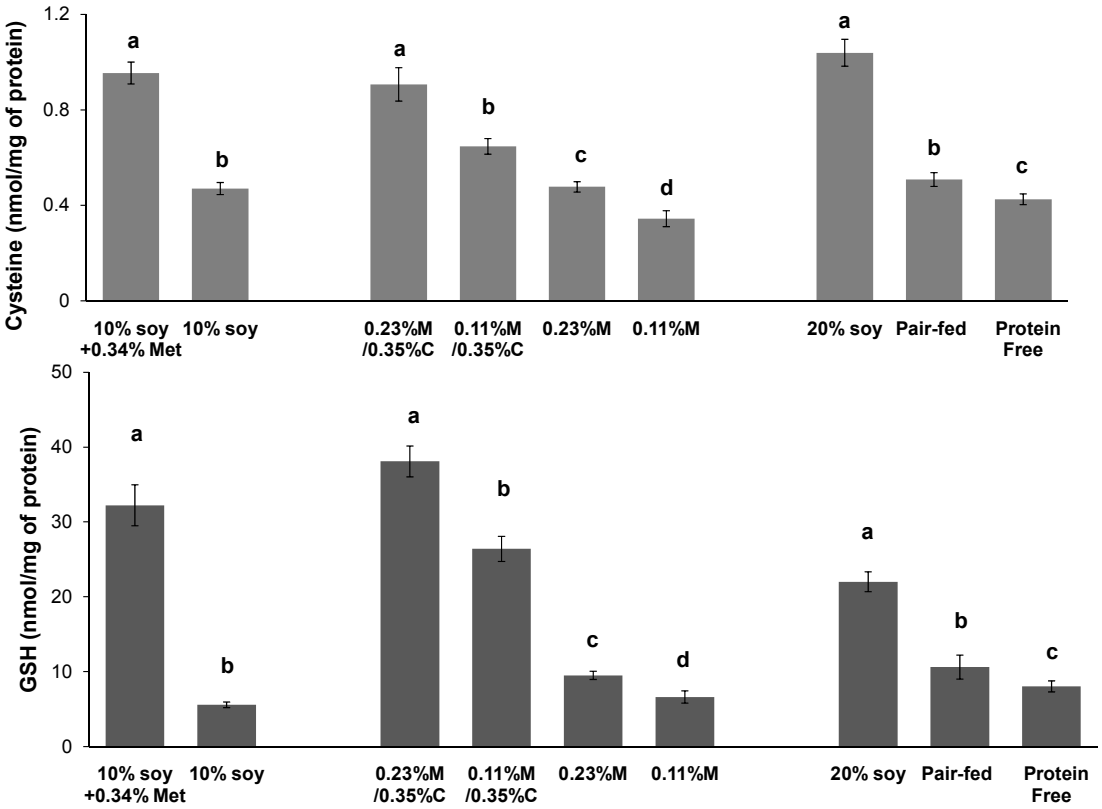

Figure 5.1. Hepatic Cysteine and Glutathione. Total cysteine and glutathione levels in liver of rats fed the assigned diet for 7 days: 10% soy +0.34%M (control), 10% soy (SAA-deficient), N=12 ; 0.23%M/0.35%C (control), 0.11%M/0.35%C (SAA-deficient), 0.23%M (SAA-deficient), 0.11%M (SAA-deficient), N=4 ; 20% soy (control), Pair-fed to protein-free (pair-fed control) and Protein-free, N=6. Values are means ± SEM. Different letter denotes statistically significant difference at $P<0.05$.

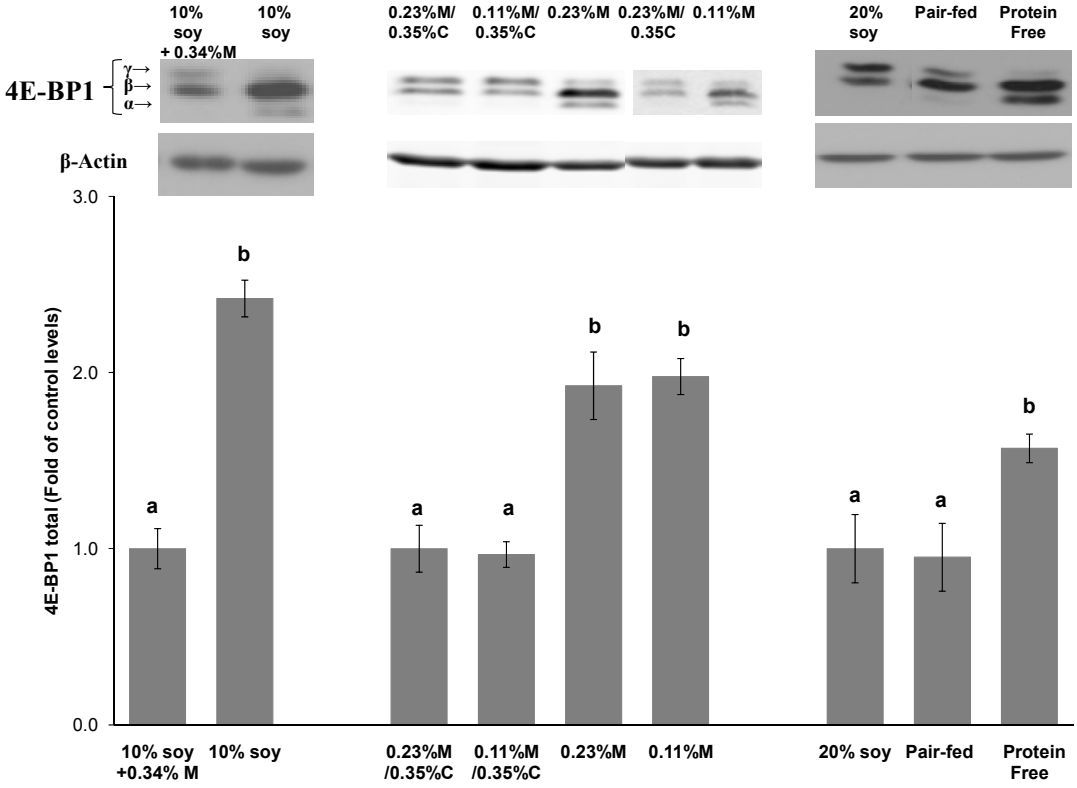

Figure 5.2. Hepatic total 4EBP1. Representative western blots and fold relative to the mean for the control group values for total levels of 4E-BP1 in liver of rats fed the following diets for 7 days: 10% soy +0.34%M (control), 10% soy (SAA-deficient), N=12 ; 0.23%M/0.35%C (control), 0.11%M/0.35%C (SAA-deficient), 0.23%M (SAA-deficient), 0.11%M (SAA-deficient), N=4 ; 20% soy (control), Pair-fed to protein-free (pair-fed control) and Protein-free, N=6. Equal amounts of total protein were loaded per lane. All values are means ± SEM. Values denoted by different letters are significantly different at $P<0.05$.

Even though protein/amino acid deficiency had a profound and consistent impact on the levels of hepatic total 4E-BP1 in rats, the impact of these deficient diets on the phosphorylation state of 4E-BP1 was less significant. More specifically, there was no difference in the phosphorylation state of hepatic 4E-BP1 between 10% soy+0.34%M and 10% soy fed rats. Even though the mean ratio of hyper/hypo-P 4E-BP1 was lowered by close to 40% in liver of rats fed amino acid deficient diets (i.e., 0.11%M/0.35%C, 0.23%M and 0.11%M) compared to control, the apparent trend for a decrease did not reach statistical significance. On the contrary, protein-free fed rats as well as their pair-fed group exhibited a significantly lower hyper/hypo-P 4E-BP1 ratio, which was 20 to 30% of control (Figure 5.3), demonstrating that the mTOR pathway was suppressed. We also assessed 4E-BP1 levels and phosphorylation status in the gastrocnemius muscle of rats fed several of the diets (i.e., the 10% soy+ 0.34%M, 10% soy, 20% soy, and the protein free groups). We did not observe a difference in either the level of total 4E-BP1 or its phosphorylation status in skeletal muscle of the 10% soy+0.34%M and the 10% soy groups (data not shown). However, we did see a striking reduction in the phosphorylation of 4E-BP1 in muscle of the rats fed the protein free diet and in muscle of the pair-fed control rats compared to the 20% soy control group fed *ad libitum*, but the total levels of muscle 4E-BP1 did not differ among the three groups. More specifically the hyper/hypo-P 4E-BP1 ratio was 8% ($P<0.001$) and 17% ($P<0.001$) of control for the protein free and the pair-fed group respectively (Figure 5.4). In general, changes in the phosphorylation state of 4E-BP1 in response to diet were similar for liver and muscle for these five treatment groups and generally reflected feed intake, whereas the increases in total 4E-BP1 were observed only in liver (not in muscle) and only in the rats more restricted in protein or amino acid intake (not in 10% soy or pair-fed groups).

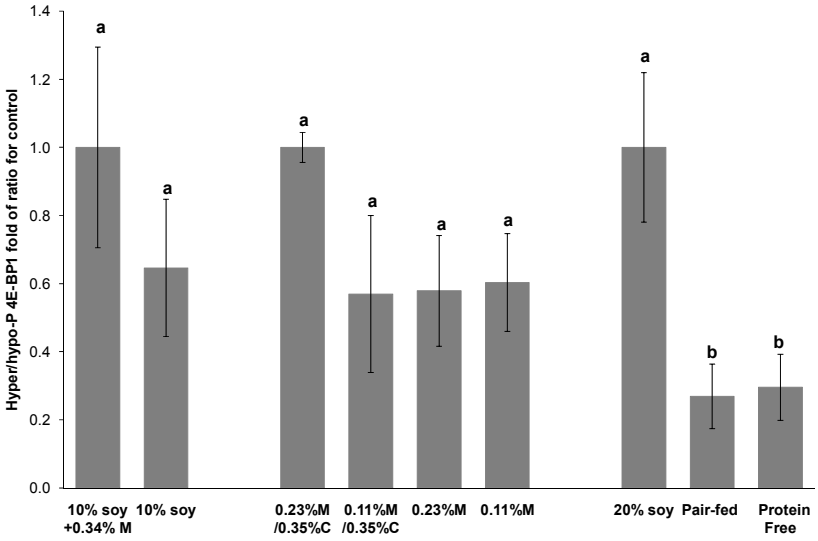

Figure 5.3. Hepatic ratio of hyper-P/hypo-P 4E-BP1. Ratio for hyper-P/hypo-P 4E-BP1 in liver of rats fed the following diets for 7 days: 10% soy +0.34%M (control), 10% soy (SAA-deficient), N=12 ; 0.23%M/0.35%C (control), 0.11%M/0.35%C (SAA-deficient), 0.23%M (SAA-deficient), 0.11%M (SAA-deficient), N=4 ; 20% soy (control), Pair-fed to protein-free (pair-fed control) and Protein-free, N=6. Equal amounts of total protein were loaded per lane. All values are relative to the mean of the appropriate control group ± SEM. Values denoted by different letters are significantly different at $P<0.05$.

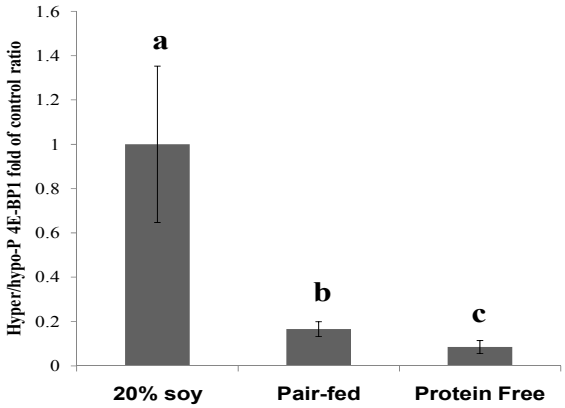

Figure 5.4. Muscle ratio of hyper-P/hypo-P 4E-BP1. Ratio of hyper-P/hypo-P 4E-BP1 in the gastrocnemius muscle of rats fed the following diets for 7 days: 10% soy +0.34%M (control), 10% soy (SAA-deficient), N=12 ; 0.23%M/0.35%C (control), 0.11%M/0.35%C (SAA-deficient), 0.23%M (SAA-deficient), 0.11%M (SAA-deficient), N=4 ; 20% soy (control), Pair-fed to protein-free (pair-fed control) and Protein-free, N=6. Equal amounts of total protein were loaded per lane. All values are relative to the mean of the appropriate control group ± SEM. Values denoted by different letters are significantly different at $P<0.05$.

The phosphorylation state of the ribosomal protein S6 (ratio of rpS6-P to total rpS6) is frequently used as a reliable indicator of mTOR activation state; S6-kinase is a direct substrate of mTOR and rpS6 is the target of S6 kinase. The ratio of rpS6-P to total rpS6 was significantly lower in liver of rats fed amino acid deficient diets (Figure 5.5), as well as in rats fed a protein-free diet and their pair-fed group. There was however no difference in the phosphorylation status of rpS6 between the 10% soy+0.34%M and the 10% soy rats. As for 4E-BP1 phosphorylation status, the most dramatic reduction occurred for rats fed the protein-free diet or that were pair-fed to those rats.

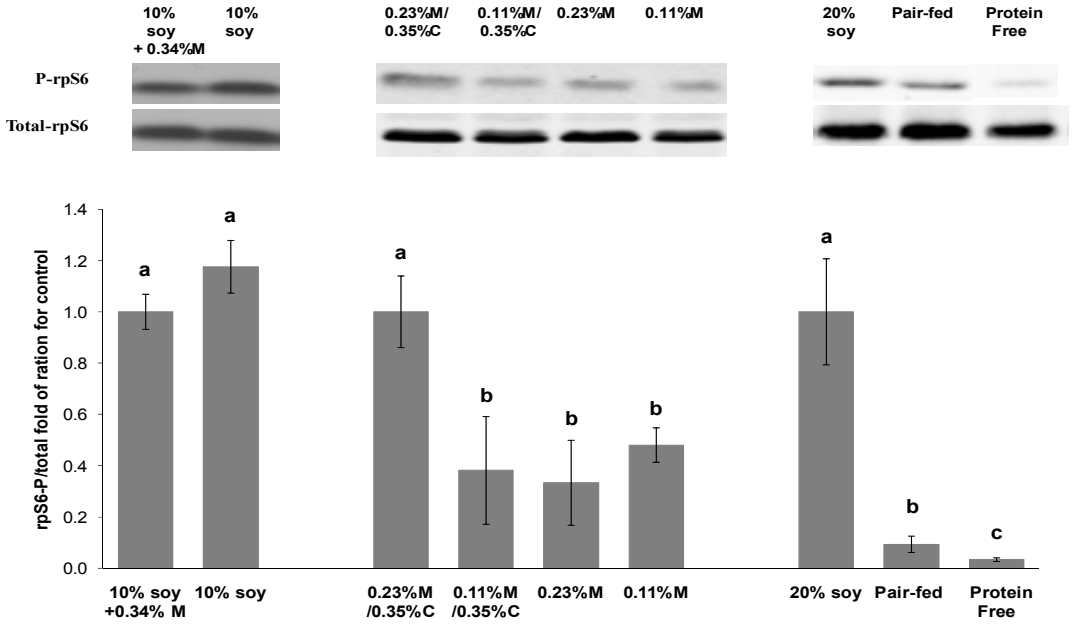

Figure 5.5. Hepatic ratio of ribosomal protein S6-P/total S6. Representative western blots and fold values for the ratio of phosphorylated rpS6 to total rpS6 in liver of rats fed the following diets for 7 days: 10% soy +0.34%M (control), 10% soy (SAA-deficient), N=12 ; 0.23%M/0.35%C (control), 0.11%M/0.35%C (SAA-deficient), 0.23%M (SAA-deficient), 0.11%M (SAA-deficient), N=4 ; 20% soy (control), Pair-fed to protein-free (pair-fed control) and Protein-free, N=6. Equal amounts of total protein were loaded per lane. All values are relative to the mean of the appropriate control group ± SEM. Values denoted by different letters are significantly different at $P<0.05$.

Phosphorylation of eIF2α was induced in liver of rats fed a 10% soy diet, as we previously reported (Sikalidis and Stipanuk, 2010). Unexpectedly, we did not observe an increase in eIF2α phosphorylation in liver of rats fed any of the other protein or sulfur amino acid deficient diets, except for those fed the 0.11%M amino acid diet. Surprisingly, rats fed the other amino acid deficient diets or the rats that consumed the protein-free diet had hepatic eIF2α-P to total eIF2α ratios that were similar to or less than those of their respective controls (Figure 5.6).

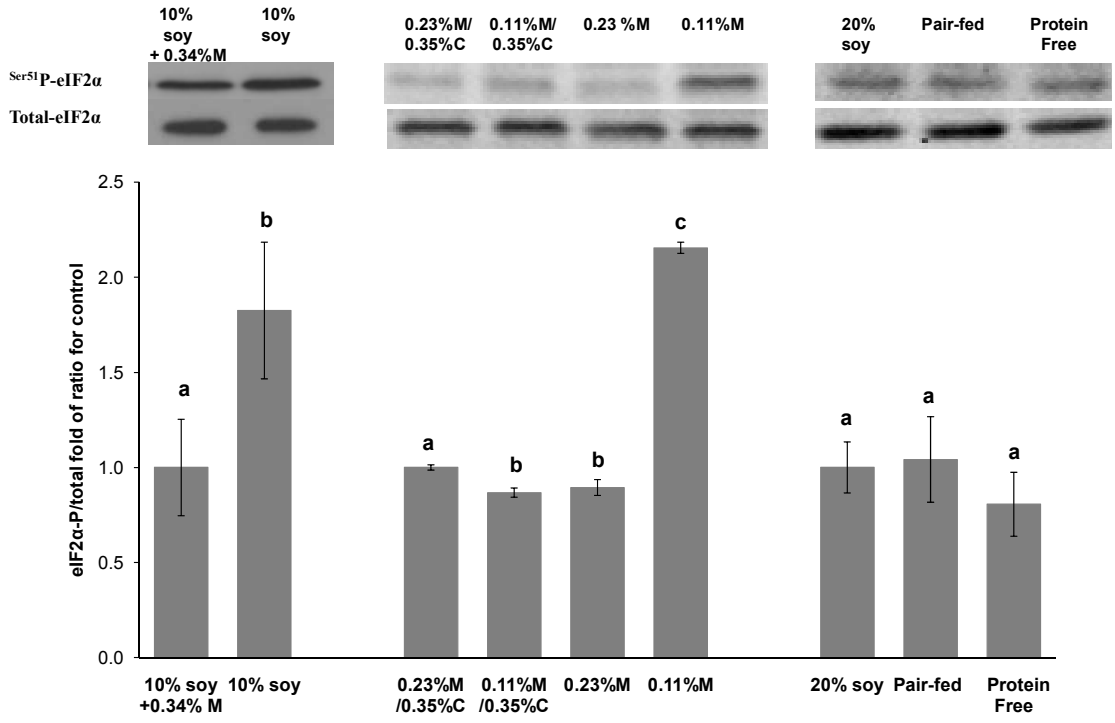

Figure 5.6. Hepatic ratio of eIF2α-P/total eIF2α. Representative western blots and fold values for the ratio of Ser^{51} phosphorylated eIF2α to total eIF2α in liver of rats fed the following diets for 7 days: 10% soy +0.34%M (control), 10% soy (SAA-deficient), N=12 ; 0.23%M/0.35%C (control), 0.11%M/0.35%C (SAA-deficient), 0.23%M (SAA-deficient), 0.11%M (SAA-deficient), N=4 ; 20% soy (control), Pair-fed to protein-free (pair-fed control) and Protein-free, N=6. Equal amounts of total protein were loaded per lane. All values are relative to the mean of the appropriate control group ± SEM. Values denoted by different letters are significantly different at $P<0.05$.

Study 2

Body weight and feed intake

In study 2, rats were fed either an adequate or a deficient amino acid based diet for up to 5 days. Rats fed the control diet consumed an average of 17.7±0.5g/d (mean of the average feed intakes for each of days 1, 3 and 5) whereas those fed the 0.11%M diet consumed an average of 7.4±1.2g/d. The pair-fed group consumed an average of 9.1±0.1g/d (pair-fed rats average intake was higher than that of the pair-fed rats due to them being given a high estimate of diet on day 1) (Table 5.4).

After only 24 h of dietary treatment, body weights were still similar for the control and the pair-fed group but the 0.11%M group exhibited a small loss of weight (4% less than control). After 3 days, the body weight for the pair-fed group was 10% lower than that of the control animals with *ad libitum* access to the complete amino acid diet, whereas the sulfur amino acid-deficient (0.11%M) group weighed 22% less than control, despite equivalent feed intake. Overall, rats on the control diet grew normally throughout the 5-day experimental period at an average rate of 6.7± 2.5g/d, the pair-fed rats mostly maintained their weight throughout the experimental period, and the 0.11%M group lost weight at a rate of -3.4±1.4 g/d between days 1 and 3 and -2.2±1.3g/d between days 3 and 5 (Table 5.4).

Table 5.4. Body weights and feed intake from study 2

	Time-point 1			Time-point 2			Time-point 3		
	1 day on diets			3 days on diets			5 days on diets		
	Complete	Pair-fed	0.11%M	Complete	Pair-fed	0.11%M	Complete	Pair-fed	0.11%M
Body weight (g) ± SEM	154.5 ±2.7[a]	157.8 ±4.7[a]	148.3 ±3.7[a]	176.3 ±2.9[a]	158.8 ±3.3[b]	138.0 ±1.6[c]	192.3 ±1.8[a]	156.0 ±2.5[b]	133.5 ±2.3[c]
Mean feed intake $(rat \times day)^{-1}$ (g) ± SEM	18.0 ±0.4[a]	12.5 ±0.1[b]	8.6 ±1.6[c]	18.3 ±0.8[a]	8.1 ±0.0[b,*]	6.8 ±1.8[b]	16.8 ±1.8[a]	6.9 ±0.0[b,*]	6.8 ±0.2[b]

*All the diet given to rats was consumed, there was no measurable spillage effect, N=4 for time-points 1&3 and N=6 for time-point 2, significance accepted at $P<0.05$.

• Feed intake values are mean of the average feed intakes for each of days 1, 3 and 5 ± SEM.

Hepatic cysteine and GSH

As shown in Figure 5.7, hepatic cysteine levels were significantly lower, compared to controls fed *ad libitum*, in rats pair-fed to controls and in rats fed the 0.11%M diet. Cysteine levels were 13% and 27% less than control, respectively, for pair-fed rats and 0.11%M rats on day 1. For day 3, the reductions were 14% (not significant) and 42%, respectively, whereas on day 5 the reductions in hepatic cysteine level compared to control were 19% (not significant) and 36%, respectively. Hepatic cysteine level in the 0.11%M group was significantly lower than that of the pair-fed control group at both day 3 and day 5, showing that the decrease in hepatic cysteine was much greater in animals fed the amino acid deficient diet than in those fed the adequate diet even when both groups consumed a deficient amount of energy (37%-44% of the intake of control rats fed *ad libitum*) (Figure 5.7).

Hepatic GSH was significantly reduced in both rats fed the 0.11%M diet and in the pair-fed control group. In general, percentage reductions in hepatic GSH levels were greater than those for cysteine but followed a similar pattern. Specifically, hepatic GSH was 35% and 45% of control in the pair-fed and 0.11%M groups, respectively, on day 1; 36% and 58%, respectively, on day 3; and 31% and 44%, respectively, on day 5 (Figure 5.7). Hepatic GSH levels were significantly lower in rats fed the amino acid deficient diet than in rats pair-fed the adequate diet, as was true for hepatic cysteine levels.

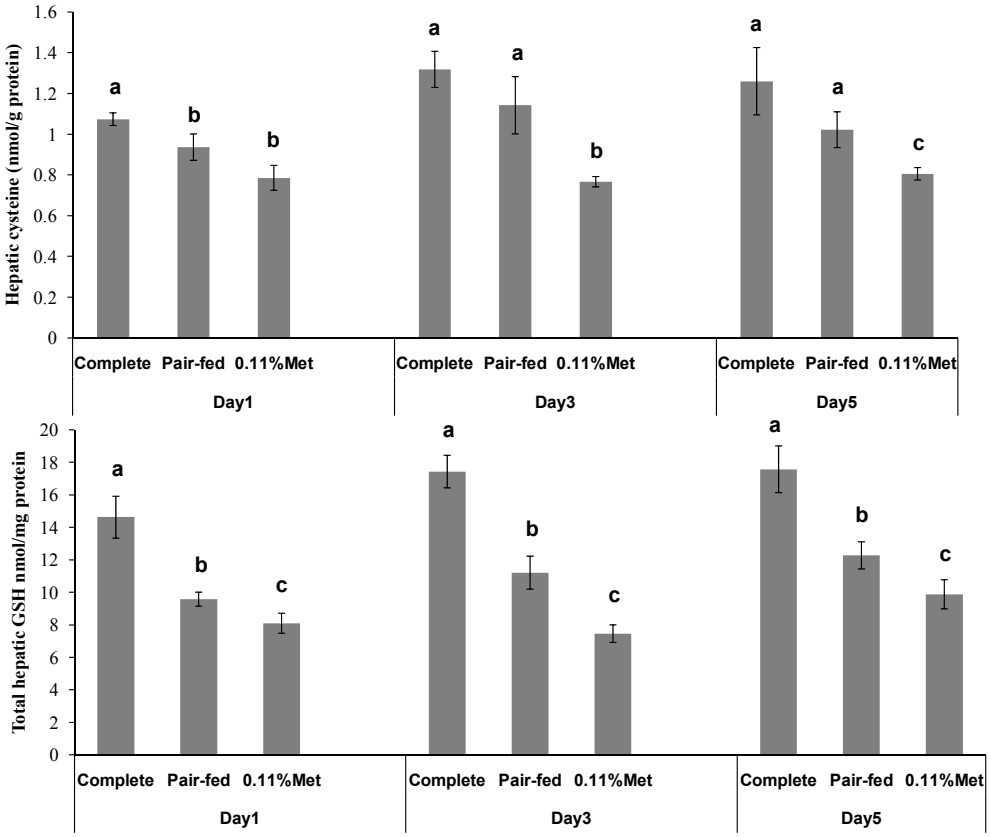

Figure 5.7. Hepatic Cysteine and Glutathione. Total cysteine and glutathione levels in liver of rats fed a 0.11%M diet with no cyst(e)ine or a complete amino acid diet with *ad libitum* access or with feed restricted to match the intake of the 0.11%M group for 1, 3 or 5 days. Values are means ± SEM, N=4. Values denoted by different letters are significantly different at $P<0.05$.

Blood parameters (glucose, plasma insulin and IGF-1)

Plasma glucose was not different among the treatment groups on day 1. On day 3 plasma glucose was 25% and 20% lower than the control level in the pair-fed and 0.11%M fed rats, respectively. It was significantly lower only for the 0.11%M group on day 5 (Table 5.5).

Table 5.5. Blood parameters from study 2

	Time-point 1			Time-point 2			Time-point 3		
	1 day on diets			3 days on diets			5 days on diets		
	Control	Pair-fed	0.11%M	Control	Pair-fed	0.11%M	Control	Pair-fed	0.11%M
Blood glucose (mg/dL)	107.3 ±7.6[a]	107.0 ±1.9[a]	98.3 ±4.6[a]	120.0 ±2.3[a]	93.0 ±4.1[b]	98.2 ±6.2[b]	106.8 ±7.6[a]	103.5 ±2.4[a]	97.0 ±2.9[b]
Plasma Insulin (ng/mL)	0.62 ±0.03[a]	0.49 ±0.05[b]	0.44 ±0.07[b]	0.84 ±0.08[a]	0.36 ±0.02[b]	0.42 ±0.06[b]	1.38 ±0.24[a]	0.59 ±0.04[b]	0.51 ±0.03[b]
Plasma IGF-1 (ng/mL)	-[*]	-[*]	-[*]	765.6 ±47.4[a]	484.3 ±56.0[b]	114.4 ±37.8[c]	709.5 ±63.9[a]	353.8 ±32.7[b]	93.7 ±17.8[c]

[*] No samples were analyzed at time-point 1.

Values are mean measurements ± SEM, N=4 for time-points 1&3 and N=6 for time-point 2, significance accepted at $P<0.05$.

Plasma insulin was significantly lower in the pair-fed group (20% lower) and in the 0.11%M group (30% lower) compared to the *ad libitum* control group after 1 day on the dietary treatments. The difference was even greater on days 3 and 5 when the plasma insulin levels of the pair-fed and 0.11%M rats were less than half of that in the controls (42% and 49%, respectively, for day 3; and 43% and 37%, respectively, for day 5) (Table 5.5).

Plasma IGF-1 was assessed at the later two time-points to allow more time for a potential change in IGF-1 plasma levels. On day 3, plasma IGF-1 was significantly lower than control in both the pair-fed and the 0.11%M fed groups (63% and 15% of control, respectively). The pattern was similar on day 5, with IGF-1 levels at 50% and 13% of control for the pair-fed and the 0.11%M fed groups, respectively (Table 5.5).

Organ weights

In absolute values, the weight of gastrocnemius muscle was approximately 10-20% and 15-30% less than control in the pair-fed and the 0.11%M groups, respectively, but when corrected for difference in body weight, there was no difference among groups in relative muscle weight at the different time-points. Liver weight was significantly lower in the pair-fed and 0.11%M groups compared to controls when expressed as a percentage of body weight: 10% lower for both the pair-fed and 0.11%M groups on day 1; 30% lower for the pair-fed group and 10% lower for the 0.11%M group on day 3; and 15% and 11% lower, respectively, on day 5 (Table 5.6). Overall, liver weight declined less in the rats fed the 0.11%M diet than in the pair-fed rats.

Table 5.6. Organ weights from study 2

	Time-point 1			Time-point 2			Time-point 3		
	1 day on diets			3 days on diets			5 days on diets		
	Control	Pair-fed	0.11% M	Control	Pair-fed	0.11%M	Control	Pair-fed	0.11%M
Liver as % of body weight[1]	4.23 ±0.05	3.81 ±0.18	3.81 ±0.21	4.42 ±0.07	3.20 ±0.08	4.01 ±0.1	4.43 ±0.23	3.80 ±0.05	4.01 ±0.09
Gastrocnemius muscle as % of body weight[1,2]	0.47 ±0.01	0.46 ±0.01	0.47 ±0.01	0.45 ±0.01	0.47 ±0.01	0.49 ±0.02	0.48 ±0.02	0.46 ±0.01	0.47 ±0.01

[1]No statistical difference among treatments and time-points.
[2]Gastrocnemius muscle was collected only from one of the rat's back legs.
N=4 for time-points 1&3 and N=6 for time-point 2, values are means ± SEM, significance accepted at $P<0.05$.

Total 4E-BP levels

Total levels of 4E-BP1 were not significantly elevated in liver of rats fed the 0.11%M diet at 1, 3 or 5 days of treatment, although they were consistently slightly higher. No increase in 4E-BP1 that approached that observed at 7 days in study 1 was observed. The reason for this is unknown (Table 5.7).

Table 5.7. Mean fold change of ISR and mTOR components from study 2

	Time-point 1			Time-point 2			Time-point 3		
	1 day on diets			3 days on diets			5 days on diets		
	Control	Pair-fed	0.11% M	Control	Pair-fed	0.11% M	Control	Pair-fed	0.11% M
eIF2α-P/total ratio	1.00 ±0.07[a]	1.27 ±0.10[a]	0.99 ±0.08[a]	1.00 ±0.03[a]	1.09 ±0.10[a]	0.81 ±0.08[a]	1.00 ±0.14[a]	1.09 ±0.07[a]	0.92 ±0.10[a]
4E-BP1 total	1.00 ±0.16[a]	1.01 ±0.24[a]	1.21 ±0.29[a]	1.00 ±0.14[a]	0.90 ±0.19[a]	1.14 ±0.18[a]	1.00 ±0.14[a]	0.85 ±0.11[a]	1.14 ±0.12[a]
4E-BP1 (γ/α+β)	1.00 ±0.36[a]	0.77 ±0.04[a]	0.71 ±0.26[a]	1.00 ±0.26[a]	0.47 ±0.17[a]	0.63 ±0.15[a]	1.00 ±0.38[a]	0.83 ±0.19[a]	0.52 ±0.08[a]
S6ribos-P/total ratio	1.00 ±0.15[a]	0.85 ±0.07[a]	0.44 ±0.03[b]	1.00 ±0.17[a]	0.77 ±0.14[a,b]	0.53 ±0.08[b]	1.00 ±0.13[a]	0.62 ±0.14[a,b]	0.48 ±0.13[b]

Values are mean fold change compared to control ± SEM, N=4 for all time-points, significance accepted at $P<0.05$.

eIF2α kinase and mTOR activation

The phosphorylation status of eIF2α was not different among treatments at any time-point in study 2 thus making it unlikely that GCN2 was activated by amino acid limitation (Table 5.7).

The ratio of rpS6-P to total rpS6 was significantly reduced in the liver of rats fed the 0.11%M diet compared to rats fed the control diet *ad libitum* at every time-point. More specifically, the rpS6r-P/total rpS6 ratio in the 0.11%M treated rats was

about half of that in the controls. The pair-fed group exhibited a ratio of rpS6-P/total rpS6 of 85%, 77% and 62% of the *ad libitum* control group on days 1, 3 and 5, respectively, and was not significantly different from either the control or 0.11%M group (Table 5.7).

The phosphorylation status of 4E-BP1 as assessed by the ratio of 4E-BP1 (γ/α+β) was 77% and 70% of control for the pair-fed and 0.11%M groups, respectively, on day 1; 47% and 63% of control on day 3; and 83% and 53% of control on day 5. Although the differences did not quite reach significance, there was a pattern similar to the one observed for rpS6 phosphorylation.

Other potential mediators of cellular responses

Because we did not observe a significant change in the phosphorylation status of eIF2α that correlated with changes in 4E-BP1 levels in either study 1 or 2, we asked whether other signaling molecules could be activated during the nutritional stress. Protein kinase C (PKC) comprises a multigene family of serine/threonine kinases that function as metabolic switches in many signal transduction pathways and are implicated in a wide range of G protein-coupled receptor and other growth factor-dependent cellular responses (Dempsey et al., 2000; Sabri and Steinberg, 2003). Therefore, we assessed the phosphorylation status of several isoforms of PKC, as it is involved in a variety of cellular responses and signaling cascades including gene expression and proliferation/growth. More specifically we assessed the phosphorylation status of PKD/PKCμ (Ser^{916}), PKD/PKCμ ($Ser^{744/748}$), PKC (pan) (βII Ser^{660}), PKCα/β II ($Thr^{638/641}$), PKCδ (Thr^{505}), PKCδ/μ ($Ser^{643/676}$), PKCμ (Thr^{538}) and PKCζ/λ ($Thr^{410/403}$). We did not observe any changes in the phosphorylation status of these PKC isoforms in liver of rats the 0.11%M diet or in liver of rats pair-fed the control diet compared to the *ad libitum* controls (data not shown). Further, we assessed

levels of REDD1, a protein that functions as a negative regulator of mTOR, but found no significant differences in REDD1 content of liver of animals fed the various diets (data not shown).

DISCUSSION

Rats fed protein/amino acid deficient diets displayed reduced growth or weight loss and low cysteine and glutathione levels

The diminished hepatic cysteine and glutathione levels of rats fed diets that were deficient in sulfur amino acids (i.e.: 10% soy, 0.11%M, 0.23%M, 0.11%M/0.35%C) or devoid of protein and in the pair-fed control group with restricted intake of complete diet demonstrated that these rats were indeed deficient in sulfur amino acids. In fact, both cysteine and glutathione levels were reduced proportionally to the degree of sulfur amino acid deficiency. The respective percent reduction of hepatic cysteine and glutathione for the 0.11%M/0.35%C, 0,23%M, 0.11%M, protein free, and pair-fed groups compared to controls were 28%, 47%, 62%, 68% and 49% for cysteine and 30%, 75%, 82%, 64% and 52% for glutathione. The same pattern was seen in the time-course study in rats fed a 0.11%M diet and in the pair-fed group on the adequate amino acid diet and was already apparent at the earliest (day 1) time-point. These observations are consistent with the well-established effects of essential amino acid-deficient diets on growth of rats. The pattern for reduction in hepatic cysteine levels was similar to that for glutathione, consistent with the previously established relationship of tissue cysteine and glutathione levels and the marked effect of cysteine availability on tissue GSH levels (Dominy et al., 2007; Stipanuk et al., 1992). This is consistent with previous reports that glutathione levels become depleted when sulfur amino acid intake is limited (Sikalidis and Stipanuk, 2010; Stipanuk et al., 1992).

The pattern of body weight reduction of the rats on the sulfur amino acid deficient diets over the 7-day experimental period and over the 5-day time-course study also paralleled the degree of sulfur amino acid deficiency. For example, rats fed the 0.11%M/0.35%C diet lost 7% of their initial weight, 0.23%M rats lost 10%, and 0.11%M rats 19% over 7 days, in contrast to a gain of 48 g (28.9% of initial body weight) over 7 days in rats fed the 0.23%M/0.35%C diet. It is well-known that rats reduce feed intake when fed an amino acid-deficient or a protein-free diet (Gietzen et al., 2006, Hao et al., 2005), but growth of rats in this study was clearly affected both by the reduced food intake and the amino acid inadequacy of the diets. For example, rats fed the protein free diet lost 17% of their initial weight whereas their pair-fed group gained approximately 1% of initial weight. Similarly, in study 2, weight loss was substantial in rats fed the 0.11%M diet whereas rats pair-fed an adequate diet were able to maintain their initial body weight. Energy intake of the two groups was similar, but the intake of methionine equivalents was quite different, 7.5 $mg \cdot d^{-1} \cdot rat^{-1}$ for the 0.11%M group and 45 $mg \cdot d^{-1} \cdot rat^{-1}$ for the pair-fed group. Furthermore, rats fed a 10% soy diet grew slower than those fed the 10% soy + 0.34%M diet even though again the feed intake was not different for these two groups.

Protein/amino acid-deficiency but not energy restriction increases total 4E-BP1 in liver but not muscle of rats

Total hepatic levels of 4E-BP1 were significantly induced in rats fed a protein-free diet but not in pair-fed rats that had the same caloric intake but received a 20% soy protein diet. Also, in rats fed the 10% soy diet, total 4E-BP1 levels were significantly increased compared to rats fed the 10% soy+0.34%M diet while feed intake was similar for the two groups. Furthermore, we saw a significant increase in the levels of total 4E-BP1 in rats that consumed the 0.11%M or the 0.23%M diet

compared to those fed the 0.11%M/0.35%C diet despite similarly reduced feed intake in all three groups. Thus, we have several comparison sets of rats with similar food intakes but different amino acid or protein intakes. In all cases, only rats with a lack of protein or essential amino acids exhibited an increased level of total 4E-BP1 protein levels. Our data suggest that diets limiting in essential amino acids (at least, sulfur amino acids) may negatively affect protein translation in the liver by elevating amounts of total 4E-BP1. It would be of interest to determine if total 4E-BP1 is elevated in liver of children with kwashiorkor versus children with marasmus, given the association of diets with low levels of protein or proteins of low quality and the suppression of plasma levels of proteins synthesized in the liver (e.g., albumin) in children with kwashiorkor.

Increased total 4E-BP1 levels in response to essential amino acid limitation have been reported in *in vitro* studies (Palii et al., 2008; Willet et al., 2009). When HepG2/C3A cells were cultured in media lacking one essential amino acid at a time, Palii et al. (2008) reported that total 4E-BP1 levels increased in response to nearly all of the single essential amino acid depletions whereas the ratio of phosphorylated/total 4E-BP1 remained unchanged (Palii et al., 2008). Work in C2C12 myoblasts by Willet et al. (2009) showed that mTOR inhibition with RAD001 led to suppression of protein synthesis and increased 4E-BP1 mRNA and protein levels, but had no effect on the phosphorylation of 4E-BP1 (Willet et al., 2009). Experiments with tumor cells (Rousseau et al, 1996) and with Drosophila (Miron et al., 2001) have demonstrated that the eIF4E-binding proteins act as negative regulators of cell growth. Other *in vitro* research (Azar et al., 2010) demonstrated that total 4E-BP1 increases significantly when cells suppress translation as they are reaching confluence. The same group (Azar et al., 2010) further showed, in various cell types, that cell cycle and protein synthesis are faster in cells where 4E-BP1 is either downregulated via siRNA

transfection, hence suggesting a link between amount of total 4E-BP1 and potency of translation suppression.

In our studies, in the gastrocnemius muscle, we saw little change in total 4E-BP1 levels in response to protein or amino acid-deficient diets, although we did observed decreases in the phosphorylation of 4E-BP1. A report of higher levels of total 4E-BP1 in skeletal muscle of aged rats suggests that regulation of total 4E-BP1 may occur in skeletal muscle under some conditions. Paturi et al. (2010) observed that 4E-BP1 total protein level was elevated by 55% in aged rats compared to young rats, whereas the 4E-BP1 phosphorylation status was not significantly different.

Rats on protein/amino acid-deficient diets exhibited suppressed mTOR signaling

The mTOR system responds to both energy (increased insulin, reduced AMPK) and increased availability of amino acids. In condition of low amino acid availability and/or decreased food intake, mTOR signaling would be expected to be suppressed based on the reports of a number of groups (Kimball et al., 2004; Proud et al., 2004; Kimball et al., 2002, Hong-Brown et al., 2005; Richardson et al., 2004; Fingar et al., 2004a and 2004b; Varry et al., 1999). We evaluated mTOR activation indirectly via the phosphorylation status of its direct phosphorylation target 4E-BP1 and the phosphorylation status of ribosomal protein S6 (rpS6, which is phosphorylated by p70S6 kinase, a direct target of mTOR). Our studies indicated that reduction of the phosphorylation of 4E-BP1 did not occur in the liver of rats fed the 10% soy protein diet, although there was substantially more total 4E-BP1, and hence more active hypophosphorylated 4E-BP1, in liver of these rats. This suggests that regulation of total 4E-BP1 protein levels may respond to amino acid limitation in the absence of changes in food intake and, hence, insulin levels and availability of exogenous fuels. However, we did observe significantly lower hyperphosphorylation of 4E-BP1 in liver

of rats fed either the protein-free diet or pair-fed a 20% soy protein diet at the reduced intake of the protein-free group. Both groups had similar ratios of hypophosphorylated to hyperphosphorylated 4E-BP1, although only the protein-free group exhibited a greater level of total 4E-BP1. In addition, a similar trend was observed in rats fed amino acid-based diets. Rats fed a marginally sulfur amino acid deficient diet (0.11%M/0.35%Cys) had reduced food intake and reduced mTOR signaling (particularly as evidenced by rpS6 phosphorylation) but no change in the hepatic total 4E-BP1 level, whereas rats fed more severely deficient diets (0.23%M or 0.11%M), which had similarly reduced food intake, exhibited an increase in total 4E-BP1 as well as suppressed hyperphosphorylation of 4E-BP1. These results suggest that the phosphorylation state of hepatic 4E-BP1, regulated by mTOR signaling, might be mainly due to the level of insulin or insulin-like growth factor signaling or fuel availability rather than to a decrease in amino acid availability, whereas total 4E-BP1 levels might be responding mainly to amino acid levels. When both protein and total energy intakes are deficient, both the increase in total 4E-BP1 and the decrease in hyperphosphorylation of 4E-BP1 would result in more active hypophosphorylated 4E-BP1and contribute to suppression of translation initiation.

Because the results of the first study suggested that insulin or insulin-like growth factor signaling were more important than amino acid availability in regulating mTOR signaling, as evidenced by phosphorylation of 4E-BP1 and rpS6, we assessed plasma insulin and IGF-1 concentrations in the subsequent study with rats fed the 0.11%M and 0.23%M/0.35%C amino acid diets. Compared to the control group fed the complete diet *ad libitum*, plasma insulin levels were lower and similar in rats fed the 0.11%M diet and in rats pair-fed the complete 0.23%M/0.35%C diet. Thus, plasma insulin levels correspond closely with reduced mTOR signaling in liver of these rats. Plasma IGF-1 levels were also significantly lower in both the 0.11%M and

pair-fed rats, compared to the control rats fed *ad libitum*, but, in marked contrast to insulin levels, the plasma IGF-1 levels in rats fed the 0.11%M diet were only 25% of those in the pair-fed control group. Thus, the dietary sulfur amino acid deficiency had a large effect on the plasma IGF-1 level, raising the question of whether IGF-1 signaling could affect 4E-BP1 protein levels.

IGF-1 has been shown to induce mTOR signaling in a variety of studies (Glass DJ, 2010; Inoki et al., 2002; Rommel et al., 2001; Baumann et al., 2009). Feng and Levine (2010) reported that the IGF-1/Akt/mTOR axis signals for cell growth in response to high levels of amino acids. It has been suggested that under stress conditions (including nutrient deprivation), when cell division procedure is more prone to errors, p53 shuts down the IGF-1/Akt pathway and subsequently the mTOR pathway by inducing the expression of an array of genes (e.g., IGF-BP3, PTEN, TSC2, AMPKβ1, sestrins 1 and 2, and REDD1) that are known to negatively regulate mTOR in order to minimize the error frequency of cell division (Feng and Lavine, 2010; Feng et al.,, 2007, 2005; Lavine et al., 2006, Ellisen et al., 2002; Stambolic et al., 2001). Although our data do not suggest a close relationship between plasma IGF-1 levels and mTOR activity, IGF-1 levels do generally parallel the degree of growth suppression, with both being more reduced in rats deficient in both amino acids and total energy than in rats deficient in energy alone. The role of p53 in mediating the IGF-1/Akt pathway suggests that p53 should be considered as a possible regulator of total 4E-BP1 protein levels, particularly if a connection with IGF-1 signaling can be demonstrated in future studies.

The GCN2/eIF2α integrated stress response pathway was not highly induced in livers of rats fed protein/amino acid-deficient diets

As GCN2 is activated during amino acid deprivation to phosphorylate eIF2α in turn to suppress global translation and induce the expression of specific proteins (Deval et al., 2009; Clemens et al., 2001), we initially hypothesized that 4E-BP1 would be one of the proteins whose expression is upregulated with phosphorylation of eIF2α. Some evidence for the interrelation of mTOR with GCN2 was provided in a study by Anthony et al. (2004), in which $GCN2^{-/-}$ mice that were fed a leucine-free diet for 6 days exhibited the expected block in hepatic eIF2α phosphorylation and, somewhat surprisingly, also failed to display the decrease in phosphorylation of S6K1 and 4E-BP1 observed in wild-type mice, suggesting that GCN2 activity is an important contributor to the nutritional regulation of the mTOR pathway (Anthony et al., 2004). Also *in vitro* studies by Lu et al. (2004) have indicated that 4E-BP mRNA levels are increased when eIF2α is phosphorylated (Lu et al., 2004). In our studies however, we only observed increased eIF2α phosphorylation levels paralleling the increase of total 4E-BP1 only in the case of rats fed a 10% soy diet and in those fed the 0.11%M diet. Interestingly, we did not see induction of eIF2α phosphorylation in the case of rats fed the protein-free or the other amino acid-deficient diets. Furthermore, we did not see upregulation of downstream targets of eIF2α phosphorylation in liver of rats fed the protein-free or other amino acid-deficient diets (e.g., ATF4, ATF3, ASNS and SLC7A11) confirming the lack of induction of GCN2 (data not shown). Our studies, therefore, suggest that increases in hepatic total 4E-BP1 are independent of eIF2α phosphorylation status although they seem to be dependent upon a deficiency or imbalance of essential amino acids in the diet.

Regulation of total eIF4E-binding protein levels represents a third mechanism for control of protein translation

Our results demonstrate that protein or amino acid-deficient diets result in an increase in the level of hepatic total 4E-BP1 but no further change in the phosphorylation state of 4E-BP1 beyond that accounted for by the associated suppression of feed intake. This results in an increase in the absolute amount of hypophosphorylated 4E-BP1 available to bind eIF4E and block cap-dependent translation initiation. We further observed that protein or amino acid deficient diets resulted in an increase of total 4E-BP1 whether or not eIF2α phosphorylation, presumably via activation of GCN2, occurred and whether or not downstream targets of the integrated stress response pathway were affected. Hence in addition to eIF2α phosphorylation and mTOR signaling via the lowered phosphorylation of 4E-BP1 in response to amino acid limitation, we propose that increased expression of total 4E-BP protein is a third mechanism for protein translation attenuation in response to amino acid limitation.

Subsequent studies are needed to address how the amino acid deficiency is sensed and what signaling pathway is involved in increased expression of 4E-BP1 as well as possible tissue specificity for this mechanism. Given the association of 4E-BP1 levels with plasma IGF-1 levels that we observed, the insulin-like growth factor regulatory network should be further considered as one possibility for regulation of 4E-BP expression and regulation of protein translation.

SUMMARY

The long-term overall objective of this work was to increase understanding of the protein and essential amino acid contribution to the regulation of protein translation and the induction of the integrated stress response by investigating cellular and animal responses to indispensable amino acid deficiency. More specifically, we initially aimed to further assess the role of eIF2α phosphorylation and downstream transcriptional regulation on the ability of mammalian cells to adapt to a lack of indispensable amino acids. We first compared the response of a hepatoma cell line (HepG2/C3A) to leucine versus cysteine deprivation to obtain a list of genes that were differentially expressed in response to a deficiency of both amino acids. By focusing on genes differentially expressed under both conditions, we aimed to identify genes that were being regulated via the eIF2α phosphorylation pathway and not by the mTOR pathway. The comparison of cells exposed to deficiencies of two different amino acids was done to facilitate selection of genes whose expression was more likely to be altered specifically in response to GCN2-induced eIF2α phosphorylation. Our gene expression studies in HepG2/C3A cells identified a number of genes involved in cell growth and division that were consistently down-regulated under conditions of either cysteine or leucine deprivation, as well as a number of genes involved in amino acid uptake, aminoacyl-tRNA synthesis, and the ISR that were consistently up-regulated. These suggest that the cell attempts to adapt to an amino acid-limited environment by facilitating more efficient utilization of the limited resource (i.e., amino acids) and up-regulating stress-response genes, while at the same time, suppressing overall protein synthesis, cell proliferation, and growth. All of these responses can be seen as consistent with cell or organismal survival under conditions of nutrient limitation.

More specifically, we observed eIF2α phosphorylation and up-regulation of protein levels for ATF4, ATF3, and ASNS in cells cultured in cyst(e)ine- or leucine-deficient medium and induction of CHOP in cells cultured in leucine-deficient, but not in cyst(e)ine-deficient medium (Lee et al., 2008; Sikalidis et al., 2010). Microarray analysis of gene expression in cells cultured under the same conditions showed upregulation of mRNA levels for ASNS, ATF3, ATF4, C/EBP-β, CHOP, SLC7A1, SLC7A11, and TRIB3. The mRNA levels for a variety of aminoacyl-tRNA synthetases (e.g., CARS, SARS, WARS) and CTH (cystathionine γ-lyase) [along with those for VLDR (very low density lipoprotein receptor), CLCN3 (chloride channel 3), and TXNIP (thioredoxin interacting protein)] were also consistently up-regulated in response to amino acid deprivation in a GCN2-dependent fashion (Lee et al., 2008; Deval et al., 2009; Sikalidis et al., 2010). The results of this study add to our understanding of how gene expression changes in response to amino acid deprivation in a human-derived cell line.

In continuation, we wanted to see if the results obtained from our *in vitro* studies would hold in an animal model. More specifically, we wanted to assess whether the eIF2α phosphorylation pathway might play a role in regulating nutrient utilization or stress responses in animals fed diets limiting in an indispensable amino acid. Because the models used to study eIF2α-kinase–mediated responses to amino acid deficiency have commonly used media or diets devoid of one or more essential amino acids, we asked whether eIF2α-kinase–mediated responses would be induced in animals fed a more typical diet that was marginal in essential amino acid composition but not as imbalanced as one in which one essential amino acid is totally absent. Our rat study with the SAA-deficient diet revealed a robust and sustained activation of the eIF2α phosphorylation/ATF4−mediated ISR in liver of rats fed a SAA-deficient diet, and our observations suggest that components of the ISR, such as induction of amino

acid transporters and aminoacyl-tRNA synthetases, may be responsible for an apparent adaptation that occurred over the first four days animals were fed the SAA-deficient diet, allowing an increase in the rate of growth over the remaining three days of the experimental period (Sikalidis and Stipanuk, 2010). Components of the ISR that are associated with apoptosis or translational recovery were not induced, suggesting that the ISR was adaptive and promoted survival and growth under conditions of modest indispensable amino acid limitation. Furthermore, our results confirm other reports (Palii et al., 2009; Lu et al., 2004b) that upregulation of 4E-BP1 expression is a downstream component of the ISR in liver and suggest that eIF2α kinase-dependent, mTOR-independent changes in 4E-BP availability may affect eIF4F complex formation in response to amino acid limitation.

Additionally to the two known mechanisms of 4E-BP hypophosphorylation and eIF2α phosphorylation in inhibiting translation initiation, recent evidence suggests the possibility of a third mechanism, the induction of 4E-BP expression. The phosphorylation of eIF2α increases the translation of a few specific existing mRNAs, including ATF4, which induces the expression of genes known to be associated with cell survival and stress resistance. Since 4E-BP is a repressor of translation initiation that aids in survival during low energy and low amino acid status, it is possible that 4E-BP is one of the proteins whose expression is induced during nutrient deficiency. To investigate the possibility of an increase in 4E-BP1 expression as a third mechanism in regulating translation initiation, 4E-BP1 protein levels were assessed in the livers and muscles of rats treated with a variety of diets aiming to create varying conditions (low energy, low protein, no protein).

In these studies, as well as in the one with SAA-deficient rats, we consistently observed an increase in total amount of 4E-BP1 in the protein free regiment but not in the energy restricted state. Furthermore the hyper/hypo-P 4E-BP1 ratio was somewhat

reduced mostly for the protein free and energy restricted group which along with the consistent markedly reduction of the Phospho/total S6ribosomal ratio suggests that the mTOR signaling was functioning as expected. As no significant changes in the phosphorylation of eIF2α were observed in this set of studies, it appears that deficiency of proteins or essential amino acids but not energy leads to increased levels of 4E-BP1 in the livers of rats but this response does not seem to be GCN2 dependent (eIF2α phosphorylation).

Further, we conducted a time-course animal study with the goal to capture the time at which the response to amino acid limitation is initiated. Although we were successful in inducing a sulfur amino acid deficiency we were unable to definitively identify a time-point at which a response initiates. We did observe however, that since the first day on amino acid deficient diet rats exhibited lower insulin and IGF-1 levels as well as reduced phosphorylation of S6ribosomal protein. These observations suggest that mTOR signaling was suppressed. We saw an increase (although not significant) in the levels of total 4E-BP1 but we did not see any change in the phosphorylation status of eIF2α.

RESEARCH QUESTIONS & FUTURE DIRECTIONS

One issue we came across in this work was the lack of agreement of cell studies for eIF2α-P/ATF4 gene targets. This situation could be attributed to the different kind of cells used in various studies (MEF wild-type, MEFs ATF4$^{-/-}$, MEF GCN2$^{-/-}$, HepG2/C3A) and different treatment/experimental design (use of tunicamycin, drug-induced activation of an artificial eIF2α kinase, withdrawal of different amino acids). It appears that the choice of cell-line and the experimental procedures influence the outcomes at a point where consensus is limited.

Another issue was the inability to repeat strong induction of eIF2α/ISR in rats with diets different from the 10% soy diet we first used. Although the experimental procedures were not very different (same strain of rats, similar age and weights, same housing conditions and diet preparation method) it seemed that the robust response of eIF2α/ISR is more a result of a subtle imbalance in the amino acid ratio/contribution in the diet, which is fairly challenging to repeat.

Determining whether phosphorylation of eIF2α by GCN2 is essential for induction of 4E-BP1 expression

Several *in vitro* studies have shown induction of gene expression for 4E-BP1 by amino acid deprivation. Actual protein levels of 4E-BP1 however, have not been extensively studied *in vitro* when amino acids are withdrawn. Our *in vivo* studies show that amino acid deficient diets can increase levels of 4E-BP1 in rats. To confirm the reported *in vitro* results (message level) and our *in vivo* observations, mRNA and protein levels of 4E-BP1 under an amino acid deprivation regiment *in vitro* (using a variety of cell-lines) could be measured.

Determining whether ATF4 induction is essential for induction of 4E-BP1 expression

If the upregulation of 4E-BP1 is dependent on GCN2 activation and subsequent eIF2α phosphorylation, it would be predicted that it is also dependent on ATF4 expression. Increased ATF4 expression may suffice to achieve the responses observed with amino acid deprivation or, alternatively, ATF4 may be necessary but not sufficient to deliver the response due to a requirement for additional GCN2/eIF2α-mediated events. Amino acid deprivation can be induced in $ATF4^{-/-}$ and wild type cells and the phosphorylation of eIF2α as well as total 4E-BP1 can be measured. Also wild type cells can be knocked-down with siRNA to see if an intermediate response in the total 4E-BP1 levels would be produced.

APPENDIX 1

Materials and Methods in detail

RNA extraction and microarray analysis

Dishes of HepG2/C3A cells cultured as described above were washed twice with ice-cold PBS, and cells were then directly lysed into denaturation solution. Total RNA was extracted from cells with an RNeasy Micro kit (Qiagen Inc., Valencia, CA). Microarray analysis was done on three individual cultures of cells for each treatment group.

These total RNA samples were labeled according to the standard one-cycle amplification and labeling protocol developed by Affymetrix (Santa Clara, CA). Labeled cRNAs were hybridized on Affymetrix GeneChip Human U133 plus 2.0 Array containing over 47,000 transcripts. Hybridized GeneChips were then stained, washed, and scanned by GeneChip Scanner 3000. The raw array data were processed by Affymetrix GCOS software to obtain detection calls and signal values. The signals were scaled to a target value of 500 with GCOS software. The scaled signal values were log2-transformed and centered by subtracting median signal from each gene across all arrays in the experiment. A t-test was applied on the normalized signal of each probe-set to look for differential genes between the two experimental conditions. A significance level cutoff was empirically established at $P \leq 0.0001$, based on the number of genes passing the cutoff, the magnitude of fold changes, and the relationship between fold changes and *P* values, to select differential gene sets. Our complete microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO, accession no. GSE13142).

Microarray results were confirmed by quantitative RT-PCR for several of the eIF2α phosphorylation/ATF4−induced genes as well as for *NQO1*, a gene that responds to oxidative stress but whose expression was not affected by amino acid deprivation, and *VSNL1*, a gene whose expression was down-regulated by amino acid deprivation. Isolated RNA was treated with DNase I, and first-strand cDNA was subsequently produced with the Invitrogen Superscript III kit and random primers. qRT-PCR was done with an Applied Biosystems 7500 Real Time PCR System, TaqMan Universal PCR Master Mix, and TaqMan gene expression assays for the specific genes (Applied Biosciences, Foster City, CA); 18S rRNA was used as the endogenous control.

Nuclear extraction procedure

To obtain nuclear extracts for measurement of CHOP expression levels, liver tissue was homogenized in cold 10 mM HEPES buffer, pH 7.9, with 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 1 mM DTT (Sha et al, 2009). Cells were allowed to swell on ice for 15 min and were then lysed with the addition of 10% NP-40 (0.6% final volume) and vigorous vortexing. The lysate was transferred to a pre-chilled tube and centrifuged at 4^{o}C for 5 min at 12,000 × g. Supernatant (cytosolic fraction) was removed and the pellet was re-suspended in ice-cold 20 mM HEPES buffer, pH 7.9, with 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT and vortexed vigorously. The extract was then centrifuged at 4^{o}C for 5 min at 12,000 × g to obtain the supernatant (nuclear fraction).

Measurement of hepatic glutathione and cysteine levels.

Total intracellular cyst(e)ine levels were measured by a modification of the method of Gaitonde (Gaitonde, 1967). Liver tissues were homogenized in 5% (w/v) sulfosalicylic acid (SSA) to form a 20% (w/v) homogenate. The homogenate was centrifuged at 4^{o}C at 18,000 × g for 20 min to collect the supernatant. The pH of the SSA supernatant was adjusted to 8.3 using 10 N NaOH, and then 500 mM dithiothreitol (DTT) was added to yield a final concentration of 5 mM. Samples were reduced with DTT for 15 min at room temperature. Following reduction, a 100 µL aliquot of sample was acidified with 0.l mL glacial acetic acid and then reacted with 0.1 mL acid ninhydrin reagent (250 mg ninhydrin dissolved in a mixture of 6 mL glacial acetic acid and 4 mL of 12 N HCl) for 10 min at 100°C. After being heated, the sample mixture was cooled on ice for 3 min and then diluted to 1.0 mL with 95% ethanol. Sample absorbance was measured at 560 nm. Cysteine levels were quantified using cysteine hydrochloride standards (0–500 µM) dissolved in 5% SSA and processed in the same manner as the samples. Testing of this procedure by our laboratory has shown it to be a highly selective and sensitive means of detecting cysteine levels between 5 and 500 µM in cell extracts (Dominy et al., 2007).

Total glutathione levels were measured using an HPLC method as described by Cereser et al, 2001. Briefly, liver tissue was homogenized in 2.5% perchloric acid to form a 20% (w/v) homogenate. The homogenate was centrifuged at 4^{o}C at 18,000 × g for 20 min to collect the supernatant. Then 90 µL of boric acid buffer (pH 9.0) was added to 10 µL of sample supernatant. Immediately 100 µL of o-phthaldialdehyde (OPA) solution was added, and the mixture was incubated at room temperature for 5 min. After the incubation step, 800 µL of 500 mM KPO_4 buffer (pH 7.0) was added, and this final mixture was analyzed by HPLC using an injection volume of 20 µL. HPLC was performed using a Nova-Pak C18 Column, 3.9×150 mm (Waters Corp.,

Milford, MA) and a gradient mobile phase generated using 50 mM KPO_4 buffer, pH 7.0 (eluent A) and 50 mM KPO_4 buffer, pH 7.0, with 3% (v/v) tetrahydrofuran and 40% (v/v) acetonitrile (eluent B). The flow rate was 1 mL/min. The mobile phase was started at 95% A, linearly changed to 70% A over 10 min, then linearly changed to 0% A over 5 min, and held at 0% A for 3 min. To regenerate the column prior to injection of the subsequent sample, the mobile phase was moved back to 95% A over 3 min and equilibrated for 8 min. Detection was conducted on a Waters 2475 fluorometer at a λ=340 nm wavelength with an emission of 420 nm.

Identification of CARS as a possible ATF4 direct target

Using the Genomatix MatInspectror on-line software (Genomatix GmbH, Munich, Germany) to perform a promoter sequence analysis for transcription factor binding sites (*in silico*), we identified an AARE (genomic sequence: GTTGCATCA) at position 516-524 upstream of the transcription start site at position 571 in the human CARS gene (Gene ID: 833, Homo sapiens chromosome 11, cysteinyl-tRNA synthetase, sequence GXP_169570). Using the same approach, we were able to identify an AARE (genomic sequence: GTTGCATCA) at position 517-525 upstream of the transcription start site at position 575 in the rat CARS gene (Gene ID: 293638, Rattus norvegicus chromosome 1, cysteinyl-tRNA synthetase, sequence GXP_1735315). Thus, CARS (Cysteinyl-tRNA synthetase) is also likely a direct target of ATF4.

APPENDIX 2

Table A1. Antibodies and dilutions used for immunoblotting described in chapters 3 & 4

Antibody	Supplier	1^{o} dilution
P-eIF2α (Ser^{51}) (119A11)*	Cell Signaling Technology	1:1000 in 5% BSA
eIF2α total*	Cell Signaling Technology	1:1000 in 5% BSA
ATF4	Gift, M.S. Kilberg	1:1000 in 5% BSA
ATF3 (C-19)	Santa Cruz Biotechnology	1:200 in 5% FFM
ASNS (C-14)	Santa Cruz Biotechnology	1:200 in 5% FFM
SLC7A11 (xCT)	Abcam Inc.	1:1000 in 5% FFM
CARS	Abcam Inc.	1:500 in 5% FFM
CTH	Abnova	1:500 in 5% FFM
CHOP (GADD153)	Santa Cruz Biotechnology	1:500 in 5% FFM
TRIB3	Santa Cruz Biotechnology	1:400 in 5% FFM
GADD34 (H-193)	Santa Cruz Biotechnology	1:400 in 5% FFM
Redd1	Cell Signaling Technology	1:1000 in 5% BSA
4E-BP1 total*	Cell Signaling Technology	1:1000 in 5% BSA
Phospho-p70 S6 Kinase	Cell Signaling Technology	1:1000 in 5% BSA
p70 S6 Kinase (total)	Cell Signaling Technology	1:1000 in 5% BSA
p-S6ribosomal protein*	Cell Signaling Technology	1:1000 in 5% BSA
S6ribosomal protein (total)*	Cell Signaling Technology	1:1000 in 5% BSA
GCLC	Neomarkers	1:2000 in 5% BSA
β-actin (control)	Cell Signaling Technology	1:1000 in 5% BSA

*If the Li-Cor system was used, antibodies were diluted in a solution 1:1 (PBS:Rockland blocking buffer) + 0.1% Tween-20).
BSA: Bovine Serum Albumin; FFM: Fat Free Milk. BSA and FFM solutions are w/v prepared in 1× TBST (Tris Buffer Saline Tween-20).
Abcam Inc., Cambridge, MA ; Abnova, Walnut, CA ; Cell Signaling Technology, Danvers, MA ; Neomarkers, Freemont, CA ; Santa Cruz Biotechnology, Santa Cruz, CA.

APPENDIX 3

Table A2. Adopted from Lee et al., 2008; Table 2, Differential expression of genes in response to cysteine deprivation[1]

		Fold Difference			
Probe id	**Gene Symbol**	**Long –Cys/ Short +Cys**	**Long – Cys/ Long + Cys**	**Short – Cys/ Long + Cys**	**Gene Title**
218098_at	ARFGEF2	1.61	1.97	1.75	ADP-ribosylation factor guanine nucleotide-exchange factor 2 (brefeldin A-inhibited)
205047_s_at	ASNS	2.43	1.56	1.35	asparagine synthetase
202672_s_at	ATF3	3.24	10.28	7.34	activating transcription factor 3
212971_at	CARS	1.98	2.69	2.28	cysteinyl-tRNA synthetase
227558_at	CBX4	1.94	2.49	2.05	chromobox homolog 4 (Pc class homolog, *Drosophila*)
212501_at	CEBPB	2.98	4.60	4.46	CCAAT/enhancer binding protein (C/EBP), β
204203_at	CEBPG	1.90	2.56	2.48	CCAAT/enhancer binding protein (C/EBP), γ
201925_s_at	DAF	2.75	4.20	3.61	decay accelerating factor for complement (CD55, Cromer blood group system)
201919_at	FLJ10618	1.90	2.65	2.57	hypothetical protein FLJ10618
204131_s_at	FOXO3A	1.80	2.84	3.11	forkhead box O3A
203725_at	GADD45A	1.78	5.59	3.79	growth arrest and DNA-damage-inducible, α
203925_at	GCLM	1.88	3.25	1.97	glutamate-cysteine ligase, modifier subunit
210587_at	INHBE	4.47	6.76	10.59	inhibin, βE
203320_at	LNK	2.65	3.41	4.40	lymphocyte adaptor protein
208786_s_at	MAP1LC3B	2.08	3.68	1.70	microtubule-associated protein 1 light chain 3β

Table A2. (Continued)

		Fold Difference			
Probe id	**Gene Symbol**	**Long –Cys/ Short +Cys**	**Long – Cys/ Long + Cys**	**Short – Cys/ Long + Cys**	**Gene Title**
236285_at	MGC16635	2.18	11.32	5.35	hypothetical protein BC009980
204538_x_at	NPIP /// LOC339047 /// LOC440341	2.39	2.76	2.09	nuclear pore complex interacting protein /// hypothetical protein LOC339047 /// similar to hypothetical protein LOC339047
207528_s_at	SLC7A11	2.92	5.61	4.93	solute carrier family 7 (cationic amino acid transporter, y^+ system), member 11
209921_at		4.56	13.77	14.86	
217678_at		4.98	9.88	11.41	
203438_at	STC2	3.00	5.99	8.30	stanniocalcin 2
218145_at	TRIB3	2.79	4.03	5.38	tribbles homolog 3 (*Drosophila*)
226181_at	TUBE1	3.40	4.52	5.88	tubulin, ε1
201266_at	TXNRD1	1.69	3.62	2.04	thioredoxin reductase 1
209822_s_at	VLDLR	3.09	3.52	3.06	very low-density lipoprotein receptor
218919_at	ZFAND1	2.11	2.42	2.46	zinc finger, AN1-type domain 1
227755_at		2.31	5.86	6.03	CDNA clone IMAGE:4077090, partial cds

[1]For inclusion in this table, a gene had to be differentially expressed with a significance level of $P < 0.0001$ for all 3 treatment comparisons.

APPENDIX 4

Table A3. Genes by category/function differentially upregulated/downregulated in amino acid-deficient HepG2/3A cells[1]

Upregulated	#genes	Downregulated	#genes
Genes with known functional AAREs	5	Cell cycle/Cell Division	26
Amino acid metabolism	5	Calcium signaling	2
Endosomal/lysosomal system	4	Cytoskeleton	1
GAPs and GEFs	2	Fatty acid and sterol metabolism	11
Growth regulation	5	Nucleotide synthesis/metabolism	8
Cytoskeleton	2	Other	16
Transcriptional regulators	3	Unknown function(s)	3
Other	7		
Unknown function(s)	18		

[1]To be included in this table, differential gene expression had to meet a statistical cutoff of $P<0.0001$.

APPENDIX 5

Supplemental materials on Chapter 4

Table S1. Genes whose expression was upregulated in HepG2/C3A cells cultured in –Leu medium[1]

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
204072_s_at	13CDNA73	hypothetical protein CG003	1.85	1.14E-05
209993_at	ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	2.05	1.86E-05
213532_at	ADAM17	a disintegrin and metalloproteinase domain 17 (tumor necrosis factor, alpha, converting enzyme)	1.30	2.18E-07
204333_s_at	AGA	aspartylglucosaminidase	1.41	6.40E-05
210517_s_at	AKAP12	A kinase (PRKA) anchor protein (gravin) 12	3.04	1.51E-05
225524_at	ANTXR2	anthrax toxin receptor 2	2.57	4.25E-06
209369_at	ANXA3	annexin A3	1.57	9.19E-05
218098_at	ARFGEF2	ADP-ribosylation factor guanine nucleotide-exchange factor 2 (brefeldin A-inhibited)	1.85	3.58E-05
209435_s_at	ARHGEF2	rho/rac guanine nucleotide exchange factor (GEF) 2	4.40	9.89E-06
225283_at	ARRDC4	arrestin domain containing 4	5.28	2.99E-06
205047_s_at	ASNS	asparagine synthetase	3.48	2.30E-07
207076_s_at	ASS	argininosuccinate synthetase	1.67	9.54E-07
202672_s_at	ATF3	activating transcription factor 3	4.00	8.21E-06
203080_s_at	BAZ2B	bromodomain adjacent to zinc finger domain, 2B	1.73	2.95E-05
226517_at	BCAT1	branched chain aminotransferase 1, cytosolic	1.98	5.52E-06
225285_at			1.87	9.42E-05
232698_at	BPIL1	bactericidal/permeability-increasing protein-like 1	1.61	7.15E-05
200920_s_at	BTG1	B-cell translocation gene 1, anti-proliferative	1.47	9.56E-06
203612_at	BYSL	bystin-like	1.35	8.56E-05
226892_at	C10orf12	chromosome 10 open reading frame 12	1.77	3.16E-05
226398_s_at	C10orf4	chromosome 10 open reading frame 4	2.15	1.01E-06
218983_at	C1RL	complement component 1, r subcomponent-like	1.80	9.01E-05
218094_s_at	C20orf35	chromosome 20 open reading frame 35	1.42	4.22E-05
224932_at	C22orf16	chromosome 22 open reading frame 16	3.81	7.23E-08
235051_at	C3orf6	chromosome 3 open reading frame 6	1.42	4.18E-05
205500_at	C5	complement component 5	1.96	1.72E-05
220088_at	C5R1	complement component 5 receptor 1 (C5a ligand)	2.13	7.35E-05
226135_at	C6orf107	chromosome 6 open reading frame 107	1.57	9.14E-05
226301_at	C6orf192	chromosome 6 open reading frame 192	1.43	8.77E-05
220755_s_at	C6orf48	chromosome 6 open reading frame 48	1.79	5.82E-05
227443_at	C9orf150	chromosome 9 open reading frame 150	2.31	1.47E-05
221865_at	C9orf91	chromosome 9 open reading frame 91	2.81	6.07E-05
213161_at	C9orf97	chromosome 9 open reading frame 97 /// chromosome 9 open reading frame 97	1.89	6.66E-05
217873_at	CAB39	calcium binding protein 39	1.43	5.89E-05

Table S1 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
212711_at	CAMSAP1	calmodulin regulated spectrin-associated protein 1	1.61	9.32E-05
221040_at	CAPN10	calpain 10	1.31	6.01E-05
212971_at	CARS	cysteinyl-tRNA synthetase	2.30	8.72E-07
227558_at	CBX4	chromobox homolog 4 (Pc class homolog, Drosophila)	2.82	2.93E-06
225010_at	CCDC6	coiled-coil domain containing 6	1.42	9.44E-05
221511_x_at	CCPG1	cell cycle progression 1	3.02	3.15E-05
218157_x_at	CDC42SE1	CDC42 small effector 1	2.37	4.13E-06
229120_s_at			2.36	6.67E-05
212501_at	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	3.34	1.22E-05
225527_at	CEBPG	CCAAT/enhancer binding protein (C/EBP), gamma	2.39	3.51E-05
204203_at			2.21	4.13E-05
213618_at	CENTD1	centaurin, delta 1	1.65	3.81E-05
204258_at	CHD1	chromodomain helicase DNA binding protein 1	1.64	9.02E-05
226803_at	CHMP4C	chromatin modifying protein 4C	2.10	9.61E-06
200811_at	CIRBP	cold inducible RNA binding protein	1.73	2.16E-05
224998_at	CKLFSF4	chemokine-like factor super family 4	1.61	3.50E-05
201734_at	CLCN3	chloride channel 3	2.44	1.17E-05
201735_s_at			2.42	2.70E-05
204576_s_at	CLUAP1	clusterin associated protein 1	1.60	1.24E-06
201161_s_at	CSDA	cold shock domain protein A	1.68	3.50E-05
208867_s_at	CSNK1A1	casein kinase 1, alpha 1	1.60	8.45E-05
204971_at	CSTA	cystatin A (stefin A)	2.66	8.00E-05
206085_s_at	CTH	cystathionase (cystathionine gamma-lyase)	3.06	1.19E-05
227520_at	CXorf15	chromosome X open reading frame 15	1.63	3.52E-05
206754_s_at	CYP2B7P1	cytochrome P450, family 2, subfamily B, polypeptide 7 pseudogene 1	2.34	6.45E-06
201925_s_at	DAF	decay accelerating factor for complement (CD55, Cromer blood group system)	5.53	5.07E-05
1555950_a_at			4.27	3.77E-06
224911_s_at	DCBLD2	discoidin, CUB and LCCL domain containing 2	1.82	5.45E-05
209383_at	DDIT3	DNA-damage-inducible transcript 3	6.68	3.76E-05
222239_s_at	DDX26	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 26	1.43	1.51E-05
221780_s_at	DDX27	DEAD (Asp-Glu-Ala-Asp) box polypeptide 27	1.68	4.30E-05
201479_at	DKC1	dyskeratosis congenita 1, dyskerin	1.31	5.89E-06
224822_at	DLC1	deleted in liver cancer 1	2.03	6.45E-06
201681_s_at	DLG5	discs, large homolog 5 (Drosophila)	1.59	4.45E-05
205963_s_at	DNAJA3	DnaJ (Hsp40) homolog, subfamily A, member 3	1.67	7.57E-05
1554078_s_at			1.55	1.84E-05
204135_at	DOC1	downregulated in ovarian cancer 1	1.69	7.07E-06
212254_s_at	DST	dystonin	1.77	9.24E-05
201041_s_at	DUSP1	dual specificity phosphatase 1	1.87	7.42E-05
226952_at	EAF1	ELL associated factor 1	1.73	3.07E-05
227404_s_at	EGR1	early growth response 1	4.54	5.22E-06
201694_s_at			3.49	1.21E-05
200597_at	EIF3S10	eukaryotic translation initiation factor 3, subunit 10 theta, 150/170kDa	1.77	5.89E-05

Table S1 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
200005_at	EIF3S7	eukaryotic translation initiation factor 3, subunit 7 zeta, 66/67kDa /// eukaryotic translation initiation factor 3, subunit 7 zeta, 66/67kDa	1.41	2.15E-05
221539_at	EIF4EBP1	eukaryotic translation initiation factor 4E binding protein 1	1.87	7.53E-06
235296_at	EIF5A2	eukaryotic translation initiation factor 5A2	2.34	2.08E-05
220161_s_at	EPB41L4B	erythrocyte membrane protein band 4.1 like 4B	1.84	3.45E-05
223427_s_at			1.74	8.64E-05
200843_s_at	EPRS	glutamyl-prolyl-tRNA synthetase	1.84	2.73E-05
235745_at	ERN1	endoplasmic reticulum to nucleus signalling 1	2.31	3.24E-05
227696_at	EXOSC6	exosome component 6	1.95	1.74E-05
225591_at	FBXO25	F-box protein 25	1.57	6.55E-05
224318_s_at	FLJ10081	hypothetical protein FLJ10081	1.38	9.15E-05
201919_at	FLJ10618	Hypothetical protein FLJ10618	2.17	3.79E-06
225327_at	FLJ10980	hypothetical protein FLJ10980	1.91	9.31E-05
228262_at	FLJ14503	hypothetical protein FLJ14503	1.75	4.26E-05
218984_at	FLJ20485	hypothetical protein FLJ20485	1.28	1.56E-05
1557828_a_at	FLJ21657	hypothetical protein FLJ21657	1.78	6.40E-05
222872_x_at	FLJ22833	hypothetical protein FLJ22833	2.22	3.86E-05
226184_at	FMNL2	formin-like 2	1.74	4.02E-05
228463_at	FOXA3	forkhead box A3	1.55	8.50E-05
224891_at	FOXO3A	forkhead box O3A	1.77	1.31E-05
223287_s_at	FOXP1	forkhead box P1	2.21	2.22E-05
218373_at	FTS	fused toes homolog (mouse)	1.51	1.52E-05
208693_s_at	GARS	glycyl-tRNA synthetase	2.21	8.04E-05
212378_at	GART	phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase	1.33	5.40E-05
210002_at	GATA6	GATA binding protein 6	1.21	5.97E-05
221577_x_at	GDF15	growth differentiation factor 15	2.88	6.37E-06
202722_s_at	GFPT1	glutamine-fructose-6-phosphate transaminase 1	2.21	3.24E-05
209248_at	GHITM	growth hormone inducible transmembrane protein	1.86	8.50E-05
203159_at	GLS	glutaminase	1.97	1.79E-05
213021_at	GOSR1	golgi SNAP receptor complex member 1	1.58	2.93E-05
208813_at	GOT1	glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1)	1.64	6.69E-06
224634_at	GPATC4	G patch domain containing 4	1.30	6.23E-05
222953_at	GPR83	G protein-coupled receptor 83	6.41	2.80E-07
209409_at	GRB10	growth factor receptor-bound protein 10	2.84	4.66E-05
235381_at	HBXAP	hepatitis B virus x associated protein	1.50	2.80E-05
200690_at	HSPA9B	heat shock 70kDa protein 9B (mortalin-2)	2.29	1.70E-05
228002_at	IDI2	isopentenyl-diphosphate delta isomerase 2	1.65	5.76E-05
202147_s_at	IFRD1	interferon-related developmental regulator 1	2.71	7.06E-05
210587_at	INHBE	inhibin, beta E	4.61	2.37E-05
202794_at	INPP1	inositol polyphosphate-1-phosphatase	1.65	1.76E-07
226216_at	INSR	Insulin receptor	1.48	7.99E-05
209098_s_at	JAG1	jagged 1 (Alagille syndrome)	2.10	9.40E-05
201648_at	JAK1	Janus kinase 1 (a protein tyrosine kinase)	1.54	7.04E-05
226352_at	JMY	Junction-mediating and regulatory protein	2.71	1.50E-05
217938_s_at	KCMF1	potassium channel modulatory factor 1	1.32	2.76E-05

Table S1 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
221125_s_at	KCNMB3	potassium large conductance calcium-activated channel, subfamily M beta member 3	5.21	5.45E-06
212395_s_at	KIAA0090	KIAA0090	1.57	8.17E-05
216913_s_at	KIAA0690	KIAA0690	2.24	9.26E-06
212942_s_at	KIAA1199	KIAA1199	2.44	1.13E-05
224826_at	KIAA1434	hypothetical protein KIAA1434	1.60	5.74E-05
233893_s_at	KIAA1530	KIAA1530 protein	1.68	1.18E-05
225770_at	KIAA1972	KIAA1972 protein	1.13	5.23E-05
212162_at	KIDINS220	kinase D-interacting substance of 220 kDa	1.27	9.69E-05
202393_s_at	KLF10	Kruppel-like factor 10	3.08	4.82E-05
205569_at	LAMP3	lysosomal-associated membrane protein 3	5.87	7.83E-06
236565_s_at	LARP6	La ribonucleoprotein domain family, member 6	3.37	5.10E-06
202386_s_at	LKAP	limkain b1	1.94	3.20E-05
209205_s_at	LMO4	LIM domain only 4	2.06	9.63E-05
221501_x_at	LOC339047	hypothetical protein LOC339047	2.25	7.87E-05
235147_at	LOC440928	hypothetical gene supported by AK096649	1.62	4.20E-05
220437_at	LOC55908	hepatocellular carcinoma-associated gene TD26	5.20	3.46E-07
226650_at	LOC90637	hypothetical protein LOC90637	1.68	5.15E-06
227145_at	LOXL4	lysyl oxidase-like 4	3.36	3.95E-05
211615_s_at	LRPPRC	leucine-rich PPR-motif containing /// leucine-rich PPR-motif containing	1.36	1.39E-06
226206_at	MAFK	v-maf musculoaponeurotic fibrosarcoma oncogene homolog K (avian)	1.81	9.33E-05
226084_at	MAP1B	microtubule-associated protein 1B	2.93	5.07E-06
208786_s_at	MAP1LC3B	microtubule-associated protein 1 light chain 3 beta	2.93	7.36E-06
201475_x_at	MARS	methionine-tRNA synthetase	2.26	7.23E-05
235141_at	MARVELD2	MARVEL domain containing 2	1.70	2.83E-05
236814_at	MDM4	Mdm4, transformed 3T3 cell double minute 4, p53 binding protein (mouse)	2.10	1.68E-05
213696_s_at	MED8	mediator of RNA polymerase II transcription, subunit 8 homolog (yeast)	1.34	3.58E-05
212535_at	MEF2A	MADS box transcription enhancer factor 2, polypeptide A (myocyte enhancer factor 2A)	1.71	2.12E-05
236285_at	MGC16635	hypothetical protein BC009980	7.04	6.98E-06
223297_at	MGC4268	hypothetical protein MGC4268	1.54	1.31E-05
219270_at	MGC4504	hypothetical protein MGC4504	3.44	9.31E-05
219959_at	MOCOS	molybdenum cofactor sulfurase	2.30	5.93E-05
219451_at	MSRB2	methionine sulfoxide reductase B2	1.75	9.43E-05
225520_at	MTHFD1L	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like	2.04	9.76E-06
233665_x_at	MTO1	mitochondrial translation optimization 1 homolog (S. cerevisiae)	1.37	6.07E-05
203037_s_at	MTSS1	metastasis suppressor 1	1.97	2.04E-05
228846_at	MXD1	MAX dimerization protein 1	3.27	6.90E-05
226275_at			2.14	1.45E-05
202431_s_at	MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	2.17	5.68E-05
201976_s_at	MYO10	myosin X	2.92	1.29E-05
211916_s_at	MYO1A	myosin IA /// myosin IA	1.56	1.56E-05
218330_s_at	NAV2	neuron navigator 2	2.12	4.67E-06
201384_s_at	NBR1	neighbor of BRCA1 gene 1	1.36	4.71E-05

Table S1 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
207700_s_at	NCOA3	nuclear receptor coactivator 3	1.35	3.00E-05
213331_s_at	NEK1	NIMA (never in mitosis gene a)-related kinase 1	1.46	5.62E-05
200759_x_at	NFE2L1	nuclear factor (erythroid-derived 2)-like 1	1.99	4.54E-05
203574_at	NFIL3	nuclear factor, interleukin 3 regulated	1.76	9.17E-07
223439_at	NKAP	NF-kappaB activating protein	2.51	1.15E-05
204538_x_at	NPIP /// LOC339047 /// LOC440341	nuclear pore complex interacting protein /// hypothetical protein LOC339047 /// similar to hypothetical protein LOC339047	2.40	4.38E-05
207563_s_at	OGT	O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase)	1.66	3.97E-05
1554503_a_at	OSCAR	osteoclast-associated receptor	2.77	1.49E-05
210401_at	P2RX1	purinergic receptor P2X, ligand-gated ion channel, 1	3.45	1.26E-07
209230_s_at	P8	p8 protein (candidate of metastasis 1)	2.20	1.25E-06
200816_s_at	PAFAH1B1	platelet-activating factor acetylhydrolase, isoform Ib, alpha subunit 45kDa	1.66	8.96E-05
204005_s_at	PAWR	PRKC, apoptosis, WT1, regulator	1.46	3.25E-05
224986_s_at	PDPK1	3-phosphoinositide dependent protein kinase-1	1.72	4.74E-05
218336_at	PFDN2	prefoldin 2	1.47	2.14E-05
209803_s_at	PHLDA2	pleckstrin homology-like domain, family A, member 2	2.99	7.89E-06
209318_x_at	PLAGL1	pleiomorphic adenoma gene-like 1	1.18	8.11E-05
218258_at	POLR1D	polymerase (RNA) I polypeptide D, 16kDa	1.13	5.66E-05
202066_at	PPFIA1	protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 1	1.54	5.44E-05
201500_s_at	PPP1R11	protein phosphatase 1, regulatory (inhibitor) subunit 11	1.67	7.36E-06
37028_at	PPP1R15A	protein phosphatase 1, regulatory (inhibitor) subunit 15A	2.87	1.14E-05
202014_at			2.22	1.45E-05
203338_at	PPP2R5E	protein phosphatase 2, regulatory subunit B (B56), epsilon isoform	1.54	5.52E-05
1555781_at	PQLC2	PQ loop repeat containing 2	1.69	3.15E-05
212217_at	PREPL	prolyl endopeptidase-like	1.94	4.26E-05
225278_at	PRKAB2	protein kinase, AMP-activated, beta 2 non-catalytic subunit	1.70	1.23E-05
1558027_s_at			1.62	1.81E-05
215707_s_at	PRNP	prion protein (p27-30) (Creutzfeld-Jakob disease, Gerstmann-Strausler-Scheinker syndrome, fatal familial insomnia)	2.04	5.79E-05
202529_at	PRPSAP1	phosphoribosyl pyrophosphate synthetase-associated protein 1	1.46	9.15E-05
202525_at	PRSS8	protease, serine, 8 (prostasin)	2.83	5.77E-06
212222_at	PSME4	proteasome (prosome, macropain) activator subunit 4	1.24	2.86E-05
224937_at	PTGFRN	prostaglandin F2 receptor negative regulator	1.30	5.70E-05
203997_at	PTPN3	protein tyrosine phosphatase, non-receptor type 3	1.89	1.41E-06

Table S1 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
201166_s_at	PUM1	pumilio homolog 1 (Drosophila)	1.42	1.04E-05
216221_s_at	PUM2	pumilio homolog 2 (Drosophila)	1.39	5.99E-05
213530_at	RAB3GAP	RAB3 GTPase-activating protein	1.78	6.18E-05
224880_at	RALA	v-ral simian leukemia viral oncogene homolog A (ras related)	1.81	1.35E-05
201712_s_at	RANBP2	RAN binding protein 2	1.30	3.77E-05
210676_x_at	RANBP2L1	RAN binding protein 2-like 1	1.49	7.01E-05
220746_s_at	RAP80	receptor associated protein 80	1.42	1.07E-05
223467_at	RASD1	RAS, dexamethasone-induced 1	3.59	8.35E-05
203250_at	RBM16	RNA binding motif protein 16	1.54	7.02E-05
218134_s_at	RBM22	RNA binding motif protein 22	1.17	8.57E-06
214113_s_at	RBM8A	RNA binding motif protein 8A	1.37	6.92E-05
213878_at	RECQL	RecQ protein-like (DNA helicase Q1-like)	2.03	9.25E-05
212116_at	RFP	ret finger protein	1.25	2.45E-05
209568_s_at	RGL1	ral guanine nucleotide dissociation stimulator-like 1	2.52	3.13E-05
219610_at	RGNEF	rho-guanine nucleotide exchange factor	1.40	1.93E-05
212120_at	RHOQ	ras homolog gene family, member Q	1.96	2.01E-05
223168_at	RHOU	ras homolog gene family, member U	1.43	9.01E-05
202130_at	RIOK3	RIO kinase 3 (yeast) /// RIO kinase 3 (yeast)	2.05	3.74E-05
212155_at	RNF187	ring finger protein 187	1.93	5.49E-05
210716_s_at	RSN	restin (Reed-Steinberg cell-expressed intermediate filament-associated protein)	2.03	1.41E-05
231894_at	SARS	seryl-tRNA synthetase	3.49	3.02E-05
200802_at			2.12	1.10E-06
213435_at	SATB2	SATB family member 2	1.87	2.90E-05
224983_at	SCARB2	scavenger receptor class B, member 2	1.54	3.79E-05
224250_s_at	SECISBP2	SECIS binding protein 2	1.55	5.97E-05
218265_at			1.44	1.62E-05
202318_s_at	SENP6	SUMO1/sentrin specific protease 6	1.63	5.69E-05
212190_at	SERPINE2	serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2	2.49	6.82E-06
202656_s_at	SERTAD2	SERTA domain containing 2	1.58	3.75E-05
36545_s_at	SFI1	Sfi1 homolog, spindle assembly associated (yeast)	3.14	2.68E-05
214402_s_at			1.89	7.82E-05
212322_at	SGPL1	sphingosine-1-phosphate lyase 1	1.40	5.79E-05
210101_x_at	SH3GLB1	SH3-domain GRB2-like endophilin B1	1.59	3.75E-05
209091_s_at			1.57	1.96E-05
209090_s_at			1.30	8.96E-05
214437_s_at	SHMT2	serine hydroxymethyltransferase 2 (mitochondrial)	1.39	3.52E-05

Table S1 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
207097_s_at	SLC17A2	solute carrier family 17 (sodium phosphate), member 2	2.96	1.97E-05
213549_at	SLC18A2	solute carrier family 18 (vesicular monoamine), member 2	1.47	2.42E-05
209610_s_at	SLC1A4	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	1.77	2.69E-05
201920_at	SLC20A1	solute carrier family 20 (phosphate transporter), member 1	1.26	3.62E-05
228497_at	SLC22A15	solute carrier family 22 (organic cation transporter), member 15	4.31	1.42E-05
217122_s_at	SLC35E2	solute carrier family 35, member E2	1.81	6.87E-05
224579_at	SLC38A1	Solute carrier family 38, member 1	1.82	1.29E-05
200924_s_at	SLC3A2	solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	2.18	9.81E-05
212295_s_at	SLC7A1	solute carrier family 7 (cationic amino acid transporter, y+ system), member 1	2.58	1.11E-05
209921_at	SLC7A11	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	4.18	6.76E-07
217678_at			3.21	2.25E-06
225516_at	SLC7A2	solute carrier family 7 (cationic amino acid transporter, y+ system), member 2	2.83	7.95E-06
203373_at	SOCS2	suppressor of cytokine signaling 2	2.44	6.61E-05
228662_at	SOCS7	suppressor of cytokine signaling 7	1.64	1.41E-05
206239_s_at	SPINK1	serine protease inhibitor, Kazal type 1	3.92	7.57E-05
1554807_a_at	SPIRE1	spire homolog 1 (Drosophila)	2.15	2.56E-05
226342_at	SPTBN1	spectrin, beta, non-erythrocytic 1	4.63	8.23E-06
200672_x_at			2.49	7.07E-05
212071_s_at			2.13	4.10E-05
203439_s_at	STC2	stanniocalcin 2	1.81	4.73E-05
228967_at	SUI1	putative translation initiation factor	1.61	6.29E-05
213090_s_at	TAF4	TAF4 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 135kDa	1.36	3.87E-05
225308_s_at	TANC	TPR domain, ankyrin-repeat and coiled-coil-containing	1.74	8.95E-05
201263_at	TARS	threonyl-tRNA synthetase	1.87	6.08E-05
226409_at	TBC1D20	TBC1 domain family, member 20	1.34	8.04E-05
221471_at	TDE1	tumor differentially expressed 1	1.31	3.43E-05
208944_at	TGFBR2	transforming growth factor, beta receptor II (70/80kDa)	1.28	3.53E-05
226628_at	THOC2	THO complex 2	1.46	5.54E-05
225698_at	TIGA1	TIGA1	2.68	3.14E-07
228256_s_at			1.93	1.38E-05
202085_at	TJP2	tight junction protein 2 (zona occludens 2)	1.26	9.91E-05
228284_at	TLE1 /// LOC389863	transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila) /// hypothetical gene supported by BC000228; NM_005077	1.87	7.50E-05

Table S1 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
236430_at	TMED6	transmembrane emp24 protein transport domain containing 6	2.25	4.38E-05
209226_s_at	TNPO1	transportin 1	1.40	5.11E-05
202241_at	TRIB1	tribbles homolog 1 (Drosophila)	2.08	1.43E-05
1555788_a_at	TRIB3	tribbles homolog 3 (Drosophila)	3.96	5.90E-06
218145_at			3.19	2.83E-05
210266_s_at	TRIM33	tripartite motif-containing 33	1.89	8.34E-05
218972_at	TTC17	tetratricopeptide repeat domain 17	2.40	6.09E-05
201010_s_at	TXNIP	thioredoxin interacting protein	3.17	2.10E-05
208174_x_at	U2AF1L2	U2(RNU2) small nuclear RNA auxiliary factor 1-like 2	1.41	5.88E-05
211285_s_at	UBE3A	ubiquitin protein ligase E3A (human papilloma virus E6-associated protein, Angelman syndrome)	1.22	2.44E-05
201535_at	UBL3	ubiquitin-like 3	1.81	3.45E-05
222991_s_at	UBQLN1	ubiquilin 1	1.33	4.64E-06
232299_at	UNQ830	ASCL830	2.00	9.80E-05
220419_s_at	USP25	ubiquitin specific protease 25	1.85	1.06E-05
221654_s_at	USP3	ubiquitin specific protease 3	1.48	2.72E-05
212066_s_at	USP34	ubiquitin specific protease 34	1.38	2.05E-05
221513_s_at	UTP14C /// UTP14A	UTP14, U3 small nucleolar ribonucleoprotein, homolog C (yeast) /// UTP14, U3 small nucleolar ribonucleoprotein, homolog A (yeast)	1.51	4.72E-05
209822_s_at	VLDLR	very low density lipoprotein receptor	3.04	4.05E-05
217742_s_at	WAC	WW domain containing adaptor with coiled-coil	1.65	2.71E-05
200629_at	WARS	tryptophanyl-tRNA synthetase	4.46	1.87E-05
200628_s_at			2.56	4.32E-05
224813_at	WASL	Wiskott-Aldrich syndrome-like	1.93	8.33E-05
218107_at	WDR26	WD repeat domain 26	1.49	2.73E-06
219001_s_at	WDR32	WD repeat domain 32	1.42	2.20E-06
221735_at	WDR48	WD repeat domain 48	1.38	4.80E-05
221712_s_at	WDR74	WD repeat domain 74 /// WD repeat domain 74	1.58	7.22E-05
212048_s_at	YARS	tyrosyl-tRNA synthetase	1.99	5.34E-05
222408_s_at	YPEL5	yippee-like 5 (Drosophila)	2.12	2.79E-05
217783_s_at			1.69	7.09E-05
210275_s_at	ZA20D2	zinc finger, A20 domain containing 2	1.68	5.98E-05
205788_s_at	ZC3H11A	zinc finger CCCH-type containing 11A	1.46	5.61E-05
223506_at	ZC3H8	zinc finger CCCH-type containing 8	3.20	1.11E-05
217367_s_at	ZHX3	zinc fingers and homeoboxes 3	1.28	5.43E-05

Table S1 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
223642_at	ZIC2	Zic family member 2 (odd-paired homolog, Drosophila)	2.03	7.54E-05
225221_at	ZKSCAN1	zinc finger with KRAB and SCAN domains 1	1.46	5.04E-05
202136_at	ZMYND11	zinc finger, MYND domain containing 11	1.43	7.58E-06
215022_x_at	ZNF11B	zinc finger protein 11B	1.77	3.49E-05
202049_s_at	ZNF262	zinc finger protein 262	1.24	3.18E-05
225539_at	ZNF295	zinc finger protein 295	1.38	3.38E-05
204175_at	ZNF593	zinc finger protein 593	1.15	3.38E-05
227822_at	ZNF605	zinc finger protein 605	1.94	2.73E-05
211257_x_at	ZNF638	zinc finger protein 638	1.33	2.40E-05
227755_at		CDNA clone IMAGE:4077090, partial cds	5.24	3.16E-06
225522_at		similar to BMP2 inducible kinase	3.12	1.23E-05
226886_at		clone 114 tumor rejection antigen mRNA, complete cds	1.97	3.38E-05
1555832_s_at		Homo sapiens, clone IMAGE:4096273, mRNA	1.81	5.95E-05
235360_at		unknown mRNA	1.81	9.65E-05
239065_at			1.76	1.27E-05
224606_at		Homo sapiens, clone IMAGE:4096273, mRNA	1.74	7.85E-07
229428_at		CDNA FLJ40725 fis, clone TKIDN1000001, highly similar to translocase of inner mitochondrial membrane 23	1.73	3.95E-05
217625_x_at		hypothetical gene supported by AK024177	1.73	8.97E-05
225935_at		FP6778	1.66	3.84E-05
234985_at			1.63	4.02E-05
212462_at			1.62	8.34E-05
226669_at			1.54	4.09E-05
227356_at		CDNA: FLJ22198 fis, clone HRC01218	1.51	6.95E-05
226949_at			1.48	6.85E-06
228661_s_at		CDNA FLJ11489 fis, clone HEMBA1001915	1.39	9.69E-05
229803_s_at		AF034176 Human mRNA (Tripodis and Ragoussis) Homo sapiens cDNA clone ntcon5 contig	1.39	7.09E-06
243553_x_at			1.16	2.38E-05

[1]To be included in this table, differential gene expression had to meet a statistical cutoff of $P<0.0001$.

Table S2. Genes whose expression was downregulated in HepG2/C3A cells cultured in –Leu medium[1]

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
1554878_a_at	ABCD3	ATP-binding cassette, sub-family D (ALD), member 3	0.42	8.83E-05
218405_at	ABT1	activator of basal transcription 1	0.67	7.64E-05
234312_s_at	ACAS2	acetyl-Coenzyme A synthetase 2 (ADP forming)	0.42	1.69E-05
209608_s_at	ACAT2	acetyl-Coenzyme A acetyltransferase 2 (acetoacetyl Coenzyme A thiolase)	0.27	3.17E-06
208950_s_at	ALDH7A1	aldehyde dehydrogenase 7 family, member A1	0.51	8.69E-05
208951_at			0.47	2.04E-05
220148_at	ALDH8A1	aldehyde dehydrogenase 8 family, member A1	0.37	2.65E-05
214687_x_at	ALDOA	aldolase A, fructose-bisphosphate	0.66	6.82E-05
238996_x_at			0.50	2.75E-06
226414_s_at	ANAPC11	APC11 anaphase promoting complex subunit 11 homolog (yeast)	0.65	2.84E-05
208722_s_at	ANAPC5	anaphase promoting complex subunit 5	0.72	6.04E-05
222608_s_at	ANLN	anillin, actin binding protein (scraps homolog, Drosophila)	0.32	5.27E-05
201590_x_at	ANXA2	annexin A2	0.64	1.01E-05
213503_x_at			0.62	3.68E-05
201302_at	ANXA4	annexin A4	0.57	1.85E-05
201301_s_at			0.54	7.14E-07
208074_s_at	AP2S1	adaptor-related protein complex 2, sigma 1 subunit	0.70	8.13E-05
206632_s_at	APOBEC3B	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3B	0.28	1.11E-06
229526_at	AQP11	aquaporin 11	0.54	3.40E-05
225171_at	ARHGAP18	Rho GTPase activating protein 18	0.36	1.84E-05
211935_at	ARL6IP	ADP-ribosylation factor-like 6 interacting protein	0.53	1.57E-05
219094_at	ARMC8	armadillo repeat containing 8	0.75	9.35E-05
210971_s_at	ARNTL	aryl hydrocarbon receptor nuclear translocator-like	0.56	8.84E-06
222912_at	ARRB1	arrestin, beta 1	0.64	7.68E-05

Table S2 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
205673_s_at	ASB9	ankyrin repeat and SOCS box-containing 9	0.33	9.02E-05
209517_s_at	ASH2L	ash2 (absent, small, or homeotic)-like (Drosophila)	0.77	8.46E-05
244519_at	ASXL1	additional sex combs like 1 (Drosophila)	0.53	2.55E-05
202961_s_at	ATP5J2	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit f, isoform 2	0.59	4.48E-05
233110_s_at	BCL2L12	BCL2-like 12 (proline rich)	0.60	3.61E-05
210334_x_at	BIRC5	baculoviral IAP repeat-containing 5 (survivin)	0.27	5.40E-05
227291_s_at	BOLA3	bolA-like 3 (E. coli)	0.72	9.96E-05
235609_at	BRIP1	BRCA1 interacting protein C-terminal helicase 1	0.38	2.66E-05
202427_s_at	BRP44	brain protein 44	0.62	5.18E-05
209642_at	BUB1	BUB1 budding uninhibited by benzimidazoles 1 homolog (yeast)	0.33	7.79E-05
201457_x_at	BUB3	BUB3 budding uninhibited by benzimidazoles 3 homolog (yeast)	0.72	4.97E-05
219067_s_at	C10orf86	chromosome 10 open reading frame 86	0.56	1.24E-05
202562_s_at	C14orf1	chromosome 14 open reading frame 1	0.41	1.32E-05
217188_s_at			0.40	4.13E-05
214264_s_at	C14orf143	chromosome 14 open reading frame 143	0.52	7.15E-05
238691_at	C14orf62	chromosome 14 open reading frame 62	0.73	6.13E-05
218493_at	C16orf33	chromosome 16 open reading frame 33	0.40	7.57E-06
221739_at	C19orf10	chromosome 19 open reading frame 10	0.76	4.12E-05
212003_at	C1orf144	chromosome 1 open reading frame 144	0.53	1.20E-05
225687_at	C20orf129	chromosome 20 open reading frame 129	0.37	5.83E-05
219512_at	C20orf172	chromosome 20 open reading frame 172	0.38	5.55E-06
224972_at	C20orf52	chromosome 20 open reading frame 52	0.59	3.70E-05
226936_at	C6orf173	chromosome 6 open reading frame 173	0.34	1.83E-05
228053_s_at	C9orf105	chromosome 9 open reading frame 105	0.71	7.35E-05
210691_s_at	CACYBP	calcyclin binding protein	0.65	8.12E-05
201381_x_at			0.65	6.01E-05
209563_x_at	CALM1	calmodulin 1 (phosphorylase kinase, delta)	0.64	3.12E-05
200935_at	CALR	calreticulin	0.42	5.90E-05
225693_s_at	CAMTA1	calmodulin binding transcription activator 1	0.69	6.75E-06
201432_at	CAT	catalase	0.64	3.81E-05
213226_at	CCNA2	cyclin A2	0.34	2.20E-05
203418_at			0.27	1.51E-05
228729_at	CCNB1	cyclin B1	0.35	9.74E-06
214710_s_at			0.35	2.70E-05

Table S2 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
266_s_at	CD24	CD24 antigen (small cell lung carcinoma cluster 4 antigen)	0.58	7.94E-05
201028_s_at	CD99	CD99 antigen	0.66	1.46E-05
203214_x_at	CDC2	cell division cycle 2, G1 to S and G2 to M	0.30	3.59E-05
210559_s_at			0.29	2.17E-05
203968_s_at	CDC6	CDC6 cell division cycle 6 homolog (S. cerevisiae)	0.30	2.39E-06
203967_at			0.27	1.88E-05
204252_at	CDK2	cyclin-dependent kinase 2	0.45	4.15E-06
211804_s_at			0.34	9.04E-05
204159_at	CDKN2C	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	0.27	5.00E-05
209714_s_at	CDKN3	cyclin-dependent kinase inhibitor 3 (CDK2-associated dual specificity phosphatase)	0.34	6.12E-05
204962_s_at	CENPA	centromere protein A, 17kDa	0.38	8.50E-05
209194_at	CETN2	centrin, EF-hand protein, 2	0.38	2.64E-06
205394_at	CHEK1	CHK1 checkpoint homolog (S. pombe)	0.42	1.20E-05
205393_s_at			0.38	1.44E-05
210416_s_at	CHEK2	CHK2 checkpoint homolog (S. pombe)	0.38	6.75E-05
201953_at	CIB1	calcium and integrin binding 1 (calmyrin)	0.58	1.59E-05
204170_s_at	CKS2	CDC28 protein kinase regulatory subunit 2	0.46	3.95E-06
1554804_a_at	CLDN19	claudin 19	0.51	4.65E-05
222409_at	CORO1C	coronin, actin binding protein, 1C	0.75	9.26E-05
203880_at	COX17	COX17 homolog, cytochrome c oxidase assembly protein (yeast)	0.52	3.53E-06
218057_x_at	COX4NB	COX4 neighbor	0.70	1.14E-05
201441_at	COX6B1	cytochrome c oxidase subunit Vib polypeptide 1 (ubiquitous)	0.62	5.63E-05
206651_s_at	CPB2	carboxypeptidase B2 (plasma, carboxypeptidase U)	0.30	4.79E-06
205081_at	CRIP1	cysteine-rich protein 1 (intestinal)	0.28	1.44E-05
201112_s_at	CSE1L	CSE1 chromosome segregation 1-like (yeast)	0.50	5.96E-06
210766_s_at			0.47	1.74E-06
228906_at	CXXC6	CXXC finger 6	0.51	4.95E-05
207843_x_at	CYB5	cytochrome b-5	0.67	5.85E-06
209366_x_at			0.64	1.93E-05
1554574_a_at	CYB5R3	cytochrome b5 reductase 3	0.49	5.67E-05
216607_s_at	CYP51A1	cytochrome P450, family 51, subfamily A, polypeptide 1	0.26	4.31E-05
202428_x_at	DBI	diazepam binding inhibitor (GABA receptor modulator, acyl-Coenzyme A binding protein)	0.42	1.63E-05
209389_x_at			0.25	3.27E-06
222889_at	DCLRE1B	DNA cross-link repair 1B (PSO2 homolog, S. cerevisiae)	0.54	3.10E-05
226980_at	DEPDC1B	DEP domain containing 1B	0.36	3.56E-05

Table S2 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
209549_s_at	DGUOK	deoxyguanosine kinase	0.74	4.27E-05
202534_x_at	DHFR	dihydrofolate reductase	0.43	5.72E-05
48808_at			0.36	1.95E-05
218285_s_at	DHRS6	dehydrogenase/reductase (SDR family) member 6	0.53	9.70E-05
203764_at	DLG7	discs, large homolog 7 (Drosophila)	0.37	9.47E-05
215253_s_at	DSCR1	Down syndrome critical region gene 1	0.81	7.79E-05
222680_s_at	DTL	denticleless homolog (Drosophila)	0.42	2.14E-06
1553984_s_at	DTYMK	deoxythymidylate kinase (thymidylate kinase)	0.43	3.54E-05
203270_at			0.39	3.54E-05
208956_x_at	DUT	dUTP pyrophosphatase	0.50	7.95E-05
209932_s_at			0.40	1.78E-06
228033_at	E2F7	E2F transcription factor 7	0.45	5.24E-05
219990_at	E2F8	E2F transcription factor 8	0.36	1.86E-05
220942_x_at	E2IG5	growth and transformation-dependent protein	0.62	3.92E-05
223193_x_at			0.60	6.19E-05
202735_at	EBP	emopamil binding protein (sterol isomerase)	0.32	2.82E-06
213787_s_at			0.29	1.24E-05
223087_at	ECHDC1	enoyl Coenzyme A hydratase domain containing 1	0.67	4.94E-06
201135_at	ECHS1	enoyl Coenzyme A hydratase, short chain, 1, mitochondrial	0.75	8.93E-05
201341_at	ENC1	ectodermal-neural cortex (with BTB-like domain)	0.49	1.94E-05
202596_at	ENSA	endosulfine alpha	0.59	4.85E-05
209527_at	EXOSC2	exosome component 2	0.67	3.95E-05
208964_s_at	FADS1	fatty acid desaturase 1	0.33	3.85E-05
208962_s_at			0.29	7.16E-05
202218_s_at	FADS2	fatty acid desaturase 2	0.57	1.69E-05
225684_at	FAM33A	family with sequence similarity 33, member A	0.56	3.38E-05
203564_at	FANCG	Fanconi anemia, complementation group G	0.45	2.19E-05
203255_at	FBXO11	F-box protein 11	0.60	1.42E-06
239358_at	FDFT1	farnesyl-diphosphate farnesyltransferase 1	0.56	1.51E-05
208647_at			0.37	9.33E-08
210950_s_at			0.30	2.55E-05
201275_at	FDPS	farnesyl diphosphate synthase (farnesyl pyrophosphate synthetase, dimethylallyltranstransferase, geranyltranstransferase)	0.37	3.38E-06
204768_s_at	FEN1	flap structure-specific endonuclease 1	0.34	3.41E-06
204767_s_at			0.28	1.66E-05
45633_at	FLJ13912	hypothetical protein FLJ13912	0.58	8.54E-05

Table S2 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
219544_at	FLJ22624	FLJ22624 protein	0.43	2.77E-05
205674_x_at	FXYD2	FXYD domain containing ion transport regulator 2	0.60	6.08E-05
203178_at	GATM	glycine amidinotransferase (L-arginine:glycine amidinotransferase)	0.40	1.51E-05
36475_at	GCAT	glycine C-acetyltransferase (2-amino-3-ketobutyrate coenzyme A ligase)	0.58	9.06E-06
204867_at	GCHFR	GTP cyclohydrolase I feedback regulator	0.31	2.44E-05
230788_at	GCNT2	glucosaminyl (N-acetyl) transferase 2, I-branching enzyme	0.52	3.79E-06
214430_at	GLA	galactosidase, alpha	0.43	5.98E-05
200681_at	GLO1	glyoxalase I	0.54	2.74E-06
200946_x_at	GLUD1	glutamate dehydrogenase 1	0.79	2.01E-05
235678_at	GM2A	GM2 ganglioside activator	0.49	9.12E-05
215891_s_at			0.44	3.24E-05
212737_at			0.36	5.83E-05
205240_at	GPSM2	G-protein signalling modulator 2 (AGS3-like, C. elegans)	0.65	2.01E-05
208336_s_at	GPSN2	glycoprotein, synaptic 2	0.48	5.89E-06
214091_s_at	GPX3	glutathione peroxidase 3 (plasma)	0.61	4.80E-05
202554_s_at	GSTM3	glutathione S-transferase M3 (brain)	0.57	9.47E-05
1557915_s_at	GSTO1	glutathione S-transferase omega 1	0.73	7.79E-05
223128_at	H17	hypothetical protein H17	0.78	3.33E-05
202487_s_at	H2AFV	H2A histone family, member V	0.58	4.73E-05
201036_s_at	HADHSC	L-3-hydroxyacyl-Coenzyme A dehydrogenase, short chain	0.43	6.49E-05
203138_at	HAT1	histone acetyltransferase 1	0.45	2.49E-05
218662_s_at	HCAP-G	chromosome condensation protein G	0.38	4.61E-05
223556_at	HELLS	helicase, lymphoid-specific	0.35	5.45E-05
202540_s_at	HMGCR	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	0.36	1.40E-05
202539_s_at			0.31	1.04E-05
205822_s_at	HMGCS1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	0.27	5.41E-05
209709_s_at	HMMR	hyaluronan-mediated motility receptor (RHAMM)	0.39	4.75E-05
222396_at	HN1	hematological and neurological expressed 1	0.68	4.40E-05
216855_s_at	HNRPU	heterogeneous nuclear ribonucleoprotein U (scaffold attachment factor A)	0.68	4.87E-05
206445_s_at	HRMT1L2	HMT1 hnRNP methyltransferase-like 2 (S. cerevisiae)	0.80	7.75E-05
209513_s_at	HSDL2	hydroxysteroid dehydrogenase like 2	0.71	1.76E-07
218026_at	HSPC009	HSPC009 protein	0.57	2.00E-06
217774_s_at	HSPC152	hypothetical protein HSPC152	0.68	7.46E-05
206855_s_at	HYAL2	hyaluronoglucosaminidase 2	0.70	6.87E-06

Table S2 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
204615_x_at	IDI1	isopentenyl-diphosphate delta isomerase 1	0.24	9.61E-06
208881_x_at			0.23	8.21E-07
203854_at	IF	I factor (complement)	0.52	2.48E-05
201163_s_at	IGFBP7	insulin-like growth factor binding protein 7	0.71	6.76E-05
201234_at	ILK	integrin-linked kinase	0.67	8.68E-05
242787_at	INCENP	Inner centromere protein antigens 135/155kDa	0.56	2.13E-05
206245_s_at	IVNS1ABP	influenza virus NS1A binding protein	0.64	2.43E-05
202503_s_at	KIAA0101	KIAA0101	0.39	4.50E-05
243136_at	KIAA0182	KIAA0182 protein	0.62	5.22E-05
221952_x_at	KIAA1393	KIAA1393	0.48	9.55E-05
218755_at	KIF20A	kinesin family member 20A	0.29	8.38E-05
204162_at	KNTC2	kinetochore associated 2	0.52	2.99E-05
211762_s_at	KPNA2	karyopherin alpha 2 (RAG cohort 1, importin alpha 1) /// karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	0.63	6.01E-05
201650_at	KRT19	keratin 19	0.48	1.10E-05
219541_at	LIME1	Lck interacting transmembrane adaptor 1	0.45	5.16E-05
200805_at	LMAN2	lectin, mannose-binding 2	0.73	4.12E-05
218574_s_at	LMCD1	LIM and cysteine-rich domains 1	0.74	2.49E-05
225614_at	LOC113174	hypothetical protein BC012010	0.56	7.90E-05
225404_at	LOC113444	hypothetical protein BC011880	0.76	1.96E-05
222622_at	LOC283871	hypothetical protein LOC283871	0.32	5.42E-05
225967_s_at	LOC284184	hypothetical LOC284184	0.65	1.30E-05
230937_at	LOC285835	hypothetical protein LOC285835	0.81	5.52E-06
224637_at	LOC400948	similar to RIKEN cDNA 2310016E02	0.62	7.41E-06
237017_s_at	LOC440286	LOC440286	0.49	2.59E-05
208107_s_at	LOC81691	exonuclease NEF-sp /// exonuclease NEF-sp	0.55	3.20E-05
202209_at	LSM3	LSM3 homolog, U6 small nuclear RNA associated (S. cerevisiae)	0.45	7.15E-05
202736_s_at	LSM4	LSM4 homolog, U6 small nuclear RNA associated (S. cerevisiae)	0.50	3.23E-05
202245_at	LSS	lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)	0.25	1.18E-05
212282_at	MAC30	hypothetical protein MAC30	0.40	9.05E-05
212279_at			0.31	7.42E-07
208309_s_at	MALT1	mucosa associated lymphoid tissue lymphoma translocation gene 1	0.69	8.11E-05
207256_at	MBL2	mannose-binding lectin (protein C) 2, soluble (opsonic defect)	0.46	4.39E-07
226238_at	MCEE	methylmalonyl CoA epimerase	0.60	1.35E-05
201555_at	MCM3	MCM3 minichromosome maintenance deficient 3 (S. cerevisiae)	0.39	2.61E-05

Table S2 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
222036_s_at	MCM4	MCM4 minichromosome maintenance deficient 4 (S. cerevisiae)	0.44	9.38E-07
201930_at	MCM6	MCM6 minichromosome maintenance deficient 6 (MIS5 homolog, S. pombe) (S. cerevisiae)	0.33	2.69E-05
224320_s_at	MCM8	MCM8 minichromosome maintenance deficient 8 (S. cerevisiae)	0.55	9.60E-05
204825_at	MELK	maternal embryonic leucine zipper kinase	0.33	6.66E-05
225568_at	MGC14141	hypothetical protein MGC14141	0.29	3.70E-05
226456_at	MGC24665	hypothetical protein MGC24665	0.27	2.40E-05
227063_at	MGC40107	hypothetical protein MGC40107	0.47	5.33E-06
228221_at	MGC45474	hypothetical protein MGC45474	0.72	2.21E-05
225799_at	MGC4677 /// LOC541471	hypothetical protein MGC4677 /// hypothetical LOC541471 protein	0.49	7.80E-05
231736_x_at	MGST1	microsomal glutathione S-transferase 1	0.78	5.62E-05
217909_s_at	MLX	MAX-like protein X	0.78	8.14E-05
226790_at	MOPT	protein containing single MORN motif in testis	0.57	7.09E-05
205235_s_at	MPHOSPH1	M-phase phosphoprotein 1	0.40	1.13E-05
225298_at	MR-1	myofibrillogenesis regulator 1	0.54	3.23E-05
204386_s_at	MRP63	mitochondrial ribosomal protein 63	0.63	6.28E-05
217907_at	MRPL18	mitochondrial ribosomal protein L18	0.70	9.17E-06
213897_s_at	MRPL23	mitochondrial ribosomal protein L23	0.61	5.50E-05
224330_s_at	MRPL27	mitochondrial ribosomal protein L27 /// mitochondrial ribosomal protein L27	0.74	9.95E-05
222993_at	MRPL37	mitochondrial ribosomal protein L37	0.71	7.32E-05
227186_s_at	MRPL41	mitochondrial ribosomal protein L41	0.66	6.31E-06
210008_s_at	MRPS12	mitochondrial ribosomal protein S12	0.50	2.35E-05
202911_at	MSH6	mutS homolog 6 (E. coli)	0.58	2.37E-06
211450_s_at			0.548	5.26E-06
213629_x_at	MT1F	metallothionein 1F (functional)	0.44	3.01E-05
217165_x_at			0.44	5.21E-05
204745_x_at	MT1G	metallothionein 1G	0.40	6.57E-05
206461_x_at	MT1H	metallothionein 1H	0.53	5.56E-05
204326_x_at	MT1X	metallothionein 1X	0.38	7.93E-06
222400_s_at	MTCBP-1	membrane-type 1 matrix metalloproteinase cytoplasmic tail binding protein-1	0.54	7.17E-05
217761_at			0.45	1.00E-06
214268_s_at	MTMR4	myotubularin related protein 4	0.70	2.01E-05
36907_at	MVK	mevalonate kinase (mevalonic aciduria)	0.48	5.38E-05
201969_at	NASP	nuclear autoantigenic sperm protein (histone-binding)	0.48	3.76E-05
202839_s_at	NDUFB7	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7, 18kDa	0.53	7.45E-05

Table S2 (Continued

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
201757_at	NDUFS5	NADH dehydrogenase (ubiquinone) Fe-S protein 5, 15kDa (NADH-coenzyme Q reductase)	0.57	7.91E-05
201840_at	NEDD8	neural precursor cell expressed, developmentally down-regulated 8	0.54	5.01E-05
219502_at	NEIL3	nei endonuclease VIII-like 3 (E. coli)	0.47	2.75E-05
201076_at	NHP2L1	NHP2 non-histone chromosome protein 2-like 1 (S. cerevisiae)	0.79	7.02E-05
218133_s_at	NIF3L1	NIF3 NGG1 interacting factor 3-like 1 (S. pombe)	0.65	5.02E-05
209104_s_at	NOLA2	nucleolar protein family A, member 2 (H/ACA small nucleolar RNPs)	0.70	1.10E-06
200701_at	NPC2	Niemann-Pick disease, type C2	0.67	2.32E-05
219347_at	NUDT15	nudix (nucleoside diphosphate linked moiety X)-type motif 15	0.49	1.31E-05
219978_s_at	NUSAP1	nucleolar and spindle associated protein 1	0.37	2.61E-05
218039_at			0.35	4.92E-05
202397_at	NUTF2	nuclear transport factor 2	0.55	1.90E-05
213599_at	OIP5	Opa interacting protein 5	0.31	2.09E-05
218162_at	OLFML3	olfactomedin-like 3	0.43	7.20E-05
205085_at	ORC1L	origin recognition complex, subunit 1-like (yeast)	0.43	6.82E-05
201014_s_at	PAICS	phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase	0.71	9.98E-05
219148_at	PBK	PDZ binding kinase	0.26	5.09E-05
227759_at	PCSK9	proprotein convertase subtilisin/kexin type 9	0.18	2.66E-05
208638_at	PDIA6	protein disulfide isomerase family A, member 6	0.62	4.66E-05
216640_s_at			0.60	3.67E-05
226452_at	PDK1	pyruvate dehydrogenase kinase, isoenzyme 1	0.63	4.36E-05
221521_s_at	Pfs2	DNA replication complex GINS protein PSF2	0.23	5.21E-06
200737_at	PGK1	phosphoglycerate kinase 1	0.65	3.54E-05
201968_s_at	PGM1	phosphoglucomutase 1	0.66	8.98E-05
213227_at	PGRMC2	progesterone receptor membrane component 2	0.70	2.46E-05
202620_s_at	PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	0.51	8.34E-05
202619_s_at			0.43	9.50E-05
205909_at	POLE2	polymerase (DNA directed), epsilon 2 (p59 subunit)	0.34	1.27E-05
211730_s_at	POLR2L	polymerase (RNA) II (DNA directed) polypeptide L, 7.6kDa /// polymerase (RNA) II (DNA directed) polypeptide L, 7.6kDa	0.44	3.78E-06
204839_at	POP5	processing of precursor 5, ribonuclease P/MRP subunit (S. cerevisiae)	0.64	3.21E-05
209482_at	POP7	processing of precursor 7, ribonuclease P subunit (S. cerevisiae)	0.55	4.25E-05
200968_s_at	PPIB	peptidylprolyl isomerase B (cyclophilin B)	0.54	9.24E-05

Table S2 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
235113_at	PPIL5	peptidylprolyl isomerase (cyclophilin)-like 5	0.37	3.99E-05
235061_at	PPM1K	protein phosphatase 1K (PP2C domain containing)	0.54	3.68E-06
204284_at	PPP1R3C	protein phosphatase 1, regulatory (inhibitor) subunit 3C	0.65	5.16E-05
200975_at	PPT1	palmitoyl-protein thioesterase 1 (ceroid-lipofuscinosis, neuronal 1, infantile)	0.70	8.93E-05
200844_s_at	PRDX6	peroxiredoxin 6	0.82	7.34E-05
205053_at	PRIM1	primase, polypeptide 1, 49kDa	0.56	2.25E-05
205628_at	PRIM2A	primase, polypeptide 2A, 58kDa	0.58	1.30E-05
204304_s_at	PROM1	prominin 1	0.68	2.73E-05
226611_s_at	PRR6	proline rich 6	0.57	2.10E-06
200786_at	PSMB7	proteasome (prosome, macropain) subunit, beta type, 7	0.82	1.85E-05
212296_at	PSMD14	proteasome (prosome, macropain) 26S subunit, non-ATPase, 14	0.80	1.15E-05
200772_x_at	PTMA	prothymosin, alpha (gene sequence 28)	0.59	1.23E-05
219654_at	PTPLA	protein tyrosine phosphatase-like (proline instead of catalytic arginine), member a	0.69	8.82E-05
203554_x_at	PTTG1	pituitary tumor-transforming 1	0.40	6.57E-05
223032_x_at	PX19	px19-like protein	0.66	3.12E-06
242414_at	QPRT	quinolinate phosphoribosyltransferase (nicotinate-nucleotide pyrophosphorylase (carboxylating))	0.72	2.16E-05
209849_s_at	RAD51C	RAD51 homolog C (S. cerevisiae)	0.56	2.12E-05
206066_s_at			0.43	1.49E-05
217775_s_at	RDH11	retinol dehydrogenase 11 (all-trans and 9-cis)	0.61	8.28E-07
203696_s_at	RFC2	replication factor C (activator 1) 2, 40kDa	0.45	7.32E-05
204127_at	RFC3	replication factor C (activator 1) 3, 38kDa	0.33	8.61E-05
204128_s_at			0.26	2.47E-05
204023_at	RFC4	replication factor C (activator 1) 4, 37kDa	0.37	2.62E-05
1552502_s_at	RHBDL2	rhomboid, veinlet-like 2 (Drosophila)	0.49	7.57E-05
212651_at	RHOBTB1	Rho-related BTB domain containing 1	0.67	5.59E-05
203022_at	RNASEH2A	ribonuclease H2, large subunit	0.39	3.82E-06
203436_at	RPP30	ribonuclease P/MRP 30kDa subunit	0.60	3.98E-06
201476_s_at	RRM1	ribonucleotide reductase M1 polypeptide	0.49	1.46E-05
201477_s_at			0.39	5.46E-06
209773_s_at	RRM2	ribonucleotide reductase M2 polypeptide	0.28	1.03E-05
203186_s_at	S100A4	S100 calcium binding protein A4 (calcium protein, calvasculin, metastasin, murine placental homolog)	0.28	4.93E-06
209146_at	SC4MOL	sterol-C4-methyl oxidase-like	0.28	1.86E-06
231059_x_at	SCAND1	SCAN domain containing 1	0.85	2.53E-05
200832_s_at	SCD	stearoyl-CoA desaturase (delta-9-desaturase)	0.47	9.65E-05

Table S2 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
211162_x_at			0.15	8.88E-05
211708_s_at			0.14	1.47E-05
222747_s_at	SCML1	sex comb on midleg-like 1 (Drosophila)	0.56	8.63E-06
201194_at	SEPW1	selenoprotein W, 1	0.59	5.35E-05
33323_r_at	SFN	Stratifin	0.58	2.87E-05
209980_s_at	SHMT1	serine hydroxymethyltransferase 1 (soluble)	0.38	7.44E-06
219733_s_at	SLC27A5	solute carrier family 27 (fatty acid transporter), member 5	0.46	8.09E-06
202497_x_at	SLC2A3	solute carrier family 2 (facilitated glucose transporter), member 3	0.29	1.93E-05
202498_s_at			0.28	7.08E-06
202499_s_at			0.20	2.49E-05
222088_s_at	SLC2A3 /// SLC2A14	solute carrier family 2 (facilitated glucose transporter), member 3 /// solute carrier family 2 (facilitated glucose transporter), member 14	0.28	8.11E-05
1555500_s_at	SLC2A4RG	SLC2A4 regulator	0.66	9.22E-05
217289_s_at	SLC37A4	solute carrier family 37 (glycerol-6-phosphate transporter), member 4	0.66	1.30E-05
219795_at	SLC6A14	solute carrier family 6 (amino acid transporter), member 14	0.60	2.45E-06
204240_s_at	SMC2L1	SMC2 structural maintenance of chromosomes 2-like 1 (yeast)	0.51	8.39E-05
201664_at	SMC4L1	SMC4 structural maintenance of chromosomes 4-like 1 (yeast)	0.54	4.33E-05
201663_s_at			0.48	2.11E-05
206055_s_at	SNRPA1	small nuclear ribonucleoprotein polypeptide A'	0.67	8.63E-06
209891_at	SPBC25	spindle pole body component 25 homolog (S. cerevisiae)	0.23	2.04E-06
201240_s_at	SPCS2	signal peptidase complex subunit 2 homolog (S. cerevisiae)	0.63	5.12E-06
209218_at	SQLE	squalene epoxidase	0.37	5.50E-05
213562_s_at			0.31	7.66E-05
213577_at			0.23	3.03E-07
218283_at	SS18L2	synovial sarcoma translocation gene on chromosome 18-like 2	0.56	6.44E-06
212625_at	STX10	syntaxin 10	0.70	9.98E-05
200740_s_at	SUMO3	SMT3 suppressor of mif two 3 homolog 3 (yeast)	0.66	3.98E-05
222977_at	SURF4	surfeit 4	0.67	2.86E-05
222979_s_at			0.62	3.59E-05
209358_at	TAF11	TAF11 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 28kDa	0.61	9.35E-05
1555724_s_at	TAGLN	Transgelin	0.52	2.30E-05
220789_s_at	TBRG4	transforming growth factor beta regulator 4	0.70	1.22E-05

Table S2 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
200085_s_at	TCEB2	transcription elongation factor B (SIII), polypeptide 2 (18kDa, elongin B) /// transcription elongation factor B (SIII), polypeptide 2 (18kDa, elongin B)	0.80	1.41E-05
226320_at	THOC4	THO complex 4	0.53	4.34E-05
202338_at	TK1	thymidine kinase 1, soluble	0.24	1.44E-05
1554408_a_at			0.21	8.91E-06
224413_s_at	TM2D2	TM2 domain containing 2 /// TM2 domain containing 2	0.60	3.63E-05
1554485_s_at	TMEM37	transmembrane protein 37	0.36	3.59E-05
1554483_at			0.34	3.27E-05
218073_s_at	TMEM48	transmembrane protein 48	0.54	3.61E-05
225182_at	TMEM50B	transmembrane protein 50B	0.54	7.91E-05
223396_at	TMEM60	transmembrane protein 60	0.76	1.98E-05
228369_at	TNRC5	trinucleotide repeat containing 5	0.73	5.38E-05
201292_at	TOP2A	topoisomerase (DNA) II alpha 170kDa	0.52	4.00E-05
213011_s_at	TPI1	triosephosphate isomerase 1	0.70	4.06E-05
222976_s_at	TPM3	tropomyosin 3	0.67	8.97E-05
203824_at	TSPAN8	tetraspanin 8	0.34	6.01E-05
204822_at	TTK	TTK protein kinase	0.48	9.49E-05
212320_at	TUBB	tubulin, beta polypeptide	0.46	2.81E-05
203272_s_at	TUSC2	tumor suppressor candidate 2	0.60	9.07E-06
224511_s_at	TXNL5	thioredoxin-like 5 /// thioredoxin-like 5	0.48	4.75E-05
202589_at	TYMS	thymidylate synthetase	0.53	3.23E-06
1554696_s_at			0.43	5.13E-07
205890_s_at	UBD	ubiquitin D	0.45	2.78E-05
223229_at	UBE2T	ubiquitin-conjugating enzyme E2T (putative)	0.27	1.73E-05
218190_s_at	UCRC	ubiquinol-cytochrome c reductase complex (7.2 kD)	0.51	7.33E-05
209103_s_at	UFD1L	ubiquitin fusion degradation 1-like	0.63	9.69E-05
225655_at	UHRF1	ubiquitin-like, containing PHD and RING finger domains, 1	0.34	2.21E-05
202330_s_at	UNG	uracil-DNA glycosylase	0.42	5.19E-05
1569532_a_at	UNQ2541	MSFL2541	0.19	1.97E-07
202090_s_at	UQCR	ubiquinol-cytochrome c reductase, 6.4kDa subunit	0.67	7.06E-05
203797_at	VSNL1	visinin-like 1	0.33	2.66E-05
203721_s_at	WDR50	WD repeat domain 50	0.75	8.31E-05
223868_s_at	WWOX	WW domain containing oxidoreductase	0.78	8.67E-05
218349_s_at	ZWILCH	Zwilch, kinetochore associated, homolog (Drosophila)	0.45	3.28E-05

Table S2 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
204026_s_at	ZWINT	ZW10 interactor	0.34	1.30E-05
229189_s_at		CDNA FLJ90295 fis, clone NT2RP2000240	0.74	4.77E-05
224752_at		Homo sapiens, Similar to hypothetical protein MGC10526, clone IMAGE:4133906, mRNA	0.69	2.04E-05
225716_at		Full-length cDNA clone CS0DK008YI09 of HeLa cells Cot 25-normalized of Homo sapiens (human)	0.68	4.02E-05
238056_at		CDNA FLJ13332 fis, clone OVARC1001813	0.63	9.23E-05
227349_at		CDNA FLJ11381 fis, clone HEMBA1000501	0.55	6.19E-05
227491_at		CDNA: FLJ22539 fis, clone HRC13227	0.47	1.84E-05
226006_at			0.46	5.59E-05
227921_at			0.31	9.53E-06

[1]To be included in this table, differential gene expression had to meet a statistical cutoff of $P<0.0001$.

Table S3. Genes whose expression was upregulated in HepG2/C3A cells cultured in –Cys medium[1]

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
205434_s_at	AAK1	AP2 associated kinase 1	1.95	1.64E-06
213353_at	ABCA5	ATP-binding cassette, sub-family A (ABC1), member 5	3.33	9.65E-06
209993_at	ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	2.34	3.83E-05
222592_s_at	ACSL5	acyl-CoA synthetase long-chain family member 5	2.21	1.06E-06
200996_at	ACTR3	ARP3 actin-related protein 3 homolog (yeast)	1.58	1.30E-05
203935_at	ACVR1	activin A receptor, type I	2.24	2.73E-05
209122_at	ADFP	adipose differentiation-related protein	2.35	1.53E-06
202912_at	ADM	adrenomedullin	1.98	4.02E-06
210517_s_at	AKAP12	A kinase (PRKA) anchor protein (gravin) 12	6.06	1.77E-06
227529_s_at		A kinase (PRKA) anchor protein (gravin) 12	6.20	2.72E-05
227530_at		A kinase (PRKA) anchor protein (gravin) 12	7.50	2.13E-05
206561_s_at	AKR1B10	aldo-keto reductase family 1, member B10 (aldose reductase)	1.59	4.20E-05
1562102_at	AKR1C1	Aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III)	3.70	1.18E-05
217626_at	AKR1C1 /// AKR1C2	aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase) /// aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III)	5.59	6.83E-05
1555854_at	AKR1C2	Aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III)	6.34	5.41E-05
201197_at	AMD1	adenosylmethionine decarboxylase 1	1.32	9.66E-05
207992_s_at	AMPD3	adenosine monophosphate deaminase (isoform E)	3.43	1.59E-05
221522_at	ANKRD27	ankyrin repeat domain 27 (VPS9 domain)	1.73	9.36E-05
205082_s_at	AOX1	aldehyde oxidase 1	3.14	2.50E-05
205083_at		aldehyde oxidase 1	3.08	7.64E-06
39248_at	AQP3	aquaporin 3	10.01	4.64E-06
206784_at	AQP8	aquaporin 8	2.10	3.11E-05

Table S3 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
205239_at	AREG	amphiregulin (schwannoma-derived growth factor)	14.00	6.41E-06
218098_at	ARFGEF2	ADP-ribosylation factor guanine nucleotide-exchange factor 2 (brefeldin A-inhibited)	1.97	8.84E-05
222518_at		ADP-ribosylation factor guanine nucleotide-exchange factor 2 (brefeldin A-inhibited)	2.09	3.48E-05
203946_s_at	ARG2	arginase, type II	2.25	3.25E-05
209435_s_at	ARHGEF2	rho/rac guanine nucleotide exchange factor (GEF) 2	5.53	4.38E-05
201880_at	ARIH1	Ariadne homolog, ubiquitin-conjugating enzyme E2 binding protein, 1 (Drosophila)	1.56	1.35E-05
217852_s_at	ARL10C	ADP-ribosylation factor-like 10C	1.90	1.67E-05
222442_s_at		ADP-ribosylation factor-like 10C	2.04	9.87E-05
205020_s_at	ARL4	ADP-ribosylation factor-like 4	1.99	3.81E-05
225283_at	ARRDC4	arrestin domain containing 4	3.84	4.86E-05
233857_s_at	ASB2	ankyrin repeat and SOCS box-containing 2	1.83	3.35E-05
205047_s_at	ASNS	asparagine synthetase	1.56	4.23E-06
210896_s_at	ASPH	aspartate beta-hydroxylase	2.78	2.90E-05
1554980_a_at	ATF3	activating transcription factor 3	4.30	6.17E-05
202672_s_at		activating transcription factor 3	10.28	1.72E-07
212536_at	ATP11B	ATPase, Class VI, type 11B	1.44	2.41E-05
202874_s_at	ATP6V1C1	ATPase, H+ transporting, lysosomal 42kDa, V1 subunit C, isoform 1	1.87	8.28E-06
242230_at	ATXN1	ataxin 1	3.88	3.77E-05
221485_at	B4GALT5	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 5	1.40	2.21E-05
224957_at	BC022357	cDNA sequence BC022357	1.52	6.23E-05
204032_at	BCAR3	breast cancer anti-estrogen resistance 3	2.29	7.63E-05
203053_at	BCAS2	breast carcinoma amplified sequence 2	1.83	5.05E-05
209311_at	BCL2L2	BCL2-like 2	1.77	3.00E-05
202710_at	BET1	BET1 homolog (S. cerevisiae)	1.70	7.69E-06
208685_x_at	BRD2	bromodomain containing 2	1.84	1.69E-05
214820_at	BRWD1	bromodomain and WD repeat domain containing 1	1.54	4.34E-05
225976_at	BTF3L4	basic transcription factor 3-like 4	1.64	9.21E-05
225334_at	C10orf32	chromosome 10 open reading frame 32	1.88	5.48E-06
226398_s_at	C10orf4	chromosome 10 open reading frame 4	2.41	9.27E-05
223059_s_at	C10orf45	chromosome 10 open reading frame 45	2.20	4.41E-05
229801_at	C10orf47	chromosome 10 open reading frame 47	2.73	7.19E-05

Table S3 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
221260_s_at	C12orf22	chromosome 12 open reading frame 22 /// chromosome 12 open reading frame 22	2.75	5.39E-05
202623_at	C14orf11	chromosome 14 open reading frame 11	1.76	3.08E-06
225679_at	C14orf35	chromosome 14 open reading frame 35	1.66	6.76E-05
224798_s_at	C15orf17	chromosome 15 open reading frame 17	2.09	4.06E-05
204700_x_at	C1orf107	chromosome 1 open reading frame 107	1.81	8.31E-05
1553333_at	C1orf161	chromosome 1 open reading frame 161	2.56	2.50E-06
215767_at	C2orf10	chromosome 2 open reading frame 10	1.69	7.90E-06
221245_s_at	C2orf31	chromosome 2 open reading frame 31 /// chromosome 2 open reading frame 31	1.43	2.62E-05
220088_at	C5R1	complement component 5 receptor 1 (C5a ligand)	9.36	2.03E-06
226135_at	C6orf107	chromosome 6 open reading frame 107	2.46	7.14E-05
224808_s_at	C7orf20	chromosome 7 open reading frame 20	1.60	5.31E-05
227443_at	C9orf150	chromosome 9 open reading frame 150	4.96	5.42E-05
212765_at	CAMSAP1L1	calmodulin regulated spectrin-associated protein 1-like 1	2.46	6.25E-05
218929_at	CARF	collaborates/cooperates with ARF (alternate reading frame) protein	2.12	8.24E-06
212971_at	CARS	cysteinyl-tRNA synthetase	2.69	9.89E-07
227558_at	CBX4	chromobox homolog 4 (Pc class homolog, Drosophila)	2.49	1.17E-05
202047_s_at	CBX6	chromobox homolog 6	2.21	9.10E-05
205627_at	CDA	cytidine deaminase	2.93	7.09E-05
202284_s_at	CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	3.17	2.62E-05
236313_at	CDKN2B	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	2.15	4.41E-06
203098_at	CDYL	chromodomain protein, Y-like	2.03	5.46E-05
203099_s_at	CDYL	chromodomain protein, Y-like	1.75	8.63E-05
212501_at	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	4.60	7.19E-07
204203_at	CEBPG	CCAAT/enhancer binding protein (C/EBP), gamma	2.56	2.40E-05
225527_at		CCAAT/enhancer binding protein (C/EBP), gamma	3.31	8.54E-06
204066_s_at	CENTG2	centaurin, gamma 2	2.06	4.03E-05
206861_s_at	CGGBP1	CGG triplet repeat binding protein 1	1.74	6.77E-06

Table S3 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
205308_at	CGI-62	CGI-62 protein	1.82	6.51E-05
201734_at	CLCN3	Chloride channel 3	2.09	3.25E-05
201735_s_at		chloride channel 3	2.04	4.82E-05
205830_at	CLGN	calmegin	2.89	1.09E-05
214683_s_at	CLK1	CDC-like kinase 1	2.01	7.92E-05
203630_s_at	COG5	component of oligomeric golgi complex 5	1.96	3.02E-05
224828_at	CPEB4	cytoplasmic polyadenylation element binding protein 4	2.37	4.02E-05
224831_at		cytoplasmic polyadenylation element binding protein 4	2.56	9.57E-05
223115_at	CRSP6	cofactor required for Sp1 transcriptional activation, subunit 6, 77kDa	1.34	8.44E-05
206085_s_at	CTH	cystathionase (cystathionine gamma-lyase)	2.90	2.74E-05
217127_at		cystathionase (cystathionine gamma-lyase)	2.86	1.11E-05
202087_s_at	CTSL	cathepsin L	1.72	9.85E-05
1557954_at	CXorf15	Chromosome X open reading frame 15	1.98	8.81E-05
201289_at	CYR61	cysteine-rich, angiogenic inducer, 61	4.63	7.57E-06
216060_s_at	DAAM1	dishevelled associated activator of morphogenesis 1	2.16	3.21E-05
201925_s_at	DAF	decay accelerating factor for complement (CD55, Cromer blood group system)	4.20	8.05E-07
201926_s_at		decay accelerating factor for complement (CD55, Cromer blood group system)	3.05	1.54E-05
224911_s_at	DCBLD2	discoidin, CUB and LCCL domain containing 2	1.96	1.74E-05
224796_at	DDEF1	development and differentiation enhancing factor 1	1.91	1.24E-05
202887_s_at	DDIT4	DNA-damage-inducible transcript 4	2.01	2.55E-05
218819_at	DDX26	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 26	2.40	7.30E-05
222543_at	DERL1	Der1-like domain family, member 1	1.74	4.13E-06
223140_s_at	DHX36	DEAH (Asp-Glu-Ala-His) box polypeptide 36	1.81	3.01E-05

Table S3 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
212854_x_at	DJ328E19.C1.1 /// FLJ20719 /// LOC200030 /// MGC8902 /// AE01 /// AG1 /// LOC440675	hypothetical protein DJ328E19.C1.1 /// hypothetical protein FLJ20719 /// AG1 protein	1.94	4.20E-05
201104_x_at		hypothetical protein DJ328E19.C1.1 /// hypothetical protein FLJ20719 /// hypothetical protein LOC200030 /// hypothetical protein MGC8902 /// AE01 mRNA /// AG1 protein /// hypothetical LOC440675	1.96	8.61E-05
214693_x_at		hypothetical protein DJ328E19.C1.1 /// hypothetical protein FLJ20719 /// hypothetical protein LOC200030 /// hypothetical protein MGC8902 /// similar to KIAA1693 protein /// AE01 mRNA /// AG1 protein /// hypothetical LOC440675	1.97	5.67E-05
210762_s_at	DLC1	deleted in liver cancer 1	2.62	7.42E-06
224822_at		deleted in liver cancer 1	2.64	5.38E-05
203811_s_at	DNAJB4	DnaJ (Hsp40) homolog, subfamily B, member 4	1.76	2.75E-05
209015_s_at	DNAJB6	DnaJ (Hsp40) homolog, subfamily B, member 6	1.58	4.74E-05
202843_at	DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9	2.04	9.87E-05
225174_at	DNAJC10	DnaJ (Hsp40) homolog, subfamily C, member 10	1.60	7.40E-05
1554966_a_at	DOC1	downregulated in ovarian cancer 1	3.67	3.08E-05
204135_at		downregulated in ovarian cancer 1	3.13	7.20E-06
215626_at	DOCK9	Dedicator of cytokinesis 9	1.31	2.95E-07
208486_at	DRD5	dopamine receptor D5	5.03	4.43E-05
212254_s_at	DST	dystonin	2.20	4.72E-05
205761_s_at	DUS4L	dihydrouridine synthase 4-like (S. cerevisiae)	1.57	2.94E-05
201041_s_at	DUSP1	dual specificity phosphatase 1	7.17	2.09E-05
201536_at	DUSP3	dual specificity phosphatase 3 (vaccinia virus phosphatase VH1-related)	2.55	3.48E-06
209457_at	DUSP5	dual specificity phosphatase 5	2.94	4.19E-06
238458_at	EFHA2	EF-hand domain family, member A2	1.39	5.90E-05
212830_at	EGFL5	EGF-like-domain, multiple 5	2.28	1.77E-05
225827_at	EIF2C2	eukaryotic translation initiation factor 2C, 2	2.30	4.60E-05
227930_at	EIF2C4	Eukaryotic translation initiation factor 2C, 4	1.79	1.26E-05
235296_at	EIF5A2	eukaryotic translation initiation factor 5A2	3.46	9.82E-07
212420_at	ELF1	E74-like factor 1 (ets domain transcription factor)	1.87	7.13E-05
222433_at	ENAH	enabled homolog (Drosophila)	1.81	5.76E-05

Table S3 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
205767_at	EREG	epiregulin	6.77	2.51E-05
206429_at	F2RL1	coagulation factor II (thrombin) receptor-like 1	5.20	2.31E-05
213506_at		coagulation factor II (thrombin) receptor-like 1	4.51	1.63E-05
227880_s_at	FAM11A	family with sequence similarity 11, member A	1.49	4.16E-05
235030_at	FAM55C	family with sequence similarity 55, member C	1.34	4.80E-05
226541_at	FBXO30	F-box protein 30	2.71	7.01E-05
47773_at	FBXO42	F-box protein 42	2.28	9.32E-06
201919_at	FLJ10618	Hypothetical protein FLJ10618	2.65	2.79E-05
225573_at	FLJ12592	putative acyl-CoA dehydrogenase	1.45	6.67E-05
219397_at	FLJ13448	hypothetical protein FLJ13448	1.62	2.36E-05
1554102_a_at	FLJ14624	hypothetical protein FLJ14624	0.74	5.18E-05
225412_at	FLJ14681	Hypothetical protein FLJ14681	1.59	8.40E-06
230009_at	FLJ21103	Hypothetical protein FLJ21103	1.52	5.99E-05
218867_s_at	FLJ21415	hypothetical protein FLJ21415	1.88	1.75E-05
222767_s_at		hypothetical protein FLJ21415	2.21	2.63E-05
226038_at	FLJ23749	hypothetical protein FLJ23749	1.40	9.99E-05
225848_at	FLJ31413	hypothetical protein FLJ31413	2.11	4.96E-05
225466_at	FLJ36874	hypothetical protein FLJ36874	1.57	5.13E-05
227856_at	FLJ39370	hypothetical protein FLJ39370	4.19	4.42E-05
208614_s_at	FLNB	filamin B, beta (actin binding protein 278)	1.76	3.97E-05
209189_at	FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	2.25	5.79E-05
204420_at	FOSL1	FOS-like antigen 1	4.89	5.02E-05
204131_s_at	FOXO3A	forkhead box O3A	2.84	2.40E-05
224889_at		forkhead box O3A	2.04	8.04E-05
223287_s_at	FOXP1	forkhead box P1	3.45	4.45E-05
224837_at		forkhead box P1	2.49	6.40E-05
224838_at		forkhead box P1	2.55	8.36E-05
227475_at	FOXQ1	forkhead box Q1	2.88	1.00E-05
207345_at	FST	follistatin	2.16	1.32E-05
226847_at		follistatin	2.51	3.76E-05
203725_at	GADD45A	growth arrest and DNA-damage-inducible, alpha	5.59	9.86E-07
218871_x_at	GALNACT-2	chondroitin sulfate GalNAcT-2	4.51	6.90E-05
222235_s_at		chondroitin sulfate GalNAcT-2	5.77	8.99E-07
212378_at	GART	phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase	1.62	6.34E-05
202922_at	GCLC	glutamate-cysteine ligase, catalytic subunit	2.57	2.12E-05

Table S3 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
202923_s_at		glutamate-cysteine ligase, catalytic subunit	2.47	3.31E-05
203925_at	GCLM	glutamate-cysteine ligase, modifier subunit	3.25	4.44E-06
219508_at	GCNT3	glucosaminyl (N-acetyl) transferase 3, mucin type	3.53	1.70E-06
203986_at	GENX-3414	genethonin 1	2.58	7.57E-05
202722_s_at	GFPT1	glutamine-fructose-6-phosphate transaminase 1	1.97	8.70E-05
1554510_s_at	GHITM	growth hormone inducible transmembrane protein	1.83	2.22E-05
203159_at	GLS	glutaminase	1.53	2.16E-05
222808_at	GLT28D1	glycosyltransferase 28 domain containing 1	1.82	6.98E-05
206917_at	GNA13	guanine nucleotide binding protein (G protein), alpha 13	4.04	1.95E-05
224761_at		guanine nucleotide binding protein (G protein), alpha 13	2.94	2.22E-05
227539_at		Guanine nucleotide binding protein (G protein), alpha 13	3.49	4.34E-06
207812_s_at	GORASP2	golgi reassembly stacking protein 2, 55kDa	1.27	1.96E-05
208813_at	GOT1	glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1)	1.83	2.92E-06
225420_at	GPAM	glycerol-3-phosphate acyltransferase, mitochondrial	1.54	4.40E-05
222953_at	GPR83	G protein-coupled receptor 83	9.89	4.11E-07
209409_at	GRB10	growth factor receptor-bound protein 10	3.07	7.38E-05
225276_at	GSPT1	G1 to S phase transition 1	1.79	1.73E-05
205930_at	GTF2E1	general transcription factor IIE, polypeptide 1, alpha 56kDa	1.47	6.78E-06
202453_s_at	GTF2H1	general transcription factor IIH, polypeptide 1, 62kDa	1.99	5.45E-05
221050_s_at	GTPBP2	GTP binding protein 2	7.04	3.67E-05
223789_s_at		GTP binding protein 2	5.85	4.68E-07
206643_at	HAL	histidine ammonia-lyase	2.09	1.14E-05
220491_at	HAMP	hepcidin antimicrobial peptide	2.29	4.38E-06
217168_s_at	HERPUD1	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	2.44	3.80E-05
215071_s_at	HIST1H2AC	histone 1, H2ac	5.43	1.27E-06
218091_at	HRB	HIV-1 Rev binding protein	1.63	8.80E-05
203202_at	HRB2	HIV-1 rev binding protein 2	1.60	8.27E-05
218936_s_at	HSPC128	HSPC128 protein	2.18	1.15E-05
202146_at	IFRD1	interferon-related developmental regulator 1	5.28	1.46E-05
202147_s_at		interferon-related developmental regulator 1	5.45	3.40E-06
201393_s_at	IGF2R	insulin-like growth factor 2 receptor	1.92	4.39E-05

Table S3 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
217489_s_at	IL6R	interleukin 6 receptor	2.28	5.80E-07
241808_at	IL7	Interleukin 7	2.18	1.51E-05
202859_x_at	IL8	interleukin 8	2.90	7.81E-05
210587_at	INHBE	inhibin, beta E	6.76	9.62E-06
224569_s_at	IRF2BP2	interferon regulatory factor 2 binding protein 2	1.34	4.95E-05
205032_at	ITGA2	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	4.68	4.37E-05
211945_s_at	ITGB1	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	1.22	4.32E-06
209098_s_at	JAG1	jagged 1 (Alagille syndrome)	5.87	5.20E-05
209099_x_at		jagged 1 (Alagille syndrome)	3.38	4.58E-05
216268_s_at		jagged 1 (Alagille syndrome)	3.72	2.24E-05
202040_s_at	JARID1A	Jumonji, AT rich interactive domain 1A (RBBP2-like)	1.64	2.16E-05
201464_x_at	JUN	v-jun sarcoma virus 17 oncogene homolog (avian)	4.31	1.32E-06
201466_s_at		v-jun sarcoma virus 17 oncogene homolog (avian)	4.83	8.13E-06
212447_at	KBTBD2	kelch repeat and BTB (POZ) domain containing 2	1.56	7.15E-05
217938_s_at	KCMF1	potassium channel modulatory factor 1	1.73	2.56E-05
201751_at	KIAA0063	KIAA0063 gene product	2.20	2.97E-05
212056_at	KIAA0182	KIAA0182 protein	1.79	3.45E-05
212474_at	KIAA0241	KIAA0241 protein	1.61	5.47E-05
200897_s_at	KIAA0992	palladin	4.25	9.51E-05
200907_s_at		palladin	4.68	2.90E-05
221802_s_at	KIAA1598	KIAA1598	1.51	6.32E-05
203390_s_at	KIF3C	kinesin family member 3C	2.37	4.05E-06
224662_at	KIF5B	kinesin family member 5B	1.71	4.46E-05
202393_s_at	KLF10	Kruppel-like factor 10	3.51	8.68E-06
208960_s_at	KLF6	Kruppel-like factor 6	3.80	6.68E-05
208961_s_at		Kruppel-like factor 6	4.19	2.95E-05
213233_s_at	KLHL9	kelch-like 9 (Drosophila)	1.45	3.27E-05
213656_s_at	KNS2	kinesin 2 60/70kDa	2.44	1.36E-05
204385_at	KYNU	kynureninase (L-kynurenine hydrolase)	3.33	8.88E-07
210663_s_at		kynureninase (L-kynurenine hydrolase)	4.06	1.11E-05
217388_s_at		kynureninase (L-kynurenine hydrolase)	3.36	2.93E-06
205569_at	LAMP3	lysosomal-associated membrane protein 3	2.97	6.37E-05
212137_at	LARP1	La ribonucleoprotein domain family, member 1	1.21	1.30E-05
226750_at	LARP2	La ribonucleoprotein domain family, member 2	1.77	9.30E-05

Table S3 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
208952_s_at	LARP5	La ribonucleoprotein domain family, member 5	1.18	8.53E-05
236565_s_at	LARP6	La ribonucleoprotein domain family, member 6	3.59	1.85E-05
223162_s_at	LCHN	LCHN protein	1.26	8.92E-05
202068_s_at	LDLR	low density lipoprotein receptor (familial hypercholesterolemia)	1.73	3.85E-05
1552546_a_at	LETM2	leucine zipper-EF-hand containing transmembrane protein 2	2.50	5.36E-06
209205_s_at	LMO4	LIM domain only 4	3.50	8.66E-06
203320_at	LNK	lymphocyte adaptor protein	3.41	3.26E-05
227586_at	LOC124491	LOC124491	1.50	7.12E-05
235191_at	LOC148189	hypothetical protein LOC148189	2.95	8.57E-06
221501_x_at	LOC339047	hypothetical protein LOC339047	2.98	2.22E-05
237737_at	LOC375010 /// LOC401131	hypothetical LOC375010 /// hypothetical LOC401131	2.53	2.58E-05
239751_at	LOC392791	hypothetical LOC392791	3.74	8.64E-05
214035_x_at	LOC399491	LOC399491 protein	2.79	6.05E-05
206741_at	LOC51066	fls485	1.63	7.23E-05
220437_at	LOC55908	hepatocellular carcinoma-associated gene TD26	2.40	4.69E-05
206584_at	LY96	lymphocyte antigen 96	6.22	3.19E-06
36711_at	MAFF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	4.19	1.39E-05
226084_at	MAP1B	microtubule-associated protein 1B	3.53	1.45E-05
208785_s_at	MAP1LC3B	microtubule-associated protein 1 light chain 3 beta	3.49	7.03E-06
208786_s_at		microtubule-associated protein 1 light chain 3 beta	3.68	3.72E-06
203353_s_at	MBD1	methyl-CpG binding domain protein 1	1.62	4.13E-06
1554544_a_at	MBP	myelin basic protein	2.33	4.74E-05
210136_at		myelin basic protein	2.56	6.83E-05
200797_s_at	MCL1	myeloid cell leukemia sequence 1 (BCL2-related)	3.12	6.09E-07
212535_at	MEF2A	MADS box transcription enhancer factor 2, polypeptide A (myocyte enhancer factor 2A)	1.96	7.25E-05
225955_at	METRNL	meteorin, glial cell differentiation regulator-like	3.40	9.65E-05
227395_at	MGC10233	Leucine rich repeat containing 35	1.73	1.83E-05
1560019_at	MGC11082	Hypothetical protein MGC11082	2.95	4.91E-06
224480_s_at	MGC11324	hypothetical protein MGC11324 /// hypothetical protein MGC11324	4.25	9.40E-08
214696_at	MGC14376	hypothetical protein MGC14376	3.54	2.85E-07
236285_at	MGC16635	Hypothetical protein BC009980	11.32	1.34E-06
1552277_a_at	MGC17337	similar to RIKEN cDNA 5730528L13 gene	2.06	3.36E-06

Table S3 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
243999_at	MGC19764	hypothetical protein MGC19764	2.10	5.57E-06
223297_at	MGC4268	hypothetical protein MGC4268	1.91	2.77E-05
55081_at	MICAL-L1	MICAL-like 1	2.23	3.00E-05
227527_at	MLL2	myeloid/lymphoid or mixed-lineage leukemia 2	1.30	8.62E-05
1557455_s_at	MOSPD1	motile sperm domain containing 1	3.93	1.84E-07
218853_s_at		motile sperm domain containing 1	3.80	3.01E-06
212859_x_at	MT1E	metallothionein 1E (functional)	1.47	8.87E-05
212250_at	MTDH	metadherin	1.39	8.17E-05
226275_at	MXD1	MAX dimerization protein 1	4.40	3.82E-06
228846_at		MAX dimerization protein 1	4.19	4.81E-07
204527_at	MYO5A	myosin VA (heavy polypeptide 12, myoxin)	1.49	7.24E-06
229432_at	NAGS	N-acetylglutamate synthase	3.42	5.97E-05
209949_at	NCF2	neutrophil cytosolic factor 2 (65kDa, chronic granulomatous disease, autosomal 2)	12.40	5.02E-07
226490_at	NHSL1	NHS-like 1	1.88	9.50E-05
223439_at	NKAP	NF-kappaB activating protein	1.53	6.13E-05
202679_at	NPC1	Niemann-Pick disease, type C1	1.51	1.94E-05
204538_x_at	NPIP /// LOC339047 /// LOC440341	nuclear pore complex interacting protein /// hypothetical protein LOC339047 /// similar to hypothetical protein LOC339047	2.76	5.28E-05
214870_x_at		nuclear pore complex interacting protein /// hypothetical protein LOC339047 /// similar to hypothetical protein LOC339047	2.86	2.31E-05
225768_at	NR1D2	nuclear receptor subfamily 1, group D, member 2	1.56	6.87E-05
201865_x_at	NR3C1	nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	2.62	2.38E-05
216321_s_at		nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	3.04	4.91E-05
202073_at	OPTN	optineurin	1.51	4.57E-05
230830_at	OSTbeta	organic solute transporter beta	1.45	9.98E-05
218196_at	OSTM1	osteopetrosis associated transmembrane protein 1	2.02	8.38E-05
222839_s_at	PAPOLG	poly(A) polymerase gamma	1.78	2.91E-05
204004_at	PAWR	PRKC, apoptosis, WT1, regulator	2.10	3.39E-05
204005_s_at		PRKC, apoptosis, WT1, regulator	2.12	5.19E-05
218718_at	PDGFC	platelet derived growth factor C	1.52	6.51E-05
205094_at	PEX12	peroxisomal biogenesis factor 12	1.94	1.98E-06
209346_s_at	PI4KII	phosphatidylinositol 4-kinase type II	1.88	7.38E-05
215236_s_at	PICALM	phosphatidylinositol binding clathrin assembly protein	1.83	3.55E-05
214152_at	PIGB	phosphatidylinositol glycan, class B	2.34	3.54E-05

Table S3 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
204285_s_at	PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1	4.41	3.47E-06
204286_s_at		phorbol-12-myristate-13-acetate-induced protein 1	3.28	4.72E-05
218224_at	PNMA1	paraneoplastic antigen MA1	2.87	2.79E-06
222572_at	PPM2C	protein phosphatase 2C, magnesium-dependent, catalytic subunit	3.30	7.59E-06
202014_at	PPP1R15A	protein phosphatase 1, regulatory (inhibitor) subunit 15A	8.56	2.89E-05
37028_at		protein phosphatase 1, regulatory (inhibitor) subunit 15A	6.17	1.79E-05
201375_s_at	PPP2CB	protein phosphatase 2 (formerly 2A), catalytic subunit, beta isoform	1.65	9.48E-05
202313_at	PPP2R2A	protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), alpha isoform	1.49	4.35E-05
229322_at	PPP2R5E	protein phosphatase 2, regulatory subunit B (B56), epsilon isoform	1.99	4.46E-05
202429_s_at	PPP3CA	protein phosphatase 3 (formerly 2B), catalytic subunit, alpha isoform (calcineurin A alpha)	1.63	2.89E-05
218329_at	PRDM4	PR domain containing 4	1.30	7.91E-05
215707_s_at	PRNP	prion protein (p27-30) (Creutzfeld-Jakob disease, Gerstmann-Strausler-Scheinker syndrome, fatal familial insomnia)	2.10	7.84E-05
225183_at	PRO0149	PRO0149 protein	1.89	2.73E-06
204447_at	ProSAPiP1	ProSAPiP1 protein	3.18	4.76E-05
203997_at	PTPN3	protein tyrosine phosphatase, non-receptor type 3	2.21	7.73E-05
222980_at	RAB10	RAB10, member RAS oncogene family	1.57	2.35E-05
222981_s_at		RAB10, member RAS oncogene family	1.79	1.29E-05
205924_at	RAB3B	RAB3B, member RAS oncogene family	2.36	6.54E-05
239202_at		RAB3B, member RAS oncogene family	2.50	7.84E-05
201047_x_at	RAB6A	RAB6A, member RAS oncogene family	1.76	5.21E-05
212561_at	RAB6IP1	RAB6 interacting protein 1	1.86	4.77E-05
210826_x_at	RAD17	RAD17 homolog (S. pombe)	1.63	8.47E-06
1556128_a_at	RASGRF2	Ras protein-specific guanine nucleotide-releasing factor 2	1.76	4.19E-05
211950_at	RBAF600	retinoblastoma-associated factor 600	2.09	7.21E-05
211686_s_at	RBM13	RNA binding motif protein 13 /// RNA binding motif protein 13	2.10	3.09E-06

Table S3 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
235004_at	RBM24	RNA binding motif protein 24	3.04	9.33E-07
201394_s_at	RBM5	RNA binding motif protein 5	2.17	9.66E-05
222666_s_at	RCL1	RNA terminal phosphate cyclase-like 1	2.82	6.56E-06
209568_s_at		ral guanine nucleotide dissociation stimulator-like 1	2.16	9.27E-05
219610_at	RGNEF	Rho-guanine nucleotide exchange factor	1.53	6.97E-05
212119_at	RHOQ	ras homolog gene family, member Q	1.71	8.23E-05
202130_at	RIOK3	RIO kinase 3 (yeast) /// RIO kinase 3 (yeast)	2.77	6.33E-05
209545_s_at	RIPK2	receptor-interacting serine-threonine kinase 2	2.34	1.62E-06
209882_at	RIT1	Ras-like without CAAX 1	2.53	5.37E-05
223886_s_at	RNF146	ring finger protein 146	2.29	2.27E-05
214023_x_at	RP11-506K6.1	tubulin, beta polypeptide paralog	2.29	5.58E-05
222514_at	RRAGC	Ras-related GTP binding C	2.30	9.12E-05
208456_s_at	RRAS2	related RAS viral (r-ras) oncogene homolog 2	2.94	2.04E-05
212590_at		related RAS viral (r-ras) oncogene homolog 2	2.52	1.53E-05
211509_s_at	RTN4	reticulon 4	1.14	6.50E-05
204268_at	S100A2	S100 calcium binding protein A2	3.22	8.99E-05
231894_at	SARS	Seryl-tRNA synthetase	4.41	1.63E-06
209486_at	SAS10	disrupter of silencing 10	1.50	5.17E-05
222669_s_at	SBDS	Shwachman-Bodian-Diamond syndrome	1.74	9.48E-05
212589_at	SCP2	Sterol carrier protein 2	2.47	7.91E-05
202071_at	SDC4	syndecan 4 (amphiglycan, ryudocan)	1.79	2.86E-05
201583_s_at	SEC23B	Sec23 homolog B (S. cerevisiae)	1.42	5.98E-05
201916_s_at	SEC63	SEC63-like (S. cerevisiae)	1.43	6.52E-05
202627_s_at	SERPINE1	serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	2.04	4.35E-06
202628_s_at		serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	2.53	8.62E-06
224928_at	SET7	SET domain-containing protein 7	1.94	4.01E-05
33323_r_at	SFN	stratifin	2.11	2.82E-05
223391_at	SGPP1	sphingosine-1-phosphate phosphatase 1	1.80	9.15E-05
209091_s_at	SH3GLB1	SH3-domain GRB2-like endophilin B1	1.82	8.15E-05
210101_x_at		SH3-domain GRB2-like endophilin B1	1.87	3.31E-06
227304_at	SHMT1	Serine hydroxymethyltransferase 1 (soluble)	1.74	6.99E-05

Table S3 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
202777_at	SHOC2	soc-2 suppressor of clear homolog (C. elegans)	1.85	3.05E-05
212811_x_at	SLC1A4	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	2.17	9.78E-05
225212_at	SLC25A25	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 25	1.91	7.96E-05
203164_at	SLC33A1	solute carrier family 33 (acetyl-CoA transporter), member 1	1.60	7.54E-05
224579_at	SLC38A1	Solute carrier family 38, member 1	2.08	9.28E-06
224580_at	SLC38A1	Solute carrier family 38, member 1	2.01	6.39E-05
218041_x_at	SLC38A2	solute carrier family 38, member 2	1.70	7.26E-05
220924_s_at		solute carrier family 38, member 2	1.68	5.81E-05
223044_at	SLC40A1	solute carrier family 40 (iron-regulated transporter), member 1	3.23	2.22E-05
212290_at	SLC7A1	solute carrier family 7 (cationic amino acid transporter, y+ system), member 1	3.25	3.38E-05
212295_s_at		solute carrier family 7 (cationic amino acid transporter, y+ system), member 1	3.37	2.09E-06
207528_s_at	SLC7A11	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	5.61	8.12E-05
209921_at		solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	13.77	2.52E-05
217678_at		solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	9.88	2.66E-06
241756_at	SMARCA2	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2	1.99	7.15E-05
210357_s_at	SMOX	spermine oxidase	3.65	1.73E-05
212666_at	SMURF1	SMAD specific E3 ubiquitin protein ligase 1	2.05	5.12E-06
227489_at	SMURF2	SMAD specific E3 ubiquitin protein ligase 2	2.36	4.11E-05
223027_at	SNX9	sorting nexin 9	2.63	3.86E-06
203373_at	SOCS2	suppressor of cytokine signaling 2	3.48	4.78E-05
212777_at	SOS1	son of sevenless homolog 1 (Drosophila)	2.03	5.73E-05
212780_at		son of sevenless homolog 1 (Drosophila)	2.01	4.33E-05
213668_s_at	SOX4	SRY (sex determining region Y)-box 4	4.85	3.66E-05

Table S3 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
210117_at	SPAG1	sperm associated antigen 1	2.38	7.44E-07
1559517_a_at	SPIRE1	spire homolog 1 (Drosophila)	2.96	9.79E-05
224995_at		spire homolog 1 (Drosophila)	4.32	8.08E-05
225018_at		spire homolog 1 (Drosophila)	3.94	6.12E-07
226342_at	SPTBN1	Spectrin, beta, non-erythrocytic 1	3.51	7.74E-05
217995_at	SQRDL	sulfide quinone reductase-like (yeast)	2.33	4.92E-05
205335_s_at	SRP19	signal recognition particle 19kDa	1.44	2.95E-05
225252_at	SRXN1	sulfiredoxin 1 homolog (S. cerevisiae)	3.63	1.29E-05
226075_at	SSB1	SPRY domain-containing SOCS box protein SSB-1	3.71	5.65E-05
203544_s_at	STAM	signal transducing adaptor molecule (SH3 domain and ITAM motif) 1	2.26	6.97E-06
203438_at	STC2	stanniocalcin 2	5.99	1.78E-06
203439_s_at		stanniocalcin 2	2.78	4.60E-05
202557_at	STCH	stress 70 protein chaperone, microsome-associated, 60kDa	4.19	7.00E-05
202558_s_at		stress 70 protein chaperone, microsome-associated, 60kDa	2.21	2.12E-05
202693_s_at	STK17A	serine/threonine kinase 17a (apoptosis-inducing)	3.76	5.65E-06
202695_s_at		serine/threonine kinase 17a (apoptosis-inducing)	4.10	2.56E-05
201061_s_at	STOM	stomatin	2.46	4.67E-07
238462_at	STS-1	Cbl-interacting protein Sts-1	2.76	2.95E-05
216985_s_at	STX3A	syntaxin 3A	2.33	7.92E-06
202021_x_at	SUI1	putative translation initiation factor	2.40	5.33E-05
212130_x_at		putative translation initiation factor	2.30	1.37E-05
212227_x_at		putative translation initiation factor	2.47	2.88E-05
201837_s_at	SUPT7L	suppressor of Ty 7 (S. cerevisiae)-like	1.54	3.96E-05
205691_at	SYNGR3	synaptogyrin 3	2.87	5.22E-05
226835_s_at	TALDO1 /// HSUP1	transaldolase 1 /// similar to RPE-spondin	1.64	1.19E-05
219682_s_at	TBX3	T-box 3 (ulnar mammary syndrome)	1.47	7.53E-05
216241_s_at	TCEA1	transcription elongation factor A (SII), 1	1.66	2.36E-05
221471_at	TDE1	tumor differentially expressed 1	1.59	3.49E-06
202720_at	TES	testis derived transcript (3 LIM domains)	3.07	1.06E-05
219292_at	THAP1	THAP domain containing, apoptosis associated protein 1	1.51	5.78E-05
212665_at	TIPARP	TCDD-inducible poly(ADP-ribose) polymerase	2.48	2.78E-06
202011_at	TJP1	tight junction protein 1 (zona occludens 1)	1.65	7.62E-05
228284_at	TLE1 /// LOC389863	transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila) /// hypothetical gene supported by BC000228; NM_005077	2.24	5.72E-05

Table S3 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
209295_at	TNFRSF10B	tumor necrosis factor receptor superfamily, member 10b	2.49	3.78E-05
210405_x_at		tumor necrosis factor receptor superfamily, member 10b	2.52	5.89E-06
218368_s_at	TNFRSF12A	tumor necrosis factor receptor superfamily, member 12A	3.79	5.98E-05
1558305_at	TNRC15	Trinucleotide repeat containing 15	1.95	2.37E-05
214774_x_at	TNRC9	trinucleotide repeat containing 9	2.43	9.83E-05
215108_x_at		trinucleotide repeat containing 9	1.98	8.11E-05
216623_x_at		trinucleotide repeat containing 9	2.45	8.34E-05
200742_s_at	TPP1	tripeptidyl peptidase I	2.03	3.01E-05
214196_s_at		tripeptidyl peptidase I	2.07	7.48E-05
202241_at	TRIB1	tribbles homolog 1 (Drosophila)	2.40	2.45E-05
1555788_a_at	TRIB3	tribbles homolog 3 (Drosophila)	5.80	5.50E-05
218145_at		tribbles homolog 3 (Drosophila)	4.03	6.86E-05
204094_s_at	TSC22D2	TSC22 domain family, member 2	1.93	1.88E-05
201758_at	TSG101	tumor susceptibility gene 101	2.11	4.51E-05
226181_at	TUBE1	tubulin, epsilon 1	4.52	4.16E-05
201008_s_at	TXNIP	thioredoxin interacting protein	4.21	2.43E-06
201009_s_at		thioredoxin interacting protein	3.53	6.72E-07
201010_s_at		thioredoxin interacting protein	3.16	5.84E-06
218794_s_at	TXNL4B	thioredoxin-like 4B	2.24	3.77E-05
201266_at	TXNRD1	thioredoxin reductase 1	3.62	8.74E-07
217825_s_at	UBE2J1	ubiquitin-conjugating enzyme E2, J1 (UBC6 homolog, yeast)	1.30	9.34E-06
201535_at	UBL3	ubiquitin-like 3	2.07	6.06E-05
222989_s_at	UBQLN1	ubiquilin 1	1.81	3.25E-05
222991_s_at		ubiquilin 1	1.77	1.97E-05
212006_at	UBXD2	UBX domain containing 2	1.76	9.34E-05
204616_at	UCHL3	ubiquitin carboxyl-terminal esterase L3 (ubiquitin thiolesterase)	1.50	5.56E-05
202893_at	UNC13B	unc-13 homolog B (C. elegans)	1.46	2.44E-05
232299_at	UNQ830	ASCL830	1.45	8.69E-05
220419_s_at	USP25	ubiquitin specific protease 25	2.04	3.96E-05
221654_s_at	USP3	ubiquitin specific protease 3	1.27	1.53E-05
223289_s_at	USP38	ubiquitin specific protease 38	2.00	6.04E-05
210513_s_at	VEGF	vascular endothelial growth factor	2.18	8.98E-05
212171_x_at		vascular endothelial growth factor	2.52	3.46E-05
209822_s_at	VLDLR	very low density lipoprotein receptor	3.52	1.83E-05
227815_at	VPS33A	Vacuolar protein sorting 33A (yeast)	1.47	5.21E-05
200629_at	WARS	tryptophanyl-tRNA synthetase	2.60	4.33E-05
210561_s_at	WSB1	WD repeat and SOCS box-containing 1	1.37	2.68E-06
212342_at	YIPF6	Yip1 domain family, member 6	1.31	5.43E-05
217783_s_at	YPEL5	yippee-like 5 (Drosophila)	3.02	2.36E-05
222408_s_at		yippee-like 5 (Drosophila)	2.85	3.93E-06
227436_at	ZA20D1	Zinc finger, A20 domain containing 1	1.82	4.87E-07

Table S3 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
213376_at	ZBTB1	zinc finger and BTB domain containing 1	1.39	7.48E-05
226962_at	ZBTB41	zinc finger and BTB domain containing 41	1.89	2.86E-07
223506_at	ZC3H8	zinc finger CCCH-type containing 8	2.21	4.74E-05
218478_s_at	ZCCHC8	zinc finger, CCHC domain containing 8	2.58	3.25E-05
218919_at	ZFAND1	zinc finger, AN1-type domain 1	2.42	9.50E-05
37254_at	ZNF133	zinc finger protein 133 (clone pHZ-13)	1.37	3.81E-05
206182_at	ZNF134	zinc finger protein 134 (clone pHZ-15)	2.78	4.74E-05
1554433_a_at	ZNF146	zinc finger protein 146	2.22	4.48E-05
206683_at	ZNF165	zinc finger protein 165	2.79	6.09E-05
219376_at	ZNF322B	zinc finger protein 322B	1.76	1.64E-06
225945_at	ZNF655	zinc finger protein 655	1.84	7.81E-05
227080_at	ZNF697	zinc finger protein 697	2.57	8.93E-05
226208_at	ZSWIM6	zinc finger, SWIM-type containing 6	2.28	3.17E-05
213567_at		Clone 23728 mRNA sequence	2.66	5.57E-05
213685_at		Gene from PAC 886K2, chromosome 1	1.79	3.03E-07
213750_at		Full length insert cDNA YH77E09	1.90	9.50E-05
224606_at		Homo sapiens, clone IMAGE:4096273, mRNA	3.92	9.07E-08
224621_at		LOC440806	1.33	2.10E-05
225522_at		Similar to BMP2 inducible kinase	2.58	1.85E-05
225811_at		Transcribed locus, weakly similar to XP_375099.1 PREDICTED: similar to hypothetical protein FLJ25224 [Homo sapiens]	1.43	7.40E-06
226223_at		CDNA FLJ14942 fis, A-PLACE1011185	2.02	8.23E-05
226520_at		Transcribed locus, weakly similar to XP_543946.1 PREDICTED: similar to chromosome 10 open reading frame 12 [Canis familiaris]	2.26	2.43E-05
226779_at		CDNA FLJ37302 fis, clone BRAMY2016009	1.57	3.87E-05
227278_at		Transcribed locus, moderately similar to XP_512541.1 PREDICTED: similar to hypothetical protein [Pan troglodytes]	2.02	2.16E-05
227452_at		Hypothetical gene supported by AK000477	2.63	8.62E-05
227492_at		Transcribed locus, weakly similar to XP_521389.1 PREDICTED: similar to UTY [Pan troglodytes]	2.86	2.32E-05
227747_at			2.81	6.15E-05

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
227755_at		CDNA clone IMAGE:4077090, partial cds	5.86	1.98E-06
232297_at			1.62	5.41E-06
241418_at		Hypothetical LOC344887	5.44	2.45E-06
242005_at			4.38	5.69E-06
243179_at		CDNA FLJ33993 fis, clone DFNES2007757	3.49	1.12E-05
64488_at		CDNA FLJ38849 fis, clone MESAN2008936	1.43	6.20E-05

[1]To be included in this table, differential gene expression had to meet a statistical cutoff of $P<0.0001$

Table S4 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
202888_s_at	ANPEP	alanyl (membrane) aminopeptidase (aminopeptidase N, aminopeptidase M, microsomal aminopeptidase, CD13, p150)	0.44	4.99E-05
201301_s_at	ANXA4	annexin A4	0.58	3.40E-05
201302_at		annexin A4	0.55	3.32E-05
218115_at	ASF1B	ASF1 anti-silencing function 1 homolog B (S. cerevisiae)	0.29	9.32E-05
218857_s_at	ASRGL1	asparaginase like 1	0.34	7.21E-05
244519_at	ASXL1	additional sex combs like 1 (Drosophila)	0.70	7.23E-05
218782_s_at	ATAD2	ATPase family, AAA domain containing 2	0.42	7.61E-05
222740_at		ATPase family, AAA domain containing 2	0.46	7.12E-05
203188_at	B3GNT6	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 6	0.20	1.29E-05
211715_s_at	BDH	3-hydroxybutyrate dehydrogenase (heart, mitochondrial) /// 3-hydroxybutyrate dehydrogenase (heart, mitochondrial)	0.51	7.56E-05
202095_s_at	BIRC5	baculoviral IAP repeat-containing 5 (survivin)	0.32	3.88E-05
219555_s_at	BM039	uncharacterized bone marrow protein BM039	0.38	7.44E-06
222118_at		uncharacterized bone marrow protein BM039	0.41	4.78E-05
209642_at	BUB1	BUB1 budding uninhibited by benzimidazoles 1 homolog (yeast)	0.36	1.56E-05
212121_at	C10orf61	chromosome 10 open reading frame 61	0.52	7.55E-05
212123_at		chromosome 10 open reading frame 61	0.61	6.79E-05
219067_s_at	C10orf86	chromosome 10 open reading frame 86	0.50	2.23E-05
202562_s_at	C14orf1	chromosome 14 open reading frame 1	0.53	5.53E-05
226597_at	C19orf32	chromosome 19 open reading frame 32	0.31	5.40E-05
218546_at	C1orf115	chromosome 1 open reading frame 115	0.57	3.45E-05
229381_at	C1orf64	chromosome 1 open reading frame 64	0.45	5.16E-05
225687_at	C20orf129	chromosome 20 open reading frame 129	0.33	1.62E-05
218709_s_at	C20orf9	chromosome 20 open reading frame 9	0.77	2.89E-05
218741_at	C22orf18	chromosome 22 open reading frame 18	0.27	9.10E-05
205654_at	C4BPA	complement component 4 binding protein, alpha	0.41	2.86E-05
201309_x_at	C5orf13	chromosome 5 open reading frame 13	0.35	4.59E-06
218233_s_at	C6orf49	chromosome 6 open reading frame 49	0.48	9.84E-05
223516_s_at		chromosome 6 open reading frame 49	0.42	7.49E-05
225777_at	C9orf140	chromosome 9 open reading frame 140	0.43	1.21E-05
200653_s_at	CALM1	calmodulin 1 (phosphorylase kinase, delta)	0.54	2.36E-05
64408_s_at	CALML4	calmodulin-like 4	0.41	4.50E-05
212669_at	CAMK2G	calcium/calmodulin-dependent protein kinase (CaM kinase) II gamma	0.69	3.21E-05

Table S4 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
203418_at	CCNA2	cyclin A2	0.27	1.28E-06
213226_at			0.31	8.69E-06
214710_s_at	CCNB1	cyclin B1	0.35	2.00E-05
228729_at			0.33	1.21E-05
208650_s_at	CD24	CD24 antigen (small cell lung carcinoma cluster 4 antigen)	0.50	9.75E-05
216379_x_at		CD24 antigen (small cell lung carcinoma cluster 4 antigen)	0.52	8.20E-05
203214_x_at	CDC2	cell division cycle 2, G1 to S and G2 to M	0.32	2.34E-06
210559_s_at		cell division cycle 2, G1 to S and G2 to M	0.27	6.80E-06
202870_s_at	CDC20	CDC20 cell division cycle 20 homolog (S. cerevisiae)	0.39	8.73E-05
205167_s_at	CDC25C	cell division cycle 25C	0.27	2.26E-05
204126_s_at	CDC45L	CDC45 cell division cycle 45-like (S. cerevisiae)	0.33	3.74E-05
203967_at	CDC6	CDC6 cell division cycle 6 homolog (S. cerevisiae)	0.19	6.98E-08
203968_s_at		CDC6 cell division cycle 6 homolog (S. cerevisiae)	0.27	9.01E-06
204510_at	CDC7	CDC7 cell division cycle 7 (S. cerevisiae)	0.39	2.41E-06
224753_at	CDCA5	cell division cycle associated 5	0.28	8.44E-06
224428_s_at	CDCA7	cell division cycle associated 7 /// cell division cycle associated 7	0.19	1.89E-06
204159_at	CDKN2C	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	0.20	7.06E-05
1555758_a_at	CDKN3	cyclin-dependent kinase inhibitor 3 (CDK2-associated dual specificity phosphatase)	0.25	1.77E-05
209714_s_at		cyclin-dependent kinase inhibitor 3 (CDK2-associated dual specificity phosphatase)	0.28	1.66E-05
204962_s_at	CENPA	centromere protein A, 17kDa	0.36	1.71E-05
207828_s_at	CENPF	centromere protein F, 350/400ka (mitosin)	0.36	1.34E-05
209194_at	CETN2	centrin, EF-hand protein, 2	0.41	2.16E-05
205394_at	CHEK1	CHK1 checkpoint homolog (S. pombe)	0.23	5.02E-06
210416_s_at	CHEK2	CHK2 checkpoint homolog (S. pombe)	0.45	8.34E-05
221675_s_at	CHPT1	choline phosphotransferase 1	0.60	5.67E-05
64900_at	CHST5 /// MGC15429	carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 5 /// hypothetical protein MGC15429	0.47	3.21E-05
218267_at	CINP	cyclin-dependent kinase 2-interacting protein	0.72	7.30E-05
209357_at	CITED2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	0.59	6.42E-05
228335_at	CLDN11	claudin 11 (oligodendrocyte transmembrane protein)	0.30	9.81E-06
1554804_a_at	CLDN19	claudin 19	0.29	1.44E-06
201774_s_at	CNAP1	chromosome condensation-related SMC-associated protein 1	0.31	1.84E-06

Table S4 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
211727_s_at	COX11	COX11 homolog, cytochrome c oxidase assembly protein (yeast) /// COX11 homolog, cytochrome c oxidase assembly protein (yeast)	0.63	2.59E-05
206651_s_at	CPB2	carboxypeptidase B2 (plasma, carboxypeptidase U)	0.07	2.50E-07
204172_at	CPOX	coproporphyrinogen oxidase	0.39	3.85E-05
1555889_a_at	CRTAP	cartilage associated protein	0.47	5.09E-05
201111_at	CSE1L	CSE1 chromosome segregation 1-like (yeast)	0.45	6.67E-05
201112_s_at		CSE1 chromosome segregation 1-like (yeast)	0.55	7.44E-06
201487_at	CTSC	cathepsin C	0.39	5.09E-05
224516_s_at	CXXC5	CXXC finger 5 /// CXXC finger 5	0.83	7.43E-05
228906_at	CXXC6	CXXC finger 6	0.35	5.53E-05
209389_x_at	DBI	diazepam binding inhibitor (GABA receptor modulator, acyl-Coenzyme A binding protein)	0.21	1.43E-05
211070_x_at		diazepam binding inhibitor (GABA receptor modulator, acyl-Coenzyme A binding protein) /// diazepam binding inhibitor (GABA receptor modulator, acyl-Coenzyme A binding protein)	0.23	8.75E-05
222925_at	DCDC2	doublecortin domain containing 2	0.87	3.54E-05
203409_at	DDB2 /// LHX3	damage-specific DNA binding protein 2, 48kDa /// LIM homeobox 3	0.53	2.19E-05
232278_s_at	DEPDC1	DEP domain containing 1	0.38	1.06E-05
235545_at		DEP domain containing 1	0.59	2.82E-05
226980_at	DEPDC1B	DEP domain containing 1B	0.28	3.18E-05
201791_s_at	DHCR7	7-dehydrocholesterol reductase	0.28	6.61E-05
202533_s_at	DHFR	dihydrofolate reductase	0.34	2.89E-05
202534_x_at		dihydrofolate reductase	0.30	2.18E-05
48808_at		dihydrofolate reductase	0.29	2.31E-05
204602_at	DKK1	dickkopf homolog 1 (Xenopus laevis)	0.39	5.69E-05
203764_at	DLG7	discs, large homolog 7 (Drosophila)	0.31	6.39E-05
209560_s_at	DLK1	delta-like 1 homolog (Drosophila)	0.60	9.55E-05
242138_at	DLX1	distal-less homeo box 1	0.34	2.85E-05
213647_at	DNA2L	DNA2 DNA replication helicase 2-like (yeast)	0.31	5.42E-05
201697_s_at	DNMT1	DNA (cytosine-5-)-methyltransferase 1	0.54	2.34E-06
221677_s_at	DONSON	downstream neighbor of SON	0.52	4.33E-05
236055_at	DQX1	DEAQ box polypeptide 1 (RNA-dependent ATPase)	0.50	1.11E-05
218585_s_at	DTL	denticleless homolog (Drosophila)	0.17	1.17E-05

Table S4 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
1553984_s_at	DTYMK	deoxythymidylate kinase (thymidylate kinase)	0.24	9.57E-05
203270_at		deoxythymidylate kinase (thymidylate kinase)	0.30	6.88E-06
208956_x_at	DUT	dUTP pyrophosphatase	0.39	2.15E-07
209932_s_at		dUTP pyrophosphatase	0.35	4.92E-05
228033_at	E2F7	E2F transcription factor 7	0.41	8.56E-05
219990_at	E2F8	E2F transcription factor 8	0.26	1.73E-05
202735_at	EBP	emopamil binding protein (sterol isomerase)	0.38	2.50E-05
213787_s_at		emopamil binding protein (sterol isomerase)	0.31	5.66E-06
201984_s_at	EGFR	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	0.44	9.83E-05
208290_s_at	EIF5	eukaryotic translation initiation factor 5	0.67	1.51E-05
208708_x_at		eukaryotic translation initiation factor 5	0.61	1.49E-06
204256_at	ELOVL6	ELOVL family member 6, elongation of long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like, yeast)	0.51	1.93E-05
201341_at	ENC1	ectodermal-neural cortex (with BTB-like domain)	0.41	2.11E-05
201719_s_at	EPB41L2	erythrocyte membrane protein band 4.1-like 2	0.27	5.96E-05
209368_at	EPHX2	epoxide hydrolase 2, cytoplasmic	0.31	1.57E-06
200043_at	ERH	enhancer of rudimentary homolog (Drosophila) /// enhancer of rudimentary homolog (Drosophila)	0.72	3.04E-05
203358_s_at	EZH2	enhancer of zeste homolog 2 (Drosophila)	0.47	1.45E-05
208962_s_at	FADS1	fatty acid desaturase 1	0.39	5.15E-06
208964_s_at		fatty acid desaturase 1	0.42	3.46E-05
222056_s_at	FAHD2A	fumarylacetoacetate hydrolase domain containing 2A	0.61	2.45E-05
225834_at	FAM72A	family with sequence similarity 72, member A	0.49	8.83E-05
203564_at	FANCG	Fanconi anemia, complementation group G	0.35	3.02E-05
211623_s_at	FBL	fibrillarin /// fibrillarin	0.60	4.08E-05
234863_x_at	FBXO5	F-box protein 5	0.49	8.99E-05
210638_s_at	FBXO9	F-box protein 9	0.60	7.98E-05
210950_s_at	FDFT1	farnesyl-diphosphate farnesyltransferase 1	0.24	1.18E-05
205650_s_at	FGA	fibrinogen alpha chain	0.38	7.65E-05
1552921_a_at	FIGNL1	fidgetin-like 1	0.50	5.92E-06
1562110_at	FLJ10006	Hypothetical protein FLJ10006	0.81	3.34E-05
213007_at	FLJ10719	hypothetical protein FLJ10719	0.34	7.44E-06
228273_at	FLJ11029	Hypothetical protein FLJ11029	0.41	2.41E-05
45633_at	FLJ13912	hypothetical protein FLJ13912	0.50	3.05E-06
1554102_a_at	FLJ14624	hypothetical protein FLJ14624	0.74	5.18E-05
215947_s_at	FLJ14668	hypothetical protein FLJ14668	0.57	3.15E-05

Table S4. Genes whose expression was downregulated in HepG2/C3A cells cultured in –Cys medium[1]

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
14033_at	ABCC6	ATP-binding cassette, sub-family C (CFTR/MRP), member 6	0.42	2.89E-06
214274_s_at	ACAA1	acetyl-Coenzyme A acyltransferase 1 (peroxisomal 3-oxoacyl-Coenzyme A thiolase)	0.38	3.77E-06
209608_s_at	ACAT2	acetyl-Coenzyme A acetyltransferase 2 (acetoacetyl Coenzyme A thiolase)	0.23	2.03E-05
201128_s_at	ACLY	ATP citrate lyase	0.63	3.34E-06
205364_at	ACOX2	acyl-Coenzyme A oxidase 2, branched chain	0.41	1.78E-05
220675_s_at	ADPN	adiponutrin	0.33	3.66E-05
211986_at	AHNAK	AHNAK nucleoprotein (desmoyokin)	0.71	4.56E-05
201675_at	AKAP1	A kinase (PRKA) anchor protein 1	0.68	9.04E-05
201900_s_at	AKR1A1	aldo-keto reductase family 1, member A1 (aldehyde reductase)	0.69	8.31E-05
214259_s_at	AKR7A2	aldo-keto reductase family 7, member A2 (aflatoxin aldehyde reductase)	0.57	8.21E-06
217791_s_at	ALDH18A1	aldehyde dehydrogenase 18 family, member A1	0.50	7.82E-06
222416_at		aldehyde dehydrogenase 18 family, member A1	0.43	2.88E-06
203608_at	ALDH5A1	aldehyde dehydrogenase 5 family, member A1 (succinate-semialdehyde dehydrogenase)	0.46	8.18E-05
221589_s_at	ALDH6A1	Aldehyde dehydrogenase 6 family, member A1	0.43	2.74E-05
208950_s_at	ALDH7A1	aldehyde dehydrogenase 7 family, member A1	0.22	2.48E-06
208951_at		aldehyde dehydrogenase 7 family, member A1	0.18	6.95E-05
201612_at	ALDH9A1	aldehyde dehydrogenase 9 family, member A1	0.47	6.77E-05
219649_at	ALG6	asparagine-linked glycosylation 6 homolog (yeast, alpha-1,3-glucosyltransferase)	0.50	3.07E-05
204294_at	AMT	aminomethyltransferase (glycine cleavage system protein T)	0.48	2.31E-06
208721_s_at	ANAPC5	anaphase promoting complex subunit 5	0.46	7.59E-05
208722_s_at		anaphase promoting complex subunit 5	0.44	2.74E-05
1552619_a_at	ANLN	anillin, actin binding protein (scraps homolog, Drosophila)	0.40	1.72E-05
222608_s_at		anillin, actin binding protein (scraps homolog, Drosophila)	0.33	7.04E-05
201051_at	ANP32A	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member A	0.74	7.09E-05
221505_at	ANP32E	acidic (leucine-rich) nuclear phosphoprotein 32 family, member E	0.56	9.32E-05

Table S4 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
219650_at	FLJ20105	FLJ20105 protein	0.48	5.26E-05
221685_s_at	FLJ20364	hypothetical protein FLJ20364	0.35	6.93E-05
220060_s_at	FLJ20641	hypothetical protein FLJ20641	0.46	3.54E-05
217872_at	FLJ20643	hypothetical protein FLJ20643	0.82	2.03E-05
234008_s_at	FLJ21736	esterase 31	0.53	2.18E-05
53071_s_at	FLJ22222	hypothetical protein FLJ22222	0.45	4.79E-05
213294_at	FLJ38348	Hypothetical protein FLJ38348	0.70	3.81E-05
218210_at	FN3KRP	fructosamine-3-kinase-related protein	0.52	8.55E-05
1773_at	FNTB	farnesyltransferase, CAAX box, beta	0.50	6.83E-05
225851_at		farnesyltransferase, CAAX box, beta	0.39	4.34E-05
202580_x_at	FOXM1	forkhead box M1	0.45	9.49E-05
223700_at	GAJ	GAJ protein	0.33	2.07E-05
213133_s_at	GCSH	glycine cleavage system protein H (aminomethyl carrier)	0.65	3.71E-05
203560_at	GGH	gamma-glutamyl hydrolase (conjugase, folylpolygammaglutamyl hydrolase)	0.34	1.40E-05
218350_s_at	GMNN	geminin, DNA replication inhibitor	0.24	3.02E-06
215690_x_at	GPAA1	GPAA1P anchor attachment protein 1 homolog (yeast)	0.54	4.00E-05
243193_at	GPC3	Glypican 3	0.27	7.94E-05
211829_s_at	GPR30	G protein-coupled receptor 30	0.59	5.94E-06
201348_at	GPX3	glutathione peroxidase 3 (plasma)	0.48	7.07E-05
214091_s_at		glutathione peroxidase 3 (plasma)	0.51	9.15E-05
201106_at	GPX4	glutathione peroxidase 4 (phospholipid hydroperoxidase)	0.62	8.94E-05
203815_at	GSTT1	glutathione S-transferase theta 1	0.52	3.89E-06
202605_at	GUSB	glucuronidase, beta	0.57	5.20E-05
200853_at	H2AFZ	H2A histone family, member Z	0.48	5.11E-05
213911_s_at		H2A histone family, member Z	0.55	6.31E-05
201035_s_at	HADHSC	L-3-hydroxyacyl-Coenzyme A dehydrogenase, short chain	0.35	9.73E-05
201036_s_at		L-3-hydroxyacyl-Coenzyme A dehydrogenase, short chain	0.22	2.56E-05
211569_s_at		L-3-hydroxyacyl-Coenzyme A dehydrogenase, short chain	0.29	8.96E-05
212789_at	hCAP-D3	KIAA0056 protein	0.44	4.27E-06
218663_at	HCAP-G	chromosome condensation protein G	0.26	9.50E-05
201209_at	HDAC1	histone deacetylase 1	0.68	4.13E-05
223556_at	HELLS	helicase, lymphoid-specific	0.29	3.69E-05
204689_at	HHEX	hematopoietically expressed homeobox	0.53	8.51E-05
213374_x_at	HIBCH	3-hydroxyisobutyryl-Coenzyme A hydrolase	0.61	6.65E-05
204754_at	HLF	Hepatic leukemia factor	0.55	3.08E-05
204755_x_at		hepatic leukemia factor	0.59	1.21E-05
208025_s_at	HMGA2	high mobility group AT-hook 2 /// high mobility group AT-hook 2	0.41	3.82E-06
200680_x_at	HMGB1	high-mobility group box 1	0.66	3.71E-05
208808_s_at	HMGB2	high-mobility group box 2	0.39	3.64E-05

Table S4 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
208668_x_at	HMGN2	high-mobility group nucleosomal binding domain 2	0.48	2.79E-05
209377_s_at	HMGN3	high mobility group nucleosomal binding domain 3	0.24	4.03E-06
200014_s_at	HNRPC	heterogeneous nuclear ribonucleoprotein C (C1/C2) /// heterogeneous nuclear ribonucleoprotein C (C1/C2)	0.75	9.85E-05
200942_s_at	HSBP1	heat shock factor binding protein 1	0.57	4.49E-05
214434_at	HSPA12A	heat shock 70kDa protein 12A	0.48	4.79E-05
200799_at	HSPA1A	heat shock 70kDa protein 1A	0.53	1.90E-05
218026_at	HSPC009	HSPC009 protein	0.41	1.04E-05
210211_s_at	HSPCA	heat shock 90kDa protein 1, alpha	0.83	2.29E-05
210619_s_at	HYAL1	hyaluronoglucosaminidase 1	0.47	1.02E-05
1555037_a_at	IDH1	isocitrate dehydrogenase 1 (NADP+), soluble	0.62	5.25E-05
201193_at		isocitrate dehydrogenase 1 (NADP+), soluble	0.61	2.15E-06
204615_x_at	IDI1	isopentenyl-diphosphate delta isomerase 1	0.32	1.36E-05
1555564_a_at	IF	I factor (complement)	0.39	2.88E-05
203854_at		I factor (complement)	0.36	6.22E-05
214022_s_at	IFITM1	interferon induced transmembrane protein 1 (9-27)	0.45	6.69E-05
223807_at	IGSF1	immunoglobulin superfamily, member 1	0.27	8.12E-05
219255_x_at	IL17RB	interleukin 17 receptor B	0.42	9.02E-06
224156_x_at		interleukin 17 receptor B	0.42	2.24E-05
203126_at	IMPA2	inositol(myo)-1(or 4)-monophosphatase 2	0.50	4.70E-05
204017_at	KDELR3	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 3	0.47	5.21E-05
202503_s_at	KIAA0101	KIAA0101	0.14	2.33E-05
211713_x_at		KIAA0101 /// KIAA0101	0.25	9.42E-05
224870_at	KIAA0114	KIAA0114 gene product	0.61	9.17E-05
213478_at	KIAA1026	kazrin	0.62	6.09E-05
221952_x_at	KIAA1393	KIAA1393	0.38	7.35E-06
218342_s_at	KIAA1815	KIAA1815	0.46	9.52E-06
218755_at	KIF20A	kinesin family member 20A	0.35	6.95E-06
211519_s_at	KIF2C	kinesin family member 2C	0.38	2.11E-05
228377_at	KLHL14	kelch-like 14 (Drosophila)	0.38	8.42E-05
201088_at	KPNA2	karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	0.56	4.57E-05
214039_s_at	LAPTM4B	lysosomal associated protein transmembrane 4 beta	0.72	1.33E-05
1552362_a_at	LEAP-2	liver-expressed antimicrobial peptide 2	0.36	2.38E-05
225614_at	LOC113174	hypothetical protein BC012010	0.54	4.40E-05
238554_at	LOC283852	hypothetical protein LOC283852	0.41	5.04E-05
231982_at	LOC284422	similar to HSPC323	0.26	1.98E-05
224637_at	LOC400948	similar to RIKEN cDNA 2310016E02	0.39	9.17E-06
224602_at	LOC401152	HCV F-transactivated protein 1	0.39	5.77E-05
226165_at	LOC401466	hypothetical gene supported by BC055092	0.61	4.72E-05
235898_at	LOC440858	LOC440858	0.52	1.85E-06

Table S4 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
209679_s_at	LOC57228	hypothetical protein from clone 643	0.55	4.60E-05
215215_s_at	LOC81691	exonuclease NEF-sp	0.50	4.64E-05
211596_s_at	LRIG1	leucine-rich repeats and immunoglobulin-like domains 1 /// leucine-rich repeats and immunoglobulin-like domains 1	0.89	5.29E-09
202904_s_at	LSM5	LSM5 homolog, U6 small nuclear RNA associated (S. cerevisiae)	0.61	9.56E-07
211747_s_at		LSM5 homolog, U6 small nuclear RNA associated (S. cerevisiae) /// LSM5 homolog, U6 small nuclear RNA associated (S. cerevisiae)	0.50	2.20E-05
202245_at	LSS	lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)	0.36	7.97E-05
219588_s_at	LUZP5	leucine zipper protein 5	0.33	1.55E-05
203007_x_at	LYPLA1	lysophospholipase I	0.80	4.97E-05
212279_at	MAC30	hypothetical protein MAC30	0.17	2.25E-06
212281_s_at		hypothetical protein MAC30	0.16	2.31E-07
212282_at		hypothetical protein MAC30	0.13	4.88E-06
1554768_a_at	MAD2L1	MAD2 mitotic arrest deficient-like 1 (yeast)	0.22	1.45E-05
203362_s_at		MAD2 mitotic arrest deficient-like 1 (yeast)	0.23	6.42E-05
209014_at	MAGED1	melanoma antigen family D, 1	0.35	4.66E-05
210018_x_at	MALT1	mucosa associated lymphoid tissue lymphoma translocation gene 1	0.66	8.66E-05
228468_at	MASTL	microtubule associated serine/threonine kinase-like	0.40	5.16E-06
207256_at	MBL2	mannose-binding lectin (protein C) 2, soluble (opsonic defect)	0.43	4.47E-05
209624_s_at	MCCC2	methylcrotonoyl-Coenzyme A carboxylase 2 (beta)	0.52	9.41E-05
220651_s_at	MCM10	MCM10 minichromosome maintenance deficient 10 (S. cerevisiae)	0.15	1.98E-05
202107_s_at	MCM2	MCM2 minichromosome maintenance deficient 2, mitotin (S. cerevisiae)	0.21	3.73E-05
201555_at	MCM3	MCM3 minichromosome maintenance deficient 3 (S. cerevisiae)	0.19	1.70E-06
212141_at	MCM4	MCM4 minichromosome maintenance deficient 4 (S. cerevisiae)	0.29	9.85E-05
216237_s_at	MCM5	MCM5 minichromosome maintenance deficient 5, cell division cycle 46 (S. cerevisiae)	0.16	2.75E-06
201930_at	MCM6	MCM6 minichromosome maintenance deficient 6 (MIS5 homolog, S. pombe) (S. cerevisiae)	0.31	4.44E-06

Table S4 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
208795_s_at	MCM7	MCM7 minichromosome maintenance deficient 7 (S. cerevisiae)	0.42	2.59E-05
210983_s_at		MCM7 minichromosome maintenance deficient 7 (S. cerevisiae)	0.30	7.70E-05
202645_s_at	MEN1	multiple endocrine neoplasia I	0.75	6.49E-05
226118_at	MGC11266	hypothetical protein MGC11266	0.52	4.43E-05
227055_at	MGC17301	hypothetical protein MGC17301	0.53	9.63E-05
226456_at	MGC24665	hypothetical protein MGC24665	0.21	2.60E-07
238465_at	MGC33648	hypothetical protein MGC33648	0.85	1.04E-05
226499_at	MGC61598	Similar to ankyrin-repeat protein Nrarp	0.51	2.88E-05
205904_at	MICA	MHC class I polypeptide-related sequence A	0.73	5.30E-05
206247_at	MICB	MHC class I polypeptide-related sequence B	0.31	1.59E-05
209585_s_at	MINPP1	multiple inositol polyphosphate histidine phosphatase, 1	0.53	4.59E-05
231746_at	MIXL1	Mix1 homeobox-like 1 (Xenopus laevis)	0.19	3.70E-05
212020_s_at	MKI67	antigen identified by monoclonal antibody Ki-67	0.59	2.63E-05
218883_s_at	MLF1IP	MLF1 interacting protein	0.32	4.46E-05
217909_s_at	MLX	MAX-like protein X	0.59	4.60E-05
218865_at	MOSC1	MOCO sulphurase C-terminal domain containing 1	0.29	3.14E-05
202472_at	MPI	mannose phosphate isomerase	0.44	2.43E-05
202911_at	MSH6	mutS homolog 6 (E. coli)	0.32	5.06E-06
211450_s_at		mutS homolog 6 (E. coli)	0.35	6.02E-05
222403_at	MTCH2	mitochondrial carrier homolog 2 (C. elegans)	0.82	6.54E-05
223172_s_at	MTP18	mitochondrial protein 18 kDa	0.54	9.12E-05
201710_at	MYBL2	v-myb myeloblastosis viral oncogene homolog (avian)-like 2	0.15	4.50E-06
203359_s_at	MYCBP	c-myc binding protein	0.47	4.24E-05
201970_s_at	NASP	nuclear autoantigenic sperm protein (histone-binding)	0.34	2.39E-05
210289_at	NAT8	N-acetyltransferase 8 (camello like)	0.46	1.26E-05
210774_s_at	NCOA4	nuclear receptor coactivator 4	0.66	7.21E-05
214279_s_at	NDRG2	NDRG family member 2	0.45	4.74E-05
209224_s_at	NDUFA2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 2, 8kDa	0.51	2.68E-06
203606_at	NDUFS6	NADH dehydrogenase (ubiquinone) Fe-S protein 6, 13kDa (NADH-coenzyme Q reductase)	0.41	1.49E-05
211080_s_at	NEK2	NIMA (never in mitosis gene a)-related kinase 2 /// NIMA (never in mitosis gene a)-related kinase 2	0.58	5.00E-05
202007_at	NID1	nidogen 1	0.42	2.93E-05
225592_at	NRM	nurim (nuclear envelope membrane protein)	0.38	7.51E-05

Table S4 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
215093_at	NSDHL	NAD(P) dependent steroid dehydrogenase-like	0.45	4.18E-05
209731_at	NTHL1	nth endonuclease III-like 1 (E. coli)	0.42	7.67E-05
224581_s_at	NUCKS	Nuclear ubiquitous casein kinase and cyclin-dependent kinase substrate	0.54	1.64E-05
218768_at	NUP107	nucleoporin 107kDa	0.57	2.01E-05
219978_s_at	NUSAP1	nucleolar and spindle associated protein 1	0.34	4.49E-05
200077_s_at	OAZ1	ornithine decarboxylase antizyme 1 /// ornithine decarboxylase antizyme 1	0.69	3.81E-06
215952_s_at		ornithine decarboxylase antizyme 1	0.63	7.25E-05
213599_at	OIP5	Opa interacting protein 5	0.26	1.11E-05
219105_x_at	ORC6L	origin recognition complex, subunit 6 homolog-like (yeast)	0.44	9.24E-06
218589_at	P2RY5	purinergic receptor P2Y, G-protein coupled, 5	0.42	6.30E-05
205719_s_at	PAH	phenylalanine hydroxylase	0.59	9.54E-05
217583_at		phenylalanine hydroxylase	0.45	6.65E-05
201014_s_at	PAICS	phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase	0.67	1.09E-05
226649_at	PANK1	pantothenate kinase 1	0.40	7.53E-05
228717_at		Pantothenate kinase 1	0.43	1.60E-05
227626_at	PAQR8	progestin and adipoQ receptor family member VIII	0.45	9.13E-05
219148_at	PBK	PDZ binding kinase	0.21	6.79E-05
205353_s_at	PBP	prostatic binding protein	0.33	1.12E-05
211941_s_at		prostatic binding protein	0.40	4.85E-05
212857_x_at	PC4	activated RNA polymerase II transcription cofactor 4	0.85	9.48E-05
201202_at	PCNA	proliferating cell nuclear antigen	0.31	5.75E-06
218014_at	PCNT1	pericentrin 1	0.37	1.32E-06
203857_s_at	PDIA5	protein disulfide isomerase family A, member 5	0.45	4.39E-05
207668_x_at	PDIA6	protein disulfide isomerase family A, member 6	0.52	3.66E-05
208638_at		protein disulfide isomerase family A, member 6	0.59	9.74E-05
221142_s_at	PECR	peroxisomal trans-2-enoyl-CoA reductase	0.43	4.09E-05
212092_at	PEG10	paternally expressed 10	0.35	1.35E-05
209242_at	PEG3	paternally expressed 3	0.42	5.01E-05
207132_x_at	PFDN5	prefoldin 5	0.72	3.29E-06
221521_s_at	Pfs2	DNA replication complex GINS protein PSF2	0.14	1.23E-05
200886_s_at	PGAM1	phosphoglycerate mutase 1 (brain)	0.59	8.91E-06
226623_at	PHYHIPL	phytanoyl-CoA hydroxylase interacting protein-like	0.51	1.61E-05
1559156_at	PIAS1	Protein inhibitor of activated STAT, 1	0.52	1.42E-05

Table S4 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
1563111_a_at	PIGX	phosphatidylinositol glycan, class X	0.62	3.43E-05
203649_s_at	PLA2G2A	phospholipase A2, group IIA (platelets, synovial fluid)	0.29	2.85E-05
202240_at	PLK1	polo-like kinase 1 (Drosophila)	0.36	8.56E-05
204886_at	PLK4	polo-like kinase 4 (Drosophila)	0.35	5.33E-05
204887_s_at		polo-like kinase 4 (Drosophila)	0.30	2.52E-05
204519_s_at	PLLP	plasma membrane proteolipid (plasmolipin)	0.26	5.06E-07
204441_s_at	POLA2	polymerase (DNA directed), alpha 2 (70kD subunit)	0.48	5.48E-05
205909_at	POLE2	polymerase (DNA directed), epsilon 2 (p59 subunit)	0.29	6.33E-05
210830_s_at	PON2	paraoxonase 2	0.78	5.20E-05
213695_at	PON3	paraoxonase 3	0.77	5.56E-06
209482_at	POP7	processing of precursor 7, ribonuclease P subunit (S. cerevisiae)	0.38	2.78E-05
235113_at	PPIL5	peptidylprolyl isomerase (cyclophilin)-like 5	0.34	6.76E-05
225203_at	PPP1R16A	protein phosphatase 1, regulatory (inhibitor) subunit 16A	0.40	3.53E-05
228010_at	PPP2R2C	protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), gamma isoform	0.34	4.48E-05
39729_at	PRDX2	peroxiredoxin 2	0.51	1.15E-05
205053_at	PRIM1	primase, polypeptide 1, 49kDa	0.34	5.45E-05
226611_s_at	PRR6	proline rich 6	0.48	9.49E-05
206102_at	PSF1	DNA replication complex GINS protein PSF1	0.14	2.66E-06
223808_s_at	PTPMT1	protein tyrosine phosphatase, mitochondrial 1	0.63	3.57E-05
203554_x_at	PTTG1	pituitary tumor-transforming 1	0.26	2.44E-05
232037_at	PUNC	putative neuronal cell adhesion molecule	0.36	1.62E-05
223032_x_at	PX19	px19-like protein	0.49	6.65E-05
222077_s_at	RACGAP1	Rac GTPase activating protein 1	0.43	9.29E-05
210216_x_at	RAD1	RAD1 homolog (S. pombe)	0.50	6.28E-05
204146_at	RAD51AP1	RAD51 associated protein 1	0.25	1.61E-05
213762_x_at	RBMX	RNA binding motif protein, X-linked	0.58	4.68E-05
225310_at		RNA binding motif protein, X-linked	0.63	6.29E-05
201485_s_at	RCN2	reticulocalbin 2, EF-hand calcium binding domain	0.62	1.62E-05
201486_at		reticulocalbin 2, EF-hand calcium binding domain	0.55	6.55E-06
209085_x_at	RFC1	replication factor C (activator 1) 1, 145kDa	0.67	9.39E-05
1053_at	RFC2	replication factor C (activator 1) 2, 40kDa	0.45	7.93E-05
203696_s_at		replication factor C (activator 1) 2, 40kDa	0.34	7.92E-06
204127_at	RFC3	replication factor C (activator 1) 3, 38kDa	0.21	3.79E-05
204128_s_at		replication factor C (activator 1) 3, 38kDa	0.19	6.17E-05

Table S4 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
204023_at	RFC4	replication factor C (activator 1) 4, 37kDa	0.25	9.36E-05
203210_s_at	RFC5	replication factor C (activator 1) 5, 36.5kDa	0.32	1.23E-05
202963_at	RFX5	regulatory factor X, 5 (influences HLA class II expression)	0.48	4.23E-05
212651_at	RHOBTB1	Rho-related BTB domain containing 1	0.48	3.17E-06
209507_at	RPA3	replication protein A3, 14kDa	0.22	6.46E-05
216505_x_at	RPS10 /// LOC388885	ribosomal protein S10 /// hypothetical LOC388885	0.85	6.85E-05
201476_s_at	RRM1	ribonucleotide reductase M1 polypeptide	0.33	6.34E-05
201890_at	RRM2	ribonucleotide reductase M2 polypeptide	0.16	1.21E-05
209773_s_at		ribonucleotide reductase M2 polypeptide	0.10	6.98E-08
1559946_s_at	RUVBL2	RuvB-like 2 (E. coli)	0.65	8.70E-05
234987_at	SAMHD1	SAM domain and HD domain 1	0.47	4.83E-05
209146_at	SC4MOL	sterol-C4-methyl oxidase-like	0.31	5.64E-05
200832_s_at	SCD	stearoyl-CoA desaturase (delta-9-desaturase)	0.38	2.76E-05
203453_at	SCNN1A	sodium channel, nonvoltage-gated 1 alpha	0.41	1.66E-06
201339_s_at	SCP2	sterol carrier protein 2	0.61	2.12E-05
201286_at	SDC1	syndecan 1	0.45	1.78E-05
222021_x_at	SDHA /// SDHAL2	succinate dehydrogenase complex, subunit A, flavoprotein (Fp) /// succinate dehydrogenase complex, subunit A, flavoprotein-like 2	0.57	4.64E-05
244163_at	SEMA3A	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A	0.42	3.17E-05
219194_at	SEMA4G	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4G	0.42	6.66E-06
1555851_s_at	SEPW1	selenoprotein W, 1	0.45	1.88E-05
230318_at	SERPINA1	Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	0.50	5.46E-05
220626_at	SERPINA10	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 10	0.46	7.46E-05
209443_at	SERPINA5	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 5	0.38	7.06E-05
206325_at	SERPINA6	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 6	0.23	4.97E-07
206386_at	SERPINA7	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 7	0.17	9.35E-08

Table S4 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
205576_at	SERPIND1	serine (or cysteine) proteinase inhibitor, clade D (heparin cofactor), member 1	0.55	5.83E-05
235339_at	SETDB2	SET domain, bifurcated 2	0.74	7.90E-05
200753_x_at	SFRS2	splicing factor, arginine/serine-rich 2	0.44	3.84E-07
200754_x_at		splicing factor, arginine/serine-rich 2	0.47	3.48E-06
214882_s_at		splicing factor, arginine/serine-rich 2	0.44	6.77E-05
201739_at	SGK	serum/glucocorticoid regulated kinase	0.46	9.48E-05
209980_s_at	SHMT1	serine hydroxymethyltransferase 1 (soluble)	0.26	6.34E-05
205339_at	SIL	TAL1 (SCL) interrupting locus	0.35	1.83E-05
207974_s_at	SKP1A	S-phase kinase-associated protein 1A (p19A)	0.68	7.32E-05
203124_s_at	SLC11A2	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2	0.65	1.70E-05
230687_at	SLC13A3	solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3	0.25	3.11E-05
227506_at	SLC16A9	solute carrier family 16 (monocarboxylic acid transporters), member 9	0.44	7.58E-05
226728_at	SLC27A1	solute carrier family 27 (fatty acid transporter), member 1	0.61	7.82E-06
202499_s_at	SLC2A3	solute carrier family 2 (facilitated glucose transporter), member 3	0.36	1.82E-05
218494_s_at	SLC2A4RG	SLC2A4 regulator	0.28	3.53E-05
202830_s_at	SLC37A4	solute carrier family 37 (glycerol-6-phosphate transporter), member 4	0.41	3.29E-06
219795_at	SLC6A14	solute carrier family 6 (amino acid transporter), member 14	0.33	3.82E-07
202219_at	SLC6A8	solute carrier family 6 (neurotransmitter transporter, creatine), member 8	0.38	1.58E-05
204240_s_at	SMC2L1	SMC2 structural maintenance of chromosomes 2-like 1 (yeast)	0.37	1.66E-05
201770_at	SNRPA	small nuclear ribonucleoprotein polypeptide A	0.48	6.59E-05
206055_s_at	SNRPA1	small nuclear ribonucleoprotein polypeptide A'	0.55	2.05E-05
203832_at	SNRPF	small nuclear ribonucleoprotein polypeptide F	0.33	2.23E-05
209891_at	SPBC25	spindle pole body component 25 homolog (S. cerevisiae)	0.24	8.99E-05
212558_at	SPRY1	sprouty homolog 1, antagonist of FGF signaling (Drosophila)	0.52	3.19E-06
227752_at	SPTLC2L	serine palmitoyltransferase, long chain base subunit 2-like (aminotransferase 2)	0.25	1.98E-05
213562_s_at	SQLE	squalene epoxidase	0.32	9.73E-05
200652_at	SSR2	signal sequence receptor, beta (translocon-associated protein beta)	0.62	1.19E-05

Table S4 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
203015_s_at	SSX2IP	synovial sarcoma, X breakpoint 2 interacting protein	0.58	6.98E-05
204092_s_at	STK6	serine/threonine kinase 6	0.54	5.75E-05
200783_s_at	STMN1	stathmin 1/oncoprotein 18	0.29	3.12E-06
213881_x_at	SUMO2	SMT3 suppressor of mif two 3 homolog 2 (yeast)	0.70	6.22E-05
41037_at	TEAD4	TEA domain family member 4	0.56	2.84E-05
214063_s_at	TF	transferrin	0.65	7.67E-05
204731_at	TGFBR3	transforming growth factor, beta receptor III (betaglycan, 300kDa)	0.59	4.36E-05
203046_s_at	TIMELESS	timeless homolog (Drosophila)	0.44	6.21E-05
1554408_a_at	TK1	thymidine kinase 1, soluble	0.27	1.87E-05
202338_at		thymidine kinase 1, soluble	0.23	3.58E-05
222477_s_at	TM7SF3	transmembrane 7 superfamily member 3	0.62	7.20E-06
213352_at	TMCC1	transmembrane and coiled-coil domain family 1	0.56	6.84E-06
1554483_at	TMEM37	transmembrane protein 37	0.22	7.39E-05
1554485_s_at		transmembrane protein 37	0.20	1.92E-05
234672_s_at	TMEM48	transmembrane protein 48	0.42	8.72E-05
203432_at	TMPO	thymopoietin	0.39	9.58E-05
218467_at	TNFSF5IP1	tumor necrosis factor superfamily, member 5-induced protein 1	0.60	5.39E-05
1552977_a_at	TNRC5	trinucleotide repeat containing 5	0.41	8.94E-05
201292_at	TOP2A	topoisomerase (DNA) II alpha 170kDa	0.32	2.41E-05
210052_s_at	TPX2	TPX2, microtubule-associated, homolog (Xenopus laevis)	0.49	3.64E-06
204033_at	TRIP13	thyroid hormone receptor interactor 13	0.32	2.60E-07
209108_at	TSPAN6	tetraspanin 6	0.38	3.63E-05
209109_s_at		tetraspanin 6	0.33	2.03E-05
203824_at	TSPAN8	tetraspanin 8	0.17	4.56E-05
212320_at	TUBB	tubulin, beta polypeptide	0.34	3.69E-06
213726_x_at	TUBB2	tubulin, beta, 2	0.51	4.74E-05
201714_at	TUBG1	tubulin, gamma 1	0.62	9.41E-06
209077_at	TXN2	thioredoxin 2	0.63	9.26E-06
202589_at	TYMS	thymidylate synthetase	0.33	7.93E-06
202954_at	UBE2C	ubiquitin-conjugating enzyme E2C	0.32	9.95E-05
223229_at	UBE2T	ubiquitin-conjugating enzyme E2T (putative)	0.33	4.53E-05
202706_s_at	UMPS	uridine monophosphate synthetase (orotate phosphoribosyl transferase and orotidine-5'-decarboxylase)	0.72	5.63E-05
202330_s_at	UNG	uracil-DNA glycosylase	0.19	5.91E-07
206771_at	UPK3A	uroplakin 3A	0.22	7.78E-05
203856_at	VRK1	vaccinia related kinase 1	0.35	4.39E-05
203797_at	VSNL1	visinin-like 1	0.18	1.78E-05
203798_s_at		visinin-like 1	0.17	4.27E-05
229551_x_at	ZNF367	zinc finger protein 367	0.34	2.42E-05

Table S4 (Continued)

Probe ID	Gene Symbol	Gene Title	Fold Difference	P
204026_s_at	ZWINT	ZW10 interactor	0.17	1.27E-05
205675_at			0.46	7.01E-05
215528_at		MRNA; cDNA DKFZp586O1318 (from clone DKFZp586O1318)	0.63	5.34E-05
221588_x_at			0.36	1.46E-05
222044_at			0.64	7.44E-05
227349_at		CDNA FLJ11381 fis, clone HEMBA1000501	0.37	9.98E-05
227350_at		CDNA FLJ11381 fis, clone HEMBA1000501	0.20	1.95E-05
228143_at			0.63	9.89E-05
229500_at			0.75	2.55E-05
230960_at		Transcribed locus	0.37	1.60E-06
231007_at	1	Transcribed locus, weakly similar to XP_375099.1 PREDICTED: similar to hypothetical protein FLJ25224 [Homo sapiens]	0.40	1.02E-06
236201_at	1	Transcribed locus	0.30	3.45E-05
238075_at	1	Transcribed locus	0.32	4.22E-05
240422_at	1	Transcribed locus	0.38	6.43E-05
241607_at	1	LOC440702	0.38	5.54E-05

[1]To be included in this table, differential gene expression had to meet a statistical cutoff of $P<0.0001$.

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