Elevated ATF4 Expression, in the Absence of Other Signals, Is Sufficient for Transcriptional Induction via CCAAT Enhancer-binding Protein-activating Transcription Factor Response Elements

Eukaryotic cells have evolved specific ways for coping with various kinds of stresses. Interestingly, several independent environmental stress conditions trigger the same downstream signaling event, phosphorylation of the eukaryotic translation initiation factor (eIF)3 2α (1–3). In mammalian cells, four eIF2α kinases have been identified. Heme-regulated inhibitor is activated by heme deprivation, oxidative, and heat stresses. PKR (protein kinase RNA-dependent) can be triggered by double-strand RNA binding and is proposed to participate in the cellular anti-viral defense. PERK (RNA-dependent protein kinase-like endoplasmic reticulum kinase) is a component of the unfolded protein response (UPR), which is triggered by endoplasmic reticulum stress. GCN2 (general control nonderepressible 2) was originally found to be activated during amino acid deprivation and can also be induced by UV irradiation and pro teasea inhibition (1, 2, 4). Once phosphorylated by one of those four independent kinases, p-eIF2α contributes to a cellular response program to respond to the perturbation or alternatively, to induce apoptosis (1). During this response, general protein synthesis declines because the guanine exchange factor eIF2B that catalyzes the formation of active eIF2-GTP from inactive eIF2α-GDP is inhibited by p-eIF2α (5). Paradoxically, under these conditions translation of some mRNA species is increased, among them is activating transcription factor 4 (ATF4).

There are two short upstream open reading frames (uORFs) within the 5’-leader of the ATF4 mRNA (6, 7). Under nonstress conditions, after translation of uORF1, sufficient eIF2-GTP makes it possible to reinitiate translation from the uORF2, which is out of frame with ATF4, and therefore ATF4 synthesis is minimized. Conversely, stress-induced p-eIF2α leads to limited eIF2-GTP and prolongs the duration for the scanning ribosome to reinitiate following uORF1. Consequently, uORF2 is skipped, and initiation at the ATF4 coding region is increased. Thus, ATF4 protein content is rapidly increased under the many conditions that lead to p-eIF2α production, including the amino acid response (AAR) following protein or amino acid limitation and the UPR following endoplasmic reticulum stress (8).

As a member of the basic region leucine zipper (bZIP) transcription factor family, ATF4 can regulate gene transcription by forming a homodimer or heterodimer with other bZIP transcription factors (9). Microarray analysis with mammalian cells has shown that, as with its yeast counterpart GCN4, ATF4 activates a large number of genes that have a wide spectrum of functions (3, 10). Several ATF4 target genes have been identi-
fied that contain a genomic sequence comprised of a half-site for C/EBP family members and a half-site for ATF members and will be referred to as a C/EBP-ATF response element (CARE) (11). Using the AAR as a model system to activate ATF4 synthesis, Chen et al. (12) have proposed a self-limiting model that consists of two stages. During the first stage, increased ATF4 synthesis leads to enhanced binding to the CARE composite sites. This binding is associated with localized histone acetylation and subsequent recruitment of the general transcriptional machinery. Among the ATF4 target genes are the transcription factors ATF3 (13, 14), CCAAT-enhancer binding protein β (C/EBPβ) (15, 16), and C/EBP homology protein (CHOP) (17). During the second stage of the ATF4 response, increased expression of ATF3, C/EBPβ, and CHOP leads to their binding at the CARE site, which causes a subsequent suppression of transcription back toward the basal level. This self-limiting model for ATF4 action, originally reported for asparagine synthetase (ASNS), has been confirmed for several other CARE-containing genes (14, 18).

Although diverse stresses merge at the phosphorylation of eIF2α and up-regulation of ATF4, upstream signaling cascades triggered by those stresses can be quite varied (1). In addition, microarray analysis has shown that a significant number of genes up-regulated during the AAR and UPR are ATF4-independent (3, 10, 19), and others are GCN2-independent (20). Although it is clear that ATF4 is a critical component of the AAR and UPR pathways, whether or not an initial stress, such as amino acid limitation, triggers other required pathways or signals that act in concert with ATF4 has not been extensively studied. One example, which has been observed in HepG2 human hepatoma cells, is the requirement of MAPK signaling for an optimal p-eIF2α/ATF4 response (21). The relative lack of information on this topic leads to the following question: Is increased ATF4 alone sufficient to trigger the AAR transcriptional program, or does amino acid limitation trigger parallel signals that enhance or are required for ATF4 action?

In the present studies we took advantage of a cell line, hereafter called ATF4–293, in which ectopic expression of ATF4 can be induced by adding tetracycline (Tet) in the absence of amino acid deprivation (22). Although endogenous ATF4 expression and the phosphorylation of eIF2α were not affected by Tet treatment, ectopically expressed ATF4 alone was able to induce transcription from several CARE-containing genes. However, analysis revealed that ASNS transcription activity was not entirely proportional to the total abundance of overexpressed ATF4.

### Table 1

| Gene                      | Forward            | Reverse            |
|---------------------------|--------------------|--------------------|
| ASNS mRNA                 | 5'-GCAAGCTGAGAACAGACCAATG-3' | 5'-TGGCTTCCATGCAATGTC-3' |
| ASNS transcription activity| 5'-CTGGGAGATCCCTTGTATCACTG-3' | 5'-CTGAAGCTTCACTACCTCCT-3' |
| CHOP mRNA                 | 5'-GCAAGCTGAGAACAGACCAATG-3' | 5'-TGGCTTCCATGCAATGTC-3' |
| C/EBPβ mRNA               | 5'-GCAAGCTGAGAACAGACCAATG-3' | 5'-TGGCTTCCATGCAATGTC-3' |
| SNAT2 mRNA                | 5'-GCAAGCTGAGAACAGACCAATG-3' | 5'-TGGCTTCCATGCAATGTC-3' |
| ATF3 mRNA                 | 5'-GCAAGCTGAGAACAGACCAATG-3' | 5'-TGGCTTCCATGCAATGTC-3' |
| ATF4 mRNA, coding region  | 5'-GCAAGCTGAGAACAGACCAATG-3' | 5'-TGGCTTCCATGCAATGTC-3' |
| ATF4 mRNA, 3'-untranslated region | 5'-GCAAGCTGAGAACAGACCAATG-3' | 5'-TGGCTTCCATGCAATGTC-3' |
| glyceraldehyde-3-phosphate dehydrogenase mRNA | 5'-GCAAGCTGAGAACAGACCAATG-3' | 5'-TGGCTTCCATGCAATGTC-3' |
| ASNS, promoter ChIP       | 5'-GCAAGCTGAGAACAGACCAATG-3' | 5'-TGGCTTCCATGCAATGTC-3' |
| ASNS, Exon 7 ChIP         | 5'-GCAAGCTGAGAACAGACCAATG-3' | 5'-TGGCTTCCATGCAATGTC-3' |

### Figure 1

**Characterization of ATF4 expression in 293-ATF4 cells.** Immunoblot analysis of ATF4 protein expression was analyzed using 40 μg of whole cell extract/lane. The blots were probed with antibodies for ATF4 and then reprobed to detect β-actin. DMEM represents the untreated control cells. In 293-ATF4 cells, synthesis of endogenous ATF4 was induced with HisOH (2 mM), and the ectopic ATF4 expression was induced with Tet at the indicated concentrations for 8 h (A). The time course from 0–8 h of ATF4 induction with either HisOH or 0.01 μg/ml Tet was compared (B). The Tet sensitivity of endogenous ATF4 was compared in parental HEK293 cells versus the 293-ATF4 cells selected to express the Tet-inducible ATF4 (C).

### Materials and Methods

**Cell Line and Cell Culture**—The ATF4–293 cell line was created by transfecting HEK293 cells with a tetracycline-inducible construct that contains the ATF4 coding region (22). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, pH 7.4) (Mediatech, Herndon, VA) at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. The medium was supplemented with 1X nonessential amino acids, 2 mM glutamine, 100 mg/ml streptomycin sulfate, 100 units/ml penicillin G, 0.25 mg/ml amphotericin B, and 10% (v/v) tetracycline-free fetal bovine serum, 25 μg/ml zeocin, and 2.5 μg/ml blasticidin. The cultures were replenished with fresh medium 12 h prior to initiating all treatments to ensure that the cells were in the basal state. To trigger endogenous ATF4 synthesis, the AAR pathway was induced by transfer of cells to medium containing 2 mM HisOH, which blocks charging of histidine onto the corresponding tRNA and thus mimics histidine dep-
Overexpression of ectopic ATF4 was induced by adding Tet at the concentrations and times indicated.

**RNA Isolation and Quantitative RT-PCR**—Total RNA was isolated with the Qiagen RNeasy kit (Qiagen), including a DNase I treatment before the final elution to eliminate genomic DNA contamination. To measure steady state mRNA, total RNA was quantified and diluted to 20 ng/μl, and a 5-μl aliquot was mixed with 62.5 pmol of PCR primers, 6.25 units of reverse transcriptase, and 2.5 units of RNase inhibitor, together with 12.5 μl of SYBR Green PCR master mix (Applied Biosystems) in 25 μl of total volume. Reverse transcription and real time PCR were both performed with a DNA Engine Opticon 3 system (Bio-Rad). The primers used are listed in Table 1. The reactions were incubated at 48 °C for 30 min followed by 95 °C for 10 min to activate the Taq polymerase and amplification of 36 cycles at 95 °C for 15 s and 60 °C for 60 s. After PCR, melting curves were acquired by a stepwise increase of the temperature from 55 to 95 °C to ensure that a single product was amplified in the reaction. Glyceraldehyde-3-phosphate dehydrogenase mRNA levels were also measured as a nonregulated internal control. Transcription activity of the ASNS gene was monitored by real-time PCR, using primers within the ASNS protein coding region, whereas the transcription activity was assessed using PCR primers that bridged an intron-exon boundary to measure the short-lived heteronuclear RNA. The results are presented as the averages ± S.D. of three independent samples for which PCR was performed in duplicate.
time quantitative PCR (RT-qPCR) as described previously (12), using a pair of primers (Table 1) that amplify across the junction of intron 4 and exon 5 such that the amount of unspliced heteronuclear RNA is measured, which has been shown to be a valid indicator of transcription activity (23).

**Immunoblotting**—For preparation of total cell extracts, the cells were washed with ice-cold PBS and then lysed with Bio-Rad Laemmli buffer supplemented with 5% 2-mercaptoethanol, 1/1000 protein inhibitor mixture (Roche Applied Science), and 1/100 phosphatase inhibitor I and II mixture (Sigma). The whole cell lysate was sonicated, and protein was quantified before separating 40 µg/lane on a 10.5–14% Tris-HCl polyacrylamide gel (Bio-Rad) and electrotransferring to a Trans-Blot nitrocellulose membrane (0.2 µm) (Bio-Rad). The membrane was stained with Fast Green to check for equal loading and then incubated with 5 or 10% blocking solution consisting of 5 or 10% (w/v) Carnation nonfat dry milk, in TBST (30 mM Tris-base, pH 7.5, 200 mM NaCl, and 0.1% (v/v) Tween 20) for 2 h at room temperature or overnight at 4 °C. The blots were washed five times for 5 min in TBST and then incubated with the appropriate peroxidase-conjugated secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for 1 h at room temperature. The blots were then washed five times for 5 min in freshly made TBST. The bound secondary antibody was detected using an Enhanced 10 chemiluminescence kit (GE Healthcare) and by exposing the blot to Biomax MR film (Kodak, Rochester, NY). The antibodies used were as follows: rabbit anti-ATF4 polyclonal antibody (1:5000; made by Cocalico Biotechnology Inc.); rabbit anti-β-actin polyclonal antibody (1:10,000; Sigma); and rabbit anti-Ser51 phospho-eIF2α and anti-total eIF2α polyclonal antibodies (9721 and 9722; 1:500 dilution; Cell Signaling, Danvers, MA).

**Chromatin Immunoprecipitation (ChIP)**—The ChIP assay was performed according to our previously published protocol (12). The ATF4 antibody was the same as used for immunoblotting, whereas other antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) as follows: ATF3 (sc-188); C/EBPβ (sc-150); RNA polymerase II (sc-899); TFIIID (TBP) (sc-204); TFIIIB (sc-274); normal rabbit IgG (sc-2027). Antibody against acetylated histone H3 (Ab1791) was purchased from Abcam (Cambridge, MA). DNA enrichment for either the ASNS promoter or exon 7 was analyzed with RT-qPCR as described above. The reaction mixtures were incubated at 95 °C for 15 min, followed by amplification at 95 °C for 15 s and 61.4 °C for 60 s for 35 cycles. All of the experiments were performed in triplicate, and each sample was subjected to PCR in duplicate. The result was described as the ratio to total input DNA at a 1:20 dilution. The primers are listed in Table 1.

**Immunostaining**—All of the steps were completed at room temperature. Before staining, the cells were cross-linked with 3.7% formaldehyde in PBS for 15 min, permeabilized with 0.1% Triton X-100 in PBS for 5 min, and blocked with 1% fetal bovine serum in a PBST solution (0.1% Tween 20 in PBS) for 1 h. The cells were incubated with ATF4 antibody (1:5000, diluted with PBST) for 2 h, washed with PBST for 5 × 5 min, and then incubated in Alexa Fluor 488 goat anti-rabbit IgG secondary antibodies (1:200; Molecular Probes, Inc., Eugene, OR) for 1 h. After washing with PBST for 5 × 5 min, the cells were stained and placed in Vectashield mounting medium with 4’,6-diamidino-2-phenylindole (Vector Laboratories, Inc., Burlingame, CA). The images were captured with a RETIGA Exi digital cam-
era (Qimaging) connected to a Diaphot Nikon fluorescence microscope.

RESULTS

Characterization of HEK293 Cells That Stably Express Ectopic ATF4—Ord et al. (22) produced a HEK293 cell line that ectopically expresses ATF4 in a Tet-inducible manner, and these cells will be referred to as ATF4−293 cells. We used the ATF4−293 cells to investigate whether or not an elevation of ATF4 protein content, independent of other possible AAR-generated signals, is sufficient to activate genes that harbor CARE enhancer sequences. ATF4 protein was increased in a concentration-dependent manner from 0.007 to 0.1 μg/ml Tet (Fig. 1A). The endogenous ATF4 protein level, obtained with 2 mM HisOH treatment, was between that observed with 0.007 and 0.01 μg/ml Tet. A time course established that the optimal time for ATF4 production by HisOH treatment was 4−8 h, whereas that for 0.01 μg/ml Tet was 8 h or more (Fig. 1B). Both the parental HEK293 cells and the ATF4−293 cells were subjected to HisOH and Tet incubations to demonstrate that Tet treatment did not induce the expression of endogenous ATF4 in the parental cells and that in the ATF4−293 cells the endogenous AAR pathway was still fully responsive as judged by HisOH induction (Fig. 1C). Measurement of the endogenous ATF4 using PCR primers specific for the ATF4 3′-untranslated region sequence, which was lacking in the transfected ATF4 construct, could be distinguished from the total ATF4 content measured by primers within the coding region (Fig. 2A). Using this approach, it was determined that like the parental cells, Tet treatment of the 293−ATF4 cells does not induce the expression of endogenous ATF4, ruling out a direct or indirect effect of the drug on either the AAR or the UPR pathways (Fig. 2B). To confirm the lack of a feedback effect of the ectopically expressed ATF4 at earlier steps in the AAR pathway, the phosphorylation of eIF2α was also monitored (Fig. 2C). Although HisOH induced p-eIF2α as expected, Tet treatment of the cells had no effect supporting the interpretation that the ectopically expressed ATF4 does not cause an increase in the endogenous ATF4 levels or any of the eIF2α kinases.

A nuclear localization sequence within the C-terminal of the ATF4 protein has been identified (24) and preliminary experiments suggested that endogenous ATF4 is quickly translocated to the nucleus after synthesis,4 but there remained the question of whether or not activation of the AAR pathway is necessary for maximal nuclear localization for the newly synthesized protein. Earlier studies showing strong ATF4 action suggested that a significant portion of the ectopically expressed protein is located in the nucleus (22). ATF4−293 cells were incubated in either HisOH or Tet to induce the synthesis of endogenous or exogenous ATF4, respectively, and then stained with ATF4 antibody (Fig. 3). There was a low level of staining of the cytoplasm in the DMEM control condition, whereas after activation of the AAR pathway, most, but not all cells, exhibited a significant amount of ATF4 restricted to the nucleus. That not all cells in a culture respond equally after activation of the AAR pathway has also been observed by monitoring C/EBPβ localization (25). Following Tet treatment, the majority of ectopically expressed ATF4 staining was associated with the nuclear compartment (Fig. 3), indicating that trafficking of ATF4 does not require additional signals from the AAR pathway.

Comparison of Transcription Mediated by Ectopic versus Endogenous ATF4—Induction of the transcription activity from the ASNS gene by Tet treatment occurred in a concentration-dependent manner (Fig. 4). The amount of Tet required to elicit the same degree of transcription as HisOH was greater than 0.02 μg/ml, a level of Tet that caused a much greater increase in ATF4 protein content than HisOH (Fig. 1). The data indicate that ATF4 alone is sufficient to activate transcription but suggest that when ATF4 protein is increased in the absence of the AAR signaling pathway, more ATF4 is required to achieve the same level of ASNS transcription.

To determine whether the difference in ASNS control by ectopically expressed versus endogenous ATF4 was also true for other AAR element-driven genes, the mRNA content was analyzed for C/EBPβ (15), sodium-dependent neutral amino acid transporter 2 (SNAT2) (26), ATF3 (14), and CHOP (20), all known to be induced by amino acid deprivation via ATF4 (Fig. 5). In each case, the level of induction by 0.01 μg/ml Tet was less than that by HisOH, and even 0.1 μg/ml Tet produced expres-

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4 C. Zhong and M. S. Kilberg, unpublished data.
sion that was only equal to (ATF3) or slightly greater than HisOH (SNAT2 and C/EBPβ). These data indicate that the effect observed for the ASNS gene is also true for other CARE-containing genes, that is, to achieve the same level of transcription, more ATF4 protein is required in the Tet-induced cells than in the cells in which the AAR pathway is activated.

Recruitment of C/EBP-ATF-binding Proteins by Ectopically Expressed ATF4—In addition to ATF4, a CARE site also binds C/EBPβ and ATF3 (12, 14). As described above, at 4–8 h after activation of the AAR pathway, there is an ATF4-dependent increased synthesis and subsequent recruitment of C/EBPβ and ATF3 to the ASNS promoter that coincides with a suppression of transcription activity (12, 14). To determine whether induction of ATF4 expression alone can trigger the recruitment of these additional C/EBP-ATF-binding proteins, ChIP analysis of the ASNS promoter was performed after HisOH or Tet treatment of HepG2 cells (Fig. 6). Induction of ATF4 protein alone still resulted in increased binding of C/EBPβ and ATF3 at 8 h, illustrating that signals other than ATF4 are not required for their recruitment. However, despite the fact that 0.01 μg/ml Tet produced significantly more ATF4 protein than did 2 mM HisOH treatment (Fig. 1), the amount of ATF4 and C/EBPβ binding was less in the ectopically expressing cells compared with the cells with the AAR activated (Fig. 6). Factor binding was minimal at a distal region (exon 7) of the ASNS gene, documenting the specificity of the promoter binding.

Recruitment of the General Transcription Machinery—In parallel with ATF4 binding, assembly of the general transcription machinery, including RNA polymerase II, occurs with 30–45 min after amino acid withdrawal from cells (12). To determine whether there are events triggered by the AAR pathway other than ATF4 synthesis that are required for efficient recruitment of the general transcription factors associated with the initiation and elongation complexes, TBP (TFIID), TFIIE, and TAF1 were analyzed by ChIP analysis of the ASNS promoter after Tet and HisOH treatment (Fig. 7). As negative controls, nonspecific IgG was used, and PCR was performed using primers against ASNS exon 7. Consistent with the increased ASNS transcription (Fig. 4), ectopically expressed ATF4 alone resulted in increased recruitment of each of the sampled factors (Fig. 7).

Therefore, the data document that ATF4 binding alone is a sufficient signal to trigger assembly of the appropriate transcription initiation and elongation complexes.

Effect of Ectopically Expressed ATF4 on Histone Modification—Histone modification is an early event associated with ATF4 binding to the ASNS promoter (12). To determine whether signals from the AAR pathway other than ATF4 binding were required to trigger these changes, histone 4 (H4) acetylation and total histone content (H3) was monitored by ChIP analysis of the ASNS promoter and, as a negative control, a distal region of the gene (exon 7). Total histone content was checked using an antibody against H3 because the commercially available antibodies against H4 are not suitable for ChIP analysis. Activation of the AAR pathway resulted in an increase in H4 acetylation at the ASNS promoter (Fig. 8). Although production of ATF4 alone also caused an increase in H4 acetylation, the effect was less than that produced by HisOH, even at 0.1 μg/ml Tet.
Elevated ATF4 expression after amino acid deprivation may also be a component of the mechanism by which a distinct subset of genes is activated by each of four different elf2α kinases, which all trigger increased de novo ATF4 synthesis. For example, we demonstrated that following UPR activation ATF4 binds to the SNAT2 gene CARE site at a level equal to that observed following AAR activation, and yet there is no increase in SNAT2 transcription by the UPR (28). The ATF4 dimerization partner for the SNAT2 CARE under these two conditions has not been established.

Although the activation of the GCN2/p-eIF2α/ATF4 signaling cascade following amino acid deprivation is well documented, a few observations have raised the possibility that additional signaling pathways may also be activated by amino acid depletion. 1) It has been reported that amino acid limitation increases certain mRNA species in a GCN2-independent fashion (29). 2) Among the GCN2-independent genes is insulin-like growth factor binding protein 1 (30). Consistent with this observation, it has been reported that rather than a CARE site, the rat insulin-like growth factor-binding protein 1 promoter contains an upstream stimulatory factor-binding element that is responsible for the amino acid-regulated transcription (31). 3) The c-Jun N-terminal kinase (32) and the MEK-ERK (21) arms of the MAPK pathway are increased in response to amino acid limitation. In the case of the MEK-ERK, it was shown in HepG2 hepatoma cells that both elf2α phosphorylation and ATF4 synthesis were dependent on a parallel ERK activation by amino acid deprivation. ERK can activate 90-kDa ribosomal S6 kinase 2 (RSK2) (33), which in turn has been shown to phosphorylate and enhance ATF4 function in osteoblasts (34). It is possible that RSK or other MAPK networks may serve to link GCN2-independent upstream signals, triggered by amino acid limitation, with the magnitude and/or the specificity of ATF4 transcriptional activity. This concept is consistent with the present observation that to achieve the same degree of gene activation, the absolute amount of ectopically expressed ATF4 is greater than that needed during amino acid limitation.

Elevated ATF4 expression after amino acid deprivation induces de novo synthesis of C/EBPβ (15, 16), ATF3 (14), and...
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CHOP (17), and these factors then bind to CARE sequences to suppress activity (11, 12, 14). Although the level of C/EBPβ binding to the ASNS promoter was reduced in the cells ectopically expressing ATF4 alone, the observation that both C/EBPβ and ATF3 are recruited to the ASNS promoter after Tet induction of ATF4, documents that their synthesis and genomic action does not require factors other than ATF4. Collectively, the results presented in this report demonstrate that ATF4 alone is sufficient to trigger the transcriptional activation and subsequent suppression program that occurs in response to amino acid deprivation of mammalian cells. However, subtle modulation of the ATF4 signal at specific genes cannot be ruled out, and the observed differences in histone modification by endogenous versus ectopic ATF4 expression illustrate that further investigation is required to fully understand the complexity of the mechanisms associated with ATF4 action.

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**REFERENCES**

1. Wek, R. C., Jiang, H. Y., and Anthony, T. G. (2006) *Biochim. Biophys. Acta* **1762**, 1–12
2. Wek, R. C., and Cavener, D. R. (2007) *Antioxid. Redox. Signal.* **9**, 2357–2371
3. Harding, H. P., Zhang, Y., Zeng, H., Novoa, I., Lu, P. D., Calfon, M., Sadri, N., Yun, C., Popko, B., Paules, R., Stojdl, D. F., Bell, J. C., Hettmann, T., Leiden, J. M., and Ron, D. (2003) *Mol. Cell* **11**, 619–633
4. Dever, T. E. (2002) *Cell* **108**, 543–556
5. Kimball, S. R. (2002) *J. Nutr.* **132**, 883–886
6. Vattem, K. M., and Wek, R. C. (2004) *Proc. Natl. Acad. Sci. U.S.A.* **101**, 11269–11274
7. Lu, P. D., Harding, H. P., and Ron, D. (2004) *J. Cell Biol.* **167**, 27–33
8. Kilberg, M. S., Pan, Y. X., Chen, H., and Leung-Pineda, V. (2005) *Annu. Rev. Nutr.* **25**, 59–85
9. Ameri, K., and Harris, A. L. (2008) *Int. J. Biochem. Cell Biol.* **40**, 14–21
10. Lee, J. I., Dominy, J. E., Jr., Sikalidis, A. K., Hirschberger, L. L., Wang, W., and Stipanuk, M. H. (2008) *Physiol. Genomics* **33**, 218–229
11. Kilberg, M. S., Shan, J., and Su, N. (2009) *Trends Endocrinol. Metab.* **20**, in press
12. Chen, H., Pan, Y. X., Dudenhausen, E. E., and Kilberg, M. S. (2004) *J. Biol. Chem.* **279**, 50829–50839
13. Pan, Y., Chen, H., Siu, F., and Kilberg, M. S. (2003) *J. Biol. Chem.* **278**, 38402–38412
14. Pan, Y. X., Chen, H., Thiaville, M. M., and Kilberg, M. S. (2007) *Biochem. J.* **401**, 299–307
15. Chen, C., Dudenhausen, E., Chen, H., Pan, Y. X., Gjymishka, A., and Kilberg, M. S. (2005) *Biochem. J.* **391**, 649–658
16. Thiaville, M. M., Dudenhausen, E. E., Zhong, C., Pan, Y. X., and Kilberg, M. S. (2008) *Biochem. J.* **410**, 473–484
17. Su, N., and Kilberg, M. S. (2008) *J. Biol. Chem.* **283**, 35106–35117
18. Lopez, A. B., Wang, C., Huang, C. C., Yaman, I., Li, Y., Chakravarty, K., Johnson, P. F., Chiang, C. M., Snider, M. D., Wek, R. C., and Hatzoglou, M. (2007) *Biochem. J.* **402**, 163–173
19. Hamamura, K., Liu, Y., and Yokota, H. (2008) *J. Bone Miner. Metab.* **26**, 231–240
20. Averous, J., Bruhat, A., Jousse, C., Carraro, V., Thiel, G., and Fafournoux, P. (2004) *J. Biol. Chem.* **279**, 5288–5297
21. Thiaville, M. M., Pan, Y. X., Gjymishka, A., Zhong, C., Kaufman, R. J., and Kilberg, M. S. (2008) *J. Biol. Chem.* **283**, 10848–10857
22. Ord, D., Meerits, K., and Ord, T. (2007) *Exp. Cell Res.* **313**, 3556–3567
23. Lipson, K. E., and Baserga, R. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9774–9777
24. Cibelli, G., Schoch, S., and Thiel, G. (1999) *Eur. J. Cell Biol.* **78**, 642–649
25. van, Huizen, R., Martindale, J. L., Gorse, M., and Holbrook, N. J. (2003) *J. Biol. Chem.* **278**, 15558–15564
26. Palii, S. S., Thiaville, M. M., Pan, Y. X., Zhong, C., and Kilberg, M. S. (2006) *Biochem. J.* **395**, 517–527
27. Siu, F., Chen, C., Zhong, C., and Kilberg, M. S. (2001) *J. Biol. Chem.* **276**, 48100–48107
28. Gjymishka, A., Palii, S. S., Shan, J., and Kilberg, M. S. (2008) *J. Biol. Chem.* **283**, 27736–27747
29. Deval, C., Chaveroux, C., Maurin, A. C., Cherasse, Y., Parry, L., Carraro, V., Milenkovic, D., Ferrara, M., Bruhat, A., Jousse, C., and Fafournoux, P. (2005) *FEBS J.* **276**, 707–718
30. Averous, J., Maurin, A. C., Bruhat, A., Jousse, C., Arliguie, C., and Fafournoux, P. (2005) *FEBS Lett.* **579**, 2609–2614
31. Matsukawa, T., Inoue, Y., Oishi, Y., Kato, H., and Noguchi, T. (2001) *Endocrinology* **142**, 4643–4651
32. López-Fontanals, M., Rodríguez-Mulero, S., Casado, F. J., Dérijard, B., and Dopazo, J. (2003) *J. Gen. Physiol.* **122**, 5–16
33. Dalby, K. N., Morrice, N., Caudwell, F. B., Avruch, J., and Cohen, P. (1998) *J. Biol. Chem.* **273**, 1496–1505
34. Yang, X., Matsuda, K., Bialek, P., Jacquot, S., Masuoka, H. C., Schinke, T., Li, L., Brancorsini, S., Sassone-Corsi, P., Townes, T. M., Hanauer, A., and Karsenty, G. (2004) *Cell* **117**, 387–398