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MAPK signaling triggers transcriptional induction of cFOS during amino acid limitation of HepG2 cells ☆

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ABSTRACT

Amino acid (AA) deprivation in mammalian cells activates a collection of signaling cascades known as the AA response (AAR), which is characterized by transcriptional induction of stress-related genes, including FBJ murine osteosarcoma viral oncogene homolog (cFOS). The present study established that the signaling mechanism underlying the AA-dependent transcriptional regulation of the cFOS gene in HepG2 human hepatocellular carcinoma cells is independent of the classic GCN2-eIF2-ATF4 pathway. Instead, a RAS-RAF-MEK-ERK cascade mediates AAR signaling to the cFOS gene. Increased cFOS transcription is observed from 4-24 h after AAR-activation, exhibiting little or no overlap with the rapid and transient increase triggered by the well-known serum response. Furthermore, serum is not required for the AA-responsiveness of the cFOS gene and no phosphorylation of promoter-bound serum response factor (SRF) is observed. The ERK-phosphorylated transcription factor E-twenty six-like (p-ELK1) is increased in its association with the cFOS promoter after activation of the AAR. This research identified cFOS as a target of the AAR and further highlights the importance of AA-responsive MAPK signaling in HepG2 cells.

1. Introduction

Amino acid (AA) deprivation in mammalian cells activates several signaling cascades collectively known as the AA response (AAR), which is characterized by translational and transcriptional induction of a wide-range of stress-related genes aimed at restoring cellular homeostasis [1–3]. The protein kinase general control non-derepressible 2 (GCN2) is currently the only well characterized sensor for AA deficiency and the GCN2-eIF2-ATF4 pathway is the predominant AAR signaling mechanism in mammalian cells. GCN2 senses AA limitation by binding uncharged tRNAs, which activates its kinase activity resulting in phosphorylation of eIF2. The p-eIF2 leads to a slower ribosomal assembly and a suppression of general translation while increasing the translation of specific mRNA species containing short upstream opening reading frames [4,5]. Activating transcription factor 4 (ATF4) is a primary target of this translational regulatory mechanism and an effector of many genes involved in the AAR [6,7]. ATF4 induces transcription by binding to C/EBP-ATF response elements (CARE) within hundreds of targeted genes, including asparagine synthetase (ASNS) and C/EBP homology protein (CHOP) [8,9]. However, emerging evidence suggests that there are many AA-responsive genes that are regulated by GCN2-independent mechanisms. This was demonstrated through a global expression array analysis of GCN2 knockout mouse embryonic fibroblasts (MEF) [10] and independently observed on a single gene basis for FOXA2 and FOXA3 [11], cJUN [12], and EGR1 [10,13]. The present report extends this list to include the FBJ murine osteosarcoma viral oncogene homolog (cFOS) gene.

An expression array investigation performed in HepG2 human hepatocellular carcinoma cells showed that AAR activation increased expression of cFOS mRNA [14] and that observation was confirmed in a subsequent study [12]. cFOS is a transcription factor and proto-oncogene involved in cellular proliferation and differentiation that contains a basic leucine zipper (bZIP) region, which facilitates DNA binding and dimerization with other bZIP proteins [15–17]. cFOS associates with the known pro-apoptotic factor CHOP [18], a well-characterized target gene for the AAR [reviewed in 1]. cFOS is a member of a larger class of genes termed “immediate early response genes” based on their rapid and transient induction following extracellular stimulation by growth factors and stress [16,19,20]. Although the functions of cFOS are diverse, context dependent, and not completely understood [15], it is clear that elevated cFOS expression can contribute to

Abbreviations: AA, amino acid; AAR, AA response; ActD, actinomycin D; ASNS, asparagine synthetase; ATF4, activating transcription factor 4; cFOS, FBJ murine osteosarcoma viral oncogene homolog; DOX, doxycycline; eIF2, eukaryotic initiation factor 2; ERK, extracellular-signal regulated kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GCN2, general control non-derepressible 2; HisOH, histidinol; JNK, JUN N-terminal kinase; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblasts; MEK, MAPK/ERK kinase; qPCR, quantitative real time PCR; TET, tetracycline

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cellular transformation and tumor growth [16,17]. However, to illustrate the complexity of its functions, cFOS has also been linked to tumor suppression in some circumstances [21].

Transcriptional regulation of the cFOS gene is induced by a wide variety of stimuli that trigger mitogen-activated protein kinase (MAPK) signal transduction pathways. For example, activation of the mitogen-activated extracellular kinase (MEK)/extracellular-regulated kinase (ERK) pathway is crucial for cFOS induction in response to growth factors, mitogens, and cell stress, most of which trigger increased transcription via a cluster of sequences in the cFOS promoter often referred to as the serum response element (SRE) [16,19,20]. Two of the sequences within the SRE region are the CArG element (CC-A/T_n-GG), known to bind serum response factor (SRF), and the E-twenty six (ETS) motif (GGA-A/T) that is bound by ternary complex factor (TCF) members, such as (ETS)-like factor 1 (ELK1) [22,23]. The induction of the cFOS gene triggered by the ERK pathway involves phosphorylation of constitutively bound SRF and/or ELK1, which is associated with chromatin remodeling and increased transcription [19,20,24,25]. One of the hallmarks of the immediate early response genes is a rapid onset of transcriptional activation that is of short duration. Typical of this group, after exposure of the cells to stimulus, a high degree of cFOS transcription occurs within 15 min and the return to near basal rate occurs within 90 min [16,19].

Although GCN2-eIF2-ATF4 is the best characterized AAR signaling pathway and the predominant mechanism for AA-responsive transcriptional control in mammalian cells, a recent ChIP-sequencing analysis for ATF4 binding sites did not identify functional ATF4-responsive genomic elements associated with the cFOS gene [26]. The present study investigated GCN2-independent AAR target genes in HepG2 human hepatocellular carcinoma cells cultured in medium deficient for the essential AA histidine to activate the AAR. The results document that cFOS was among a number of genes that are induced in a GCN2- and ATF4-independent process following AA limitation. For cFOS in particular, AA-responsive transcription was dependent on the RAS-RAF-MEK-ERK arm of MAPK signaling. Association of the ERK-phosphorylated transcription factor p-ELK1 with the cFOS promoter was increased after activation of the AAR, whereas the abundance of total or phosphorylated SRF was not increased. The latter result is consistent with additional data distinguishing the induction by AA limitation from that of serum replenishment. The results indicate that the ELK1 transcription factor and ETS genomic sequences must be added to the list of AA-responsive genomic signaling mechanisms that contribute to the overall AAR program in mammalian cells. Furthermore, this work extends our understanding of the role that MAPK pathways play during amino acid stress.

2. Materials and methods

2.1. Reagents

Actinomycin D (ActD) (#A1410), thapsigargin (TG) (#T9033), and tetracycline (TET) (#T3258) were from Sigma-Aldrich Co. (St. Louis, MO). All PCR primers used were obtained from Sigma-Aldrich and are listed in Table 1. The siRNA siGENOME SMARTpool constructs for non-targeting siRNA Pool #2 (siCtrl) (#D-001206-14-05), siH-RAS (#M-004142-00-0005), siK-RAS (#M-005069-00-0005), siN-RAS (#M-003919-00-0005), si-ERK1 (#L-003592-00-0005), and siERK2 (#L-003555-00-0005), siELK1 (#L-003885-00-0005) were purchased from Dharmacon/Thermo Scientific. Transient siRNA transfections with 25 nM for each siRAS member or 50 nM each for siERK1 + siERK2 (a total of 100 nM) were performed in 12-well plates according to the manufacturer's protocol using DharmaFECT4 Transfection Reagent (T-2004-02) 48-72 h prior to activating the AAR. The following inhibitors were diluted in dimethylsulfoxide. MEK inhibitor (PD98059, #P215) and c-RAF inhibitor (GW5074, #G6416) were obtained from Sigma-Aldrich. The p38 inhibitor (SB203580, #559389) was from EMD Millipore and the JNK inhibitor (SP600125, #S1076) was from Selleck Chemicals (Houston, TX).

Table 1
qPCR Primers and shRNA Sequences (Human).
Primers were obtained from Sigma-Aldrich and shRNA constructs from Open Biosystems/Thermo Scientific.

qPCR Primers:	
GAPDH mRNA	Sense, TTGGTATCGTGGAAGGACTC
	Anti-sense, ACAGTCTTCTGGGTGGCAGT
cFOS mRNA	Sense, GGAGGAGGGAGCTGACTGATA
	Anti-sense, GGCAATCTCGGTCTGCAA
cFOS hnRNA	Sense, ATGGAGGTGATGGCAGACACTTTTAC
	Anti-sense, TCTTATTCCTTTCCCTTCGGATTCTC
ASNS mRNA	Sense, GCAGCTGAAAGAAGCCCAAGT
	Anti-sense, TGTCTTCCATGCCAATTGCA
ATF4 mRNA	Sense, GGGACAGATTGGATGTTGGAGA
	Anti-sense, ACCCAACAGGGCATCCAAGT
GCN2 mRNA	Sense, GAAATGGTAAACATCGGGCAAACTC
	Anti-sense, TTCACAAGAGCCAGGAGAATCTTCAC
ERK1 mRNA	Sense, CGCTTCCGCCATGAGAATGTC
	Anti-sense, CAGGTCAGTCTCCATCAGGTCCTG
ERK2 mRNA	Sense, CGTGTTGCAGATCCAGACCATGAT
	Anti-sense, TGGACTTGGTGTAGCCCTTGGAA
ATF3 mRNA	Sense, GAGCGGAGCCTGGAGCAAAA
	Anti-sense, GGGGACGATGGCAGAAGCACT
CHOP mRNA	Sense, CATCACCACACCTGAAAGCA
	Anti-sense, TCAGCTGCCATCTCTGCA
EGR1 mRNA	Sense, AGAAGGACAAGAAAGCAGACAAAAGTGT
	Anti-sense, GGGGACGGGTAGGAAGAGAG
cJUN mRNA	Sense, TTCTATGACGATGCCCTCAACGC
	Anti-sense, GCTCTGTTTCAGGATCTTGGGGTTAC
CAT1 mRNA	Sense, TCATCTGGAGGCAGCCCGAG
	Anti-sense, CATCATGAGATAGACGTTCACGAAGATG
IL-8 mRNA	Sense, TCTCTTGGCAGCCTTCCTGATTTC
	Anti-sense, GGGGTGGAAAGGTTTGGAGTATGT
KRAS mRNA	Sense, CTAGAACAGTAGACACAAAACAGG
	Anti-sense, CGAACTAATGTATAGAAGGCATC
HRAS mRNA	Sense, TACGGCATCCCCTACATCGAGAC
	Anti-sense, CACCAACGTGTAGAAGGCATCCTC
NRAS mRNA	Sense, GAGTTACGGGATTCCATTCATTGAAAC
	Anti-sense, TGGCGTATTTCTCTTACCAGTGTGTAAAA
ELK1 mRNA	Sense, CTGACCCCATCCCTGCTTCCTA
	Anti-sense, GAAGTGAATGCTAGGAGGCAGCG
ChIP Primers:	
P1	Forward: TTTCACCTCTGCCTGTGACAGGG,
	Reverse: GGGGATTCGTGGAACTGGGC
P2	Forward: CCATCCCCGAAACCCCTCAT,
	Reverse: GCGTGTCCTAATCTCGTGAGCATTT
P3	Forward: GTGGTTGAGCCCGTGACGTTTA,
	Reverse: TCTTGGCTTCTCAGATGCTCGC
P4	Forward: GTAAGGCAGTTTCATTGATAAAAAGCGAG,
	Reverse: CACTTGCTTGAAAGGGGGTTTGTTATA
P5	Forward: CCAACCTGCTGAAGGAGAAGGAAA,
	Reverse: GATCAAGGGAAGCCACAGACATCTC
P6	Forward: GCATTGTGGTTTCTGGTTTCTCTAATACC,
	Reverse: CCCACTTCCGCCCACTATAAACTG
Anti-sense shRNA:	
shCtrl (non-silencing)	CUUACUCUCGCCCAAGCGAGAG
shATF4 (TRIPZ)	UAAACUUUCUGGGAGAUGG
shGCN2 (GIPZ)	UCAUUGCAAUCUUCAUCCU

2.2. Cell culture

HepG2 and a HepG2 subclone (C3A) human hepatocellular carcinoma cells, HC-04 immortalized human hepatocytes, U87 human glioblastoma cells, and HEK293T human embryonic kidney cells were cultured in high glucose (4.5 g/L) Dulbecco's modified Eagle's medium (DMEM, pH 7.4) supplemented with 10% fetal bovine serum, 1X non-essential AAs, 2 mM glutamine, 100 μg/ml streptomycin sulfate, 100 units/ml penicillin G, and 0.25 μg/ml amphotericin B in a 37 °C incubator with 100% humidity and 5% CO2. For experimental treatment, cells were plated at a density

of 1.0 x 10^6 cells per 60 mm dish or at 0.1 x 10^6 cells per well in 12-well plates. For transient siRNA experiments, cells were plated at 0.25 x 10^6 per well in 12-well plates and incubated for 48-72 h prior to activating the AAR. Complete DMEM medium plus serum was replenished 12 h prior to initiating any experimental treatments to maintain a basal nutritional state. Cells were then cultured in complete DMEM medium or in DMEM medium deficient for L-histidine ("DMEM-His") (obtained from US Biologicals, #D9801-02) to activate the AAR. For a few experiments (as indicated), the AAR was activated by incubation of the cells in complete DMEM containing with 2 mM L-histidinol (HisOH), which blocks the charging of histidine onto its cognate tRNA, thus mimicking histidine deprivation without actually depleting cellular histidine levels [27]. For stable knock down studies, HepG2 cells transduced with lentivirus constructs were plated at a density of 2.5 x 10^5 cells per 60 mm dish to permit shRNA expression and then treated with DMEM or DMEM-His at a similar confluence as the cells in all other experiments. The HEK293T-ATF4 cell line (with tetracycline (TET)-inducible ATF4 expression) was a gift from Dr. Tonis Ord [28], (Institute of Molecular and Cell Biology, Tartu University, Tartu, Estonia). The HEK293T-ATF4 cells were stably transfected at 5 μg/60 mm dish with a constitutively-active MEK1 (MEK^{CA}) construct [29] or as a control, green fluorescent protein (GFP-pcDNA3.1), using a calcium phosphate protocol [30]. The cells to be transfected were incubated with the plasmids overnight, washed twice with PBS, replenished with complete DMEM/serum, and incubated for another 36 h prior to activation of the AAR.

2.3. Lentiviral preparation, transduction, and HepG2 clonal selection

The TRIPZ inducible non-silencing lentiviral shRNA control (RHS4743) and shATF4 (RHS4696-99703331, clone ID: V2THS_132755) plasmids and the GIPZ non-silencing lentiviral shRNA control (RHS4346) and shGCN2 (RHS4430-101133792, clone ID: V3LHS_350194) plasmids were obtained from Thermo Scientific/Open Biosystems and the antisense RNA sequences are listed in Table 1. All lentiviral plasmids were prepared according to the manufacturer's protocol and purified with the HiSpeed Plasmid Midi Kit (Qiagen, 12643). HEK293T-ATF4 cells were transiently transfected using the Trans-Lentiviral shRNA Packaging Kit with calcium phosphate (Thermo Scientific/Open Biosystems, TLP5912) according to the manufacturer's protocol. HepG2 cells were transduced with each viral preparation according to the manufacturer's protocol, except that the volume of virus-containing supernatant was doubled and the volume of serum-free medium was reduced accordingly. Greater than 50% transduction efficiency was achieved as visualized by fluorescent microscopy 48 h after transduction. Puromycin dihydrochloride (Sigma-Aldrich, #P8833) (2.5 μg/ml) was used for selection and limiting dilution was subsequently used to select and expand clonal cell lines. TRIPZ transduced cells were induced with 2.0 μg/ml doxycycline (DOX) (Sigma-Aldrich, D9891). The clonal cell lines demonstrating the most efficient knockdown were characterized and used for further experimental analysis.

2.4. RNA isolation and quantitative real-time PCR (qPCR)

Total RNA was isolated from cells using TRIzol Reagent (Life Technologies, #15596018) according to the manufacturer's protocol. RNA samples were prepared from a minimum of three separate dishes for each condition within an experiment and each experiment was repeated with independent batches of cells. The cDNA was prepared using 1 μg RNA per sample with the qScript cDNA Synthesis Kit (Quanta, #95047-100) according to the manufacturer's protocol. The quantitative real-time PCR (qPCR) was performed with SYBR Green (Applied Biosystems/life Technologies, #4309155) according to the manufacturer's protocol and detected using a CFX Connect Real-Time System (BioRad, #185-5201) with the following protocol: 95 °C for 10 min, 95 °C for 15 sec, 60 °C for 60 sec, repeat steps 2-3 for 39 more cycles. This reaction was followed by denaturing at 95 °C for 15 sec and then heating from 55 °C to 98 °C in 0.5 °C increments for 5 sec each to produce a melting curve to ensure that only a single product was obtained. Where possible, the steady state RNA levels were measured using primers from two adjoining exons that cross a large intron to minimize the contribution of genomic DNA and the transcription activity was measured using primers that cross an intron-exon boundary to measure the short-lived hnRNA [31]. The PCR primers used are listed in Table 1.

2.5. Protein isolation and immunoblotting

After washing cells with PBS, whole cell protein was extracted by lysing cells with RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM Na_2EDTA, 0.5% sodium deoxycholate, 1.0% Triton X-100, and 0.1% sodium dodecyl sulfate) supplemented with Pierce Protease and Phosphatase Inhibitor Mini Tablets (Thermo Scientific, #88668). Immunoblotting was performed as described previously [32]. The mouse monoclonal anti-p-ERK (#sc-7383), rabbit polyclonal anti-total ERK1/2 (#sc-94), and the rabbit polyclonal anti-cFOS antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) The rabbit anti-β-actin polyclonal antibody (#A2066) from Sigma-Aldrich. The rabbit polyclonal anti-ATF4 (#ABE387) was from EMD Millipore. A goat anti-rabbit IgG-HRP conjugate (BioRad, #170-6515) and a goat anti-mouse IgG-HRP conjugate (Santa Cruz, #sc-2005) were used as the secondary antibodies (BioRad, #170-6515). Bound secondary antibody was detected using Pierce ECL Western Blotting Substrate (Thermo Scientific, #32106) according to the manufacturer's protocol followed by chemiluminescent imaging on autoradiography film.

2.6. Chromatin immunoprecipitation (ChIP)

ChIP analysis was performed according to a previously published protocol [32]. HepG2 cells were seeded at a density of 1.5 X 10^7 per 150 mm dish with DMEM medium and cultured for approximately 36 h, which includes a transfer to fresh DMEM/serum during the final 12 h prior to treatment. Immunoprecipitation was performed with one of the following antibodies. Rabbit anti-RNA Polymerase II polyclonal antibody (#sc-899), rabbit anti-serum response factor (SRF, #sc-335), and, as a non-specific negative control, a normal rabbit IgG (#sc-2027) purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The total ELK1 (#9182) and S103 phospho-SRF (#4261) antibodies were purchased from Cell Signaling, whereas the S383 phospho-ELK1 antibody was obtained from Abcam (Boston, MA, #32799). Immunoprecipitated DNA was analyzed with qPCR using primers listed in Table 1. The ChIP results are presented as the ratio to input DNA and are the averages of assays performed in triplicate.

2.7. Statistical analysis

The results obtained were analyzed using Student's t-test (2-tailed) and p values ≤ 0.05 were considered statistically significant. All values are expressed as the means $\pm$ standard deviation of at least triplicate assays to measure technical variation and typically multiple experiments were performed with independent batches of cells to assess biological reproducibility.

3. Results

3.1. GCN2-independent induction of AA-responsive genes

To test the hypothesis that some AAR target genes in HepG2 cells are activated independently of GCN2, a clonal cell line was generated that stably expresses shGCN2. Based on work in Gcn2 knockout mice, it appears that loss of the kinase does not cause major detrimental effects in the absence of stress [33,34]. The growth properties and morphology of the HepG2-shGCN2 cells were similar to those of cells selected for expression of a control shRNA sequence (HepG2-shCtrl). Using

information from an expression array analysis in HepG2 cells [14], several genes were chosen to analyze the contribution of GCN2 after incubation in DMEM lacking histidine (DMEM - His). As shown by the examples in Fig. 1, with regard to GCN2-dependency, three categories of genes were observed: 1) genes for which GCN2 knockdown had a major negative effect (ASNS, CHOP, ATF4), 2) genes that were partially affected (ATF3, CAT1), and 3) genes for which the loss of GCN2 had no effect or were slightly enhanced in their AA responsiveness (IL-8, EGR1, cJUN, cFOS). With regard to the latter group, cJUN was not unexpected in that previous studies had shown that its AAR induction was independent of ATF4 [12]. The results shown in Fig. 1 extend that observation to also exclude GCN2 as a necessary step in cJUN activation. Given the fundamental nature of cFOS in controlling cell growth and its ability to function as an oncogene, it was chosen for further study.

3.2. Mechanisms of cFOS regulation in HepG2 cells during AA deprivation

The AAR-associated cFOS transcription activity, examined by qPCR of heteronuclear RNA (hnRNA), was clearly increased by 4 h and reached a maximum of about 15-fold by 8 h (Fig. 2A). Steady state mRNA also increased by several fold within 4 h of histidine removal from the medium, peaked at about 8 h, and then declined to a plateau of about 12- to 15-fold induction between 12 and 24 h (Fig. 2A). The increase in cFOS protein was evident, but much less pronounced than the corresponding mRNA content. The maintenance of an elevated steady state mRNA at 24 h in the presence of a decline to the DMEM level for transcription activity suggested that the cFOS mRNA may be stabilized during the AAR. cFOS mRNA was measured by qPCR following incubation of cells in DMEM - His medium for 8 h and then transfer to DMEM ± His in the presence of 5 μM actinomycin D to block further mRNA synthesis (Fig. 2B). A modest increase in mRNA half-life occurred in response to AA limitation. It has been documented that HuR binding to mRNA species during the AAR can lead to increased half-life [35,36]. Thus, whereas mRNA stabilization was a contributing factor, the primary mechanism for increased cFOS expression during the AAR appeared to be enhanced transcription.

To test the cell specificity of cFOS induction, a number of other cell types were screened. cFOS transcriptional induction was also observed to occur in the HepG2 subclone C3A cells (Fig. 2C), but the induction was weaker than for the parental HepG2 cell line (Fig. 2A). The American Type Culture Collection (ATCC) website describes C3A cells as being selected to exhibit more hepatocyte-like properties than parental HepG2 cells. It is possible that the magnitude of the cFOS induction is related to transformation because non-transformed, immortalized HC-04 human hepatocytes showed little or no induction (Fig. 2C). In a transformed cell line from a different tissue, U87 human glioblastoma cells, the AAR-induced increase in cFOS expression was similar in magnitude to that observed in HepG2 cells, regardless of whether the cells were incubated in histidine-free medium or in DMEM containing 2 mM of the histidine deprivation mimetic HisOH (Fig. 2D). There was a temporal difference between the HepG2 and U87 cells in that the induction in the U87 cells occurred within 1 h and was quite transient (Fig. 2D). The rapid kinetics in the U87 cells is reminiscent of the extensively-characterized "serum response" for cFOS [16,19]. Induction of cFOS by growth factors, cytokines, and other extracellular signals can be illustrated by the serum response, a rapid, but transient induction triggered by refeeding of serum-starved cells or exposure to one of the blood borne messengers. This response is mediated by MEK-ERK signaling and the increased transcription following serum treatment occurs within minutes and lasts for only an hour or so. The serum response should occur in control DMEM medium. Indeed, after the medium change to DMEM ± His a small increase in cFOS mRNA at 1 h was observed in the DMEM incubated U87 cells (Fig. 2D). However, the AAR induction was clearly superimposed on the serum effect and much greater in magnitude. To further investigate the possible relationship between the serum response and the AAR, the regulation of cFOS in

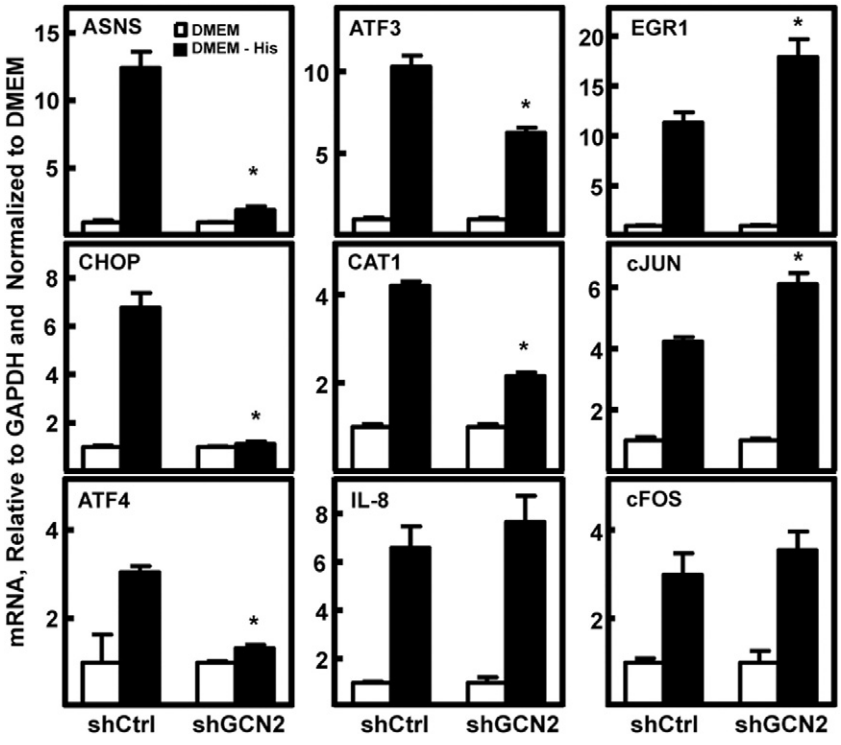

Fig. 1. AA responsive genes show a wide variation in their dependence on GCN2 in HepG2 human hepatocellular carcinoma cells. To survey AAR target genes for dependence on GCN2, HepG2 clonal cell lines were generated that stably express either a control shRNA (shCtrl) or an shRNA specific for GCN2 (shGCN2). The cells were incubated in complete DMEM or DMEM deficient for histidine (DMEM-His) for 8 h and then steady state mRNA levels for the indicated genes were measured by qPCR. GAPDH mRNA, which is not affected by the AAR, was used as an internal control and results shown are the means ± SD of assays in triplicate. An asterisk indicates that the DMEM - His value is significantly different ($p \leq 0.05$) than the DMEM control.

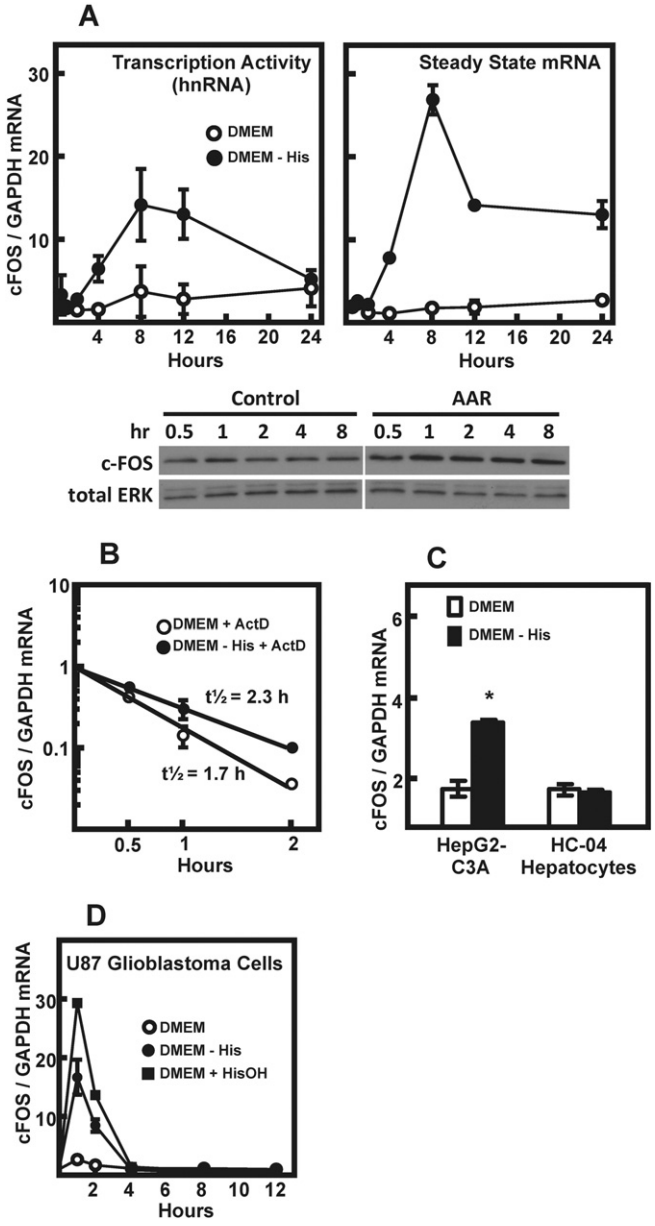

Fig. 2. Mechanism of cFOS regulation in HepG2 cells during the AAR. (Panel A) HepG2 human hepatocellular carcinoma cells were incubated in complete DMEM or DMEM deficient for histidine (DMEM-His) for 0-24 hours as indicated and cFOS transcription activity as measured by hnRNA or cFOS steady state mRNA were assayed by qPCR. cFOS protein levels were assessed by immunoblotting. (Panel B) Cells were incubated in medium deficient for histidine (DMEM-His) for 8 h and then transferred to complete DMEM or DMEM - His for an additional 2 h, both containing 5 μM actinomycin D (ActD). cFOS mRNA was measured by qPCR and data were plotted as the logarithm of mRNA content versus time following transfer to ActD containing medium. (Panel C) The HepG2 subclone, HepG2-C3A, and non-transformed, immortalized human hepatocytes (HC-04) were incubated in complete DMEM or DMEM - His for 8 h and cFOS steady state mRNA was measured by qPCR. An asterisk indicates that the DMEM - His value is significantly different ($p \leq 0.05$) than the DMEM control. (Panel D) U87 human glioblastoma cells were incubated in complete DMEM, DMEM - His, or complete DMEM ± 2 mM HisOH for 0-12 h as indicated and then cFOS steady state mRNA was measured by qPCR. In all panels, GAPDH mRNA, which is not affected by the AAR, was used as an internal control and results shown are the means ± SD of at least triplicate assays.

HepG2 cells was measured during the first 4 h after transfer to fresh DMEM ± His containing 10% FBS (Fig. 3A). A transient serum response of about 2-fold peaked at 1 h and returned to the basal state by 2 h in the cells incubated in control DMEM medium. Removing histidine from the medium did not further enhance the induction at 1 h. In contrast, the AAR-dependent induction appeared to begin between 2 and 4 h

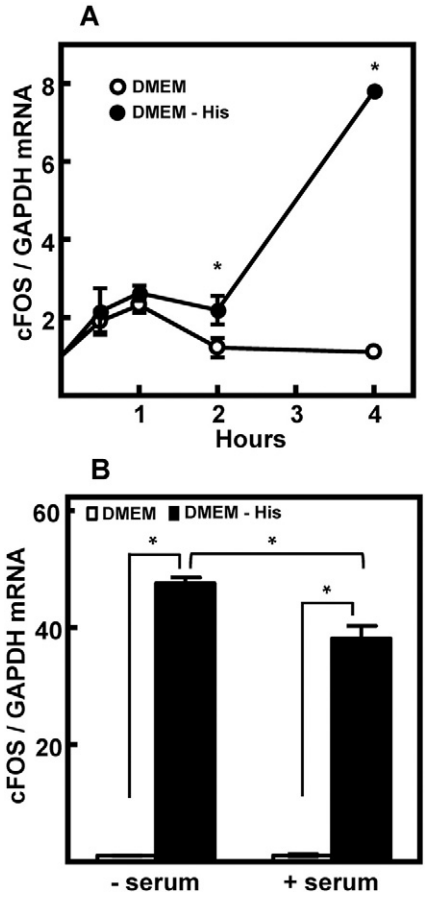

Fig. 3. The effect of serum on AAR regulation of cFOS. (Panel A) HepG2 cells were incubated in DMEM ± His for 0-4 h as indicated and cFOS steady state mRNA was measured by qPCR. (Panel B) Following overnight serum starvation, HepG2 cells were transferred to fresh medium either lacking 10% FBS (- serum) or containing 10% FBS (+ serum), as indicated. The cells in both serum treatment groups were incubated in DMEM ± 2 mM HisOH for 6 h and then cFOS steady state mRNA was measured by qPCR. For both panels, GAPDH mRNA, which is not affected by the AAR, was used as an internal control and results shown are the means ± SD of assays in triplicate. An asterisk indicates that the values are significantly different ($p \leq 0.05$).

(Fig. 3A), consistent with the response shown in Fig. 2A. To further establish that the AAR-dependent induction of cFOS in HepG2 cells is independent of serum, HepG2 cells were incubated overnight (16 h) in serum-free medium and then treated for 6 h with HisOH in the presence or absence of serum. The results illustrate that the AA-responsiveness of the cFOS gene was not dependent on serum; in fact, the induction was slightly suppressed by the presence of serum (Fig. 3B).

3.3. cFOS AAR regulation is GCN2-ATF4 independent

GCN2 is the only known sensor for AA deficiency in mammalian cells, and given that the GCN2-eIF2-ATF4 pathway is considered the primary AAR pathway [2,3], its relationship to cFOS induction was examined. Consistent with the data of Fig. 1, in HepG2-shGCN2 cells, the GCN2 mRNA levels were effectively knocked down by approximately 80% compared to the HepG2-shCtrl or parental HepG2 cells (Fig. 4A). As assayed by immunoblotting, AAR associated ATF4 protein induction was largely abolished in the HepG2-shGCN2 cells (data not shown). Despite the efficient knock down of GCN2, cFOS induction was unaffected following activation of the AAR (Fig. 4A). As a further test for the lack of a role for the GCN2-eIF2-ATF4 pathway, a HepG2-shATF4 clonal cell line, expressing a doxycycline (DOX) inducible shRNA against ATF4 was tested (Fig. 4B). After DOX treatment, there was a significant reduction in both ATF4 and ASNS mRNA levels. In contrast, no effect on cFOS

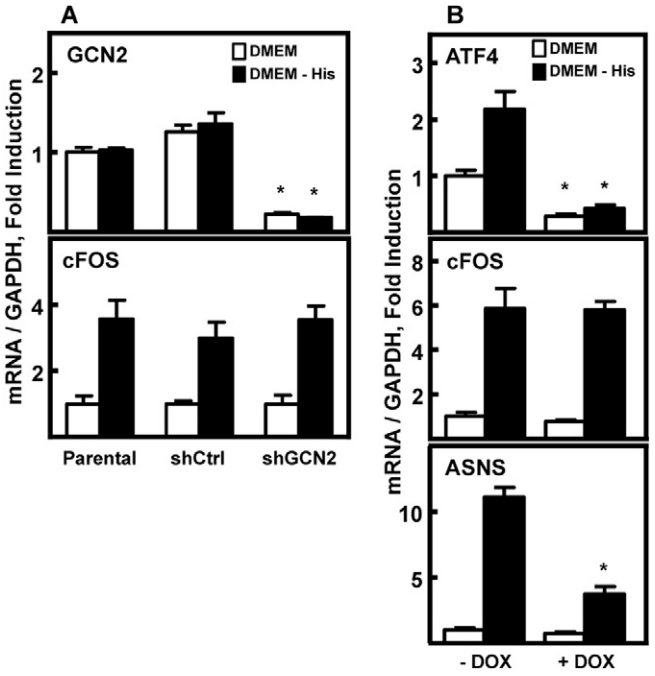

Fig. 4. AAR regulation of cFOS is GCN2- and ATF4-independent. (Panel A) Parental HepG2 cells, and two HepG2-derived clonal cell lines, one stably expressing a non-silencing shRNA (shCtrl) and another expressing shRNA against GCN2 (shGCN2) were incubated in DMEM ± His for 8 h. RNA was isolated and GCN2, cFOS, and GAPDH steady state mRNA was measured by qPCR. (Panel B) HepG2-derived clonal cells stably expressing an inducible shRNA against ATF4 under the control of doxycycline were cultured in the absence (-DOX) or presence (+DOX) of the drug for 22 days and then incubated in complete DMEM ± His for 8 h. ATF4, cFOS, ASNS, and GAPDH steady state mRNA was measured by qPCR. For both panels the results shown are the means ± SD of assays performed in triplicate. An asterisk indicates that the shGCN2 value is significantly different ($p \leq 0.05$) than the shCtr (Panel A) or the + DOX value is different than the - DOX value (Panel B).

induction by the AAR was observed (Fig. 4B). Together these data demonstrate that cFOS induction during the AAR is independent of the classic GCN2-eIF2-ATF4 pathway.

3.4. AAR induction of cFOS is MEK-ERK dependent

cFOS is known to be induced by a wide variety of cellular stresses that induce one or more of the MAPK pathways [16,19]. The three major MAPK signaling pathways (MEK-ERK, p38, and JNK) were screened using chemical inhibitors to identify signaling transduction leading to cFOS induction following AAR activation. PD98059, which inhibits MEK activity, completely blocked cFOS induction during the AAR, while the JNK inhibitor blocked the induction slightly and inhibition of p38 activity actually increased cFOS mRNA content following AA limitation (Fig. 5A). A concentration curve for PD98059 inhibition showed that even at 2.5 μM the MEK inhibitor blocked the AAR-associated increase in cFOS mRNA induction by about 85% (Fig. 5B) and the increase in p-ERK (Fig. 5C). As an independent method to determine the contribution of MEK-ERK signaling to the regulation of the cFOS gene, ERK1/2 was subjected to knockdown in HepG2 cells using siRNA oligonucleotides (Fig. 5D). Knock down of ERK1/2 caused a significant reduction in the basal (DMEM) and AAR-induced level of cFOS expression. As a negative control, there was little or no decrease in the induction of the ATF4-dependent ASNS gene. To further investigate the role of MEK signaling, MEK deficient HEK293T-ATF4 cells [12], which showed no significant induction of cFOS following AAR activation (Fig. 5E), were stably transfected with constitutively active MEK (MEK^{CA}). MEK^{CA} ectopic expression caused a 10-fold increase in basal cFOS expression that was further enhanced following AAR activation (Fig. 5E). The HEK293T-ATF4 cell line used for these studies had been previously transfected to stably express ATF4 under the control of tetracycline (TET) [28]. Treatment of these cells with TET to induce ATF4 leads to an induction of ATF4-responsive genes such as ASNS and CHOP [37]. In the present studies, induction of ATF4 caused no change in cFOS induction, with or without MEK^{CA} expression (Fig. 5E). Collectively, these results provide evidence for the conclusion that cFOS induction during the AAR is independent of GCN2-ATF4 signaling, but instead is linked to the MEK-ERK cascade.

3.5. AA deficiency induces cFOS through the RAS-RAF-MEK-ERK arm of MAPK signaling

After establishing that MEK-ERK signaling is required for cFOS induction during the AAR, additional upstream signaling molecules were investigated. RAF proteins are common regulators of MEK and c-RAF has been previously shown to induce cFOS expression in response to numerous stimuli [38,39]. cFOS induction during the AAR was examined in the presence of the c-RAF inhibitor GW5074, which abolished cFOS induction in a concentration dependent manner (Fig. 6A). Signaling to c-RAF is often through the RAS family of small GTPases [16,40]. cFOS induction during AAR activation was monitored after suppressing the expression of individual RAS family members with siRNA (Fig. 6B). Analysis of the mRNA species for all three RAS forms illustrates the specificity of the knock down (Fig. 6C). Knock down of H-RAS or N-RAS effectively suppressed cFOS induction to levels that were 29% and 34% respectively, compared to the non-targeting siControl. In contrast, knock down of K-RAS showed no significant effect (Fig. 6B). Collectively, these results demonstrate that cFOS induction during activation of the AAR is dependent on the RAS-RAF-MEK-ERK arm of MAPK signaling.

3.6. The AAR increases cFOS promoter-association of selected transcription factors and recruits Pol II.

To investigate the role of specific transcription factors through which AA-responsive ERK regulates the cFOS gene, two principle proteins known to mediate ERK actions, E-twenty six- like factor 1 (ELK1) and serum response factor (SRF), were investigated by chromatin immunoprecipitation (ChIP). Consistent with the hnRNA analysis indicating a transcriptional control mechanism, induction of the AAR increased the recruitment of RNA Pol II to the cFOS promoter (Fig. 7). Both ELK1 [19,23] and SRF [24] proteins are constitutively bound to the cFOS gene within the first 500 bp upstream of the transcription start site and in response to specific stimuli each can be activated by *in situ* phosphorylation. ChIP analysis of HepG2 cells confirmed the constitutive binding of total ELK1 and SRF in the region of the cFOS proximal promoter, and PCR primers targeted to upstream and downstream regions across the gene locus illustrated the specificity of that binding (Fig. 7). AA limitation did not alter the amount of total ELK1 association and caused a slight reduction in total SRF bound at the cFOS promoter. With regard to phosphorylation, the AAR led to a small reduction of p-SRF likely due to the loss of total SRF. In contrast, there was a substantial increase of more than 4-fold in the amount of p-ELK1 associated with the promoter region (Fig. 7). These results are consistent with the known signaling of ERK to the cFOS gene through p-ELK1 [41] and indicate that p-ELK1 contributes to the AAR transcriptional program.

3.7. cFOS induction is dependent on the ELK1 transcription factor.

To provide additional evidence that ELK1 is an important factor in the AA regulation of the EGR1 gene, HepG2 cells were transfected with a control siRNA or siRNA specific for ELK1.After activating the AAR with HisOH treatment for 8 h, the expression of both ELK1 and cFOS was measured (Fig. 8). Consistent with the known mechanism for ELK1 action, phosphorylation of bound ELK1 at the target gene, the level of ELK1 mRNA was unchanged by the AAR. There was a strong knock down of ELK1 expression by the specific siRNA,

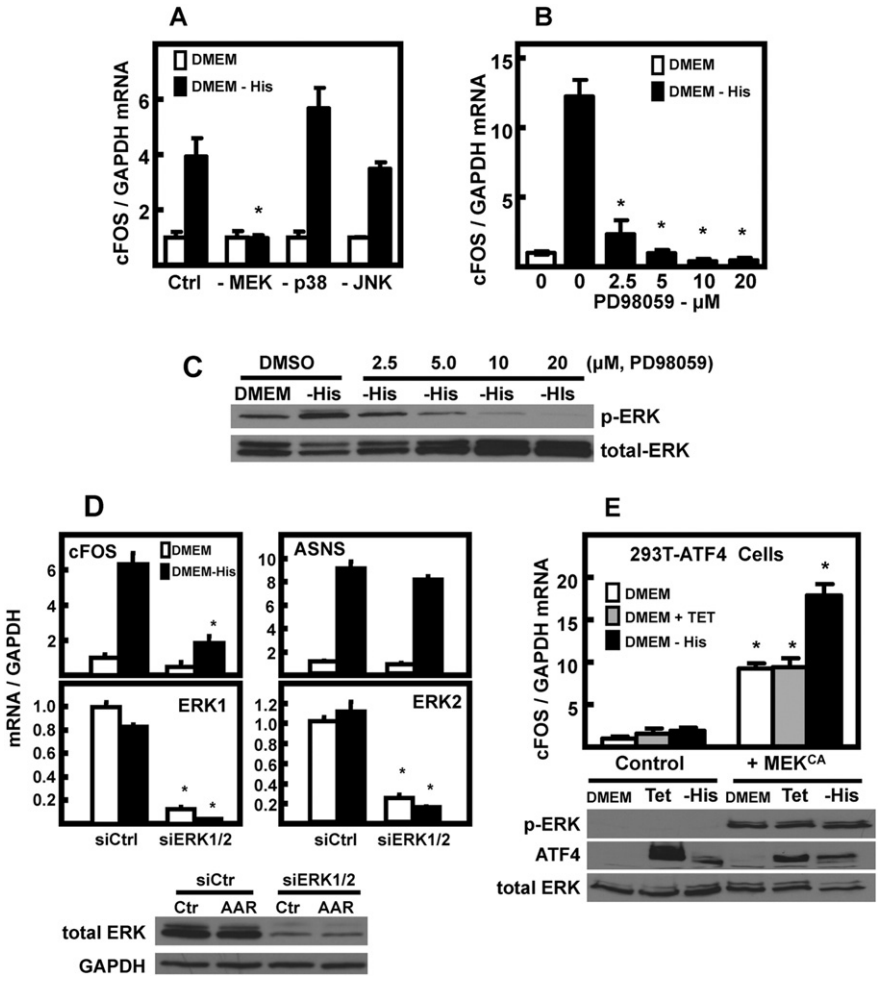

Fig. 5. cFOS AAR regulation is MEK-ERK dependent. (Panel A) HepG2 cells were pre-incubated for 1 h in the presence of DMSO (Ctrl), 20 μM PD98059 (- MEK), 10 μM SB203580 (-p38), or 10 μM SP600125 (- JNK). Cells were then incubated in DMEM ± His for 8 h, with the inhibitor. cFOS and GAPDH mRNA was measured by qPCR. (Panels B and C) HepG2 cells were pre-incubated in the presence of DMSO or PD98059 and then incubated in DMEM ± His for 8 h with PD98059. cFOS and GAPDH steady state mRNA was measured by qPCR (Panel B) or p-ERK and total ERK protein levels were assayed by immunoblotting (Panel C). (Panel D) HepG2 cells were transiently transfected with 100 nM of non-targeting siRNA (Ctrl) or siERK1/2 (50 nM siERK1 + 50 nM siERK2) and cultured for 72 h. Cells were then incubated in DMEM ± His for 8 h. ERK, cFOS, ASNS, and GAPDH steady state mRNA was measured by qPCR. The knockdown of ERK1/2 was confirmed by immunoblotting. (Panel E, upper) HEK293T-ATF4 (Control) or HEK 293 T-ATF4 cells expressing a constitutively active MEK (MEK^{CA}) were incubated in DMEM, DMEM + tetracycline (+TET), or DMEM - His for 8 h. The cFOS and GAPDH steady state mRNA was measured by qPCR. (Panel E, lower) The expression of p-ERK and ATF4 protein was assessed by immunoblotting using total ERK as the loading control. For all mRNA panels, the data shown are the means ± SD of assays in triplicate. An asterisk indicates that the experimental condition is significantly different ($p \leq 0.05$) from the corresponding control.

which corresponded with a 65% reduction of cFOS mRNA in the presence of HisOH. These data are consistent with those of Fig. 7 documenting increased phosphorylation of ELK1 at the cFOS gene and indicate that ELK1 is an important factor in AA-regulated cFOS expression.

4. Discussion

This study documents the regulation of cFOS induction by AA deprivation in HepG2 human hepatocellular carcinoma cells and provides several novel observations pertaining to the broader topic of AA stress. 1) The data extend a previous observation from an expression array that cFOS is induced by the AAR [14]. 2) The cFOS induction by AA limitation is primarily due to increased transcription and peaks at 8 h, long after the well known serum responsiveness of this gene has returned to the basal state. Furthermore, the AAR associated control does not require the presence of serum. 3) Activation of the cFOS gene is independent of the classic GCN2-eIF2-ATF4 pathway of the AAR. 4) The cFOS induction requires the RAS-RAF-MEK-ERK arm of MAPK signaling and MEK-ERK activation is both necessary and sufficient for AAR induction of cFOS mRNA. 5) The AAR-dependent induction of cFOS transcription was associated with phosphorylation of promoter-bound ELK1 and enhanced recruitment of Pol II to the promoter. 6) In contrast to the serum response for the cFOS gene, phosphorylation of promoter-bound SRF was not observed.

The GCN2-eIF2-ATF4 pathway is the classic signaling mechanism for AA limitation that was first characterized in yeast, and later documented in mammalian cells [42,43]. GCN2 remains the only well characterized sensor of intracellular AA availability, and most of the mammalian AAR induced genes that have been characterized to date contain a C/EBP-ATF response element (CARE) that mediates ATF4 activation [7]. However, an expression array analysis in Gcn2 knockout mouse embryonic fibroblasts documented a substantial number of AA-responsive genes that were Gcn2-independent [10]. Subsequently, a growing list of GCN2- and/or ATF4-independent AAR target genes have been confirmed, including FOXA2/3 [11], cJUN [12], and EGR1 [10,13]. Most of these examples are linked to activation of one or more of the MAPK pathways, as has the GCN2-independent pathway leading to phosphorylation of ATF2 by JNK2 [44]. Collectively, the data emerging from numerous laboratories indicates that in mammalian cells the GCN2-eIF2-ATF4 pathway is likely a principle mechanism for detecting and responding to AA limitation, but the AAR is actually a collection of pathways that

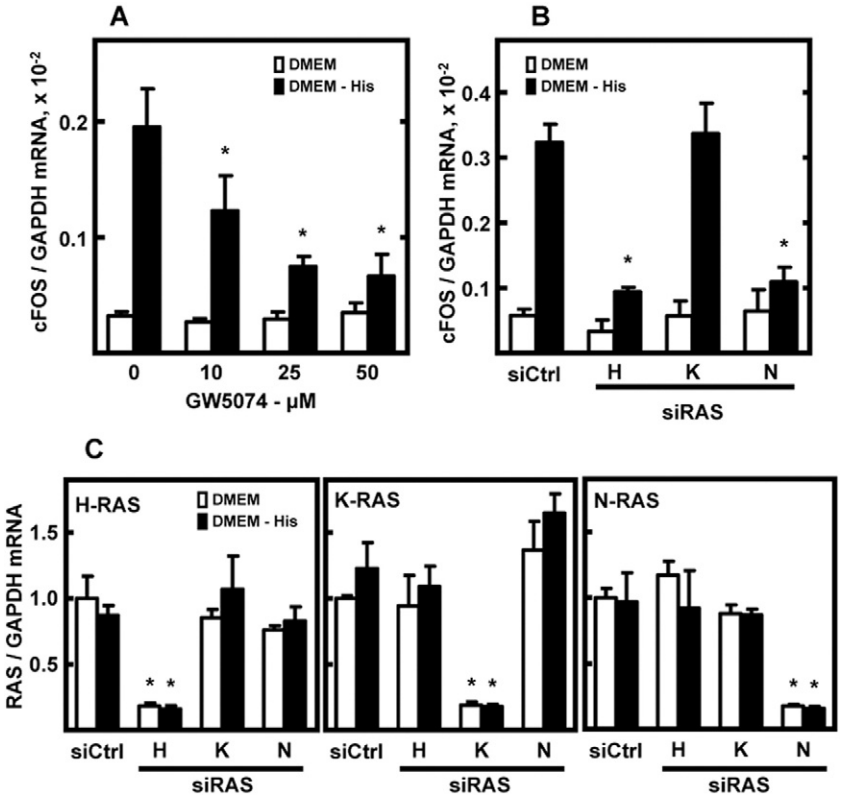

Fig. 6. AAR regulation of cFOS is dependent on c-RAF, H-RAS, and N-RAS. (Panel A) HepG2 cells were pre-incubated in the presence of the c-RAF inhibitor GW5074 at the indicated concentrations and then transferred to complete DMEM or DMEM-His for 8 h, in the continued presence of GW5074. cFOS and GAPDH steady state mRNA was measured by qPCR. (Panel B) HepG2 cells were transiently transfected with 25 nM of non-targeting siRNA (Ctrl), siRNA against H-RAS (H), siRNA against K-RAS (K), or siRNA against N-RAS (N) and cultured for 48 h. Cells were then incubated in DMEM ± His for 8 h and then steady state mRNA was measured by qPCR for: cFOS (Panels A and B); H, K, and N RAS (Panel C); or GAPDH. For all panels, the results shows are the means ± SD of triplicate assays. An asterisk indicates that the experimental condition is significantly different ($p \leq 0.05$) from the corresponding control.

are activated and modulate the cellular response in a cell specific manner.

The induction of cFOS by the AAR is quite different from the induction of cFOS by growth factors, cytokines, and other extracellular signals that is can be illustrated by the serum response. First, it does not require serum or the associated growth factors. Second, transcription is not increased until about 2-4 h after initiating the AAR, well past the time that the serum-induced activation has returned to the basal state. Finally, induction of the transcriptional activity of the gene itself lasts for at least 12 h and the steady state mRNA appears to be stabilized for even longer period of time. All of these observations suggest that the mechanism by which the gene is controlled by intracellular AA supply differs substantially from that by the growth factors associated with the serum response. Interestingly, in transformed cells, the normally transient nature of immediate early response gene expression is sometimes replaced by a more sustained expression [45]. cFOS is elevated in 30% of all human cancers, including HCC, but it is rarely directly mutated in cancer [46]. These observations point to abnormal regulation of upstream signaling events as the likely control of cFOS in transformed cells, a hypothesis consistent with the known elevation of the MEK-ERK pathway in many tumors [16,38]. Given the nutrient deprivation and activation of the AAR in tumors [47–49], further activation by AA deprivation may be superimposed on the high basal level of cFOS. In this circumstance, nutrient supply may significantly impact regulation of cFOS expression.

A number of transcription factors are the terminal effectors for nuclear ERK and among these are SRF and ELK1, which are constitutively bound to the cFOS promoter and activated by ERK-mediated phosphorylation *in situ* in a cell and stimulus-specific manner [19,20,23]. The present results show that neither total nor phosphorylated SRF association with the gene is largely changed by the AAR. In contrast, the total abundance of constitutively bound ELK1 was unchanged by the AAR, but the association of p-ELK1 with the cFOS promoter was increased substantially. These results are consistent with known regulation by ERK-ELK1 signaling and add further evidence that the mechanism of cFOS activation by the AAR differs from that the serum response involving p-SRF [24]. The importance of ELK1 in the AAR control of cFOS was confirmed by documenting a significant reduction of cFOS induction in cells transfected with siRNA specific for ELK1.

RAS is typically activated by GDP-GTP exchange following association with adaptor proteins that are linked to plasma membrane tyrosine kinase receptors [38,50]. RAF is then recruited to the RAS-containing complex, increasing its inherent kinase activity. RAF phosphorylates MEK and thus, triggers the downstream cascade of MEK-ERK-ELK1 [19,20]. The data presented in this report indicates that RAS and RAF are required for AAR-driven activation of the FOS gene in HepG2 cells. These data, along with the observation that GCN2 knockdown has no deleterious effect on FOS induction, strongly suggest that an unknown amino acid sensor exists and may trigger a step upstream of RAS. All three members of the RAS protein family are homologous and some redundancy is known to occur [51]. However, the results following siRNA knockdown of each RAS form revealed that both H-RAS and N-RAS contribute to AAR signaling in HepG2 cells, whereas K-RAS does not. The reason for this selectivity is not immediately obvious. Despite extensive research on the RAS proteins, the functional differences are still not well understood. Examples of such differences that set K-RAS apart from the other two include the observation that H-RAS and N-RAS undergo palmitoylation that may regulate the microdomain localization within the plasma membrane, whereas K-RAS does not [38]. K-RAS, but not H-RAS and N-RAS, binds calmodulin [51], but there is no known link between cellular calcium levels and the AAR. K-RAS deficient mice die during embryogenesis, where as single or

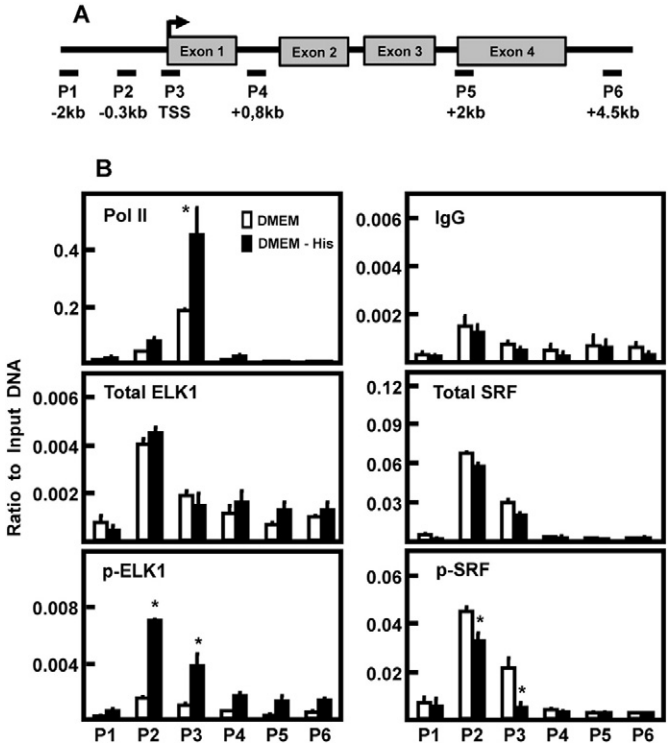

Fig. 7. Transcription factor association with the cFOS promoter in response to the AAR. (Panel A) The locations of primers (labeled P1-P6) used to analyze the transcription factor binding to the human cFOS gene are illustrated relative to the transcription start site (arrow) and the coding region of the gene. The scheme is not drawn to scale. The primer sequences are listed in Table I. (Panel B) HepG2 cells were incubated in DMEM ± His 8 h and then the cells were subjected to ChIP analysis with antibodies specific for RNA Pol II, total SRF, p-SRF, total ELK1, p-ELK1, and a non-specific IgG as a negative control. The data are plotted as the ratio to the input DNA and are the averages ± standard deviations for at least three samples. An asterisk indicates that the DMEM - His value is significantly different ($p \leq 0.05$) than the DMEM control.

double knockouts of H-RAS and N-RAS are viable [51]. Among the three members, mutations in K-RAS are by far the most common in human cancers, but RAS mutations are not highly associated with hepatocellular carcinomas [51]. Further research will be necessary to discover the details regarding RAS specificity within the AAR.

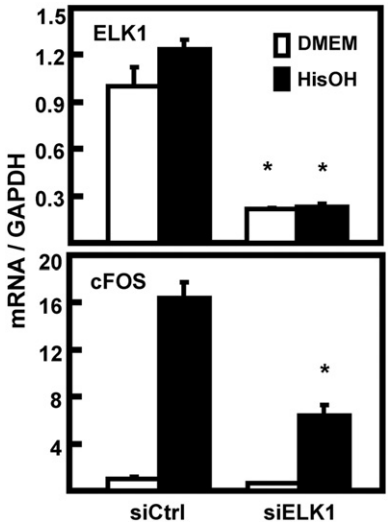

Fig. 8. AAR dependent induction of cFOS is dependent on ELK1. HepG2 cells were transiently transfected with 100 nM of non-targeting siRNA (Ctrl) or siRNA against ELK1 and cultured for 48 h. Cells were then incubated in DMEM ± His for 8 h and then steady state mRNA was measured by qPCR for ELK1, cFOS, or GAPDH. The results shown are the means ± SD of triplicate assays. An asterisk indicates that the experimental condition is significantly different ($p \leq 0.05$) from the corresponding control.

In summary, the AA-dependent transcriptional induction of the cFOS gene in HepG2 cells occurred via a signaling mechanism that was independent of the GCN2-eIF2-ATF4 pathway. Evidence for a RAS-RAF-MEK-ERK-ELK1 cascade was presented illustrating that the collection of pathways that make up the AAR includes MAPK signaling to the cFOS gene. As a consequence, the ERK-phosphorylated ETS family member pELK1 must be added to the list of factors that mediate the regulatory transcription network of the AAR.

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