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Dickkopf homolog 1, a Wnt Signaling antagonist, is transcriptionally up-regulated via an ATF4-independent and MAPK/ERK-dependent pathway following amino acid deprivation

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Abstract

Amino acid response (AAR) pathway is activated when cells are deprived of amino acids. In the present study, using the human colon cancer cell line SW480, we observed that DKK1, an antagonist of the Wnt pathway, was significantly induced at the mRNA level after the removal of amino acids from the medium. Addition of the amino alcohol histidinol, which prevents the formation of histidyl-tRNA^{His}, also increased DKK1 mRNA to a level similar to that observed when cells were deprived of all amino acids. Transcriptional activity and stability of DKK1 mRNA were both increased in the amino acid-deprived condition. The induction of DKK1 gene expression was confirmed by the increased immunofluorescent staining of the DKK1 protein in the amino acid deprived condition. Although Chromatin Immunoprecipitation assays showed increased RNA Polymerase II binding at the *DKK1* promoter in amino acid-limited conditions, ATF4 binding to the promoter is absent. Luciferase reporter assays did not detect any functional AARE within the *DKK1* gene structure. Knockdown of ATF4 by siRNA did not affect the increase of DKK1 mRNA during amino acid limitation. Inhibition of ERK phosphorylation abolished the induction of DKK1. Our study revealed that *DKK1* is a novel target gene in the response to amino acid deficiency and that the expression of *DKK1* is up-regulated through an ATF4-independent and an ERK-dependent pathway.

Keywords

amino acid deficiency; ATF4, DKK1; phosphorylated ERK; transcription; Wnt

1. Introduction

The availability of amino acids is critical to the maintenance of body and organ protein homeostasis [1]. At the cellular level, AAR pathways are activated in response to amino acid deficiency. The GCN2/ATF4 pathway is one of the well-documented AAR pathways involved in the regulation of gene expression in response to amino acid availability [2]. In this pathway, amino acid limitation induces the phosphorylation of eukaryotic initiation factor α (eIF α), which leads to a general decrease in translation of mRNA but an increase of

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specific amino acid responsive genes such as activating transcription factor 4 (ATF4) [3]. Elevated ATF4 activates many downstream genes including asparagine synthetase (ASNS) [4], ATF3 [5] and system A amino acid transporter 2 (SNAT2) [6]. Emerging evidence shows that the activation of the GCN2/ATF4 pathway plays critical role in tumor cell survival [7, 8].

Mitogen-activated protein kinase (MAPK) pathways are signaling cascades by which cells respond to extracellular stimuli. One of the three major MAPK pathways, the ERK pathway, is initiated by the activation of Raf and subsequent MEK and ERK kinases [9]. A recent study demonstrated that MEK/ERK signaling is required for phosphorylation of eIF α in human hepatoma cells by amino acid limitation [10].

The Wnt signaling pathway plays an important role in development and carcinogenesis [11]. Wnt is a combination of two homologous gene families, the *wingless* gene functioning during *Drosophila* embryogenesis [12], and the *int* genes identified as the vertebrate counterpart [13]. The canonical Wnt pathway in mammalian cells is activated when Wnt proteins bind to cell-surface receptors of the Frizzled family, thus activating the Dishevelled proteins, which then block the β -catenin degradation complex (GSK3 β /APC/AXIN). This results in the translocation of β -catenin from the cytosol to the nucleus and the initiation of the downstream transcription [14]. Aberrantly activated Wnt signaling was observed in over 90% of colorectal cancers [15]. DKK1, a Wnt antagonist, blocks the Wnt/ β -catenin pathway through competitive binding to the Wnt co-receptor LPR5/6 and the transmembrane protein Kremen1/2 [16–19]. Consistent with the state of Wnt signaling, DKK1 expression was down-regulated in human colon tumors [20].

Our laboratory previously reported that the expression of one of the Wnt signals, WNT5a, was reduced during amino acid deprivation in human colon cancer cell line SW480 [21]. This was accompanied with the reduction of the intracellular Wnt signal β -catenin. In the present study, our results showed the up-regulation of one of the antagonists, DKK1, of the Wnt signaling pathway by amino acid deprivation. Therefore the Wnt signaling pathway may be responsive to environmental signals through the amino acid response pathway. The increased expression of DKK1 is mediated in an ATF4-independent and MAPK/ERK-dependent manner.

2. Materials and Methods

2.1. Cell culture and amino acid deprivation treatments

Human colon adenocarcinoma cell line SW480 was obtained from American Type Culture Collection (Manassas, VA) and was cultured in standard Minimum Essential Medium (MEM) supplemented with 10% (v/v) fetal bovine serum (Mediatech; Herndon, VA) and 100 IU/mL Penicillin, and 100 μ g/mL Streptomycin (Mediatech; Herndon, VA). Cells were maintained at 37 °C in 95% humidity and 5% CO₂. For amino acid deprivation treatment, cells were first seeded at a density of 1.5×10^6 cells per 60-mm dish, grown for 24 h, and then given fresh media 14 h before treatments. Amino acid deprivation treatment was performed by incubating the cells in MEM or in amino acid-free MEM (MEM-AA, Cell Media Facility at the University of Illinois at Urbana-Champaign, Urbana, IL) supplemented by 10% (v/v) dialyzed fetal bovine serum (Sigma-Aldrich; St. Louis, MO). For single amino acid deprivation treatment, the above-mentioned regular MEM was made without adding the amino acid histidine (MEM-His). When inhibitors were used, an aliquot of either the stock solution or the solvent as the control was added directly to the cells in fresh media. Histidinol (HisOH) and Actinomycin D (ActD) were purchased from Sigma-Aldrich while U0126 was purchased from Cell Signaling Technology (Danvers, MA).

2.2. Quantitative real-time RT PCR

Total RNA was isolated using TRI Reagents (Sigma-Aldrich). cDNA was generated using high-capacity cDNA kit (Applied Biosystems; Foster City, CA). Real-time PCR was performed in a 20 μ L reaction volume containing 12.5 μ L POWER SYBR green master mix (2 \times , Applied Biosystems), 5 μ mol/L of each primer, and 25 ng cDNA template by cycling at 95 $^{\circ}$ C for 10 min followed by 40 cycles of 95 $^{\circ}$ C for 15 sec and 60 $^{\circ}$ C for 1 min on a 7300 real-time PCR system (Applied Biosystems). After amplification, melting curves were acquired stepwise from 55 to 95 $^{\circ}$ C to ensure that a single product was amplified in the reaction. The primers, synthesized by Integrated DNA Technologies, are as follows: *DKK1*, sense 5'- GATCATAGCACCTTGGATGGG -3', antisense 5'- GGCACAGTCTGATGACCGG -3'; *ASNS*, sense, 5'- GCAGCTGAAAGAAGCCCAAGT-3', antisense 5'-TGTCTTCCATGCCAATTGCA-3'; *ATF3*, sense 5'- GCTGTCACCACGTGCAGTATCTCA -3', antisense 5'- CTGTTCTCTCTTGTGACAAGC -3'; *ATF4*, sense 5'- TGAAGGAGTTCGACTTGGATGCC -3', antisense 5'- CAGAAGGTCATCTGGCATGGTTTC -3'; *p21*, sense 5'- CCGGACTGTGATGCGCTAATG -3', antisense 5'- CTCGGTGACAAAGTCGAAGTTC -3'; *L7a*, sense 5'- TTTGGCATTGGACAGGACATCC -3', antisense 5'- AGCGGGCCATTTCAAAAG -3'. *L7a* was used as an internal control to normalize raw data.

2.3. Western blotting

Cells were lysed with 1 \times Laemmli Buffer [62.5mmol/L Tris-HCl, pH6.8, 2% SDS, 10% Glycerol v/v, 0.01% Bromophenol blue, 5% 2- mercaptoethanol, 1 \times protease inhibitors (Roche, Indianapolis, IN) and 1 \times phosphatase inhibitors (Sigma-Aldrich)]. Protein concentration was quantified by Lowry protein assay. Equal amounts of samples were size-fractionated on a 12% Tris-HCl polyacrylamide gel and transferred to a PVDF membrane (Bio-Rad, Hercules, CA). PVDF membranes were then incubated with blocking solution [Tris-buffered saline/Tween (TBST), 20 mmol/L Tris-HCl, pH 7.6, 137 mmol/L NaCl, and 0.1% v/v Tween-20] containing 5% (w/v) Carnation nonfat dry milk for 1 h at room temperature. Primary antibodies were diluted in blocking solution according to manufacturers' instructions and incubated for 3 h at room temperature. Subsequently, the membranes were washed with TBST 5 times for 5 min each time. HRP-conjugated secondary antibody was diluted 1:10,000 in blocking solution containing 1% (w/v) Carnation nonfat dry milk and incubated for 1 h at room temperature. After 5 washes of 5 min each with TBST, the membranes were exposed to the enhanced chemiluminescence reagent SuperSignal West Dura (Pierce; Rockford, IL). Signals were detected and quantified using ChemiDoc XRS imaging system (Bio-Rad). For primary antibodies, ATF4 (sc-200) and phosphorylated ERK (p-ERK, sc-7383) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and phosphorylated eIF2 α (p-eIF2 α , #9721) was purchased from Cell Signaling Technology (Danvers, MA). Secondary antibodies were purchased from Bio-Rad. For normalization of loading and blotting, a non-specific band from ATF4 antibody was used. Data were presented as ratio to the non-specific band.

2.4. Chromatin immunoprecipitation assay

For ChIP analysis, 1.5×10^7 cells were seeded per 150-mm dish. Amino acid deprivation treatment was performed as above. Cells from three 150-mm dishes were used for each treatment and later pooled together as one sample. At 8 h after treatment, protein and DNA were cross-linked by adding formaldehyde directly to the culture medium to a final concentration of 1%. Cross-linking was stopped 10 min later by adding 2 mol/L glycine to a final concentration of 0.125 mol/L. Cross-linked chromatin was solubilized by sonication using a Sonic Dismembrator (Model 100, Fisher Scientific Co.) for five bursts of 40 s at

50% of maximum power output with 2 min cooling on ice between each burst. The chromatin immunoprecipitation procedure used was previously described [22]. Briefly, solubilized chromatin from three dishes of cells was diluted 1:5 to 1:10 with dilution buffer and each portion was incubated with 2 μ g of antibody. Normal rabbit IgG was used as a negative control for the immunoprecipitation procedure. Antibodies against ATF4 (sc-200) and Pol II (sc-899) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Acetyl-histone H3 (06-599) and acetyl-histone H4 (06-866) were from Upstate Biotechnology (www.millipore.com). The antibody-bound complex was precipitated by Protein A-Sepharose beads (Zymed). The DNA fragments in the immunoprecipitated complex were released by reversing the cross-linking at 65 °C for 5 h and purified using a QIAprep Spin Miniprep Kit (Qiagen). Purified DNA was analyzed by quantitative real time PCR with primers covering the *DKK1* proximal promoter region -95 to -28 (sense, 5'-GGCTTTGTTGTCTCCCTCCCAAG-3' and antisense, 5'-CCACCGCGGCTGCCTTTATA-3'), and the *ASNS* proximal promoter region -87 to -22 (sense, 5'-TGGTTGGTCTCGCAGGCAT-3' and antisense, 5'-CGCTTATACCGACCTGGCTCCT-3').

2.5. Transcriptional rate analysis

To measure the transcriptional activity of the *DKK1* gene, the method of Sandoval et al. [23] was used, which relies on ChIP analysis to examine Pol II binding at the coding region distal to the promoter. Primers within a downstream region away from the transcription start site were used to monitor the transcription rate according to the quantity of DNA bound to Pol II. A higher transcription rate is represented as more Pol II binding at the given time point. Primers used to measure the transcriptional activity were: *DKK1* +710 to +809 (sense, 5'-ATCTGTCTCGCCTGCAGGAAGC-3' and antisense, 5'-AAGGGAGCTTTCAGGACTCACCAT-3').

2.6. Short interfering RNA (siRNA) assay

The siRNA for control Hypoxanthine-guanine phosphoribosyltransferase and ATF4 were purchased from IDT DNA technologies (www.idtdna.com) and the DharmaFECT-4 transfection reagent was purchased from Dharmacon, Inc. (Lafayette, CO). Cells were seeded into 6-well plates at a density of 3×10^5 cells/well in MEM and grown for 16 h. Transfection was performed according to the instructions from Dharmacon, using 3 μ L of DharmaFECT-4 and 20 nmol/L per well final siRNA concentration. Cells were treated with transfection reagent for 48 h, then rinsed with PBS, given fresh MEM, and cultured for another 12 h. The medium was then removed and replaced with control MEM or amino acid free MEM. Total RNA and protein extracts were isolated at 8 h after the treatment and analyzed by quantitative real-time RT-PCR and immunoblotting, respectively.

2.7. Construction of *DKK1* promoter and enhancer sequences for reporter assay

The *DKK1* gene sequence was obtained from The Ensembl Project using the accession number ENSG00000107984. Partially truncated promoter regions were cloned into the pGL3-basic vector (Promega; Madison, WI) as following: P1 (-960/ +23), P2 (-264/+2173), P3 (-349/ +23), P4 (-733/ +23), and P5 (-555/ +23). Enhancer regions covering predicted amino acids response elements (AARE) were cloned into the pGL3-promoter vector (Promega) as following: E1 (+1109/ +1305), E2 (+1210/ +1406), E3 (+1860/ +2060), E4 (+2036/ +2253), E5 (-3000/ -2701), and E6 (+2915/ +3101). The human *ASNS* promoter region (-115/ +1) was used as a positive control. The resulting clones were confirmed by DNA sequencing at the DNA sequencing facility at the University of Illinois at Urbana-Champaign, Urbana, IL. Plasmids were harvested and purified using Wizard SV plasmid midi-prep kit (Promega).

2.8. Luciferase reporter assay

Cells were plated at a density of 1×10^5 cells / well in 24-well plates (Costar; Cambridge, MA) 24 h before transfection. One μg plasmid and 5 ng pRL-SV40 were co-transfected into each well using Superfect transfection reagent (Qiagen; Valencia, CA) following manufacturer's instructions. Amino acid deprivation treatment was started at least 16 h after transfection and lasted for 8 h. Cells were then lysed with PLB buffer and subjected to one round of freeze-thaw. Luciferase activity was measured using Dual-Luciferase Assay (Promega) using a luminometer (Fermomaster FB12, Zylux Corporation; Huntsville, AL). For the ATF4 over-expression experiment, reporter-gene plasmid was co-transfected with plasmid containing ATF4 cDNA sequence or empty vector itself following the same procedure as mentioned above.

2.9. Immunofluorescent staining

Cells were plated at a density of 0.3×10^6 cells / well of 6-well plates with glass cover slips in the wells. Subsequent treatments were performed following the above-mentioned procedures. At the end of each treatment, cells were washed with $1 \times \text{PBS}$ for 3 times and fixed by 4% Paraformaldehyde treatment for 30 min on the cover slips, followed by 0.5% Triton X-100 for 15 min for permeabilization. Image-iT FX signal enhancer (Invitrogen) was used to block the samples for 30 min and anti-DKK1 antibody (Abcam 61304, 1:250) was applied to the cover slips for 2 hours at 37°C . A goat anti-rabbit antibody with Alexa Fluor 647 (1:200 dilution, Invitrogen) was applied to the cover slip for 45 min in a dark chamber. The samples were counterstained with Hoechst (Invitrogen) for 15 min and washed off by $1 \times \text{PBS}$. Cover slips were mounted onto microscope slides using Prolong Gold antifade reagent (Invitrogen). Pictures were taken using the Zeiss Axiovert 200M microscope (Zeiss).

2.10. Statistical analysis

Standard student's t test was performed for each experiment. Each data point represents the mean of at least three independent experiments. Error bars represent calculated standard deviations from the mean. *P* values were calculated using student's t-test and asterisks indicate $P < 0.05$.

3. RESULTS

3.1. DKK1 mRNA level was increased in SW480 after amino acid deprivation

We have shown previously that amino acid deprivation in human colon cancer cell line SW480 caused reduction of Wnt signaling through the down-regulation of WNT5a [21]. In the present study, we investigated the expression of DKK1, an antagonist of the Wnt pathway. Using real time PCR, we observed that the DKK1 mRNA level was significantly increased in amino acid-deprived SW480 colon cancer cells (Fig. 1a). The induction peaked at approximately 8 h and then decreased almost to the initial level within 24 h. To investigate the contribution from either transcriptional up-regulation or increase of mRNA stability by amino acid limitation, we monitored the transcription rate by following the binding of RNA Polymerase II within the DKK1 coding region [23]. There was a 3-fold increase of transcriptional activity in amino acid-deprived cells (Fig. 1b). For mRNA stability assay, SW480 was deprived from total amino acids for 8 h and then treated with MEM, MEM-AA, or MEM-AA plus the p-ERK inhibitor, U0126, with all media containing $5 \mu\text{mol/L}$ Actinomycin D. The addition of Actinomycin D blocked further transcription; thereby allowing the observation of changes in mRNA stability. In the amino acid-deprived condition, the half-life of DKK1 mRNA increased to 2.25 h compared to 1 h in MEM (Fig. 1c). More importantly, the addition of the phosphor-ERK inhibitor U0126 in the amino acid

deprived media did not change the half life of the DKK1 mRNA, indicating that phosphorylation of ERK may not be involved in the regulation of mRNA stability of DKK1.

3.2. DKK1 expression was increased by histidinol treatment to the similar level as the amino acid deprivation

Amino alcohol histidinol (HisOH) prevents the formation of histidinyI-tRNA^{His} and could be used to test the effect from histidinyI-tRNA^{His} blockade. Results showed that serial concentrations of HisOH also increased DKK1 mRNA to a level similar to that observed when cells were amino acid-deprived (Fig. 2a), illustrating that tRNA charging is involved. Typical AAR pathway responsive genes, such as ASNS, ATF3 and p21^{waf1}, were also activated in a similar manner (Fig. 2a). Likewise, protein expression of ATF4, p-eIF2 α and p-ERK was induced under amino acid deprivation and by the addition of 16 mmol/L of HisOH (Fig. 2b, 2c). The induction of DKK1 gene expression was confirmed by increased immunofluorescent staining of the DKK1 protein in both the amino acid deprived condition and the MEM+HisOH condition where histidine availability was blocked (Fig. 2d). A parallel treatment where DKK1 protein was overexpressed by in vitro transfection was used as a positive control to show the increased staining for the DKK1 protein by immunohistochemistry (Fig. 2d).

3.3. The effect from HisOH treatment on the induction of DKK1 was not due to osmolarity change

To confirm the effect from HisOH treatment, SW480 cells were treated with complete MEM, MEM without any amino acids (MEM-AA), MEM without histidine (MEM-His), MEM with 16 mmol/L histidine (MEM+His), or MEM with 16 mmol/L HisOH (MEM+HisOH, Fig. 3). MEM+His treatment was included in this set of the experiments to eliminate potential effect from the potential change in osmolarity when 16 mmol/L of HisOH was added in the MEM+HisOH treatment. Results showed that MEM-AA, MEM-His, and MEM+HisOH treatments were all effective in inducing the amino acid response (Fig. 3). Specifically, mRNA level of both DKK1 and ATF4 was upregulated similarly under either total amino acid starvation or histidine deprivation (by -His or +HisOH, Fig. 3a). ATF4 protein level increased in the three amino acid-deprived conditions as well (Fig. 3b). In contrast to the MEM+HisOH, no induction on either DKK1 or ATF4 expression was observed when the same concentration of histidine was used in the treatment media, eliminating the possibility of an osmolaric shock.

3.4. The induction of DKK1 mRNA by amino acid deprivation involved phosphorylation of ERK

To examine the possible involvement of ERK signaling pathway in the induction of DKK1, the MEK specific inhibitor U0126 was used. Blocking of phosphorylation of ERK with U0126 at 5 μ mol/L totally abolished the induction of DKK1 (Fig. 4a), indicating the dependence on the MEK/ERK pathway for DKK1 expression. On the other hand, p-eIF2 α , ATF4 and the expression of target genes ATF3 and p21 were partially decreased (Fig. 4a, b, and c). Interestingly, the positive control for amino acid responsive pathway, ASNS mRNA expression, was not affected by U0126 treatment (Fig. 4a). The dependence of DKK1 gene expression on the phosphorylation of ERK was further confirmed using immunofluorescent staining of the DKK1 protein (Fig. 4d). Immunofluorescent staining clearly showed the induction of DKK1 protein by amino acid deprivation. The addition of U0126 blocked the induction of DKK1 protein and decreased the level to what is observed in the amino acid-containing condition (Fig. 4d).

3.5. DKK1 gene lacked a functional responsive element to amino acid deprivation

To identify a functional responsive element to amino acid limitation, we performed reporter gene assays. Using Vector NTI (Invitrogen), we analyzed a region of the DKK1 gene sequence consisting of the upstream 3 kb to the downstream 3 kb. The software predicted 5 putative AARE-like elements according to the consensus AAREs (NSRE-1: TGATGAAAC; NSRE-2: RTTKCATCA) [24]. We individually cloned 5 truncated promoter fragments of the DKK1 gene and cloned them into the pGL3-basic luciferase reporter vector and 6 DNA fragments containing the putative AARE-like elements into the pGL3-promoter vector. The constructs are tested for luciferase reporter activity. Results showed that none of the promoter regions nor any of the other predicted enhancer fragments contained a functional AARE (see supplementary file). Furthermore, when ATF4 was co-expressed with reporter plasmids containing a *DKK1* promoter region or a putative AARE, the luciferase assay showed that this exogenous, over-expressed ATF4 could not induce DKK1 expression (see supplemental file). Luciferase reporter plasmid containing *ASNS* (-115 to +1) promoter fragment was used as a positive control and it showed the expected, reported response to the amino acid starvation (supplemental file) [22, 24].

3.6. Transcription of DKK1 was increased without ATF4 binding at the promoter

To define the possible role of transcription factor binding and chromatin modification in amino acid regulated control of DKK1 expression, we investigated the binding of ATF4 and Pol II as well as local intensity of acetyl-histone H3 and acetyl-histone H4 at the proximal promoter region of *DKK1* and *ASNS* using chromatin immunoprecipitation. The results showed that Pol II binding to the promoters of both *ASNS* and *DKK1* was increased under amino acid deprived condition, indicating that both *ASNS* and *DKK1* were transcriptionally activated (Fig. 5). However, in contrast to the increased ATF4 binding to the *ASNS* promoter under amino acid deprived condition, ATF4 binding to the *DKK1* promoter was similar to the binding level of the negative control IgG in either MEM or MEM-AA condition, which indicated that the ATF4 binding to the *DKK1* promoter is not present. There was no significant difference in acetyl-histone H3 or acetyl-histone H4 level at the promoter of *DKK1* in amino acid free condition compared to complete MEM. On the other hand, both acetylated histones were increased at the *ASNS* promoter region by amino acid deprivation (Fig. 5).

3.7. Induction of DKK1 mRNA expression was independent of the expression of ATF4

To further confirm the independence of DKK1 expression on ATF4, we investigated the expression of DKK1 when ATF4 level were knocked down by an ATF4-specific siRNA in both MEM and MEM-AA conditions. During amino acid deprivation, both ATF4 mRNA and protein were decreased by the ATF4 siRNA to a level below the well-nourished control siRNA-treated cells (Fig. 6, ctrl siRNA in MEM). Control siRNA treatment did not change the ATF4 induction by amino acid deprivation. The induction of the ATF4 responsive-, AAR-target gene *ASNS* was almost completely blocked by the ATF4 siRNA treatment (Fig. 6c). However, during amino acid limitation, the silencing of ATF4 did not affect the elevated DKK1, ATF3, and p21 mRNA expression (Fig. 6c) and even resulted in an increase of DKK1 mRNA. At the same time, both p-eIF2 α and p-ERK remained elevated when ATF4 was blocked by siRNA (Fig. 6a and b).

4. DISCUSSION

The present study demonstrates the link between the amino acid response pathways and the Wnt signaling pathway. We showed that DKK1, an antagonist of the Wnt pathway, is unique in its regulation, in that its expression was up-regulated by amino acid deprivation in the human colon cancer cell line SW480. The induced expression was partly due to mRNA

stabilization and partly due to transcriptional activation. The transcriptional control of DKK1 expression is mediated through an ATF4-independent and MAPK/ERK-dependent manner. Histone acetylation at the promoter region under the amino acid deprived condition also contributes to the induction of the gene expression for DKK1.

AAR pathways are known for regulating specific cellular responses when there is limitation of amino acid availability. Our previous report has shown that Wnt5a, another Wnt signaling related gene, was down-regulated in the SW480 colon cancer cell line by amino acid deprivation [21]. Nuclear β -catenin, the major intracellular factor of canonical Wnt signaling, was also decreased, which is consistent with the down-regulation of the whole Wnt signaling pathway following amino acid deprivation [21]. In the present study, we observed that an antagonist of the Wnt pathway, DKK1, was up-regulated under the amino acid deprived conditions. Therefore, it is possible that the down-regulation of Wnt signaling was also a result of the up-regulation of DKK1. DKK1 has wide and complex effects on cell progression and differentiation [25–28]. The induced DKK1 by amino acid limitation is likely a link between the Wnt signaling in cancer development in response to nutritional stresses.

Amino acid response pathway has been shown to affect gene expression at multiple stages of gene expression [3]. Specifically amino acid limitation has been shown to regulate gene expression of the transcription factor ATF3 by ways of transcriptional control and mRNA stability [5, 29, 30]. These studies illustrated the amino acid-induced transcriptional upregulation of ATF3 which is mediated through the AARE by ATF4 [5]. At the same time, ATF3 expression is induced by amino acid limitation through stabilization of its mRNA by increased interaction with the mRNA-binding protein HuR1 [30]. We showed that DKK1 expression is responsive at both transcriptional level and mRNA stability, further confirmed the multiplicity of amino acid control of gene expression. The involvement of mRNA stabilizing machinery in the regulation of DKK1 during amino acid limitation is one of the critical components for amino acid regulation that warrants further investigation.

We hypothesized that the amino acid response of *DKK1* transcription was mediated through the classic GCN2/ATF4 pathway, as previously reported for a number of amino acid responsive genes, including *SNAT2* system A amino acid transporter [10] and *ASNS* [8]. We therefore targeted our study at identifying an AARE on the *DKK1* genomic sequence through reporter gene analysis. However, we did not identify any amino acid responsive elements at either proximal and distal promoter regions or downstream regions as in *SNAT2* and *C/EBPbeta*. It is entirely possible that we have not yet included the specific region related to the amino acid deficiency condition. On the other hand Pali et al [31] reported that total amino acid deprivation (KRB based medium) did induce a portion of transcription of the *SNAT2* gene that is AARE-independent. Similarly in the present study, the single amino acid deprivation, either by histidine elimination or by the addition of histidinol, provides the similar induction of *DKK1* transcription as the total amino acid limitation. However when the entire *DKK1* gene was specifically mapped for a potential AARE using overexpression of ATF4, we still could not identify any element that is responsive to ATF4. In future research, we may need to focus the search of an amino acid responsive element on the newly proposed C/EBP-ATF response element (CARE) [4]. On the other hand, based on the results from both over-expression and knockdown of ATF4 and from the ChIP assay, it is highly likely that the increased transcription of *DKK1* was regulated by a GCN2/ATF4-independent pathway that requires activation of p-ERK. Therefore, the *DKK1* gene may not contain an AARE that is responsive to ATF4-dependent induction. As proposed recently, multiple pathways exist in response to amino acid deficiency [32]. The pathways proposed include the GCN2/ATF4 pathway, the signaling pathway leading to ATF2 activation, and the GCN2-independent pathway. From the evidence presented here, we propose that the

SW480 cell line is utilizing the ATF4-independent pathway. It has been reported that, in HepG2 cells, blockage of ERK phosphorylation totally eliminated the phosphorylation of eIF2 α and induction of ATF4 [10]. This response indicated the interdependence in HepG2 cells between ERK signaling and GCN2/ATF4 for amino acid responsiveness. In the present study in SW480 cells, blockage of ERK phosphorylation did not completely eliminate the induction of ATF4 and p-eIF2 α but the induction of *DKK1* gene expression, on the other hand, is completely eliminated. This indicates the dependence of *DKK1* induction on the MEK/ERK signaling through phosphorylation of ERK by amino acid deprivation. On the other hand, inhibition of ATF4 expression did not affect the phosphorylation of ERK and eIF2 α . And the induction of *DKK1* gene expression remains unaffected, as long as there is signaling from phosphorylated ERK. We tried going back up the ATF4 signaling pathway and tested GCN2 dependence. When GCN2 mRNA is knocked down by siRNA, we did not observe any reduction in *DKK1* mRNA level induced by amino acid deprivation (data not shown). Since the induction of *ASNS* was also only partially reduced in this experiment, we need to utilize a better system to knock down GCN2 level for future investigation (data not shown). Further studies are needed then to decipher the ATF4-independent pathway to illustrate the transcription factor that is responsive to the amino acid regulation and that relays the activating signal from the phosphorylation of ERK.

The histone modifications at the promoter regions have been related to critical regulations of mammalian gene expression. Specifically, amino acid deprivation has been shown to induce acetylation of histone H3 and H4 on the *ASNS* promoter [22]. For *ASNS* gene, the temporal mapping of the amino acid response showed that the acetylation of both histone H3 and H4 coincided with the increased binding of ATF4 at *ASNS* promoter when there was the histidine deprivation [22]. This is also confirmed in the current study when the total amino acids were removed. For another amino acid responsive gene *CHOP*, a single amino acid limitation (-Leu) induced an increased acetylation of histone H4 and H2B but not H3 [33]. These amino acid-induced histone modifications are shown to be dependent on ATF2 binding at the promoter of *CHOP* gene [33]. In future studies, it is therefore important to identify the transcription factor that is responsible for the induction of *DKK1* transcription and to examine the needs for a regionally modified histone profile.

In summary, our present study identifies *DKK1* as a gene with a novel response to amino acid deprivation in a colon cancer cell line. The transcriptional control of *DKK1* by amino acid deprivation in this study defines a novel amino acid responsive pathway that is independent of ATF4 expression and dependent on the phosphorylation of ERK. Further experiments are needed to characterize the cascades of this signaling pathway. Future studies will also investigate the interactions between the Wnt signaling pathway and the amino acid responsive pathways, as this may be a critical component during colon cancer development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AAR	amino acid response
AARE	amino acids response elements
ATF	activating transcription factor
ASNS	asparagines synthetase
DKK1	dickkopf homolog 1
eIFα	eukaryotic initiation factor 2 alpha
ERK	extracellular signal-regulated kinase
GCN2	general control non-derepressible 2
HisOH	histidinol
MAPK	mitogen-activated protein kinase
p-eIF2α	phosphorylated eukaryotic initiation factor 2 alpha
p-ERK	phosphorylated extracellular signal-regulated kinase
Pol II	RNA polymerase II
Raf	rapidly accelerated fibrosarcoma protein

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Research Highlights

- The Wnt signaling pathway was affected by amino acid deprivation through the induction of DKK1
- Transcriptional regulation of DKK1 by amino acid deficiency was by the phosphorylation of ERK
- Knockdown of ATF4 does not inhibit the induction of DKK1 mRNA
- The study demonstrated the interactions among Wnt, AAR, and MAPK/ERK signaling pathways in a colon cancer cell line
- DKK1 is regulated by a novel amino acid response pathway that is independent of the master regulator ATF4.

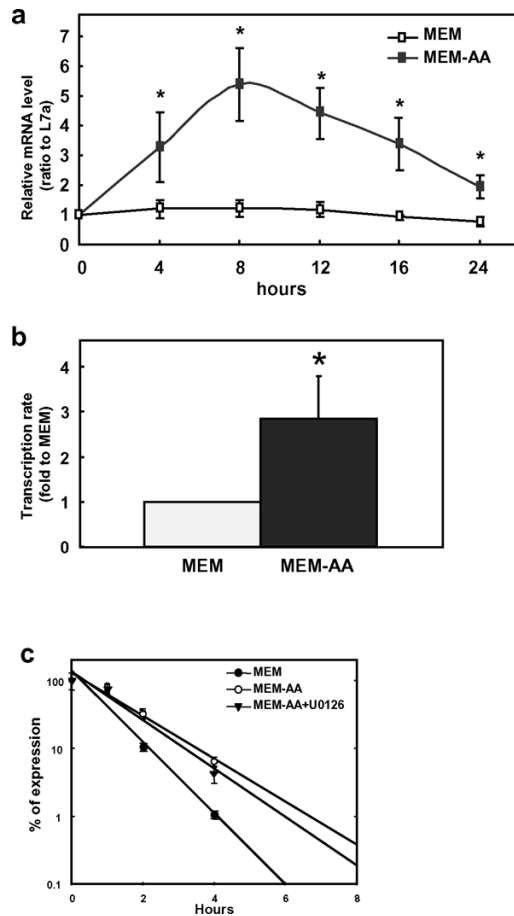


Fig. 1. *DKK1* gene expression was induced by amino acid deprivation

a) Time course of the total amino acid deprivation treatment. SW480 was grown in complete medium (MEM) and amino acid deficiency medium (MEM-AA) for 0–24 h. Relative mRNA level was determined by quantitative real time RT-PCR. Data points were normalized by internal control *L7a* and to time zero. **b)** Transcription rate of *DKK1* gene during amino acid deprivation. ChIP analysis was performed to examine Pol II binding at the coding region distal to the promoter. Data were presented as ratio to MEM. **c)** mRNA stability of *DKK1* following amino acid deprivation. Each data point represents the mean \pm S. D of three independent experiments. Asterisks (*) represent statistical significance compared to values in MEM ($P < 0.05$).

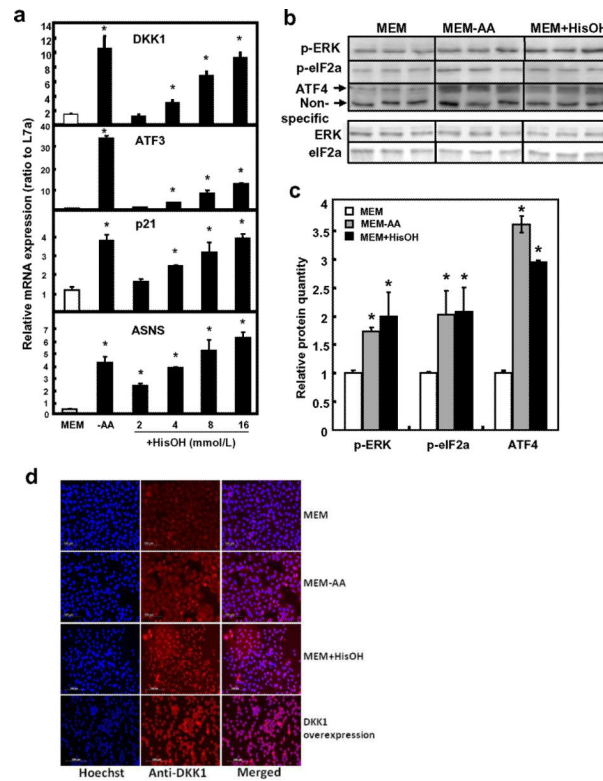


Fig. 2. Expression of mRNA and protein in SW480 after histidinol treatment

a) mRNA level was quantified in human colon cancer cell line SW480 after histidinol (HisOH) treatment. SW480 cells were grown in MEM, MEM without amino acid, and MEM containing 2, 4, 8, or 16 $\mu\text{mol/L}$ histidinol for 8 h. Relative mRNA level was determined by quantitative real time RT-PCR. Data were normalized by the internal control L7a. **b**) A representative blot from Western blotting analysis for ATF4, p-eIF2 α and p-ERK protein expression. **c**) Protein level was quantified in SW480 by Western blotting with 16 mmol/L HisOH treatment. A non-specific band was used as the loading control to normalize the raw data. Each bar represents the mean \pm S. D. of three independent experiments. Asterisks (*) represent statistical significance compared to values in MEM ($P < 0.05$). **d**) The MEM-, MEM-AA-, and MEM+HisOH-treated cells were fixed on glass cover slips and subsequently used for immunofluorescent staining. DKK1 protein was analyzed after immunofluorescent staining using an antibody against DKK1 protein and an Alexa Fluor 647-labeled secondary antibody (red). The cover slips were also counterstained with Hoechst 33342 fluorescent staining for nucleus (blue). The two pictures were overlaid to show DKK1 staining on top of the nuclear staining (merged). Cells treated with in vitro transfection of DKK1-containing plasmid was used as a positive control for the staining of DKK1 protein.

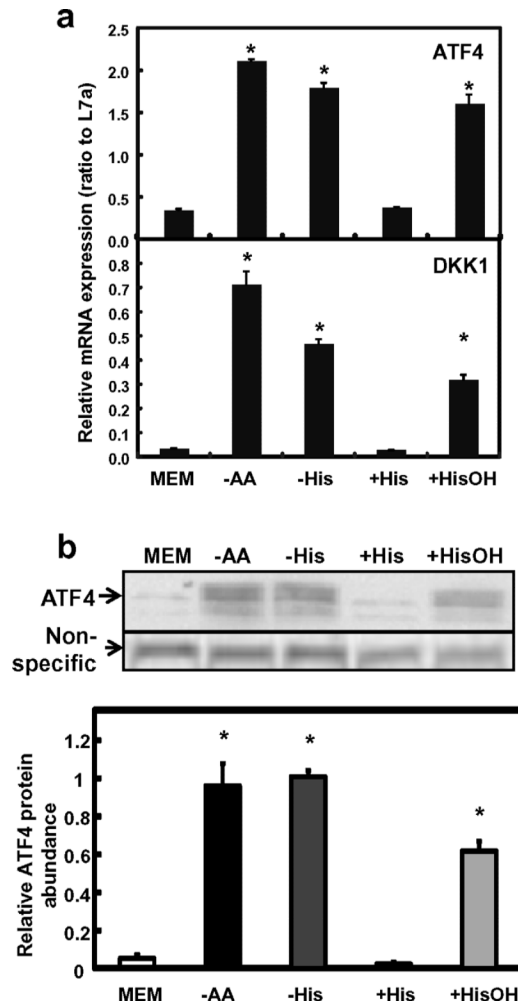


Fig. 3. DKK1 expression by total or single amino acid deprivation

SW480 was treated with either MEM (complete), -AA (total amino acid deprivation), and -His or +HisOH (single amino acid limitation) for 8 h before samples were collected and analyzed. MEM+His was used as a negative control to amino acid starvation treatment (+His). **a**) mRNA expression of ATF4 and DKK1. Real time RT-PCR was performed for gene expression. *L7a* was used as the internal control and to normalize the data. Each bar represents the mean \pm S. D. of three samples. Asterisks (*) represent statistical significance compared to values in MEM ($P < 0.05$). **b**) ATF4 protein expression analyzed by Western blotting. A non-specific band was used as the loading control to normalize the raw data. Each bar represents the mean \pm S. D. of three independent samples. Asterisks (*) represent statistical significance compared to values in MEM ($P < 0.05$).

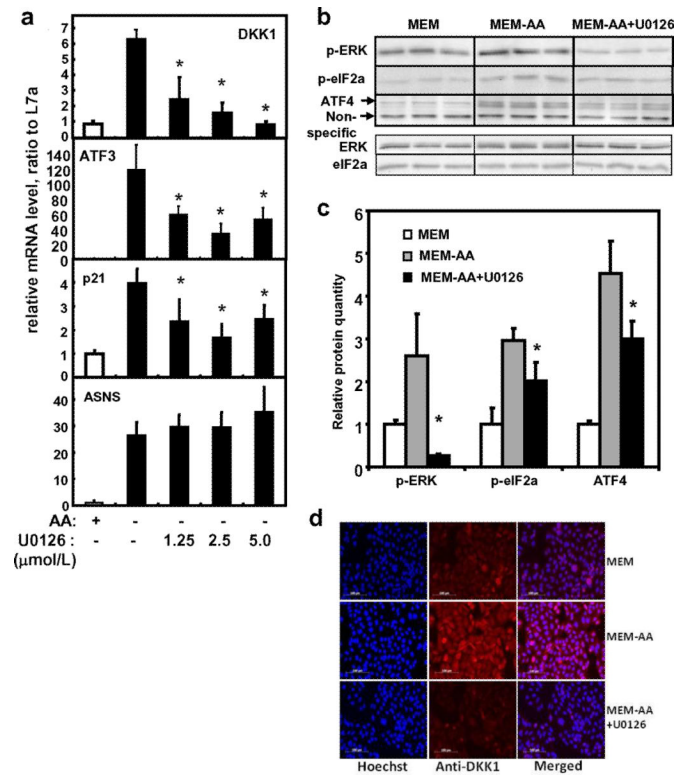


Fig. 4. Expression of mRNA and protein in SW480 after U0126 treatment

a) mRNA expression after U0126 treatment. SW480 cells were treated with either control DMSO (MEM), MEM-AA, or MEM-AA containing 1.25, 2.5, or 5 $\mu\text{mol/L}$ of the MEK1/2 inhibitor U0126 for 8 h. Relative mRNA level was determined by quantitative real time RT-PCR and normalized to DMSO control. **b**) Western blot analysis after U0126 treatment. ATF4, p-eIF2 α , and p-ERK proteins were analyzed by Western blot analysis in SW480 after U0126 treatment. Total ERK and total eIF2 α were also analyzed to ensure that the changes observed for p-ERK and p-eIF2 α are specific for phosphorylated forms. **c**) Protein abundance after U0126 treatment. Protein level of ATF4, p-eIF2 α , and p-ERK was quantified after Western blot analysis in SW480 cells treated with U0126. A non-specific band was used as the loading control to normalize the raw data. Each data point represents the mean \pm S. D. of at least three independent samples. For data from the U0126 treated samples, asterisks (*) represent statistical significance compared to values in amino acid deprived condition (MEM-AA, $P < 0.05$). **d**) DKK1 protein expression. The MEM-, MEM-, and MEM-AA+U0126-treated cells were fixed on glass cover slips and subsequently used for immunofluorescent staining. DKK1 protein was analyzed after immunofluorescent staining using an antibody against DKK1 protein and an Alexa Fluor 647-labeled secondary antibody (red). The cover slips were also counterstained with Hoechst 33342 fluorescent staining for nucleus (blue). The two pictures were overlaid to show DKK1 staining on top of the nuclear staining (merged).

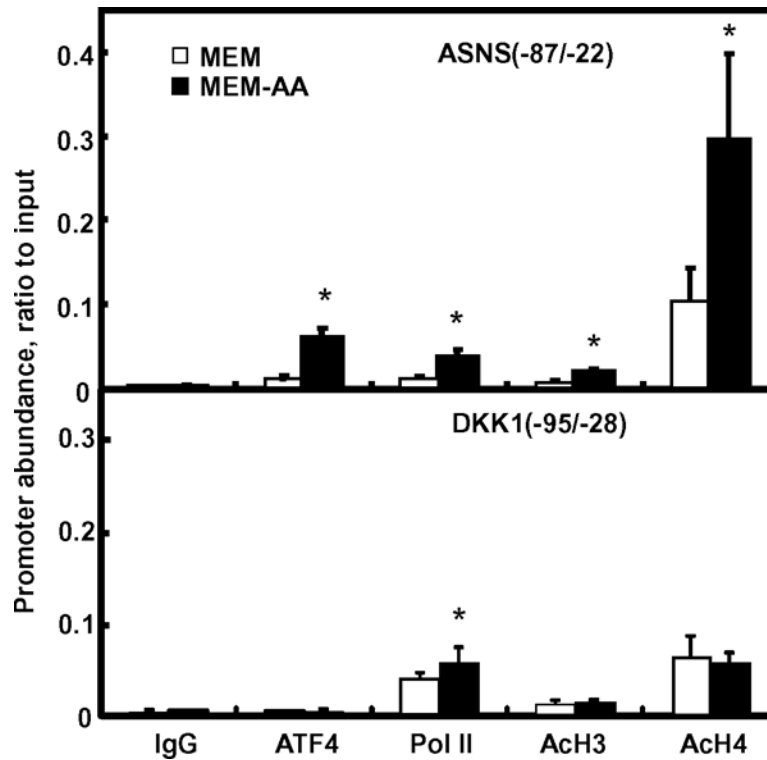


Fig. 5. Protein association at the promoter region of *DKK1*
 SW480 cells were grown in MEM or MEM-AA for 8 h. Samples were collected for chromatin immunoprecipitation assay. Primers located at the proximal promoter region of either *DKK1* (-95/-28) or *ASNS* (-87/-22) were used in the real time PCR to quantify the association of promoter DNA to ATF4, Pol II, acetyl-histone H3, and acetyl-histone H4. Normal rabbit IgG was used as a negative control (IgG). Each bar represents the mean \pm S. D. from three independent experiments. Asterisks (*) indicate statistical significance compared to values in MEM ($P < 0.05$).

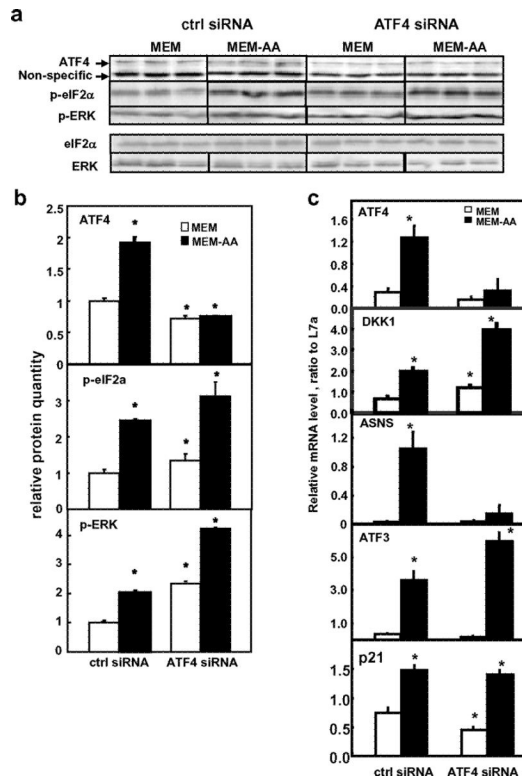


Fig. 6. Effects of ATF4 siRNA transfection in SW480

SW480 cells were transfected with control (ctrl siRNA) and ATF4 siRNA for 48 h. Culture media were changed to fresh MEM 12 h before treatment for 8 h in either MEM or MEM-AA. **a**) Representative blots of Western blotting analysis for ATF4, p-eIF2 α and p-ERK protein expression. Total eIF2 α and total ERK were examined as negative control. **b**) Quantification of ATF4, p-eIF2 α , and p-ERK protein levels. Relative protein level was presented after normalization to the non-specific band observed. **c**) Relative mRNA level was determined by quantitative real time RT-PCR and normalized to the internal control L7a. Each data point represents the mean \pm S. D. Asterisks (*) represent statistical significance compared to values in control siRNA in MEM ($P < 0.05$).