Original Article
Downregulation of TRIB3 enhances the sensitivity of lung cancer cells to amino acid deprivation by suppressing AKT activation

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Abstract: Tribbles pseudokinase 3 (TRIB3), a member of the mammalian Tribbles family, is implicated in multiple biological processes. This study aimed to investigate the biological functions of TRIB3 in lung cancer and its effect on amino acid-deprived lung cancer cells. TRIB3 mRNA expression was elevated in lung cancer tissues and cell lines compared to normal lung tissues and cells. TRIB3 knockdown markedly reduced the viability and proliferation of H1299 lung cancer cells. Deprivation of amino acids, particularly arginine, glutamine, lysine, or methionine, strongly increased TRIB3 expression via ATF4 activation in H1299 lung cancer cells. Knockdown of TRIB3 led to transcriptional downregulation of ATF4 and reduced AKT activation induced by amino acid deprivation, ultimately increasing the sensitivity of H1299 lung cancer cells to amino acid deprivation. Additionally, TRIB3 knockdown enhanced the sensitivity of H1299 cells to V-9302, a competitive antagonist of transmembrane glutamine flux. These results suggest that TRIB3 is a pro-survival regulator of cell viability in amino acid-deficient tumor microenvironments and a promising therapeutic target for lung cancer treatment.

Keywords: AKT, amino acid depletion, ATF4, lung cancer, TRIB3

Introduction

Lung cancer is the leading cause of cancer-related death worldwide [1]. Despite major advances in therapeutic options for lung cancer, some major challenges, such as optimal combination therapy, resistance to targeted therapy and immunotherapy, and identification of novel promising targets, remain [2].

Tribbles pseudokinase 3 (TRIB3), a scaffold protein belonging to the Tribbles family of serine/threonine pseudokinases, participates in multiple cellular signaling pathways, including cell growth, proliferation, and differentiation [3, 4]. In addition, TRIB3 serves as a stress sensor that responds to various microenvironmental cues, such as endoplasmic reticulum stress, hypoxia, and nutrient deprivation [5-7]. Several studies have found that TRIB3 is elevated in multiple cancers, including breast, liver, lung, and colorectal cancers, and is closely associated with cancer development and poor prognosis [8-11]. However, some studies have reported that higher TRIB3 protein level is associated with improved prognosis in patients with breast cancer, and that TRIB3 upregulation inhibits the AKT/mTORC1 axis, exerting antitumor effects [12, 13]. Thus, the biological functions and mechanisms of action of TRIB3 in cancer remain controversial. Currently, the expression of TRIB3 in lung cancer and its association with the clinicopathological features remain unclear.

Cancer cells often exhibit metabolic reprogramming, including enhanced rates of glutaminoly-

sis and fatty acid synthesis, and increased uptake of glucose to sustain their characteristic uncontrolled growth [14]. Amino acids are involved in a broad range of cellular functions including redox homeostasis, proliferation, protein and nucleic acid synthesis, and bioenergetic support [15]. Recent studies have highlighted the importance of amino acids as metabolites and metabolic regulators that support tumorigenesis [16]. Due to increased metabolic demands, cancer cells consume large amounts of amino acids to sustain their proliferation [17]. Therefore, interfering with amino acid availability represents a promising and novel anticancer strategy that has proven to be a cancer-specific Achilles heel [18]. Preclinical and clinical studies using amino acid depletion strategies for cancer treatment have been conducted [15].

In the present study, we investigated the biological functions of TRIB3 and its effects on amino acid-deprived lung cancer cells. Our results showed that TRIB3 is significantly upregulated in lung cancer tissues compared to normal tissues. Downregulation of TRIB3 markedly decreased the viability and proliferation of lung cancer cells, suggesting its potential involvement in lung cancer progression. Our results further showed that TRIB3 is a prosurvival regulator of cell viability in amino acid-deficient tumor microenvironments, and thus represents a promising target for lung cancer therapy.

Materials and methods

Cell culture and reagents

The normal human bronchial epithelial (HBE) cell line and seven human lung cancer cell lines (H69, A549, H460, Lu99, H358, H1299, and EBC-1) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA), and cultured in the recommended growth medium. TissueScan lung cancer tissue qPCR panel (Lung Cancer cDNA array II #HLRT502) containing cDNAs from 48 tissues including all four lung cancer stages (stage I, n = 25; stage II, n = 6; stage III, n = 10; stage IV, n = 2) and normal controls (n = 5) was obtained from OriGene Technologies (Rockville, MD, USA). Thiazolyl blue tetrazolium bromide (MTT, #M2128) was purchased from Sigma-Aldrich (Merck KGaA; Darmstadt, Germany). The selec-

tive glutamine transporter ASCT2 inhibitor V-9302 (#HY-112683) was purchased from MedChemExpress (Monmouth Junction, NJ, USA).

Amino acid deprivation

Media without individual amino acids were prepared using amino acid-free Roswell park memorial institute (RPMI)-1640 (#LM011-127, Welgene, Gyeongsangbuk-do, Republic of Korea) with the addition of combinations of amino acids diluted from the following stock solutions: L-arginine (#LS087-01), L-asparagine (#LS081-01), L-aspartic acid (#LS082-01), L-cystine (#LS088-01), L-glutamine (#LS002-01), L-glutamic acid (#LS083-01), glycine (#LS-089-01), L-histidine (#LS090-01), L-hydroxy-proline (#LS084-01), L-isoleucine (#LS091-01), L-leucine (#LS092-01), L-lysine (#LS093-01), L-methionine (#LS086-01), L-phenylalanine (#LS094-01), L-proline (#LS085-01), L-serine (#LS095-01), L-threonine (#LS096-01), L-tryptophan (#LS097-01), L-tyrosine (#LS098-01), and L-valine (#LS099-01). The medium was supplemented with 10% dialyzed fetal bovine serum (FBS; 10,000 MW Cut-off, #26400-044, Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA).

Transient transfection

ATF4 gene was cloned into the eukaryotic expression plasmids, pEGFP-C1 with encoding GFP. Small interfering RNAs (siRNAs) for ATF4 #1 (#sc-35112), TRIB3 #1 (#sc-44426), and control #1 (CTL; #sc-37007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). siRNAs for ATF4 #2 (#J-005125-13-0010) and CTL #2 (#D-001210-01-05) were purchased from Dharmacon (Horizon; Waterbeach, UK). Control #3 (#SN-1012) siRNA was purchased from Bioneer (Daejeon, Republic of Korea), while siRNAs for TRIB3 #2 (5'-CGCUG-ACCGUGAGAGGAAG-3') [19] and TRIB3 #3 (5'-GGACCUGAGAUACUCAGCU-3') [20] were synthesized by Bioneer. Transfection with plasmids and siRNAs was performed using Lipofectamine LTX Reagent with PLUS Reagent (#15338, Invitrogen; Thermo Fisher Scientific Inc.) and Lipofectamine RNAiMAX (#13778, Invitrogen; Thermo Fisher Scientific Inc.), respectively, according to the manufacturer's instructions.

Cell viability assay

Cell viability was determined by measuring mitochondrial activity using the MTT assay. Following treatment of the cells with the drugs, MTT reagent was added. The cells were solubilized in DMSO and the amount of MTT converted into the purple formazan product was determined by measuring the absorbance at 570 nm.

Colony formation assay

H1299 cells were transfected with CTL or TRIB3 siRNAs for 24 h. Cells were reseeded at densities of 1,000 (1×), 2,000 (2×), and 3,000 (3×) cells/well in a 6-well plate. Colony formation was monitored over ten days. Colonies were stained using the Diff-Quick staining reagent (#38721, Sysmex, Kobe, Japan).

RNA extraction, reverse transcription (RT), and polymerase chain reaction (PCR)

RNA was isolated from the cells using TRIzol reagent (#15596018, Invitrogen; Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. cDNA was synthesized from 2 μg RNA using M-MLV reverse transcriptase (#28025021, Invitrogen; Thermo Fisher Scientific Inc.). The following specific primers were used for PCR: TRIB3 #1 (5'-GCCACTGC-CTCCCGTCTTG-3' and 5'-GCTGCCTTGCCCGA-GTATGA-3'; 539-bp product) [21], TRIB3 #2 (5'-CGGAGCCTGTCCTGCTCTTC-3' and 5'-GTA-GGGTGGTCC TAGCCATAC-3'; 298-bp product), ATF4 (5'-AGTCGGGTTTGGGGGCTGAAAG-3' and 5'-TGGGGAAAGGGGAAGAGGTTGTAA-3'; 437-bp product) [21], β-actin (5'-GGATTCCTATGTGGG-CGACAG-3' and 5'-CGCTCGGTGAGGATCTTCA-TG-3'; 438-bp product) [22], and GAPDH (5'-TGAAGGTCGGAGTCAACGGATTTGGTC-3' and 5'-CATGTGGGCCATGAGGTCCAC-3'; 983-bp product) [23]. Where indicated, RT-PCR images were quantified using ImageJ software (version 1.52a; National Institute of Health, Bethesda, MD, USA).

Real-time PCR

Real-time PCR was performed using TaqMan gene expression assays (Applied Biosystems; Thermo Fisher Scientific Inc.) on Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific Inc.). Primers/probes for ATF4 (assay ID: Hs00909569_g1), TRIB3 (assay ID: Hs01082394_m1), and β-actin (assay ID: Hs01060665_g1) were used. The thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. Relative quantification values for each target gene were calculated by the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Western blotting was performed as previously described [24]. The following antibodies were used: anti-p-AKT (Ser 473) (#9271) and anti-AKT (#9272) obtained from Cell Signaling Technology (Beverly, MA, USA), anti-GFP (#sc-9996) obtained from Santa Cruz Biotechnology, and anti-β-actin (#A5316) antibody obtained from Sigma-Aldrich (Merck KGaA). Where indicated, the western blotting results were analyzed using ImageJ software (version 1.52a; National Institute of Health).

Statistical analysis

The data are expressed as the mean ± standard deviation of three independent experiments. Statistical differences were measured by Student's t-test for two groups or a one-way analysis of variance (ANOVA) for more than two groups followed by Tukey's test, using GraphPad Prism software (version 9.0; San Diego, CA, USA); diffenrences at $P<0.05$ were considered statistically significant.

Results

TRIB3 expression is upregulated in lung cancer tissues and cell lines

Lung cancer tissue qPCR panel was used to evaluate TRIB3 mRNA expression in lung cancer and normal lung tissues. The panel included cDNA from 48 tissues covering the four stages of lung cancer (stage I, n = 25; stage II, n = 6; stage III, n = 10; and stage IV, n = 2) and normal controls (n = 5). TRIB3 mRNA expression levels were higher in lung cancer tissue samples than in normal lung tissue samples (**Figure 1A**). We further analyzed TRIB3 mRNA expression in the human bronchial epithelial cell line HBE and the lung cancer cell lines H69, A549, H460, Lu99, H358, H1299, and EBC-1. Lung cancer cells showed higher TRIB3 mRNA expression compared with HBE cells (**Figure**
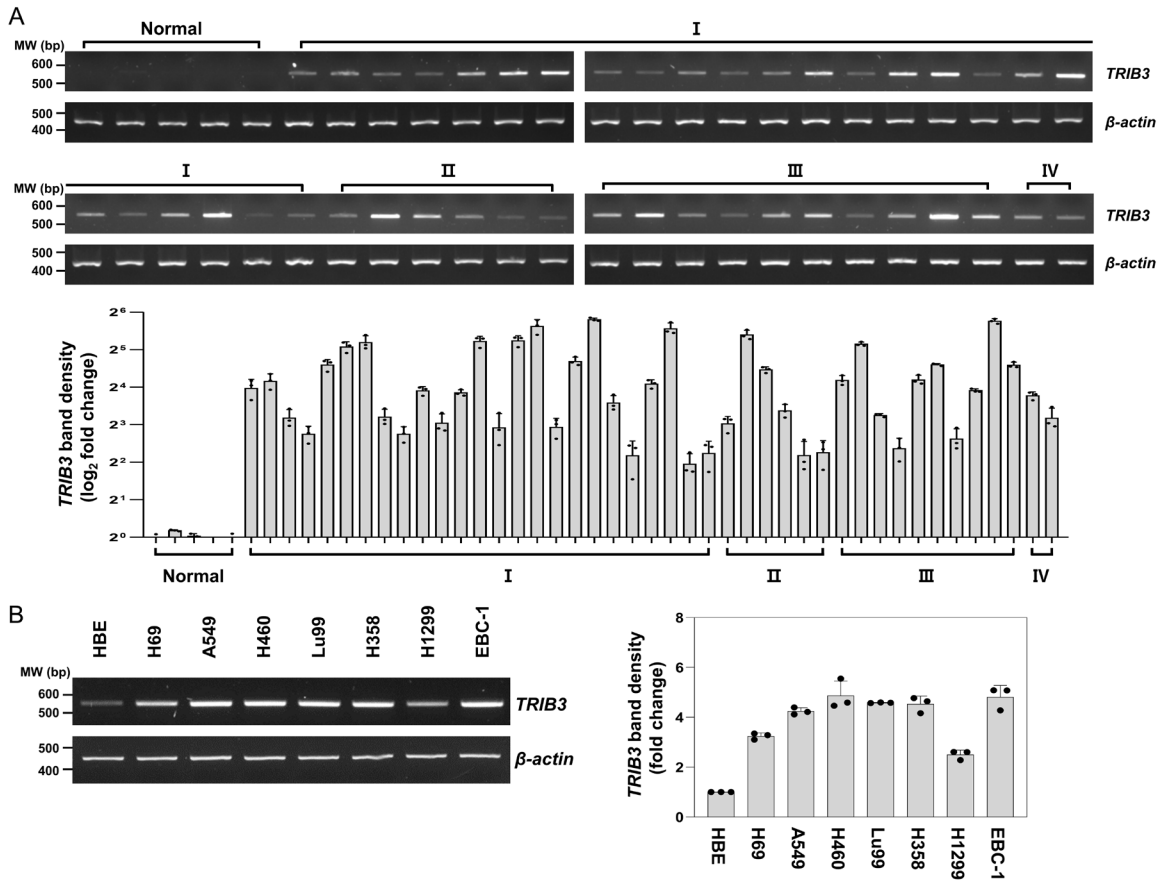
Figure 1. TRIB3 expression is elevated in lung cancer tissues and cells. A. TRIB3 mRNA levels were determined by PCR using an OriGene cDNA array consisting of normal lung tissue (n = 5) and lung cancer tissue of different pathological grades (stage I, n = 25; stage II, n = 6; stage III, n = 10; and stage IV, n = 2). PCR products were visualized on 2% agarose gel containing ethidium bromide (upper panel). The band intensities were quantified by ImageJ software, normalized to β-actin, and presented as log2-fold-change relative to normal lung tissue (low panel). B. TRIB3 mRNA levels of HBE, H69, A549, H460, Lu99, H358, H1299, and EBC-1 cell lines were assessed by RT-PCR analysis (left panel). Band intensities were quantified using ImageJ software, normalized to β-actin, and plotted as fold-change relative to HBE cell line (right panel).

1B). These data suggest that TRIB3 is upregulated in lung cancer tissues and cells.

TRIB3 expression under individual amino acid-deprivation conditions

TRIB3 serves as a stress sensor that responds to various tumor microenvironments [5-7]. We investigated TRIB3 mRNA expression in H1299 cells deprived of individual amino acid for 6 or 24 h, except for cystine, as 24 h deprivation of cystine caused considerable cell death, thus impeding RNA acquisition. Instead, we subjected the cells to cystine deprivation for 12 h. Compared to control cells, TRIB3 mRNA expression exhibited >5-fold increase in H1299 cells deprived of arginine, glutamine, lysine, or methionine for 6 or 24 h (**Figure 2A** and **2B**).

Additionally, H1299 cells subjected to isoleucine deprivation for 24 h showed >5-fold increase in TRIB3 mRNA expression compared to control cells (**Figure 2A** and **2B**). Deprivation of other the amino acids including asparagine, proline, serine, threonine, tryptophan, tyrosine, or valine also increased TRIB3 mRNA expression by 1.2-2.0-fold compared to control cells (**Figure 2A** and **2B**). In contrast, no noticeable change in TRIB3 mRNA expression was observed when the cells were deprived of aspartic acid, cystine, glutamic acid, glycine, histidine, hydroxyproline, leucine, or phenylalanine (**Figure 2A** and **2B**). These data suggest that TRIB3 expression varies in response to deprivation of individual amino acids in H1299 lung cancer cells.

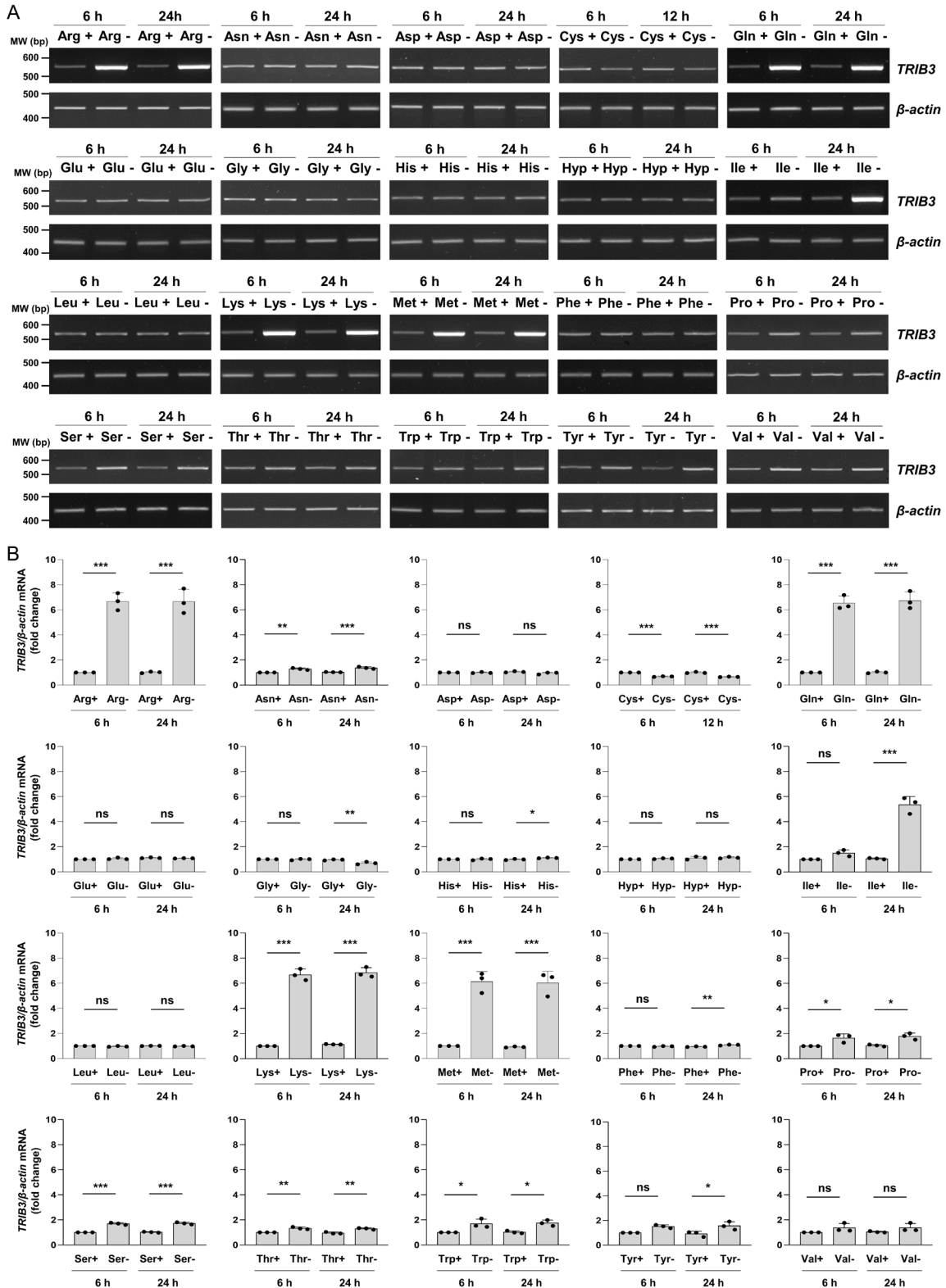# TRIB3 knockdown enhances the cell sensitivity to amino acid deprivation

Figure 2. TRIB3 mRNA expression in individual amino acid-deficient condition. A. H1299 cells were deprived of each of the 19 amino acids, excluding cystine, for 6 or 24 h, while cystine deprivation was conducted for 6 or 12 h. TRIB3 mRNA level was determined by using RT-PCR analysis. B. The band intensities of TRIB3 mRNA were quantified by ImageJ software, normalized to β-actin, and the data are presented as the fold change relative to CTL (n = 3; *P<0.05; **P<0.01; ***P<0.001; ns, not significantly different). Arg, arginine; Asn, asparagine; Asp, aspartic acid; Cys, cystine; Gln, glutamine; Glu, glutamic acid; Gly, glycine; His, histidine; Hyp, hydroxyproline; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine.
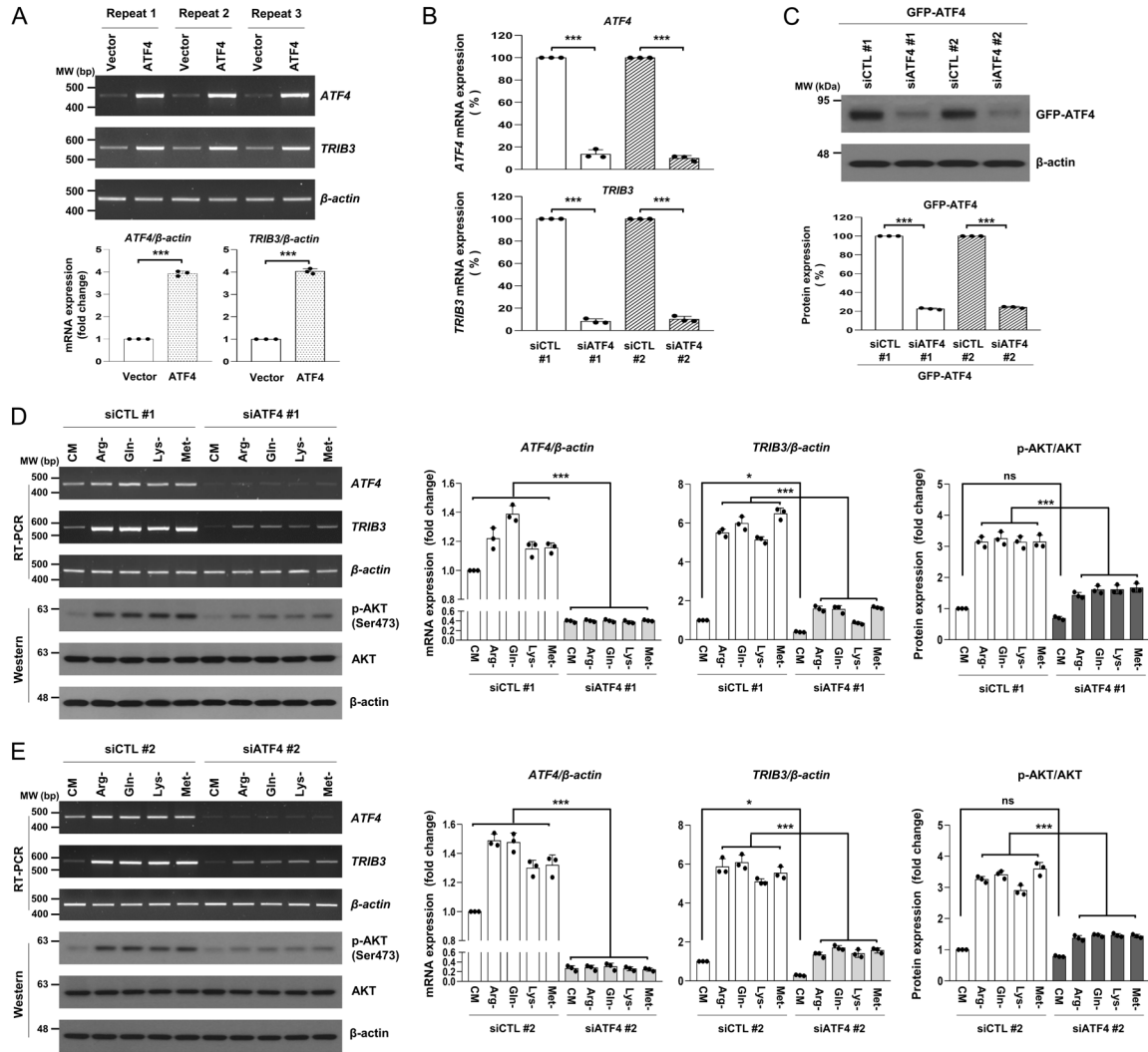
Figure 3. Amino acid deprivation upregulates the TRIB3 expression in an ATF4-dependent manner. A. H1299 cells were transfected with an empty vector or GFP-tagged ATF4 for 36 h. mRNA levels were assessed using RT-PCR analysis. B. H1299 cells were transfected with CTL or ATF4 siRNAs for 36 h. mRNA expression levels were determined by quantitative real-time PCR. Data were normalized to β-actin and presented as percentage of their respective controls. C. H1299 cells were co-transfected with CTL or ATF4 siRNAs along with GFP-ATF4 plasmids for 36 h. Protein levels were determined by western blot analysis. D, E. H1299 cells were transfected with CTL or ATF4 siRNAs for 24 h and then deprived of the indicated amino acids for 12 h. mRNA and protein band intensities were quantified using ImageJ software, normalized to β-actin, and plotted as fold-change relative to CTL (n = 3; *P<0.05; ***P<0.001; ns, not significantly different). Arg, arginine; CM, complete medium; CTL, control; Gln, glutamine; Lys, lysine; Met, methionine.

TRIB3 upregulation following amino acid deprivation is dependent on ATF4

Previous studies have revealed that the upregulation of TRIB3 during cellular stress is mediated by the binding of ATF4 to the TRIB3 promoter [5, 25]. To investigate the involvement of ATF4 in regulating TRIB3, we examined TRIB3 mRNA expression in H1299 cells transiently transfected with plasmids encoding GFP-ATF4 or siRNAs targeting ATF4. The transfection efficiency of ATF4 plasmids or siRNAs was confirmed by observing the corresponding increase or decrease in ATF4 mRNA levels (**Figure 3A** and **3B**). Additionally, we validated the transfection efficiency of ATF4 siRNAs by confirming the decrease in GFP-ATF4 protein levels in cells overexpressing GFP-tagged ATF4 (**Figure 3C**). TRIB3 mRNA levels were found to be upregulated by ATF4 overexpression, while downregu-

lated by ATF4 knockdown (**Figure 3A** and **3B**). Next, we investigated TRIB3 mRNA expression in H1299 cells whether ATF4 is critical for the upregulation of TRIB3 in response to arginine, glutamine, lysine, or methionine deprivation. ATF4 siRNA abrogated ATF4 induction following the deprivation of these amino acids (**Figure 3D** and **3E**). Furthermore, ATF4 knockdown resulted in the downregulation of TRIB3 mRNA expression induced by the deprivation of these amino acids (**Figure 3D** and **3E**). The increase in p-AKT Ser473 levels following deprivation of these amino acids was inhibited by ATF4 siRNA treatment (**Figure 3D** and **3E**). These data suggest that amino acid deprivation-induced TRIB3 expression is regulated by ATF4.

TRIB3 knockdown enhances the sensitivity of lung cancer cells to amino acid deprivation by suppressing AKT activation

To determine the role of TRIB3 in lung cancer cells, colony formation and cell viability assays were performed in H1299 cells following siRNA-mediated TRIB3 knockdown. The transfection efficiency of TRIB3 siRNAs was confirmed by observing the decrease in TRIB3 mRNA levels (**Figure 4A**). H1299 cells treated with TRIB3 siRNA exhibited decreased colony formation and cell viability compared to those treated with control siRNA (**Figure 4B** and **4C**). TRIB3 knockdown also enhanced the decrease in cell viability induced by deprivation of arginine, glutamine, lysine, or methionine (**Figure 4C**). Interestingly, TRIB3 knockdown decreased ATF4 mRNA expression induced by the deprivation of arginine, glutamine, lysine, or methionine (**Figure 4D** and **4E**). In addition, TRIB3 knockdown attenuated the upregulation of p-AKT induced by deprivation of these amino acids (**Figure 4D** and **4E**). To examine the impact of elevated AKT phosphorylation on cell viability under amino acid deprivation, we treated H1299 cells with MK-2206, an AKT inhibitor, in the absence of arginine, glutamine, lysine, or methionine. Previous studies have reported that MK-2206 exhibits antitumor activity alone and in combination with chemotherapy in solid tumors [26, 27]. As shown in **Figure 4F** and **4G**, MK-2206 inhibited AKT phosphorylation induced by deprivation of these amino acids and further enhanced the decrease in cell viability caused by their deprivation. These data suggest that TRIB3 knock-

down enhances the sensitivity of H1299 cells to amino acid deprivation by suppressing AKT activation.

TRIB3 knockdown enhances the sensitivity of lung cancer cells to V-9302

The small molecule V-9302, an inhibitor of the ASCT2 amino acid transporter, exhibits promising antitumor activity by interfering with glutamine uptake [28]. We investigated whether TRIB3 knockdown enhanced the sensitivity of H1299 lung cancer cells to V-9302. In cells treated with V-9302, we observed an increase in ATF4 and TRIB3 mRNA levels, accompanied by elevated levels of p-AKT Ser473 protein (**Figure 5A**). siRNA-mediated knockdown of TRIB3 not only attenuated the V-9302-induced upregulation of ATF4 mRNA and p-AKT protein, but also enhanced the V-9302-induced decrease in cell viability (**Figure 5B** and **5C**). These data suggest that TRIB3 knockdown enhanced the sensitivity of lung cancer cells to V-9302 by inhibiting AKT activation.

Discussion

Lung cancer is the leading cause of cancer morbidity and mortality [1, 29]. Although major progress has been made in understanding the mechanisms that lead to lung cancer and in developing new surgical and chemotherapeutic approaches, the clinical outcomes remain unsatisfactory and most patients eventually relapse, emphasizing the importance of identifying novel biomolecules and signaling pathways involved in lung cancer progression [30]. Emerging evidence suggests that TRIB3 expression is associated with poor prognosis and is higher in multiple cancers [8-12]. In the present study, TRIB3 mRNA expression was markedly higher in lung cancer tissues compared with normal tissues (**Figure 1A**). We also found that the expression of TRIB3 mRNA was increased in lung cancer cells, and that knockdown of TRIB3 in H1299 lung cancer cells reduced colony formation and cell viability (**Figures 1B**, **4B** and **4C**). These findings support previous studies showing that TRIB3 is overexpressed in lung cancers and functions as an oncogene [31].

Tumor cells have a higher amino acid demand than normal cells because they proliferate more rapidly [32]. Therefore, limiting the availability of amino acids constitutes an attractive
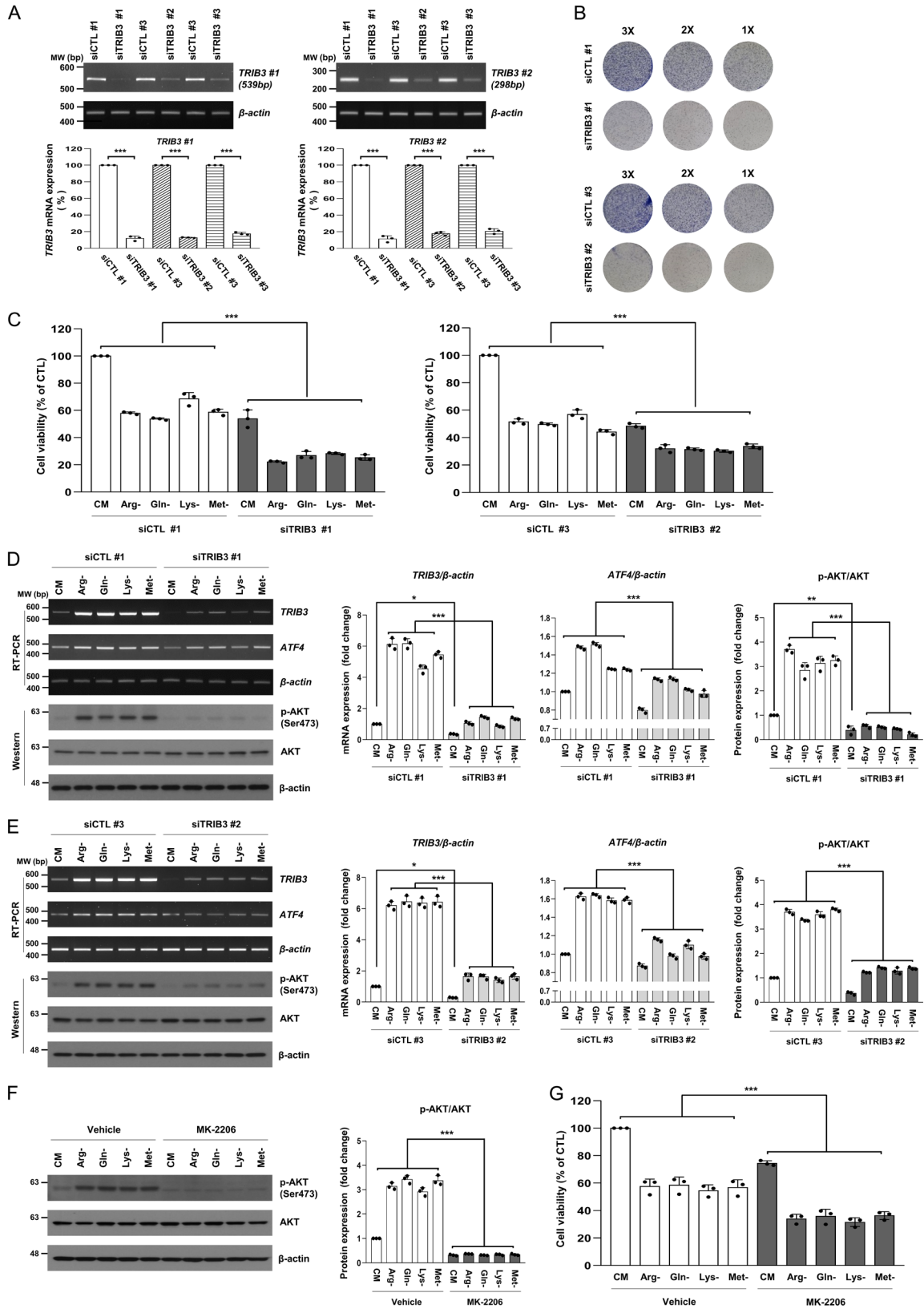
Figure 4. TRIB3 knockdown enhanced the sensitivity of lung cancer cells to amino acid deprivation by suppressing AKT activation. A. H1299 cells were transfected with CTL or TRIB3 siRNAs for 48 h. mRNA levels were assessed using RT-PCR analysis. B. H1299 were transfected with CTL or TRIB3 siRNAs for 24 h and then the transfected cells

were reseeded at a density of 1,000 (1×), 2,000 (2×), and 3,000 (3×) cells/well in a 6-well plate. Colony formation was monitored over the following ten days. C. H1299 cells were transfected with CTL or TRIB3 siRNAs for 24 h and then deprived of the indicated amino acids for 24 h. Cell viability was measured using the MTT assay. D, E. H1299 cells were transfected with CTL or TRIB3 siRNA for 24 h and deprived of the indicated amino acids for 12 h. mRNA and protein levels were assessed using RT-PCR and western blot analyses, respectively. F. H1299 cells were treated with 5 µM MK-2206 in combination with the indicated amino acid deprivations for 12 h. Protein levels were determined by western blot analysis. G. H1299 cells were treated with 5 µM MK-2206 in combination with the indicated amino acid deprivations for 24 h. Cell viability was measured using the MTT assay. mRNA and protein band intensities were quantified using ImageJ software, normalized to β-actin, and plotted as fold-change relative to CTL (n = 3; *P<0.05; **P<0.01; ***P<0.001). Arg, arginine; CM, complete medium; CTL, control; Gln, glutamine; Lys, lysine; Met, methionine.
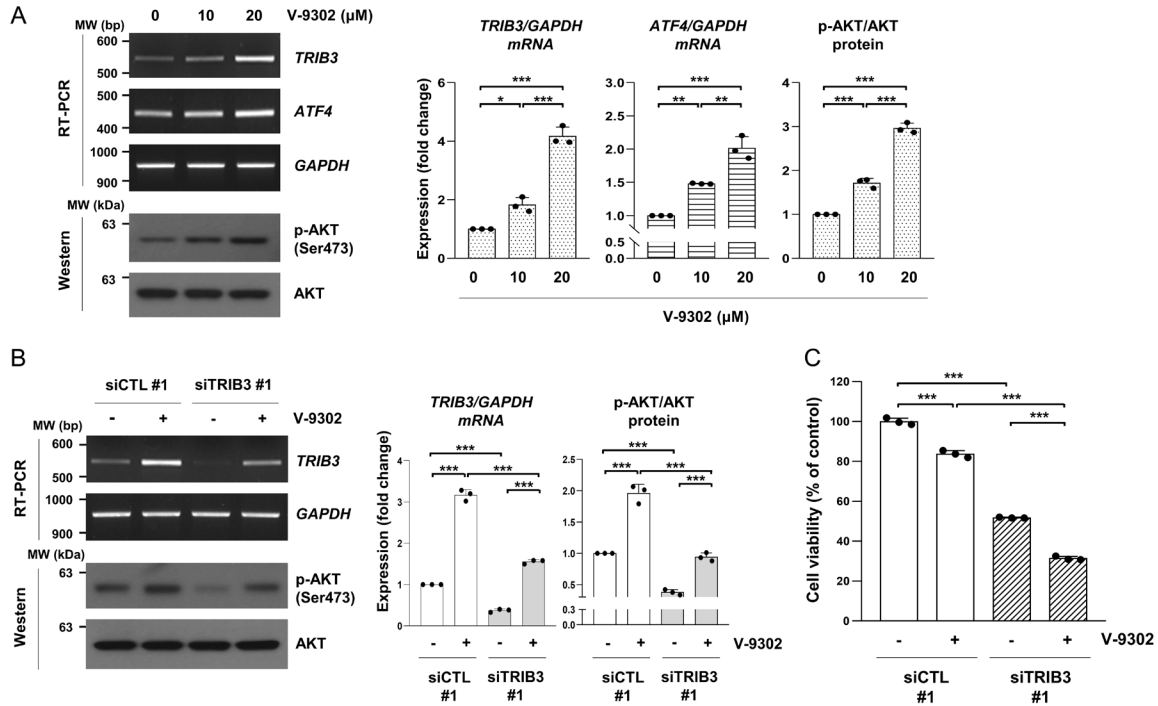

Figure 5. TRIB3 knockdown increases the sensitivity of lung cancer cell to V-9302 by suppressing AKT activation. A. H1299 cells were treated with the indicated concentrations of V-9302 for 12 h. B. H1299 cells were transfected with CTL or TRIB3 siRNA for 24 h, and then treated with 20 µM V-9302 for 12 h. C. H1299 cells transfected with CTL or TRIB3 siRNAs for 24 h were treated with 20 µM V-9302 for 24 h. Cell viability was measured using the MTT assay. mRNA and protein levels were assessed using RT-PCR and western blot analyses, respectively. mRNA and protein band intensities were quantified using ImageJ software, normalized to β-actin, and plotted as fold-change relative to CTL (n = 3; *P<0.05; **P<0.01; ***P<0.001).

option for cancer treatment. We investigated the expression of TRIB3 in H1299 lung cancer cells deprived of each of the twenty amino acids. TRIB3 mRNA expression significantly increased by >5-fold in H1299 cells deprived of arginine, glutamine, lysine, or methionine for 6 or 24 h, as well as isoleucine for 24 h, compared to control cells (**Figure 2A** and **2B**). Deprivation of each of 15 amino acids, excluding the aforementioned five amino acids, resulted in either a <2-fold increase in TRIB3 mRNA expression or no significant change in expression compared to controls (**Figure 2A** and **2B**). These data suggest that TRIB3 expression var-

ies in response to the deprivation of individual amino acids in lung cancer cells.

ATF4 is an important regulator of amino acid metabolism and is activated in response to amino acid deprivation [33, 34]. In diverse microenvironments, ATF4 binds to the C/EBP-ATF response element within the TRIB3 promoter, leading to the upregulation of TRIB3 [5, 12, 25]. Therefore, we investigated whether ATF4 is involved in amino acid deprivation-induced TRIB3 expression. ATF4 overexpression resulted in an increase in TRIB3 mRNA expression (**Figure 3A**), whereas the knock-

down of ATF4 led to a decrease in TRIB3 mRNA expression induced by the deprivation of arginine, glutamine, lysine, and methionine (**Figure 3D** and **3E**). These data suggest that ATF4 appeared to play a role in inducing TRIB3 expression under amino acid deprivation.

AKT activation is involved in the metabolic adaptation of cancer cells under extreme nutrient deprivation [35, 36]. Consistent with our prior study [37], a significant increase in AKT phosphorylation at Ser473 was observed in H1299 cells deprived of arginine, glutamine, lysine, or methionine (**Figure 3D** and **3E**). Additionally, increased AKT phosphorylation resulting from deficiencies in these amino acids was alleviated in H1299 cells following ATF4 silencing (**Figure 3D** and **3E**), suggesting that amino acid deprivation might induce ATF4, leading to AKT activation. Next, we investigated the involvement of TRIB3 in amino acid deprivation. TRIB3 knockdown reduced AKT phosphorylation in media lacking arginine, glutamine, lysine, or methionine (**Figure 4D** and **4E**). Interestingly, TRIB3 knockdown also diminished ATF4 mRNA expression induced by the deprivation of these amino acids (**Figure 4D** and **4E**). Moreover, TRIB3 knockdown significantly decreased cell viability in the absence of these amino acids (**Figure 4C**). Our findings contradict previous reports suggesting that TRIB3 inhibits AKT through direct binding and inhibition of phosphorylation [12, 13]. Shen et al. recently reported the involvement of TRIB3 in promoting the survival of oral squamous cell carcinoma cells through activation of AKT phosphorylation. This contradiction suggests that the role of TRIB3 in regulating AKT signaling might be context-dependent or multifaceted. Taken together, our findings suggest that TRIB3 is a pro-survival regulator of cell viability in amino acid-deficient tumor microenvironments, and is a promising target for lung cancer therapy.

Acknowledgements

Disclosure of conflict of interest

None.

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