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TRAF1 is critical for regulating the BRAF/MEK/ERK pathway in non-small cell lung carcinogenesis

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Abstract

Tumor necrosis factor receptor (TNFR)-associated factor 1 (TRAF1) is a unique TRAF protein that can interact directly or indirectly with multiple TNFR family members, regulatory proteins, kinases, and adaptors that contribute to its diverse functions in specific tissues. However, the role of TRAF1 in non-small cell lung cancer (NSCLC) remains unknown. In this study, we report that TRAF1 is overexpressed in human lung cancer cells and tissues. TRAF1 expression level inversely correlated with patient survival probability. Loss of TRAF1 decelerated tumor invasion in a urethane-induced lung carcinogenesis mouse model. Furthermore, TRAF1 expression affected TRAF2-mediated BRAF Lys48-linked ubiquitination, which was followed by the inhibition of growth and differentiation, and the induction of death in lung cancer cells. Overall, our work suggests that TRAF1 plays a novel role in the regulation of the BRAF/MEK/ERK signaling pathway in NSCLC and offers a candidate molecular target for lung cancer prevention and therapy.

Keywords

TRAF1; NSCLC; BRAF; ubiquitination; urethane-induced lung cancer

Introduction

Lung cancer is a leading cause of death worldwide. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancers, which is considered to be an insidious disease because it produces no symptoms until it is well advanced (1,2). The aggressiveness of NSCLC and its resistance to common therapies remain intractable issues. Hence, the

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elucidation of mechanism-based biomolecules underlying the initiation and progression of lung cancers and the identification of novel therapeutic targets are clinically very important.

Tumor necrosis factor receptor (TNFR)-associated factors (TRAFs) are signaling intermediates for TNFR superfamily members and are known to modulate various signaling pathways, such as the MAPK, NF-κB and Akt pathways (3–6). In contrast to other family members (TRAF2-7), the TRAF1 protein lacks an N-terminal RING finger, and the zinc fingers are not arranged in CART (C-rich associated with RING and TRAF) domains (7). The unique structure of TRAF1 does not diminish its functions and TRAF1 reportedly can interact directly or indirectly with multiple TNFR family members, regulator proteins, kinases, and adaptors, thereby contributing to its diverse functions in specific tissues under physiological conditions (6). Evidence has demonstrated that TRAF1 is involved in a number of signaling interactions and its expression is up-regulated in some human cancers (8). However, the functional roles of TRAF1 in those processes have not been fully understood and especially are less well studied in lung carcinogenesis.

BRAF is a member of the RAF family of serine/threonine protein kinases, which also includes ARAF and CRAF (9). Among these RAF members, BRAF is the most frequently mutated *RAF* oncogene in many cancers. It serves as a central intermediate in the mitogen-activated protein kinase (MAPK) pathways, participating in the control of various cellular processes, including proliferation, differentiation, angiogenesis, senescence, and apoptosis (10). Additionally, MEK1 and MEK2 are the only known substrates of BRAF compared with other RAFs, making BRAF a preferential candidate for investigating the effects of MAPK signal transduction in tumorigenesis (11,12). Approximately 0.8%-8% BRAF mutations are reportedly found in lung carcinomas. The majority of BRAF mutations are V600E, which are present in approximately 1.3% of NSCLCs (13). Therefore, the degradation of BRAF induced by targeting a potential pathogenic gene (i.e., *TRAF1*), which is related to NSCLC, might assist in the treatment of lung cancers in clinical practice. Until now, a relationship between TRAF1 and BRAF has not been reported.

In the present study, we examined the function of TRAF1 in lung carcinogenesis. We demonstrated a role for TRAF1 in NSCLC proliferation and apoptosis. Then we determined the mechanisms of TRAF1 in the regulation of lung carcinogenesis. Moreover, we identified an oncogenic role of TRAF1 in a urethane-induced lung cancer model using TRAF1 knockout mice. Our findings suggested that TRAF1 could be a novel target in lung carcinogenesis.

Materials and Methods

Reagents and antibodies

Cell culture media, gentamicin, penicillin, and L-glutamine were all obtained from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) was from Gemini Bio-Products (West Sacramento, CA). Tris, NaCl, and SDS for molecular biology and buffer preparation were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies to detect TRAF1 (sc-1830), β-Actin (sc-47778), BRAF (sc-166), GAPDH (sc-25778), NF-κB (p50) (sc-7178), Bcl-2 (sc-7382) and Lamin B (sc-6216) were from Santa Cruz Biotechnology,

Inc. (Santa Cruz, CA). Anti-V5 (R96025), V5-HRP (R961-25) and His-HRP (R94125) were from Invitrogen. Anti-HA (901503) was obtained from Covance (Emeryville, CA) and anti-HA-HRP (12013819001) was purchased from Roche (Indianapolis, IN). Anti-Flag (F-3165) was from Millipore. p-BRAF (#2696), NF-κB (p65) (#3034), p-MEK (#9121), MEK (#9122), p-ERK (#9101), ERK (#9102), p-c-Jun (#2361), c-Jun (#9165), p-c-Fos (#5348), c-Fos (#2250), caspase-3 (#9662), c-caspase-3 (#9661), PARP (#9542), c-PARP (#9541) and Bax (#2772) antibodies were purchased from Cell Signaling Technology (Danvers, MA).

Construction of expression vectors

Expression constructs, including Flag-BRAF, V5-TRAF1, Flag-TRAF2, HA-Lys-48-ubiquitin were obtained from Addgene (Cambridge, MA). Packaging vectors, containing pMD2.0G, psPAX, or pcDNA3.1 were also from Addgene and the pcDNA4/His max vector was obtained from Invitrogen. To construct the His-tagged expression vector of TRAF2 and HA-tagged expression vector of BRAF, the DNA sequences corresponding to His-TRAF2 (aa 1-98), His-TRAF2 (aa 99-271) and His-TRAF2 (aa 272-501), the regulatory domain of HA-BRAF (aa 1-400) and kinase domain of HA-BRAF (aa 401-766) were amplified by PCR. The HotStarTaq Master Mix Kit (Qiagen, Cat. No. 203443) was used for PCR. We designed specific primers for His-TRAF2 (aa 1-98) F: 5′AAGGATCCAGATGGCTGCAGCTAGC GTGACCC3′, R: 5′AACTCGAGTCACCTGCGGGCAGCATTATCTGGG3′; His-TRAF2 (aa 99-271) F: 5′AAGGATCCAG ATGGAGGTGGAGAGCCTGCCG3′, R: 5′AAGCGGCCGCTC ACCTCTGCAGGAGCTCTGACC3′; His-TRAF2 (aa 272-501) F: 5′AAGGATCCAGATGTG CGAGAGCCTGGAGAAGAAGACG3′, R: 5′AACTCGAGTTAGAGCCCTGTCAGGTCCA CAATG3′; HA-BRAF (aa 1-400) F: 5′AACTCGAG ATGGCGGCGCTGAGCGGTGGC3′, R: 5′AAGGATCCTCAAGCAGACAAACCTGT GGTTGATCCTCCATCAC3′; HA-BRAF (aa 401-766) F: 5′AATCTAGAATGACCCCCCCTGCCTCATTACC3′, R: 5′AAGGTACCTCAG TGGACAGGAAACGCACCATAT3′. The His-TRAF2 (aa 1-98) and His-TRAF2 (aa 272-501) PCR products were digested with BamH I/Xho I and the His-TRAF2 (aa 99-271) PCR product was digested with BamH I/Not I following instructions provided by the manufacturer. The constructs were then inserted into the corresponding sites of pcDNA4/His max (Invitrogen) to generate the expression plasmids encoding His-TRAF2 (aa 1-98), His-TRAF2 (aa 99-271) and His-TRAF2 (aa 272-501). To generate an expression vector for HA-tagged BRAF, we amplified BRAF DNA by PCR and then the HA-BRAF (aa 1-400) PCR product was digested with Xho I/BamH I and the HA-BRAF (aa 401-766) PCR product was digested with Xba I/Kpn I following instructions provided by the manufacturer and inserted into the corresponding sites of pcDNA3.1 from Addgene. Additionally, the lentivirus plasmids shTRAF1 (#1, TRCN00000 56885; 5′-AAACCCAGGGCTGCCTTGGAAAAG-3′, #2 TRCN0000056883; 5′-AAACCCAG GGCTGCCTTGGAAAAG-3′) were purchased from GE Healthcare Dharmacon (Open Bio System). The pLKO.1-puro Non-Target shRNA Control Plasmid DNA (shNT) was purchased from Sigma-Aldrich. All constructs were confirmed by restriction enzyme mapping, DNA sequencing and the Blast program.

Cell culture and transfection

All cells were purchased from American Type Culture Collection (ATCC; Manassas, VA) except the gefitinib-resistant HCC827 (HCC827GR5) cell line, which was kindly provided by Dr. Pasi A. Jänne (Harvard Medical School, Boston, MA) and authenticated by the Dr. Pasi A. Jänne group (14). The cells were routinely screened to confirm mycoplasma negative status and to verify the identity of the cells by Short Tandem Repeat (STR) profiling before being frozen. Each vial was thawed and maintained for a maximum of 2 months. Enough frozen vials of each cell line were available to ensure that all cell-based experiments were conducted on cells that had been tested and in culture for 8 weeks or less. Cells were cultured at 37°C in a 5% CO_2 humidified incubator following the ATCC protocols. MRC5 human normal lung fibroblasts were grown in Eagle's minimum essential medium (MEM) with 10% fetal bovine serum (FBS) and 1% antibiotics. A549 human lung cancer cells were cultured with F-12K medium containing 10% FBS and 1% antibiotics. All other human lung cancer cells were grown in RPMI-1640 medium supplemented with 10% FBS and 1% antibiotics. HEK293T cells (stably expressing the SV40 large T antigen in HER293 cells) were purchased from the American Type Culture Collection and cultured at 37°C in a humidified incubator with 5% CO_2 in Dulbecco's modified Eagles's medium (DMEM, Corning; Manassas, VA) supplemented with 10% FBS (Corning) and 1% penicillin streptomycin (Gen DEPOT; Barker, TX). When cells reached 60% confluence, transfection was performed using iMFectin DNA Transfection Reagent (GenDEPOT, Inc.) following the manufacturer's instructions. The cells were cultured for 36 to 48 h and proteins were extracted for further analysis.

Lentiviral infection

To generate knockdown TRAF1 cells, the lentiviral expression vector of TRAF1 (shTRAF1) or pLKO.1-puro Non-Target shRNA Control Plasmid DNA (shNT) and packaging vectors (pMD2.0G and psPAX) were transfected into HEK293T cells using iMfectin Poly DNA transfection reagent (GenDEPOT) following the manufacturer's suggested protocols. The transfection mix in 10% FBS/DMEM without antibiotics was incubated with cells for 12 h, then 10 mL of fresh medium with antibiotics (penicillin/streptomycin) were added. Viral supernatant fractions were collected at 48 h and filtered through a 0.45 μm syringe filter followed by infection into the appropriate cells together with 8 μg/mL polybrene (Millipore, Billerica, MA). After infection overnight, the medium was replaced with fresh complete growth medium containing the appropriate concentration of 1.5 μg/mL puromycin and cells incubated for an additional 24 h. The selected cells were used for experiments.

Ubiquitination assay

The ubiquitination assay was performed as described previously (15). HEK293T cells were transfected with combinations of expression vectors, including V5-TRAF1, Flag-TRAF2, Flag-BRAF, and HA-Lys48-ubiquitin. At 48 h after transfection, cells were treated with MG132 (20 μM) for 4 h before harvest. The proteins were extracted using lysis buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 1% SDS, 1 mM DTT) and boiled for 10 min before cellular debris were removed by centrifugation. The lysates were immunoprecipitated as

described above and IP was conducted with anti-BRAF. Bound proteins were eluted in SDS sample loading buffer and subjected to immunoblotting.

Quantitative real-time PCR

Knockdown TRAF1 and control H1299 lung cancer cells (1×10^6) were plated into 100-mm dishes, cultured overnight and then harvested. Trizol Reagent (Invitrogen) was used for total RNA extraction and *TRAF1* gene expression was analyzed with 100 ng of total RNA. TRAF1-specific real-time primer was: F:5′CTACTGTTTTCCTTTACTTACTACACCTC AGA-3′; R:5′ATCCAGACAACTGTTCAAACTGATG-3′; and a glyceraldehyde 3-phosphate dehydrogenase-specific real-timer primer was: F: 5′CTCTGCTCCTCCTGTTCGAC3′; R: 5′GCCCAATACGACCAAATCC3′. These were amplified by quantitative one-step real-time PCR using the TaqMan RNA-to-C_T 1-step kit (Applied Biosystems, Foster City, CA) following the manufacturer's suggested protocols. The C_T values of *TRAF1* gene expression were normalized with the C_T values of *glyceraldehyde 3-phosphate dehydrogenase* as an internal control to monitor equal RNA utilization.

Animals and carcinogen treatment

All animal studies were performed and approved by the University of Minnesota Institutional Animal Care and Use Committee (IACUC). BALB/c wild-type (WT) and BALB/c TRAF1 knockout (TRAF1 KO) mice were purchased from the Jackson Laboratory. The mice were housed and bred under virus- and antigen-free conditions. Mice were genotyped by standard PCR analysis according to the Jackson Laboratory genotyping protocol with 5′-GCCAGAGGCCACTTGTGTAG-3′, 5′-CAGAACCCCTTGCCTAATCC-3′ and 5′-TCCTAGAGGCCTGCTGCTAA-3′ as the primers. Mice (6 weeks old) were divided into 4 groups: 1) WT-vehicle-treated; 2) TRAF1 KO-vehicle-treated (6 males and 6 females each group); 3) WT-urethane-treated; 4) TRAF1 KO-urethane-treated (11 males and 11 females each group). The urethane-treated groups were subjected to a single intraperitoneal (i.p.) injection of urethane (1g/kg in 1 × PBS, Sigma) or vehicle (1 × PBS) once a week for 7 weeks. Mice were monitored every day and weighed once a week. Mice were euthanized by CO_2 asphyxiation at 6 months after the first injection of urethane or when moribund. Tumors macroscopically visible on the pleural surface of the lungs were counted and lungs were harvested for further analysis. Tissue lysates were prepared from pooled lung tumor nodules or normal lung tissue from each mouse of each group. Three sets were prepared for each group and each lane shows 1 set of pooled samples by Western blotting.

Protein-protein docking of BRAF and TRAF1/2

First the three-dimensional (3-D) structures of BRAF and TRAF were downloaded from the Protein Data Bank (PDB) (16). The PDB entries are 1UWH (17) for BRAF and 3M0D (18) for TRAF1/2. The 3-D First Fourier Transform (FFT)-based protein docking algorithm of HEX 8.00 (19) was then used for docking experiments to determine the possible binding mode between BRAF and TRAF1/2. We selected 100 sorted docked configuration possibilities for further analysis.

Immunohistochemical analysis of a tissue array and mouse lung tissues

A human lung tissue array (BC041115C) was purchased from US Biomax, Inc. (Rockville, MD). A Vectastain Elite ABC Kit obtained from Vector Laboratories (Burlingame, CA) was used for immunohistochemical staining according to the protocol recommended by the manufacturer. Mouse lung tissues were embedded in paraffin for examination. Sections were stained with hematoxylin and eosin (H&E) and analyzed by immunohistochemistry. Briefly, all specimens were deparaffinized and rehydrated. To expose antigens, samples were unmasked by submerging each into boiling sodium citrate buffer (10 mM, pH 6.0) for 10 min, and then treated with 3% H_2O_2 for 10 min. Each slide was blocked with 10% goat serum albumin in 1 × PBS in a humidified chamber for 1 h at room temperature. Then, slides were incubated with a TRAF1 antibody (1:100) and mouse lung tissue sections were hybridized with BRAF (1:100), c-Jun (1:100), or phosphorylated c-Jun (1:50) at 4°C in a humidified chamber overnight. The slides were washed and hybridized with the secondary antibody from Vector Laboratories (anti-rabbit 1:150 or anti-mouse 1:150) for 1 h at room temperature. Slides were stained using the Vectastain Elite ABC Kit (Vector Laboratories, Inc.). After developing with 3,3′-diaminobenzidine, the sections were counterstained with hematoxylin and observed by microscope (×200) and then analyzed using the Image-Pro PLUS (v.6) computer software program (Media Cybernetics, Inc.).

Additional methods

Immunoblotting, immunoprecipitation, and Western blotting were performed using standard techniques. Proliferation was estimated by MTS assay anchorage-independent growth was measured by soft agar assay. Flow cytometry was used to assess levels of apoptosis. Additional details are included in Supplementary Materials and Methods.

Statistical analysis

All quantitative data are expressed as mean values ± standard deviation (S.D.) of at least 3 independent experiments or samples. Significant differences were determined by a Student's *t* test or one-way ANOVA. A probability value of $p < 0.05$ was used as the criterion for statistical significance.

Results

TRAF1 is overexpressed in human NSCLC and associated with NSCLC patient survival probability

NSCLC is the most common type of all lung cancers and numerous oncogenes are associated with this disease. TRAF1 is a unique member amongst the TRAF family that can be activated and its mRNA expression is restricted to spleen, lung, and testis in mouse tissues (20). However, TRAF1 expression in human NSCLC has not been well defined. Initially, we determined the protein expression level of TRAF1 in lung cancer cell lines and in a human lung cancer tissue array. Results indicated that increased expression of TRAF1 occurs in several human NSCLC cell lines compared with normal MRC5 lung cells (Fig. 1A). In addition, the tissue array analysis confirmed that TRAF1 is overexpressed in lung adenocarcinoma and squamous cell carcinomas (Fig. 1B). Analysis of the TCGA database

also showed that TRAF1 is highly expressed in primary tumors (Fig. 1C). These data suggest that TRAF1 might play an important role in lung tumorigenesis. We used KM plotter to analyze the association between elevated TRAF1 expression and patient survival probability (Fig. 1D, E). Results clearly indicate that the survival probability of patients with high TRAF1 expression is significantly lower than patients with low TRAF1 expression. Findings also suggest that TRAF1 is a critical molecule in lung carcinogenesis and is associated with lung cancer patient survival probability.

Knockdown of TRAF1 attenuates proliferation of NSCLC cells

Aberrant cell proliferation is a hallmark of cancer and elimination of uncontrolled cell differentiation contributes to cancer treatment (21). In this study, we hypothesize that TRAF1 might be a crucial controller of cell growth, thereby modulating cell transformation. Therefore, to examine the function of TRAF1 in NSCLC, we used two different shTRAF1 sequences to generate TRAF1 knockdown H1299 and H1975 lung cancer cells (Fig. 2A). MTS and anchorage-independent cell growth assays were performed to evaluate the effect of knocking down TRAF1 expression on cell transformation. The results show that TRAF1 silencing decreased the absorbance reading at 492 nm, indicating that cell proliferation is attenuated (Fig. 2B). However, knockdown of TRAF1 in H358 cells, which express a very low level of TRAF1, did not significantly affect the cell proliferation (Supplementary Fig. S1A and B). Similarly, colony formation was reduced in TRAF1 knockdown cells (Fig. 2C), which indicated that blocking TRAF1 expression dramatically reduces cell transformation ability.

Knockdown of TRAF1 induces cellular apoptosis

During the progress of transformation, cell growth is regulated not only by proliferation, but also by cell death or apoptosis. Evidence indicates that many TRAFs play roles in coordinating apoptosis through the NF-κB pathway (22). Although TRAF1 might regulate cell death indirectly, it was also identified as an inducer of NF-κB that might form a protein complex that directly blocks activation of apoptosis (23). Apparently, TRAF1 is up-regulated in cancer cells enhancing its anti-apoptotic function. In this report, our apoptosis assay results showed that TRAF1 silencing resulted in increased apoptosis in both H1299 and H1975 lung cancer cells (Fig. 3A, B). In contrast, knocking down TRAF1 expression did not significant affect apoptosis in H358 cells, which express very low levels of TRAF1 (Supplementary Fig. S1C). Concurrently, we examined a special class of proteases inside the cell that are associated with apoptosis. Not surprisingly, H1299 and H1975 lung cancer cells infected with shTRAF1 generated higher levels of pro-apoptotic proteins, including c-caspase-3, PARP and Bax and lower levels of anti-apoptotic Bcl-2 (Fig. 3C). Overall, TRAF1 is involved in intracellular signaling pathways that modulate cell survival and confer resistance to apoptosis.

Knocking down TRAF1 decreases BRAF protein levels followed by downstream inhibition of signaling

The RAF-MEK-ERKs cascades and downstream signaling to NF-κB, c-Jun, and c-Fos can result in changes in cell proliferation, differentiation, or apoptosis (23–26). Interestingly, our findings revealed that knocking down TRAF1 expression in H1299 and H1975 lung cancer

cells impaired BRAF phosphorylation and also decreased the total protein level of BRAF, which led to restraint of downstream MEKs and ERKs activation (Fig. 4A). In addition, results indicated that after knocking down TRAF1, both cytosolic levels of NF-κB and its nuclear translocation were diminished (Fig. 4B). Moreover, knocking down TRAF1 in H1299 and H1975 lung cancer cells resulted in a reduction of c-Fos and c-Jun expression (Fig. 4C). These results suggest that the decrease in NF-κB, c-Fos, and c-Jun occurred after inhibition of the BRAF-MEK-ERKs cascades when blocking TRAF1 in NSCLC cells. Our results indicate that decreased BRAF expression could be a key regulatory element in the function of TRAF1 in NSCLC. Next, we examined the expression level of BRAF in lung cancer cell lines and results indicated that BRAF is also highly expressed in lung cancer cells compared with MRC5 normal lung cells (Fig. 4D). Knockdown of TRAF1 decreased the protein level of BRAF (Fig. 4A), but did not affect the RNA expression level (Supplementary Fig. S2). This suggested that the regulation of TRAF1 in lung cancer development might be involved in BRAF ubiquitination and degradation.

TRAF1 blocks TRAF2-mediated BRAF Lys48-linked ubiquitination

Protein ubiquitination is an essential post-translational modification that regulates a multitude of cellular functions, including cell cycle, differentiation, and signal transduction (27,28). Ubiquitination is sequentially modulated by 3 classes of proteins, including E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases (29). E3 ligases fall into two main families, containing either a RING (i.e., really interesting new gene) finger domain or a HECT (i.e., homologous to E6-AP COOH terminus) domain. Structurally, TRAF1 does not have an N-terminal RING finger domain, and therefore cannot act as an E3 ligase to directly regulate BRAF protein degradation. Therefore, identifying a “bridge” between TRAF1 and BRAF was considered to be a priority in this study. Many reports indicate that TRAF1 and TRAF2 preferentially form the TRAF1:$(TRAF2)_2$ heterodimeric complex that is involved in various cellular physiologies (18,20,30). In addition, TRAF2 has been shown to act as an E3 ligase that mediates ubiquitination of several proteins (31–33), which suggested that TRAF2 might possibly be an E3 ligase of BRAF that is involved in the process of protein degradation.

To investigate the relationship between TRAF1, TRAF2 and BRAF, the endogenous BRAF and TRAF1 or BRAF and TRAF2 interaction was studied in H1299 cells (Fig. 5A). The result demonstrated that TRAF1 is a protein-binding partner with TRAF2, but not BRAF, whereas TRAF2 can bind with BRAF. The relationship between TRAF1, TRAF2 and BRAF was studied by exogenously expressing these proteins in HEK293T cells. The results showed that TRAF1 decreased the binding affinity between TRAF2 and BRAF (Fig. 5B). We found that TRAF2 (aa 99-271; aa 272-501; Fig. 5C) can bind with the kinase domain of BRAF (aa 401-766; Fig. 5D). Moreover, the protein-docking model also showed that TRAF2 could bind with BRAF. Additionally, the binding range is similar to the binding range between TRAF2 and TRAF1, which indicates that TRAF1 can attenuate the binding affinity between TRAF2 and BRAF (Fig. 5E). We then used FastContact 2.0, a free energy scoring tool for protein-protein complex structures, to analyze the free energy of the docking data (34). The results showed that the complex of TRAF2 and BRAF is characterized by an electrostatic energy of −15.73 kcal/mol and a desolvation free energy of 11.42 kcal/mol. The

complex of TRAF1 and TRAF2 is characterized by an electrostatic energy of −30.73 kcal/mol and a desolvation free energy of -24.68 kcal/mol. Finally, the complex of TRAF1/TRAF2 and BRAF is characterized by an electrostatic energy of 0.11 kcal/mol and a desolvation free energy of 0.00 kcal/mol. Based on this information, we found that the binding affinity between TRAF1 and TRAF2 is higher than the binding affinity between TRAF2 and BRAF. If TRAF1 binds with TRAF2, the TRAF1/TRAF2 complex will have difficulty binding with BRAF. This analysis provides us with an explanation as to why TRAF1 can disrupt the BRAF/TRAF2 interaction and regulate BRAF stability.

Evidence indicates that BRAF expression levels can be mediated by both Lys63- and Lys48-linked polyubiquitination followed by proteasome-dependent activation or degradation (35,36). Based on the formation of TRAF1/TRAF2 heterodimeric complexes and the interaction between TRAF2 and BRAF, we also determined that TRAF1 could decrease the TRAF2-mediated BRAF Lys48-linked ubiquitination (Fig. 5F), but did not affect Lys63-linked ubiquitination (Supplementary Fig. S3). These results indicated that TRAF2 is a novel E3 ligase of BRAF and the involvement of TRAF1 leads to decreased BRAF ubiquitination. To examine BRAF stability, the shNT- and shTRAF1-transfected H1299 and H1975 cells were cultured and treated with cyclohexamide (CHX). Cells were harvested and protein levels were visualized by Western blotting. The results showed that the half-life of the BRAF protein in TRAF1-deficient H1299 cells was around 40 min, whereas the half-life of the BRAF protein in control H1299 cells was around 60 min (Fig. 5G). The half-life of the BRAF protein in TRAF1-deficient H1975 cells was around 50 min, whereas the half-life of the BRAF protein in control H1975 cells was around 100 min (Supplementary Fig. S4). Overall, TRAF1 binding with TRAF2 decreases BRAF ubiquitination and stabilizes BRAF, which activates MEK and ERKs mediation of lung cancer cell growth, apoptosis and lung tumorigenesis (Figure 5H).

Loss of TRAF1 attenuates tumor invasion in urethane-induced lung carcinogenesis

The findings thus far prompted us to investigate the functional significance of TRAF1 in lung tumorigenesis. Therefore, we used a urethane-induced lung carcinogenesis mouse model to confirm our hypothesis that TRAF1-deficient mice would be more susceptible to chemical induction of lung tumors compared to wild-type mice. Urethane is a chemical carcinogen present in tobacco leaves and tobacco smoke. Urethane-induced lung cancer has been well-characterized and accepted as a model for human lung adenocarcinoma. In lung cancer research, the urethane model of lung cancer has been widely used, particularly in BALB/c mice (37,38). In the present study, BALB/c wild-type (WT) and BALB/c TRAF1 knockout (TRAF1 KO) mice received 7 consecutive weekly i.p. injections of 1 g/kg urethane and TRAF1 WT and KO control mice received vehicle (1 × PBS) once a week for 7 weeks. The results indicated that at 6 months after the first urethane administration, a dramatically decreased number of lung tumors occurred in TRAF1 KO mice compared with WT mice (Fig. 6A, Supplementary Fig. S5A). Tumor multiplicity averaged 5.7 ± 1.7 tumors in the TRAF1 KO mice, but 31.4 ± 6.4 in WT mice (***$p < 0.001$) with no significant difference between males and females (Fig. 6B). Not surprising, Western blot data revealed that the protein levels of p-BRAF, BRAF, p-MEK, p-ERKs, p-c-Jun, and c-Jun were substantially decreased in the tumor tissues from the TRAF1 KO mice compared to WT mice (Fig. 6C).

Additionally, the histological examination after hematoxylin and eosin (H&E) staining showed that the tumors in WT mice lost normal alveolar architecture, exhibited an increased nuclear/cytoplasmic ratio, and cytologic atypia, which were identified as adenomas. However, tumors from TRAF1 KO mice displayed only a few adenomas, and lungs from this group retained a majority of the normal alveolar architecture (Fig. 6D). Additionally, the expression of BRAF, p-c-Jun and c-Jun was detected and analyzed by immunohistochemistry. Results indicated that TRAF1 KO mice showed lower expression of these markers compared with WT mice (Fig. 6D). Although the body weight of the mice in the urethane-treated groups showed no significant difference (Supplementary Fig. S5B), the WT-urethane–treated mice showed signs of illness as evidenced by overall behavior, and the survival rate of this group was lower than the control groups ($*p < 0.05$; Supplementary Fig. S5C). In contrast, the survival rate of the TRAF1 KO-urethane group was not significantly different compared with the normal control group. The TRAF1 expression in all lung tissue of all mice was determined by PCR (Supplementary Fig. S5D). Overall, our findings indicated that inhibition of TRAF1 signaling limited tumor invasion in urethane-induced lung carcinogenesis.

Discussion

Lung cancer is the leading cause of cancer death worldwide. Targeted therapy has emerged as an impressive approach for lung cancer that depends on activated oncogenes and the downstream signaling cascades. The present study demonstrates that TRAF1 is a critical molecule in NSCLC development. TRAF1 might a potential therapeutic target for NSCLC.

TRAF1 is a crucial adaptor molecule that plays a role in the assembly of receptor-associated signaling complexes. Compared with other members of TRAF family, *TRAF1* mRNA expression is only detectable in a few tissues, including lung, spleen, and testis (20). These findings suggested that TRAF1 is unique and may function in specific organs. Our results showed that TRAF1 is overexpressed in human lung cancer cell lines and tissues (Fig. 1). To study the function of TRAF1, we generated stable TRAF1 knockdown H1299 and H1975 lung cancer cell lines. We demonstrated that silenced TRAF1 dramatically suppresses proliferation (Fig. 2) and induces apoptosis (Fig. 3) of lung cancer cells. These results suggest that TRAF1 is required for the malignant phenotype of human lung cancer cells and thus a better understanding of the mechanisms by which TRAF1 regulates lung cancer development is needed.

Evidence indicates that TRAF1 activation requires RAF1 activation, and RAF1 can form heterodimers with BRAF, which contribute to the activation of the ERKs pathway (39,40). Interestingly, our findings revealed that knocking down TRAF1 expression in H1299 and H1975 lung cancer cells impaired BRAF phosphorylation and reduced BRAF total protein levels, which led to a decrease in BRAF downstream signaling (Fig. 4A). In addition, NF-κB signaling cascades (Fig. 4B) and c-Fos and c-Jun expression (Fig. 4C) were also decreased by knocking down TRAF1. The RAF-MEK-ERK kinase pathway is known to modulate cellular responses to growth signals. As an apical kinase of the ERKs pathway, BRAF can be stimulated followed by activation of MAP kinase/ERKs signaling, which can result in increased cell proliferation, differentiation, or apoptosis (41). Many studies have

demonstrated that *NF-κB* is a downstream target gene of MEK/ERKs, and it can mediate TRAF1 activation by TNF ligands and other cytokines (23,25,42). Similarly, c-Jun and c-Fos are involved in cell growth, transformation and death (43). Previous studies indicated that MAPK signaling activation results in elevated c-Fos and c-Jun expression (44). Our results indicate that decreased BRAF expression could be the key regulatory element for the function of TRAF1 in NSCLCs. Although BRAF has been considered to be an important oncogene in tumorigenesis, the BRAF inhibitory mechanisms still remain unclear. Interestingly, knockdown of TRAF1 decreased the protein level of BRAF (Fig. 4A), but did not affect its RNA expression level (Supplementary Fig. S2). Therefore, TRAF1 regulation in lung cancer development might be involved in BRAF ubiquitination and degradation. Because TRAF1 lacks the RING domain, we concluded that TRAF1 could not function as an E3 ubiquitin ligase that would directly mediate BRAF ubiquitination. However, many studies have demonstrated that TRAF1 possesses multiple functions by interacting with other TNFR family members and several intracellular proteins, such as CD30, 4-IBB, OX40, TRAF2, NIK, ASK1 and TRIP (20,45–48). TRAF1 could act as both a negative and positive modulator of signaling by certain TNF family receptors, possibly in a cell type-dependent manner (7,49). Surprisingly, we found that TRAF2 can directly bind with BRAF and act as a novel E3 ubiquitin ligase, mediating BRAF Lys48-linked ubiquitination (Fig. 5F). Accumulating evidence indicates that TRAF1 and TRAF2 can form homodimeric complexes that are involved in various cytokine signaling networks (18,20). Therefore, although the degradation of BRAF might be indirectly controlled by TRAF1, our results are consistent with the idea that TRAF1 might play a role in BRAF Lys48-linked ubiquitination through the formation of a heterotrimer with TRAF2. Interestingly, our data illustrated that TRAF1 can associate with TRAF2, which blocks the binding of TRAF2 with BRAF, leading to stabilizing levels of BRAF. Notably, the half-life of TRAF1 is shorter in TRAF1 knock down cells (Fig. 5). Overall, the present study showed for the first time that the E3 ligase TRAF2 directly binds to BRAF to modulate BRAF Lys48-linked ubiquitination, and the involvement of TRAF1 suppressed TRAF2 modulation of BRAF degradation, resulting in inhibition of BRAF downstream signaling.

We also focused on investigating the function of TRAF1 in an urethane-induced lung tumor model. These mice primarily develop non-small cell lung tumors following urethane exposure (50). We showed that knockout of TRAF1 significantly decreased the development of urethane-induced lung tumors compared with WT mice (Fig. 6A, B). Additionally, Western blotting and immunohistochemistry data detected reduced expression of p-BRAF, BRAF, p-MEK, p-ERKs, p-c-Jun and c-Jun in TRAF1 KO mouse tissues (Fig. 6C, D). This suggests that loss of TRAF1 decreases urethane-induced lung tumor development and alleviates the severity of lung injury induced by urethane. Notably, the oncogenic role of TRAF1 in NSCLC is supported by this solid *in vivo* evidence.

Based on our results, we believe that TRAF1 is a potential target for NSCLC therapy and prevention because the probability of survival of patients with high TRAF1 expression is significantly lower than patient with low TRAF1 expression (Fig. 1D, E). Also knocking down the expression level of TRAF1 in NSCLC cell lines significantly decreased the growth of NSCLC and induced cellular apoptosis (Fig. 2 and 3). Also, the knock out of TRAF1 in mice significantly prevented urethane-induced NSCLC development in mice (Fig. 6). We

hypothesize that the high expression of TRAF1 could be a predictive biomarker coupled with the downstream signaling pathway because the high expression of TRAF1 in lung cancer cells was also associated with a high expression of BRAF (Fig. 1 and 3). Also, knocking down TRAF1 expression in lung cancer cells significantly decreased the expression level of BRAF and reduced the activation of downstream signaling pathways (Fig. 3). The present study showed that the binding of TRAF1 with TRAF2 is important for activation of the BRAF/MEK/ERKs signaling pathway and NSCLC development. In future studies, we will attempt to identify inhibitors that disrupt the TRAF1 and TRAF2 binding complex to inhibit the development of NSCLC. BRAF inhibitors are already used in the clinic to treat BRAF-mutant NSCLC. However, inhibitors against the TRAF1/TRAF2 complex combined with BRAF inhibitors could potentially increase the efficiency of inhibition compared with BRAF inhibitors alone. In addition, treatment with BRAF inhibitors only, frequently results in drug resistance. These inhibitors might be able to overcome the BRAF drug resistance in the treatment of BRAF mutant NSCLC. We will conduct future experiments to support our hypothesis.

In summary, the present study revealed that TRAF1 acts as a positive regulator of the RAF-MEK-ERKs pathway by stabilizing levels of BRAF in pulmonary tumorigenesis. Notably, we found that TRAF1 expression affects TRAF2-mediated BRAF ubiquitination, followed by cellular responses, including growth, differentiation, and death. Although we established TRAF2 as a novel E3 ligase of BRAF, and the involvement of TRAF1 impacts BRAF stability, the mechanism by which the specific binding site associates with BRAF still remains unclear and further studies will be required. These findings, which collectively indicate that TRAF1 plays a novel role in the regulation of BRAF in NSCLCs, suggest that therapeutic approaches designed to inhibit TRAF1 function might represent an attractive lung cancer prevention and therapeutic strategy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Significance

Findings identify TRAF1 as a new therapeutic target for non-small cell lung cancer

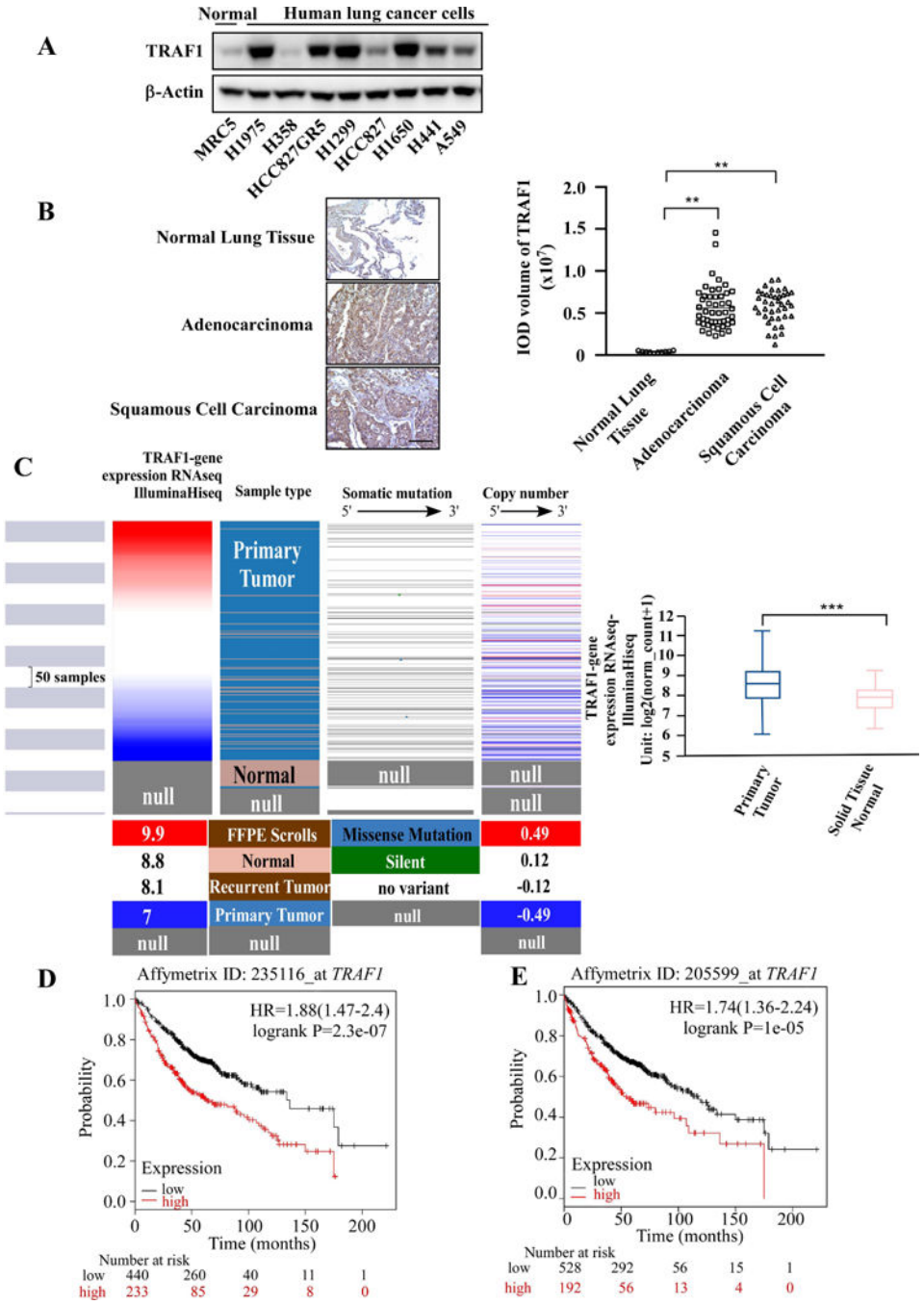

Figure 1. TRAF1 expression is up-regulated in human lung cancer and is associated with lung cancer patient survival probability

A, expression of TRAF1 in human normal and lung cancer cell lines. B, immunohistochemical analysis of TRAF1 protein expression in normal and lung cancer tissues. TRAF1 protein detection was accomplished using DAB (brown) staining and nuclei were counterstained with hematoxylin (blue). Density scores were obtained from each sample and statistical significance was determined by one-way ANOVA. Tissues include normal (n = 10), adenocarcinomas (n = 48), and squamous cell carcinomas (n = 41) and scale bar = 100 μm. C, a heat map of TRAF1 mRNA expression, amplifications and

mutations from the TCGA lung adenocarcinoma database (n = 706). The expression level of TRAF1 mRNA is compared between primary tumors (n = 514) and solid normal tissues (n = 59). Data were analyzed using UCSC Xena (http://xena.ucsc.edu/). D and E, Kaplan-Meier survival curves relative to TRAF1 expression were generated for lung cancer (KM plotter, http://kmplot.com/analysis). The desired Affymetrix IDs are validated (D) 235116_at *TRAF1* and (E) 205599_at *TRAF1.*

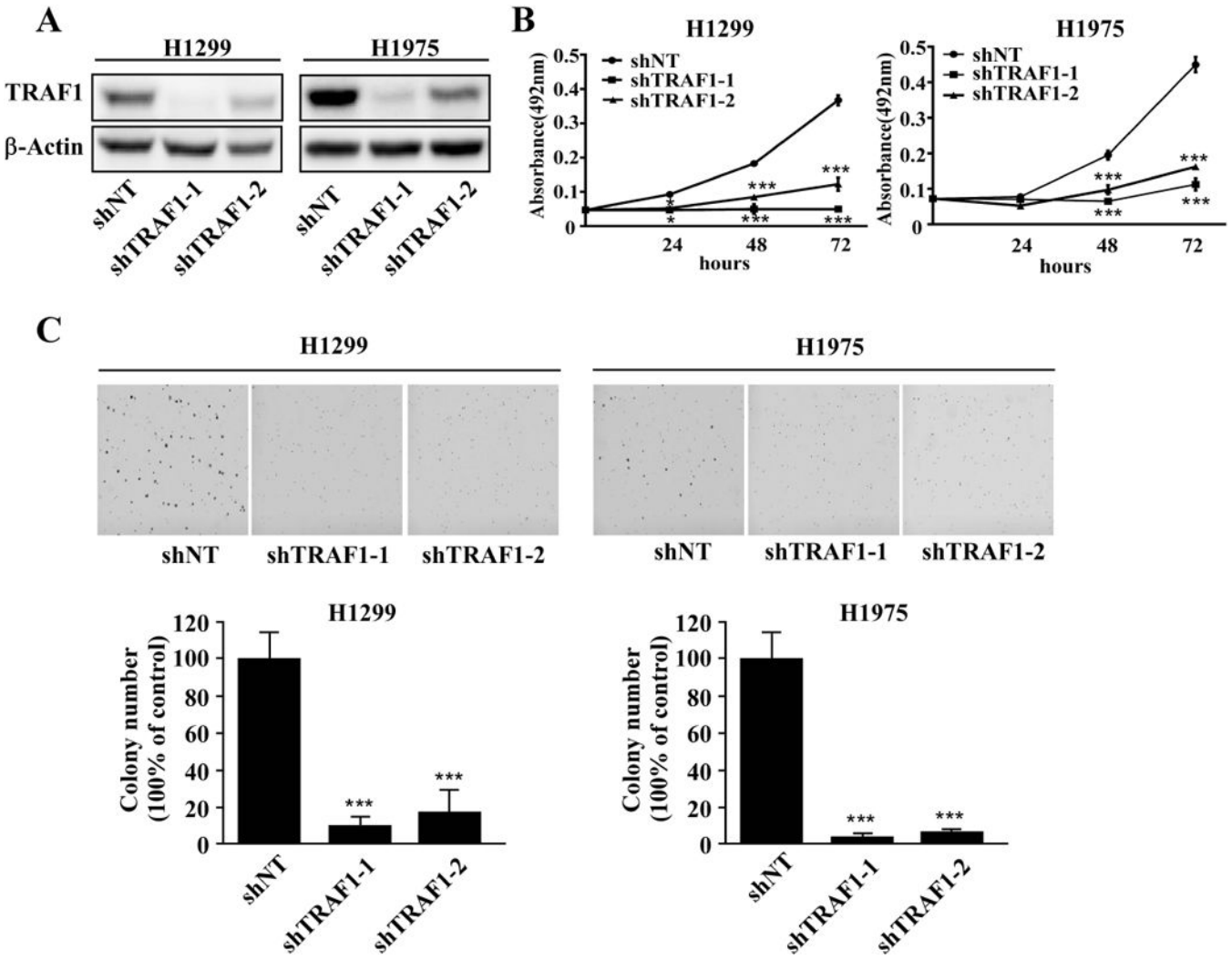

Figure 2. Knockdown of TRAF1 inhibits H1299 and H1975 lung cancer cell growth

A, H1299 and H1975 lung cancer cells with stable knockdown of TRAF1 were established. The expression of TRAF1 was determined by Western blotting. B, knockdown of TRAF1 decreases proliferation of H1299 and H1975 lung cancer cells. Cell growth was determined at 24, 48, and 72 h using the MTS assay. C, knockdown of TRAF1 reduces anchorage-independent growth of H1299 and H1975 lung cancer cells. H1299 and H1975 cells stably expressing shNT or shTRAF1 were incubated in 1.25% agar. Colonies were counted using a microscope and the Image-Pro Plus (v.6) computer software program. Data are presented as mean values ± S.D. from triplicate experiments. Statistical differences were evaluated using the Student's *t* test. The asterisks indicate a significant difference between TRAF1 knockdown and control cells (*, $p < 0.05$ and ***, $p < 0.001$).

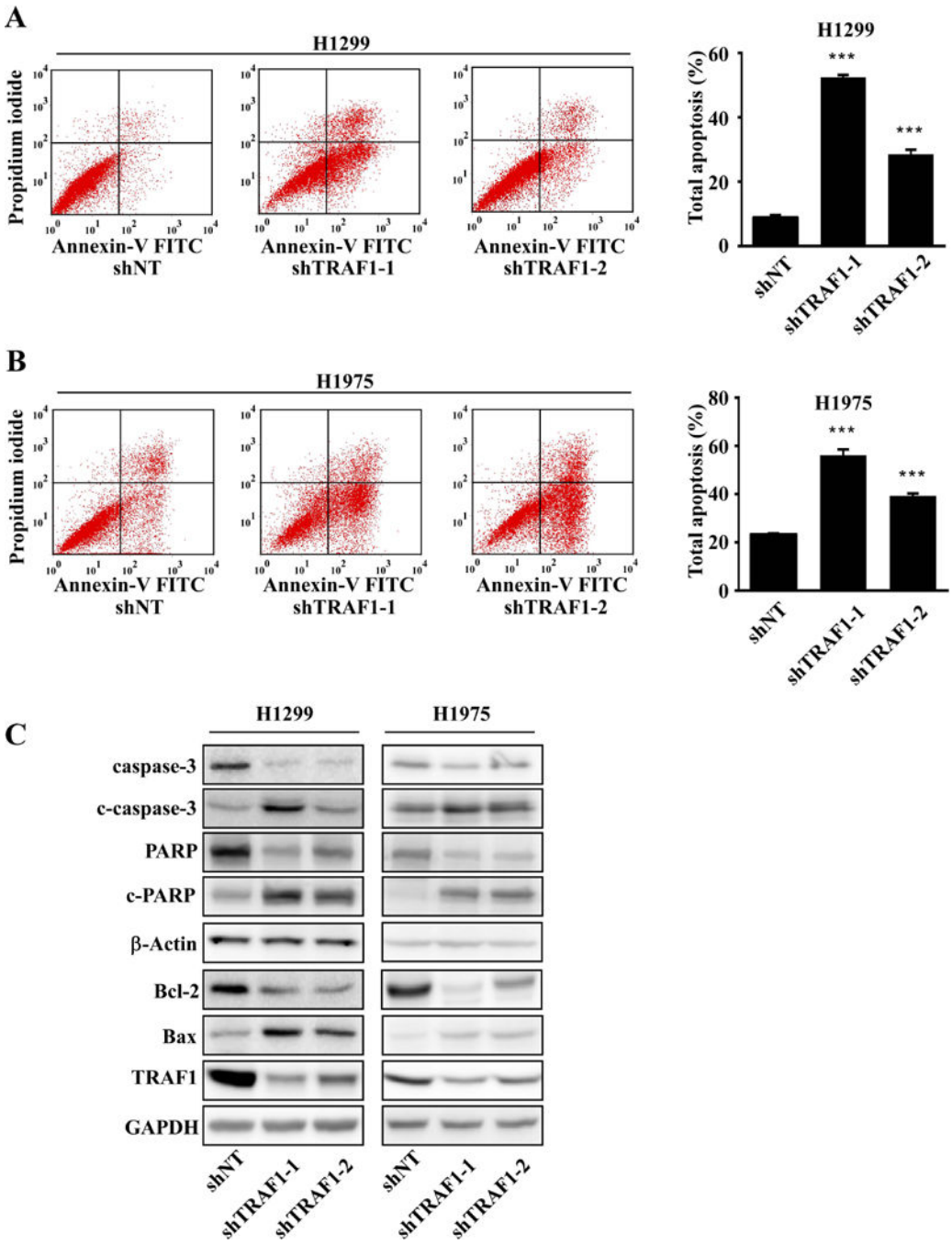

Figure 3. Knockdown of TRAF1 induces apoptosis of H1299 and H1975 lung cancer cells

A and B, knockdown of TRAF1 was established in H1299 and H1975 lung cancer cells and cells were stained with annexin V. Apoptosis was determined by flow cytometry and data are quantified (*right panels)* and the asterisks (***) indicate a significant increase in apoptosis in cells with TRAF1 knockdown ($p < 0.001$). C, cells with TRAF1 knockdown exhibit increased expression of pro-apoptotic proteins and decreased expression of anti-apoptotic proteins.

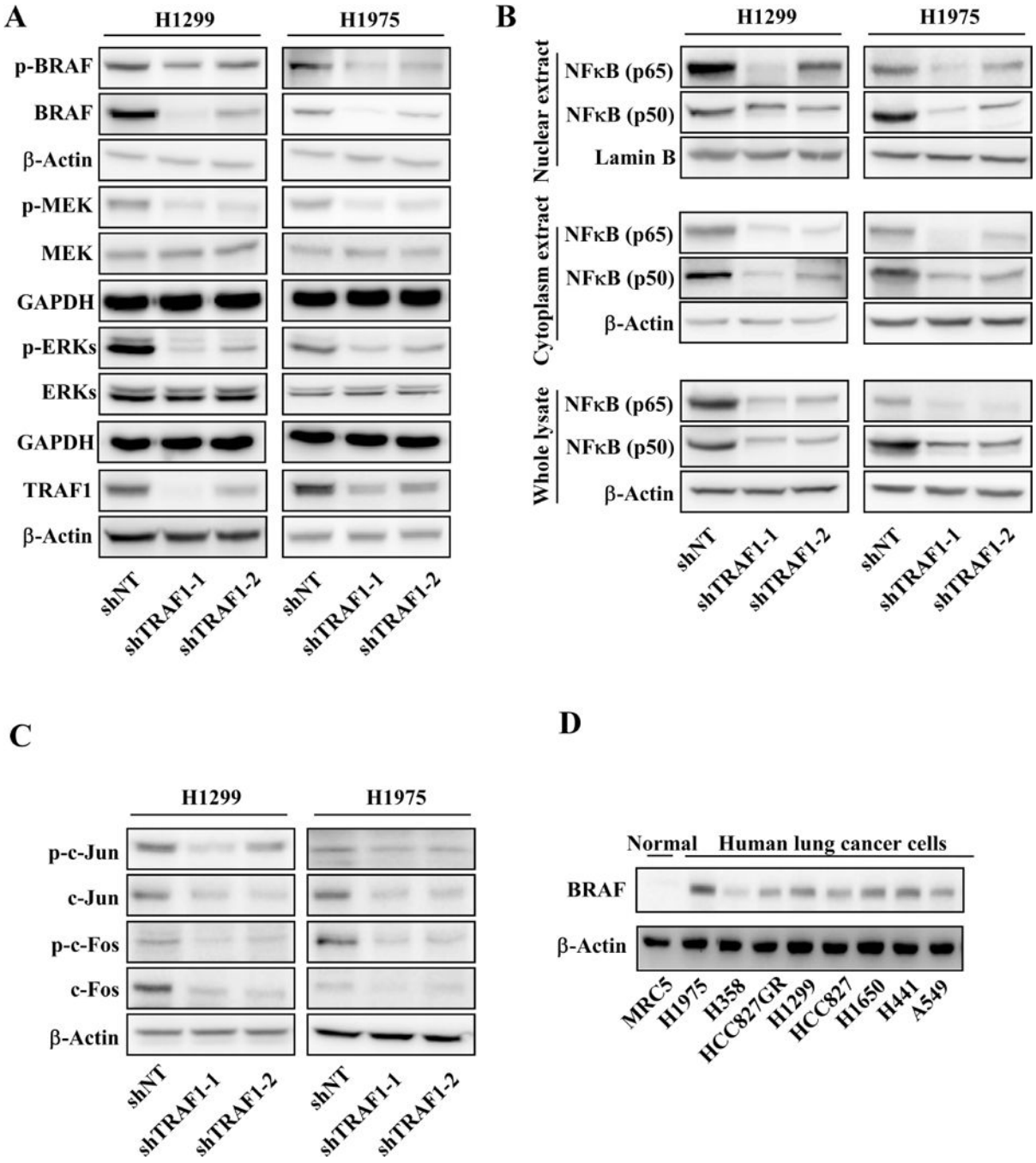

Figure 4. Knockdown of TRAF1 regulates BRAF protein levels to inhibit downstream signaling

A, knockdown of TRAF1 in H1299 and H1975 lung cancer cells impairs BRAF phosphorylation and reduces BRAF total protein levels, which affects downstream signaling. B, after knockdown of TRAF1, NF-κB (p50/p65) was decreased in both the nucleus and cytoplasm. C, after TRAF1 silencing, c-Fos and c-Jun expression was attenuated. D, BRAF expression is higher in lung cancer cell lines compared to normal MRC5 lung cells.

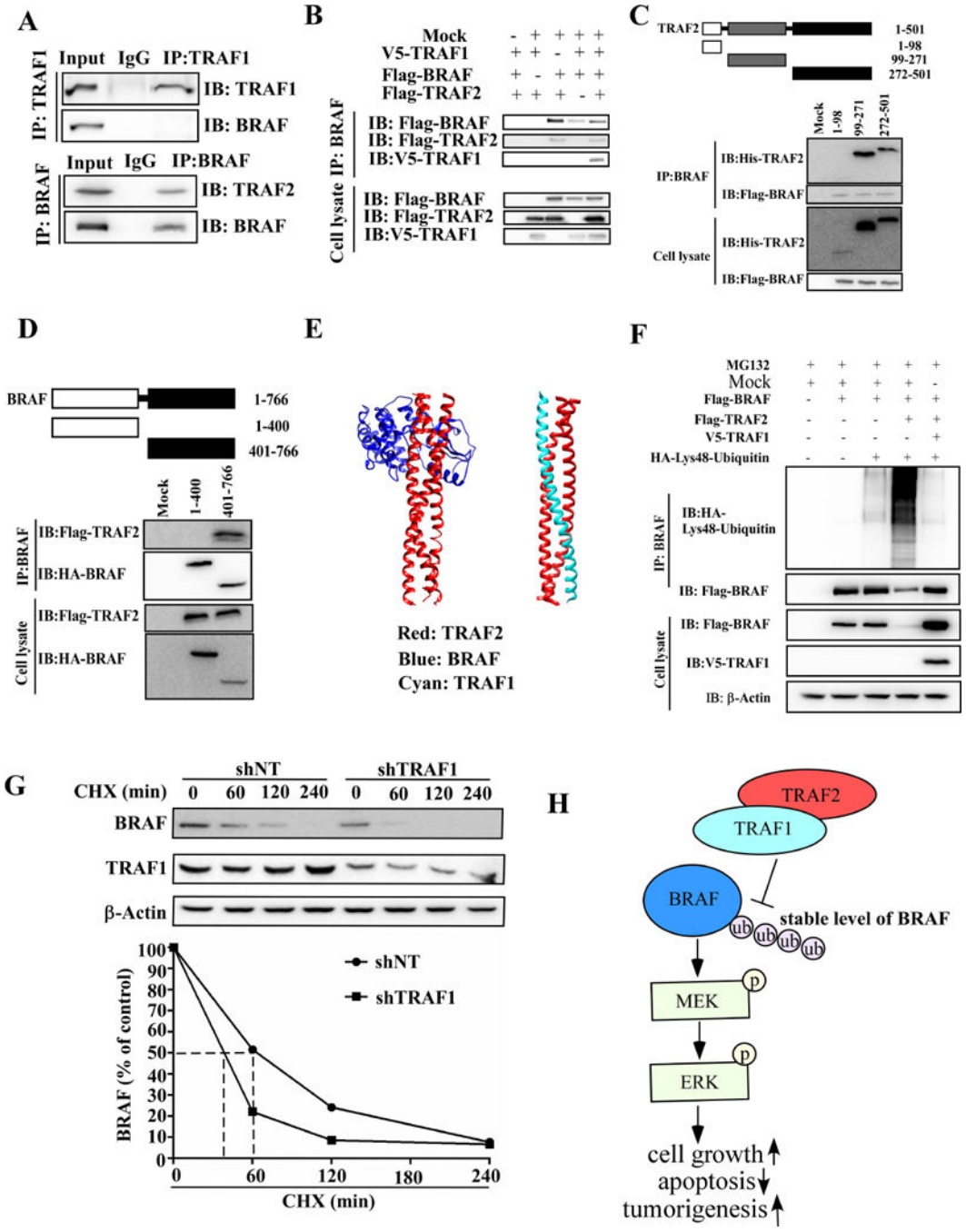

Figure 5. TRAF1 obstructs TRAF2 intermediate BRAF Lys48 linked ubiquitination

A, H1299 cell lysates were immunoprecipitated with anti-TRAF1, anti-BRAF or control IgG. The immunoprecipitated complex was detected by Western blotting with anti-TRAF1, anti-BRAF or anti-TRAF2. B, Flag-BRAF, Flag-TRAF2, V5-TRAF1 and Mock plasmids were co-transfected into HEK293T cells as indicated. At 48 h after transfection, proteins were extracted. The Flag- or V5-tagged proteins were immunoprecipitated with anti-BRAF and Western blotting was performed. C, His-TRAF2 (aa 1-98), His-TRAF2 (aa 99-271), His-TRAF2 (aa 272-501), Flag-BRAF or Mock was transfected into HEK293T cells. At 48

h after transfection, cell lysates were harvested and the His or Flag-tagged proteins were immunoprecipitated with anti-BRAF. Western blotting was performed. D, the HA-BRAF regulatory domain (aa 1-400), kinase domain (aa 401-766) or Mock plasmid was transfected into 293T cells. At 48 h after transfection, cell lysates were harvested and the Flag- or HA-tagged proteins were immunoprecipitated with anti-BRAF. Western blotting was performed. E, modeling of TRAF1 or BRAF binding with TRAF2. TRAF1 is colored cyan, TRAF2 is colored red and BRAF is colored blue. F, Flag-BRAF, Flag-TRAF2, V5-TRAF1, and HA-lys48-Ubiqutin and Mock were co-transfected into 293T cells as indicated. At 44 h after transfection, cells were treated with MG132 (20 μM) for 4 h and proteins were then extracted. The HA- or Flag-tagged proteins were immunoprecipitated with anti-BRAF. Western blotting was performed and ubiquitinated BRAF proteins were visualized with anti-HA. G, the shNT- and shTRAF1-transfected H1299 cells were cultured and treated with cyclohexamide (CHX). Cells were harvested at 0, 60, 120, and 240 min and protein levels were visualized by Western blotting. H, TRAF1 binding with TRAF2 decreases BRAF ubiquitination and stabilizes BRAF, which activates MEK and ERKs effects on lung cancer cell growth, apoptosis and NSCLC tumorigenesis.

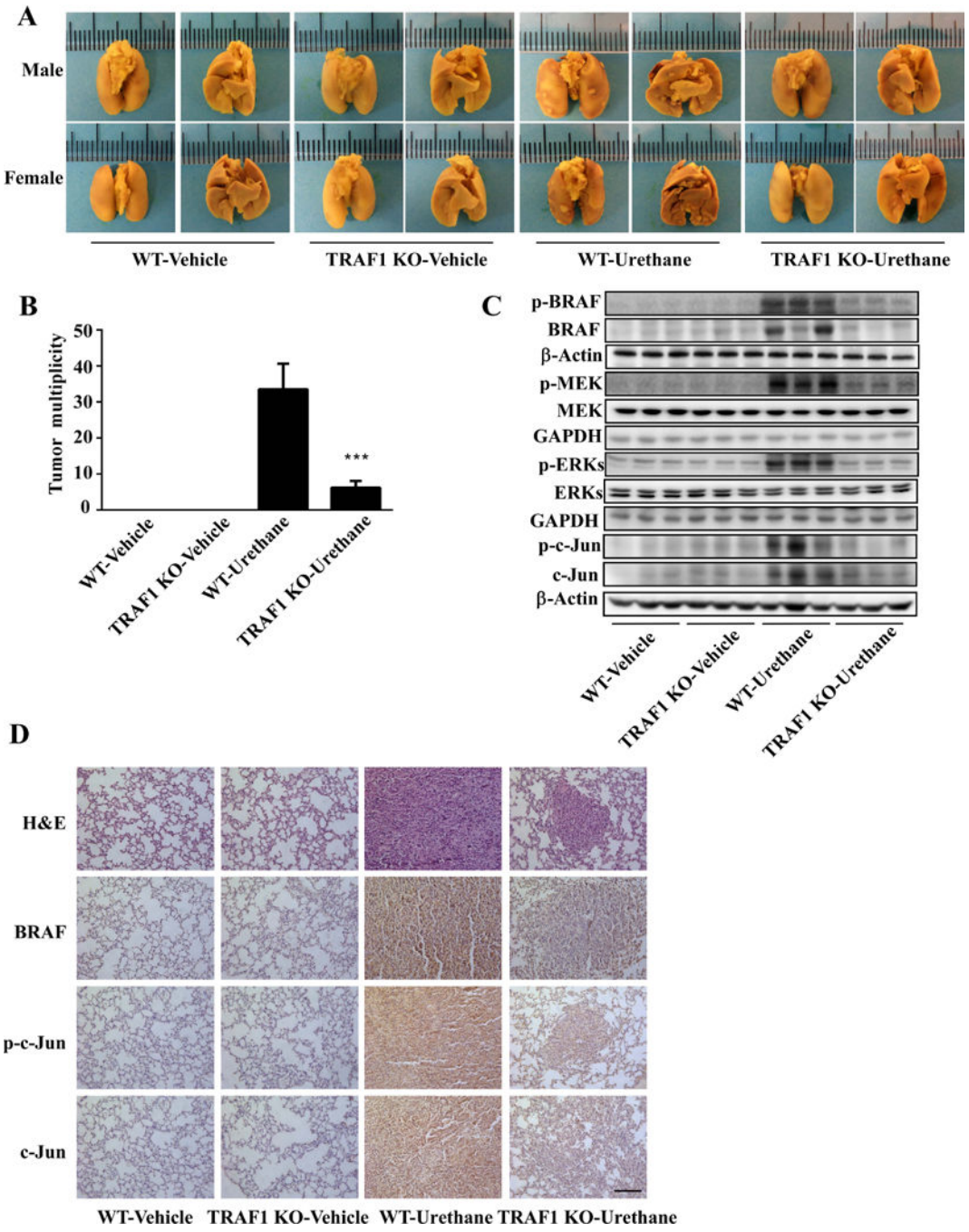

Figure 6. TRAF1 inhibits urethane-induced lung carcinogenesis

A, BALB/c wild-type (WT) and BALB/c TRAF1 knockout (TRAF1 KO) mice was used. Urethane (1 g/kg in 1 × PBS) or vehicle only was intraperitoneally (i.p.) administered weekly for 7 weeks. Lungs were collected at 6 months after first urethane treatment. B, tumor multiplicity averaged 5.7 ± 1.7 tumors in the TRAF1 KO group treated with urethane and 31.4 ± 6.4 in the urethane-treated WT group (*** $p < 0.001$) with no significant difference between male or female mice. C, protein levels of p-BRAF, BRAF, p-MEK, p-ERKs, p-c-Jun, and c-Jun were substantially decreased in the tumor tissues of the TRAF1

KO mice compared to the WT mice. The tissue lysates were prepared from pooled lung tumor nodules or normal lung tissue from each mouse of each groups. Three sets were prepared for each group and each lane shows 1 set of pooled samples subjected to Western blotting. D, lung samples were harvested and stained with H&E. Immunohistochemistry analysis was used to determine the levels of BRAF, phosphorylated c-Jun and total c-Jun in lungs from urethane-treated mice compared with those treated with vehicle.