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Original Article

The oncogenic role of TRIP13 in regulating proliferation, invasion, and cell cycle checkpoint in NSCLC cells

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Abstract: TRIP13 (thyroid hormone receptor interacting protein 13) AAA-ATPase has been reported to be involved in the metaphase checkpoint in human breast cancer, prostate cancer, and cervical cancer. However, the expression pattern and biologic role of TRIP13 in non-small cell lung cancer (NSCLC) remained unknown. In our present study, real-time PCR and western blot were used to detect the expression level of TRIP13 in NSCLC tissues and cell lines. We found that the expression levels of TRIP13 mRNA and protein were significantly upregulated in cell lines and lung tissues. Knockdown of TRIP13 by lentivirus inhibited cell proliferation and invasion in both A549 and H1299 cells. Furthermore, flow cytometry, western blot and immunoprecipitation showed that the MCC complex was disassembled and cells became arrested in metaphase, when TRIP13 was inhibited. In conclusion, here we first report that TRIP13 acts as a tumor promoter in regulating cell proliferation, invasion, and cell cycle checkpoint in NSCLC cells and may be a clinically useful marker for the diagnosis and treatment of lung cancer.

Keywords: Non-small cell lung cancer (NSCLC), TRIP13, cell cycle, checkpoint

Introduction

Lung cancer is the most common cause of cancer mortality worldwide, and non-small cell lung cancer (NSCLC) accounts for more than 85% of lung cancer cases [1]. NSCLC is mostly caused by tobacco smoking [2] and common signs of NSCLC include cough, dyspnea, weight loss, shortness of breath, and chest pain. In China, lung cancer has replaced liver cancer as the first cause of death among malignancies in 2008 [3]. Despite new advances in NSCLC formation, progression, and therapy, the cause and the molecular mechanism of NSCLC progression is still unclear.

TRIP13 (thyroid hormone receptor interacting protein 13) AAA-ATPase was first found associated with thyroid hormone receptor in a hormone-independent fashion through yeast two-hybridization [4]. Recent research found it in Caenorhabditis elegans [5, 6], Drosophila melanogaster [7], rice [8], and mouse [9-11], TRIP13 or its orthologs are involved in meiotic recombination. Metaphase checkpoint is a protective system to ensure chromosome segregation accurately by preventing defective kinetochore-microtubule attachment in mitosis and functions as a mechanism to ensure proper cell division and inhibit cancer progression. TRIP13 is also expressed in many somatic tissues and reportedly is involved in the mitotic metaphase checkpoint in human breast cancer, prostate cancer, and cervical cancer [12-15]. Moreover, TRIP13 participates in metaphase checkpoint through binding p31-met to regulate a MAD2 conformation switch, and it was also reported that TRIP13 promoted error-prone nonhomologous end joining (NHEJ) and induced chemo resistance in head and neck cancer [16]. However, the effect of TRIP13 in NSCLC has not been reported. Thus, we investigated whether TRIP13 contributed to the metastatic behavior of NSCLC cells.

In the current study, we found that TRIP13 was robustly increased in NSCLC tissues and cell lines. Knockdown of TRIP13 inhibits, and ectopic expression of TRIP13 promotes, the invasive phenotype of NSCLC. Furthermore, we demonstrated that TRIP13 downregulation promotes metaphase cell cycle arrest in NSCLC. Taken together, our results suggest that TRIP13 acts as a tumor promotor and its upregulation may play an important role in the development and progression of NSCLC.

Materials and methods

Clinical samples and immunohistochemistry

11 para-carcinoma and carcinoma tissues were taken from newly diagnosed non-small cell lung cancer patients in the Department of Respiratory Medicine, The Third Affiliated Hospital of Xinjiang Medical University. All the patients gave informed consent for use of the samples for molecular studies.

Paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated in a graded series of ethanol followed by heat-induced epitope retrieval in citrate buffer (pH 6.0). Sections were incubated at 4°C overnight with monoclonal antibodies against TRIP13 (Ab127004, Abcam). Immunostaining was performed using ChemMate DAKO EnVision Detection Kit, Peroxidase/DAB, Rabbit/Mouse (code K 5007, DakoCytomation, Glostrup, Denmark) according to the manufacturer's instructions. Subsequently, sections were counterstained with hematoxylin (Dako) and mounted in dimethylbenzene.

Cell lines, cell culture and transfection

Human normal lung epithelial cell line BEAS-2B and non-small cell lung cancer cell lines A549, H1299, H460 and H1975 were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cell lines were maintained in RPMI-1640 medium supplemented with 10% FBS, 1% Penicillin-Streptomycin, cultured at 37°C in 5% CO_2 humidified atmosphere. All cell culture related reagents were purchased from Hyclone.

Lentivirus package and infection

The short hairpin RNA ACCTCACAAGAACGTCAACAGCAATCAAGAGTTGCTGTTGACGTTCTTGTTT targeting the open reading frame of TRIP13 was constructed into pLV3-EGFP. The scramble sequence ACCTCGCACAATGAGCAACAAACATCAAGAGTGTTTGTTGCTCATTGTGCTT constructed into pLV3-EGFP used as a control, does not target any gene in humans. The plasmids contained puromycin and GFP and were constructed by Genepharma (Shanghai, China). The plasmids pLV3-EGFP, psPAX2, pMD2.G were co-transfected into 293T with Lipo3000 (Invitrogen) according to the manufacturer's procedure. Cell were cultured 48 h after transfection. We collected the medium and harvested the lentivirus by Lenti-X concentrator kit according to the manufacturer's procedure (Clonetech).

5×10^4 H1299 and A549 were seeded into a plate separately, and cultured overnight. The serum free medium was changed and we added the lentivirus with multiplicity of infection 50. After incubation for 6 h, the medium was replaced with 10% FBS medium. After 3 days' culture, 2 μg/mL puromycin was added and we cultured for 2 weeks to screen puromycin-positive cells. The efficiency of inhibition was measured by real-time PCR and western blot.

MTT assay

For cell proliferation assay, 2000 cells/well were seeded triplicate in 96-well plate. The plate was incubated for 1 to 5 days in 37°C in a 5% CO_2 humidified chamber. 10 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 10 mg/mL) were added and incubated at 37°C for 2 h. MTT was discarded and we added 100 μL Dimethyl Sulphoxide (DMSO, Sigma) to each well. The absorbance at 595 nm was measured using a microplate reader (Thermo Fisher).

Cell colony forming assay

600 cells per well were seeded into a 6-well plate and incubated at 37°C in a 5% CO_2 humidified chamber and exchanged medium for 3 days. After 14 days, we discarded the medium and washed with PBS. 0.1% crystal violet in methanol was used for staining for about 5 min at room temperature; slides were washed with water and dried in baking oven at 55°C. Cell clones were counted according to the usual criterion of 50 cells or more per colony.

Protein extraction and western blot

Cells and tissues were lysed in RIPA buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40,

0.5% sodium deoxycholate, 1 mM PMSF and protease inhibitor cocktail) and measured by BCA protein assay kit (Beyotime, Shanghai, China). Equal amounts of protein were separated by SDS-PAGE, and trans-blotted onto PVDF membrane by semi-dry transfer. The protein was blocked in 5% skimmed milk for 1 h in room temperature and incubated with indicated antibody. The ECL plus western blot detection system (GE Lifescience) was used to detect bound antibodies. Antibodies used in the experiment: TRIP13 (Ab127004, Abcam), β-actin (ab3280, Abcam), Cyclin A (ab38, Abcam), Cdk2 (ab32147, Abcam), Chk2 (6334, Cell Signaling), p-Chk2 (Thr68, 2197, Cell Signaling), Cdc2 (9116, Cell Signaling), p-Cdc2 (Tyr15, 4539, Cell Signaling), Mad2 (ab70383, Abcam), BubR1 (ab4637, Abcam), Cdc20 (14866, Cell Signaling), goat anti-mouse IgG HRP (m21001, Abmart, Shanghai, China), goat anti-rabbit IgG HRP (m21002, Abmart, Shanghai, China).

Immunoprecipitation

5×10^6 cells were harvested and lysed with RIPA buffer. Cleared cell lysates were collected after centrifuging, incubated, and rotated overnight at 4°C with 1 μg anti-BubR2 or anti-Cdc20. Then 30 μl protein A/G-conjugated magnetic beads (Pierce) were added to the samples, incubated, and rotated for 3 h at 4°C. Next, we collected and washed the co-IP protein with magnetic separation according to the manufacturer's procedure. The protein samples were resuspended with 100 μL 1 × SDS-loading buffer, heated at 100°C for 10 min, and separated by SDS-PAGE.

RNA extraction and real-time PCR

Total RNA was isolated from cells or human tissue samples by using TRIzol reagent (Takara, Dalian, China). The first-strand complementary miRNA was synthesized using oligo dT from total RNA using the PrimeScript RT master mix Perfect Real Time (Takara, Dalian, China). mRNA expression level was detected by real time PCR using SYBR green (Takara, Dalian, China) on Applied Biosystems Stepone plus real time PCR system. β-actin served as loading control. The primers of TRIP13 and β-actin used were as follows: Trip13: primer F-CAGCAGCACTGCAAAGAAAG primer R-CATCAAACTCAGTCCATGTGTAATC β-actin [17]: primer F-CTACGTCGCCCTGGACTTCGAGC primer R-GATGGAGCCGCCGATCCACACGG.

Flow cytometric analysis

Cell cycle was assessed by FACScalibur flow cytometry (Becton Dickinson, San Jose, CA, USA). Samples for cell cycle were harvested in trypsin and fixed in 70% ethanol in PBS at 4°C overnight, washed twice with PBS, and stained by propidium iodide (PI) with RNase (550825, Becton Dickinson, San Diego, CA, USA) for 30 min in the dark. Fluorescent emissions were collected through a FL2 band-pass filter.

Cell invasion assay

Cell invasion assay was performed using a transwell chamber with a polyethylene terephthalate filter membrane containing 8.0 μm pores in 24-well plates (Millipore). The chamber was coated with matrigel (Becton Dickinson, Bedford, MA), 2×10^4 cells in 100 μL serum-free medium were seeded onto the upper part of a transwell chamber. The lower chamber was filled with 1 mL 10% FBS medium. After culturing for 24 h, medium was discarded and the non-invading cells were removed with a cotton swab. Invasive cells located on the lower surface of chamber were stained with 0.1% crystal violet (Sigma).

Statistical analysis

The results were determined as mean ± SEM of three independent experiments by using statistical software package (SPSS 12.0). *P*-values, calculated by Student's T test, that were < 0.05 were considered significant.

Results

Upregulation of TRIP13 in NSCLC cell lines and tissues

To elucidate the expression level of TRIP13 in NSCLC para-carcinoma and carcinoma tissues, we obtained 11 para-carcinoma and carcinoma tissues from newly diagnosed patients. Real-time PCR was conducted to measure the expression level of TRIP13. As shown in **Figure 1A**, TRIP13 mRNA expression level of carcinoma was 2.3 times higher than para-carcinoma. Consistent with PCR results, NSCLC tissues showed much higher TRIP13 expression compared to para-carcinoma tissues by IHC (**Figure 1B**). We then analyzed the relationship between TRIP13 expression and clinical characteristics.

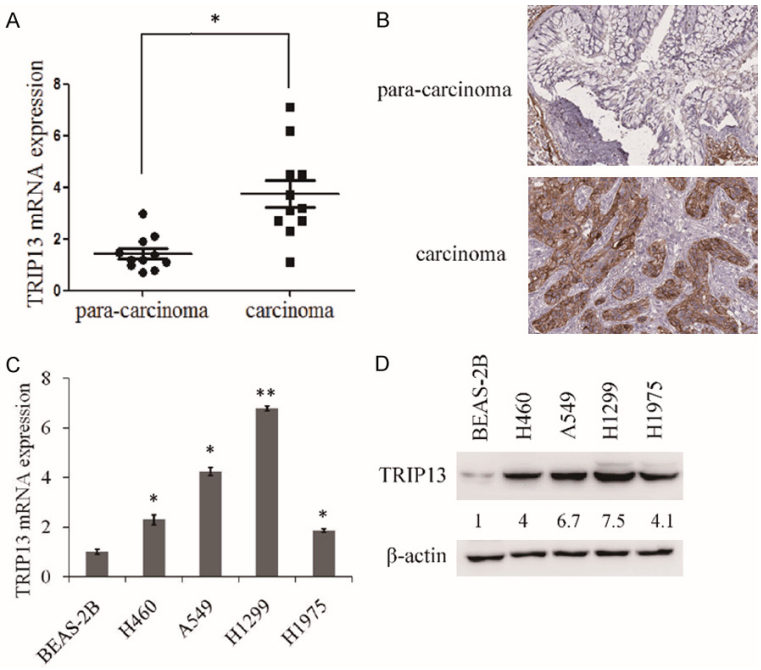

Figure 1. Upregulation of TRIP13 in NSCLC cell lines and tissues. A. Real-time PCR analysis of TRIP13 expression in 11 lung carcinoma and para-carcinoma tissues (means ± SD; *P < 0.05; Student's t test). B. Immunohistochemical staining of TRIP13 in lung carcinoma and para-carcinoma tissues. C. Real-time PCR analysis of TRIP13 expression in normal epithelial lung cell line and various tumor cell lines. D. Western blotting analysis of TRIP13 in normal epithelial lung cell line and NSCLC cell lines. β-actin served as the loading control (means ± SD; *P < 0.05; **P < 0.005, Student's t test).

Table 1. Association between TRIP13 expression and clinicopathologic characteristics of 11 NSCLC patients

Variables	Cases (n = 11)		TRIP13		*P* value*
	n	%	+ (9)	-(2)	
Age (years)					
≤ 65	6	54.54	5	1	0.889
> 65	5	45.46	4	1	
Sex					
Male	8	72.72	7	1	0.156
Female	3	27.28	2	1	
Histologic type					
Adenocarcinoma	9	81.81	8	1	0.232
Squamous cell carcinoma	2	18.19	1	1	
Tumor status					
T1-T2	9	81.81	8	1	0.036
T3-T4	2	18.19	1	1	
TNM stage					
I	3	27.28	3	0	0.003
II-III	8	72.72	6	2	

Abbreviations and note: TNM, tumor-node-metastasis. *Pearson's χ^2 test.

As shown in **Table 1**, there was no significant correlation of TRIP13 expression with age (P = 0.889), gender (P = 0.156) and histologic type (P = 0.232). However, high expression of TRIP13 was significantly correlated with primary tumor status (P = 0.036) and advanced TNM stage (I versus II + III, P = 0.003).

Furthermore, we explored the expression level of TRIP13 in NSCLC cell lines. We performed real-time PCR and western blot to measure TRIP13 expression levels in the human normal lung epithelial cell line BEAS-2B and NSCLC cell line A549, H1299, H460 and H1975. As depicted in **Figure 1C** and **1D**, compared to BEAS-2B, the expression levels of TRIP13 in the NSCLC lines were all upregulated with respect to both mRNA and protein. We found that TRIP13 was lower expressed in lung adenocarcinoma compared to the non-cancerous tissues (Supplementary Figure 3A and 3B). These findings suggested-TRIP13 might participate in NSCLC progression.

Knockdown of TRIP13 by lentivirus inhibits proliferation and invasion in NSCLC cell lines

To investigate whether TRIP13 expression has any effect on the growth of NSCLC, RNAi was conducted to generate TRIP13 knock-down (TRIP13 kd) cell lines. Lentivirus was infected into A549 and H1299 cells. Cells were also infected with LV-NC as a negative control. To determine the function of TRIP13, A549, A549-LV-NC, H1299, or H1299-LV-NC cells were used as controls in all assays.

The TRIP13 mRNA level in A549 TRIP13 kd and H1299 TRIP13 kd cells was determined by real-time PCR to confirm whether TRIP13 expression was

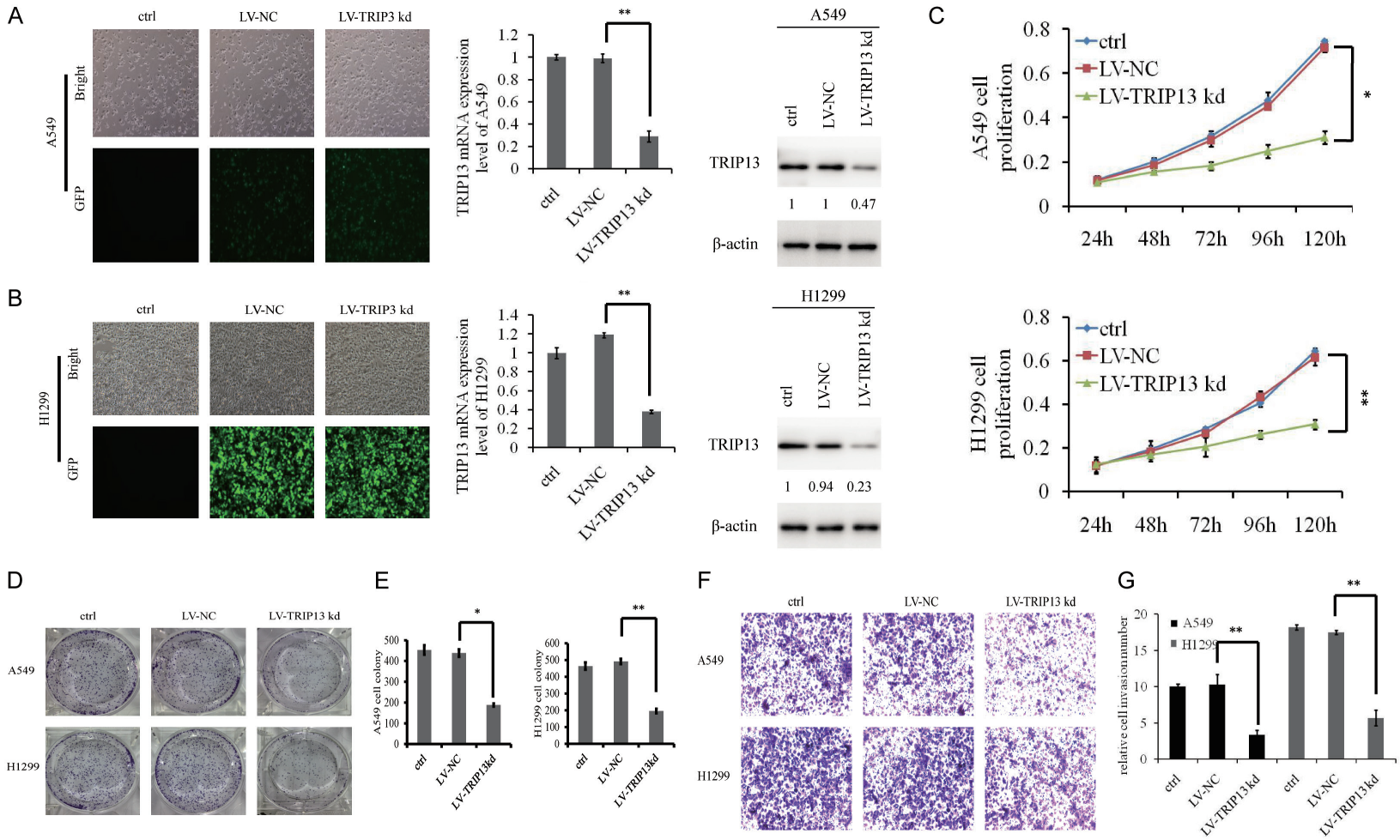

Figure 2. Knockdown of TRIP13 by lentivirus inhibits proliferation and invasion in NSCLC cell lines. A, B. Knockdown efficiency of TRIP13 by lentivirus analyzed by real-time PCR and western blot in A549 and H1299. C. Downregulated TRIP13 inhibit lung cancer cell line H1299 and A549 proliferation. The proliferation curves were measuring the absorbance at 592 nm by MTT assay. (ctrl: control; LV-NC: negative control; LV-TRIP13 kd: TRIP13 knockdown, **P < 0.01, compared to LV-NC). D. Representative micrographs of crystal violet-stained cell colonies. E. Representative quantification of crystal violet-stained cell colonies. Knockdown of TRIP13 inhibited lung cancer cell line colony formation. F. Invasion assay was performed with transwell inserts coated with Matrigel. G. The cell number of invaded cells was counted in randomly selected fields and presented as bar graphs (means ± SD; *P < 0.05; **P < 0.005, Student's t test).

specifically inhibited through RNAi in A549 and H1299 cells. As shown in **Figure 2A**, TRIP13 expression in A549 TRIP13 kd cells was inhibited by 62.1% compared to cells infected with lentivirus-NC. We found similar results in H1299 TRIP13 kd cells. TRIP13 mRNA expression in these cells was also inhibited by 71.3% as compared to NC cells (**Figure 2B**). TRIP13 protein expression in these cells was detected by western blotting. As shown in **Figure 2A** and **2B**, TRIP13 protein was much highly expressed in control cells than that of A549 TRIP13 kd and H1299 TRIP13 kd cells.

To assess the role of TRIP13 in regulating cell proliferation, we performed the MTT assay. The results showed that TRIP13 down regulation significantly inhibited the proliferation rate of A549 and H1299 cells (**Figure 2C**), and this was further confirmed by a colony formation assay (**Figure 2D** and **2E**). To detect the role of TRIP13 in the malignant behavior of NSCLC cells, we performed in vitro invasion assays. Transwell assays revealed that TRIP13 downregulation sharply inhibited the invasion ability of A549 and H1299 cells (**Figure 2F** and **2G**). This suggests a fundamental role of TRIP13 as a tumor promotor in NSCLC cells.

Furthermore, we quantified and compared the number of apoptotic cells in A549 and A549-LV-NC cells. Interestingly, the percentage of apoptotic cells in the TRIP13 knockdown group is 19.3% on average, whereas that of the control group is only 3.6%, indicating that knockdown of TRIP13 can promote cell apoptosis in A549 cells (Supplementary Figure 1A and 1B). We found similar results in H1299 cells (Supplementary Figure 1C and 1D). In addition, we also investigated the effect of TRIP13 knockdown in human normal lung epithelial cells, where results showed that the proliferation and apoptotic rates were not affected in normal lung epithelial cells compared to control groups (Supplementary Figure 2A-C).

Downregulation of TRIP13 promotes G2/M phase arrest in NSCLC cells

TRIP13 was reported to be involved in metaphase checkpoint in human breast cancer, prostate cancer, and cervical cancer, so we explored whether TRIP13 was involved in cell cycle regulation in NSCLC cells.

To detect the effect of TRIP13 on cell cycle progression, the progressions of A549 TRIP13 kd, H1299 TRIP13 kd, A549 LV-NC, H1299 LV-NC, A549, and H1299 cells were analyzed by flow cytometry. As shown in **Figure 3A** and **3C**, the percentage of cells entering G2/M phase was significantly increased in the A549 TRIP13 kd cell line. A similar result was observed in H1299 TRIP13 kd cells (**Figure 3B** and **3D**). Taken together, our data showed that TRIP13 was a key molecule in regulating G2/M phase transition in both A549 and H1299 cells.

TRIP13 regulates metaphase checkpoint related genes in NSCLC cells

The G2/M transition is guarded by at least two checkpoint control pathways including G2/M checkpoint and metaphase checkpoint. We first examined some key regulators, such as Chk2, Cdc2, Cdk2 and Cyclin A, which has been implicated in the control of the G2/M checkpoint in mammals [18-20]. Furthermore, we conducted western blot assay to measure the expression level of G2/M checkpoint related proteins and found the protein level of Chk2, Cdc2, Cdk2, and Cyclin A were not affected obviously in A549 and H1299 TRIP13 kd cells compared to control groups (**Figure 4A**).

Therefore, we presumed that downregulation of TRIP13-induced G2/M phase arrest might be regulated by metaphase checkpoint during the G2 to M phase transition in NSCLC cells. Results from the western blot assay indicated that TRIP13 downregulation significantly enhanced the protein level of Mad2, an important mitotic checkpoint complex (MCC) protein for regulating the G2/M transition, but the protein levels of BubR2 and Cdc20 were not influenced obviously (**Figure 4B**). Interestingly, the immunoprecipitation (IP) pull-down assay shown enhanced Mad2 interaction with BubR2 and Cdc20 when TRIP13 was downregulated in NSCLC cells (**Figure 4C** and **4D**), indicating that TRIP13 could promote the transition from metaphase to anaphase by inhibiting MCC complex formation.

Discussion

In our present study, the function of TRIP13 in NSCLC cells was investigated. We found that TRIP13 was up-regulated in NSCLC cells and tissues, compared with that in non-neoplastic

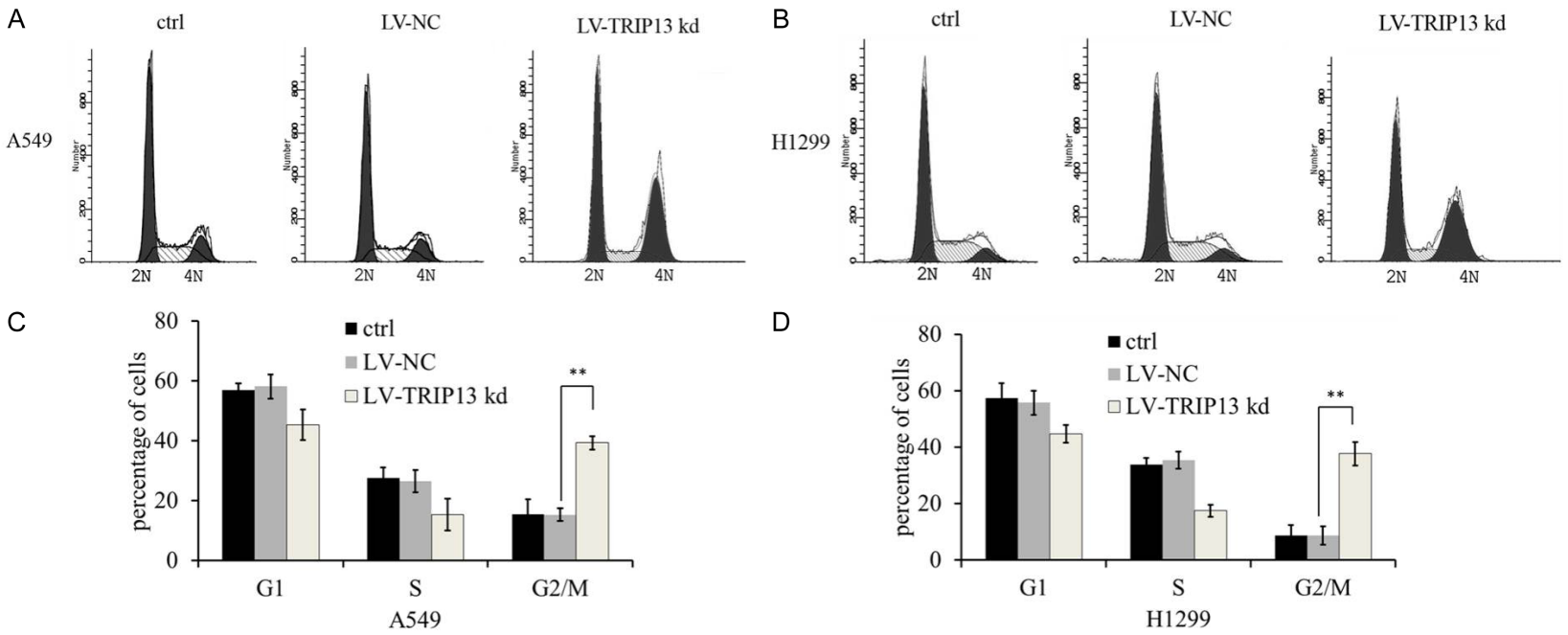

Figure 3. Downregulation of TRIP13 promotes G2/M phase arrest in NSCLC cells. A, B. Cell cycle distribution of A549 and H1299 was found by staining with PI and measured by flow cytometry in FL2 channel. C, D. Representative quantification of propidium iodide-stained cell numbers. Downregulation of TRIP13 promotes lung cancer cell arrest in the G2/M phase. (**P < 0.01, compared to LV-NC).

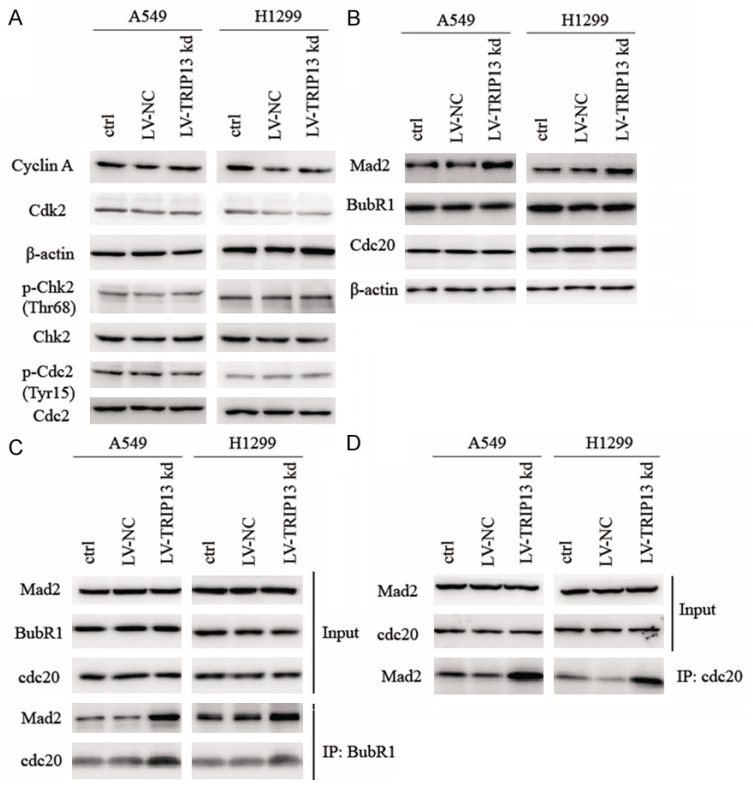

Figure 4. TRIP13 regulates metaphase checkpoint related genes in NSCLC cells. A. Downregulation of TRIP13 by lentivirus did not influence the expression level of G2/M checkpoint-related protein. B. The protein expression levels of the metaphase checkpoint-related protein were analyzed by immunoblotting. C, D. The immunoprecipitation results showed downregulation of TRIP13 promoted Mad2, Cdc20 and BubR1 complex release.

lung specimens and human normal lung epithelial cell line BEAS-2B. Moreover, the growth and invasion abilities of A549 and H1299 cells declined when TRIP13 expression was inhibited by specific TRIP13 shRNA. We suggest that TRIP13 promotes G2-M progression by inhibiting MCC complex formation.

In humans, TRIP13 is located at chromosome 5p15 and encodes a protein of 432 amino acids. TRIP13 belongs to the AAA + ATPase family of chaperone proteins that facilitate assembly or degradation of protein complexes that regulate diverse cellular functions. TRIP13 has been reported as an oncogene only in head and neck cancer. Consistent with previous study, our observations showed that TRIP13 functions as an oncogene regulating NSCLC cell tumorigenicity in vitro.

A recent network analysis of protein-protein interactions in mitosis suggested that TRIP13 is a kinetochore protein that interacts with the mitotic spindle. Cell cycle checkpoints are a protective mechanism to ensure cell proper division and proliferation, including a G1/S checkpoint, S checkpoint, G2/M checkpoint, and metaphase checkpoint. Metaphase checkpoint is a protective system to ensure chromosome proper division and inhibit cell tumor aggression. Previous studies have reported that TRIP13 could promote cell cycle progress in HeLa and breast cancer cells [12, 13]. Accordingly, our FACs analysis confirmed that downregulation of TRIP13 promotes G2/M phase arrest in NSCLC cells.

Metaphase checkpoint is a key protective system to ensure proper chromosome division. It is regulated by mitotic checkpoint complex (MCC) and ubiquitin ligase anaphase promoting complex/cyclosome (APC/C) [21, 22]. The molecular mechanism of TRIP13-regulated metaphase checkpoint in NSCLC had been unclear. Our results showed that of the important molecules in the regulation at G2/M transition, Mad2 was upregulated in TRIP13-knockdown cells. Furthermore, immunoprecipitaion results indicated that TRIP13 could promote the transition from metaphase to anaphase by inhibiting MCC complex formation.

In summary, we first found that TRIP13 acts as an oncogene in regulating NSCLC cell tumorigenicity in vitro. TRIP13 promoted cell growth by accelerating cell cycle progression. Moreover, TRIP13 regulated metaphase to anaphase progression by inhibiting Mad2 expression. TRIP13 was mainly involved in the metaphase checkpoint through regulating MCC complex disassembly in NSCLC cell lines. Our data suggests that TRIP13 is a new potential target in NSCLC and future studies need to investigate its molecular mechanisms.

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Disclosure of conflict of interest

None.

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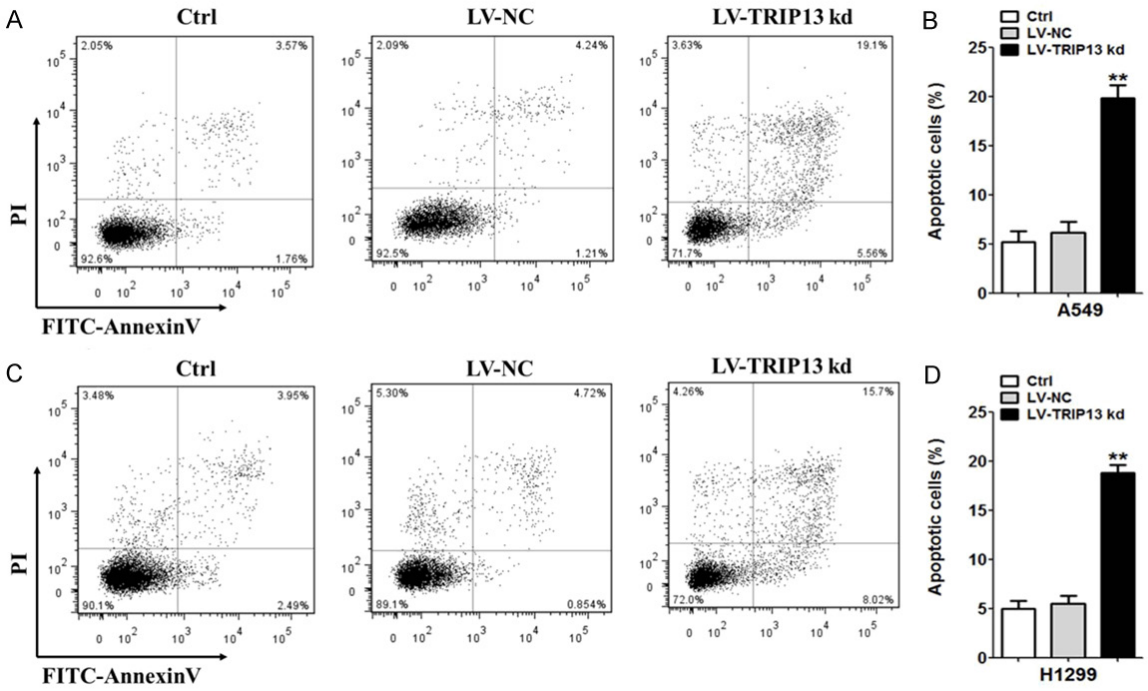
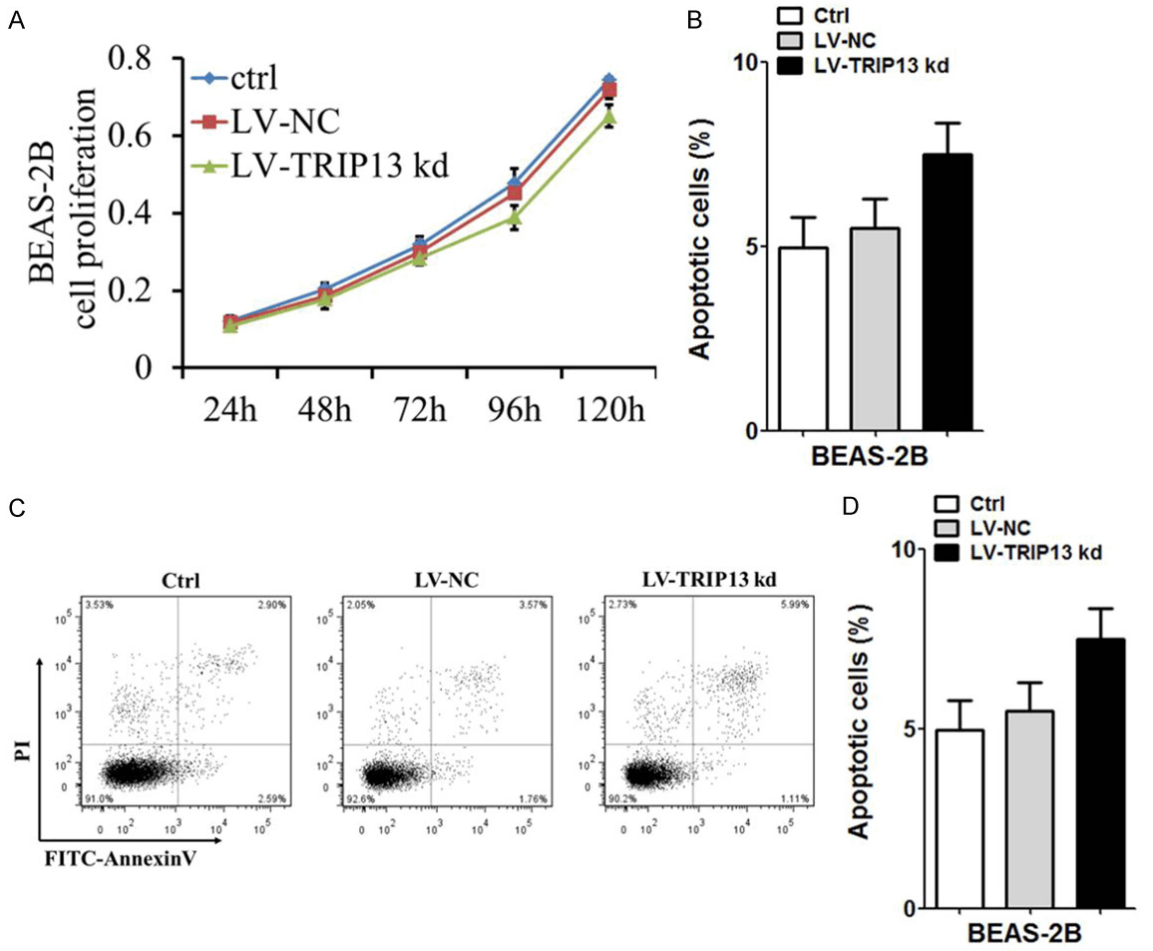
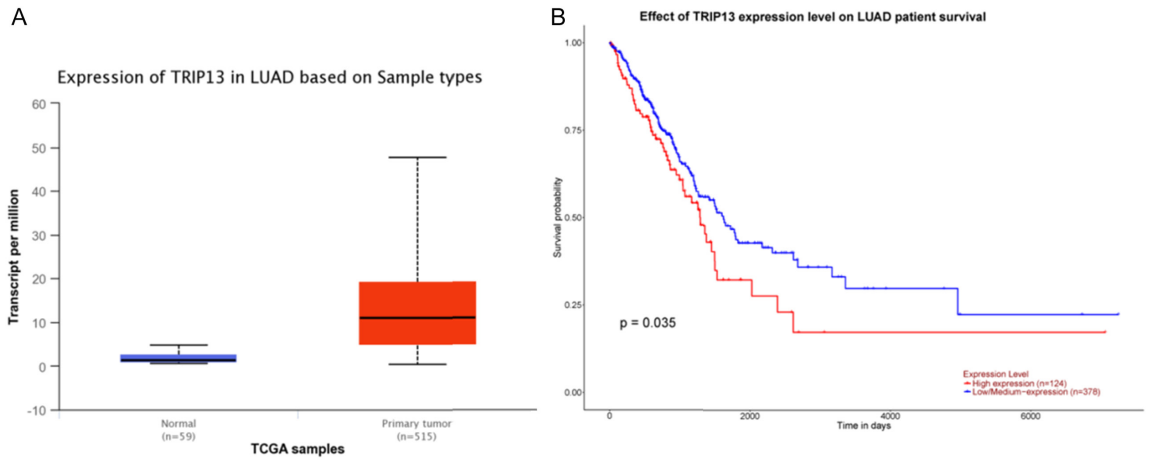


Supplementary Figure 1. Knockdown of TRIP13 by lentivirus promotes apoptosis in NSCLC cell lines. Downregulated TRIP13 promotes apoptotic rates of lung cancer cell lines H1299 and A549. Cell apoptosis was assessed by flow cytometry assay 48 h post-transfection (A-D). The percentage of apoptotic cells is shown in the bar graphs. Values represent the mean ± SEM (*P < 0.05; **P < 0.01).



Supplementary Figure 2. Effect of shTRIP13 on proliferation and apoptosis in human normal lung epithelial cells. The proliferation and apoptotic rates were not affected in normal lung epithelial cells compared to control groups. The proliferation curves measured the absorbance at 592nm by MTT assay (A, B). The percentage of apoptotic cells is shown in the bar graphs (C, D).



Supplementary Figure 3. The transcriptome expression data from lung cancer patients were analyzed in The Cancer Genome Atlas (TCGA) database (http://www.cancergenome.nih.gov) including 515 lung adenocarcinoma (LUAD). A. The RNA expression levels of TRIP13 gene in LUAD. B. Kaplan-Meier curves of overall survival were stratified by TRIP13 expression in LUAD.