

Original Article**Molecular Mechanism of TRAF6 in Malignant Proliferation of Human NK/T Cell Lymphoma Cell HANK1**

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Competing interests: The authors declare no competing interest.

Abstract. Background: Ubiquitination affects cancer progression by regulating both tumor-suppressing and tumor-promoting proteins in cancer. The current study sought to evaluate the role of TNF receptor-associated factor 6 (TRAF6) in the malignant proliferation of the human NK/T cell lymphoma cell line HANK1.

Methods: TRAF6 and MST1 expression levels in HANK1, KHYG-1, and SNK-6 cells were determined by RT-qPCR and Western blot analysis, followed by transfection of si-TRAF6 into HANK1 cells. Cell viability and proliferation were assessed by cell-counting kit-8 and 5-Ethynyl-2'-deoxyuridine (EdU) assays, and proliferating cell nuclear antigen (PCNA) expression levels in cells were determined by Western blot analysis. After that, cells were treated with MG132, followed by analysis of the binding of TRAF6 to macrophage stimulating 1 (MST1) via co-immunoprecipitation and the ubiquitination level of MST1 via the ubiquitination assay. The functional rescue experiment was performed with si-MST1 and si-TRAF6 in cells.

Results: TRAF6 was upregulated in HANK1 cells. Inhibition of TRAF6 reduced cell viability and the number of EdU-positive cells, and downregulated PCNA expression. TRAF6 binds to MST1 to promote ubiquitination-mediated degradation of MST1. After MG132 treatment, the ubiquitination level of MST1 declined. Silencing MST1 abolished the inhibition of TRAF6 on malignant proliferation of HANK1 cells.

Conclusion: TRAF6 was upregulated in HANK1 cells and bound to MST1 to promote ubiquitination-mediated degradation of MST1, consequently facilitating the malignant proliferation of HANK1 cells.

Keywords: Human NK/T cell lymphoma; Ubiquitination; TRAF6; MST1; HANK1; Malignant proliferation; PCNA; MG132.

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Introduction. Natural killer (NK)/T-cell lymphoma associated nasal malignancy, with distinct prevalence in (NKTL) is one type of Epstein-Barr virus (EBV)- Asian and Latin American.¹ NKTL is characterized by

marked aggressiveness and metastasis, proneness to therapeutic resistance, and a high relapse rate.^{2,3} Hence, current therapies, including chemotherapy, radiotherapy, and immunotherapy, show very poor treatment outcomes in NKTL patients in advanced stage.⁴ For example, treatments for systemic EBV-positive T-cell lymphoma and extranodal NKTL include the HLH-2004 chemotherapy regimen, hematopoietic stem cell transplantation, and potential targeted therapy.⁵ With the improvements in genomic and transcriptomic analyses of NKTL, targeted therapy is considered a promising strategy for the treatment of NKTL.⁶ Given the poor long-term prognosis of NKTL,⁷ it is imperative to identify effective molecules targeting NKTL cell functions to provide more targets for the treatment of NKTL.

Tumor necrosis factor receptor-associated factor 6 (TRAF6) is an E3 ubiquitin ligase and an adaptor molecule in tumor necrosis factor receptor-mediated signaling pathways.^{8,9} Of note, TRAF6 is found to be ectopically expressed in 59.6% of all tumors, including uterine fibroids, colon, gastric, breast, and glioma tumors, and affects cancer progression by regulating cancer cell proliferation, apoptosis, invasion, and migration.^{10,11} Increasing evidence has demonstrated that TRAF6 activates the inflammatory and pyroptotic responses of NK/T cells, thus disturbing the homeostasis of NK/T cells.^{12,13} Interestingly, TRAF6 can potentiate the malignant behaviors of NKTL cells by acting as an activator of nuclear factor κ B (NF- κ B).¹⁴ Although previous studies have mainly focused on the oncogenic role of TRAF6 in B cell lymphoma,¹⁵⁻¹⁷ its role in NKTL remains poorly understood.

Ubiquitination is a ubiquitin ligase-mediated posttranslational modification of proteins to target them for proteasomal degradation or non-degradative signaling, leading to alterations in tumor-suppressing and tumor-promoting proteins in cancer.¹⁸ TRAF6 catalyzes ubiquitination to degrade cancer-related proteins to regulate cancer progression.¹⁹⁻²¹ As known before, macrophage stimulating 1 (MST1) is a core component of the Hippo pathway that plays a role in the anti-cancer immune response.²² TRAF6 can bind to MST1 and degrade it via ubiquitination.²³ More importantly, MST1 is noted to be downregulated in NKTL tissues and acts as a suppressor of NKTL cell growth,²⁴ which provides the feasibility of exploring the downstream mechanism of TRAF6 in NKTL cells.

Taking the above evidence into consideration, we speculated that TRAF6 plays a role in NKTL cell proliferation via ubiquitination-mediated degradation of MST1. Consequently, our study is conducted to analyze the molecular mechanism of TRAF6 in the malignant proliferation of NKTL cells and offer a novel theoretical basis for the treatment of NKTL.

Methods and materials.

Cell culture and treatment. ANK1 cells were provided by Shanghai Yaji Biological Co., Ltd (Shanghai, China), KHYG-1 cells were obtained from the JCRB cell bank (Tokyo, Japan), and SNK-6 and normal NK cells were provided by American Type Culture Collection (ATCC, Manassas, VA, USA). HANK1 cells were inoculated in Iscove's modified Dulbecco medium (Gibco, Grand Island, NY, USA) containing 20% fetal bovine serum (FBS) and 100 U/mL interleukin-2 (IL-2). SNK-6 and normal NK cells were inoculated in Roswell Park Memorial Institute medium-1640 (RPMI-1640, Gibco) containing 10% FBS and 700 U/mL IL-2. KHYG-1 cells were inoculated in RPMI-1640 medium supplemented with 10% FBS and 100 U/mL IL-2. All cells were cultured under an environment of 37°C and 5% CO₂.

Cell transfection. Three strands of small interfering RNAs (siRNAs) targeting TRAF6 (si-TRAF6#1, si-TRAF6#2, and si-TRAF6#3), three strands of siRNAs targeting MST1 (si-MST1#1, si-MST1#2, and si-MST1#3), and their negative control (si-NC) were procured from Gene Pharma (Shanghai, China) and transfected into HANK1 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for 48 h before the subsequent experiments.

Cell counting kit-8 (CCK-8) method. HANK1 cell proliferation was evaluated using the CCK-8 method. Transfected HANK1 cells were seeded into the 96-well plate (1×10^4 cells/well) and cultured at 37 °C with 5% CO₂. On days 1, 2, and 3 of incubation, cells in each well were incubated with 10 μ L of CCK-8 reagent (Dojindo, Kumamoto, Japan) for 4 h. Afterwards, the value of optical density at a wavelength of 450 nm was quantified using a microplate reader (Bio-Rad, Hercules, CA, USA). The experiment was repeated three times.

5-Ethynyl-2'-deoxyuridine (EdU) staining. EdU staining for cell proliferation was conducted using the EdU kit (Beyotime, Beijing, China). In brief, HANK1 cells were seeded into the 24-well plate (2×10^4 cells/well). Then, HANK1 cells were incubated with the EdU reagent for 3 h, fixed with 4% paraformaldehyde for 15 min, and treated with 0.3% Triton X-100 for 15 min. After that, HANK1 cells were incubated with the Click Reaction Mixture in the dark at room temperature for 30 min and then treated with 4',6-diamidino-2-phenylindole for another 30 min. Afterwards, cells were observed under a fluorescence microscope (Olympus, Tokyo, Japan) and the results were displayed as a percentage. The experiment was repeated three times.

Co-immunoprecipitation (Co-IP) assay. In the Co-IP assay, HANK1 cells were lysed in the lysis buffer consisting of 150 mmol/L NaCl, 50 mmol/L Tris-HCl

(pH = 7.6), 1% NP-40, 1 mmol/L ethylene diamine tetraacetic acid, 1 mmol/L NaF, 1 mmol/L Na₃VO₄, 1 mmol/L phenylmethanesulfonylfluoride, and 0.1% protease inhibitor cocktail. After centrifugation, the supernatant was collected and incubated with the antibody against TRAF6 (ab137452, Abcam, Cambridge, MA, USA) or IgG (ab133470, Abcam) at 4°C for 30 min. Then, the supernatant was blended with protein A/G Plus-Agarose beads (Santa Cruz Biotechnology, Santa Cruz, MA, USA) using a shaker at 4°C overnight. After three washes, the lysates were boiled in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) protein buffer, followed by determination of the MST1 protein level using Western blot analysis. The experiment was repeated three times.

Ubiquitination assay. HANK1 cells were treated with 20 µM MG132. After 4 h, cells were collected and lysed using the lysis buffer. Then, lysates were incubated with the antibody against MST1 (ab264127, Abcam) or IgG (ab133470, Abcam) and then incubated with protein A/G plus agarose beads at 4°C overnight. Next, the ubiquitination level of MST1 (ab19247, Abcam) was determined by Western blot analysis. The experiment was repeated three times.

Real-time quantitative polymerase chain reaction (RT-qPCR). According to the instructions provided by Invitrogen, the total RNA was extracted from cultured cell lines using the TRIzol reagent. Then, 1 µg of total RNA was reverse transcribed into the complementary DNA using the PrimeScript RT Reagent kit (Invitrogen). RT-qPCR was performed using SYBR green real-time Master Mix (TaKaRa, Dalian, China) on the 7500 Sequence Detection system (Applied Biosystems, Foster City, CA, USA). The relative gene expression was normalized to GAPDH and calculated using the 2^{-ΔΔCt} method.²⁵ Primers used in PCR are shown in **Table 1**. The experiment was repeated three times.

Table 1. PCR primer sequences.

Gene	Sequence (5'-3')
TRAF6	F: ATCCCACGGAACCCAAAAGG
	R: CTCCGAAGGCTACCCATGTC
MST1	F: GTGCGGGAGAGTGAGATGTG
	R: AGTTGTGGGTAAAGCAGGCA
GAPDH	F: ATGGTTTACATGTTCCAATATG
	R: TTA CTCTTGAGGCCATGTGG

Western blot analysis. Cultured cell lines were lysed in the radioimmunoprecipitation assay buffer (Beyotime), and lysates were ultrasonically processed, cultured on ice for 30 min, and centrifuged at 4°C and 12000 g for 15 min. The protein density was quantified using the bicinchoninic acid kit (Beyotime). Protein samples were

isolated using 8% SDS-PAGE, transferred onto nitrocellulose membranes, and immediately blocked with 2% bovine serum albumin for one hour. After that, membranes were incubated at 4°C overnight with antibodies against proliferating cell nuclear antigen (PCNA) (ab92552, 1:1000), TRAF6 (ab137452, 1:500), MST1 (ab264127, 1:500), and β-actin (ab8227, 1:1000) and then incubated with the secondary antibody (1:2000, ab205718) for 1 h. Western blots were visualized using the enhanced chemiluminescence SuperSignal reagent (Thermo Fisher Scientific, Waltham, MA, USA). The grayscale value of target bands was analyzed using ImageJ software. All antibodies were provided by Abcam. The representative original bands are provided in the Supplementary file. The experiment was repeated three times.

Statistical analysis. Statistical analysis and graphing of data were performed with SPSS21.0 statistical software (IBM Corp, Armonk, NY, USA) and GraphPad Prism 8.0 software (GraphPad Software Inc., San Diego, CA, USA). Data complied with normal distribution and homogeneity of variance. The t-test was performed for pairwise comparisons of data, and one-way analysis of variance (ANOVA) was performed for multi-group comparisons of data, followed by Tukey's multiple comparison test for the post-test of data. The value of *P* < 0.05 referred to statistical significance.

Results.

TRAF6 is upregulated in HANK1 cells. TRAF6 has been reported to be expressed at high levels in NKTL cells.¹⁴ However, its role in HANK1 cell proliferation remains elusive. Firstly, we determined TRAF6 expression levels in NTKL cell lines and found that TRAF6 was upregulated in NTKL cell lines relative to normal NK cells, with HANK1 cells showing significantly increased expression of TRAF6 (**Figure 1A-B**).

TRAF6 downregulation suppresses the malignant proliferation of HANK1 cells. To explore the impact of TRAF6 on HANK1 cell proliferation, HANK1 cells were transfected with TRAF6 siRNAs (si-TRAF6#1, si-TRAF6#2, and si-TRAF6#3) to downregulate TRAF6 expression in cells (*P* < 0.05, **Figure 2A- B**). si-TRAF6#2 was found to have the highest knockdown efficiency, and as a result, was selected for the subsequent experiment. The ensuing experiments further showed that after knockdown of TRAF6, the protein levels of proliferation marker PCNA were decreased (*P* < 0.05, **Figure 2B**), cell potential for proliferation was weakened (*P* < 0.05, **Figure 2C**). The number of EdU-positive cells was reduced (*P* < 0.05, **Figure 2D**). These findings suggested that TRAF6 downregulation suppresses malignant proliferation of HANK1 cells.

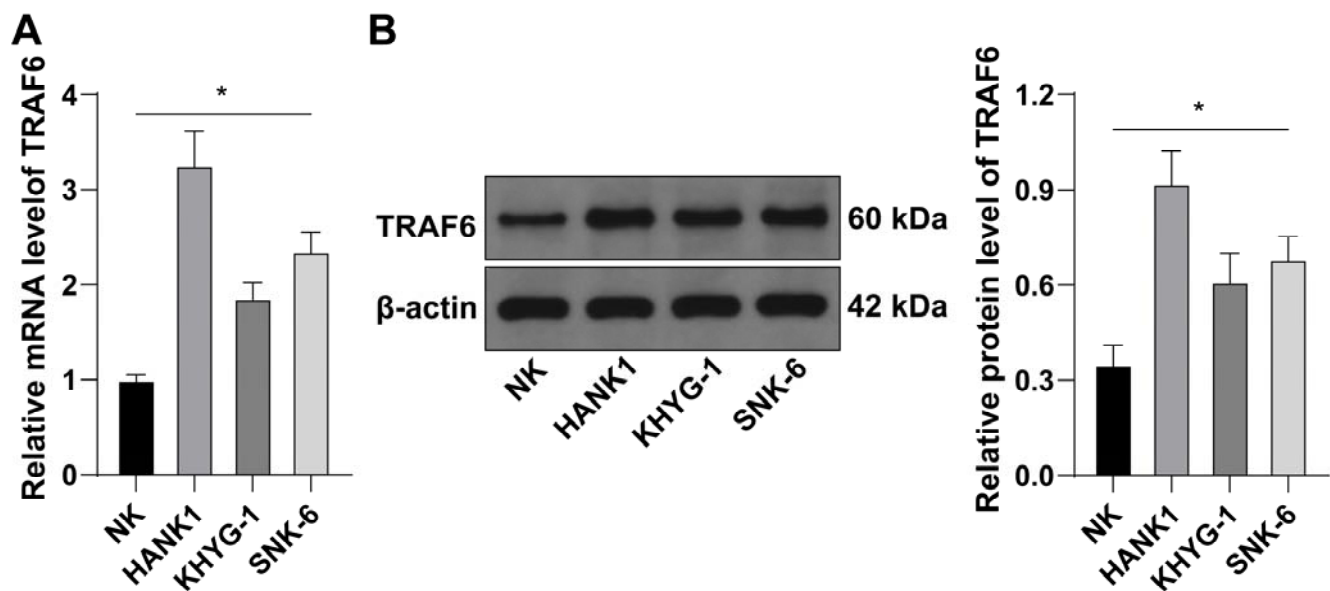


Figure 1. TRAF6 is upregulated in HANK1 cells. NK cells were used as the negative control. **A-B:** Expression levels of TRAF6 in NTKL cell lines were determined by RT-qPCR (**A**) and Western blot analysis (**B**). Data were presented as mean $\pm$ standard deviation. Multiple comparisons in panels **A-B** were analyzed using one-way ANOVA, followed by Tukey's multiple comparisons test. * $P < 0.05$.

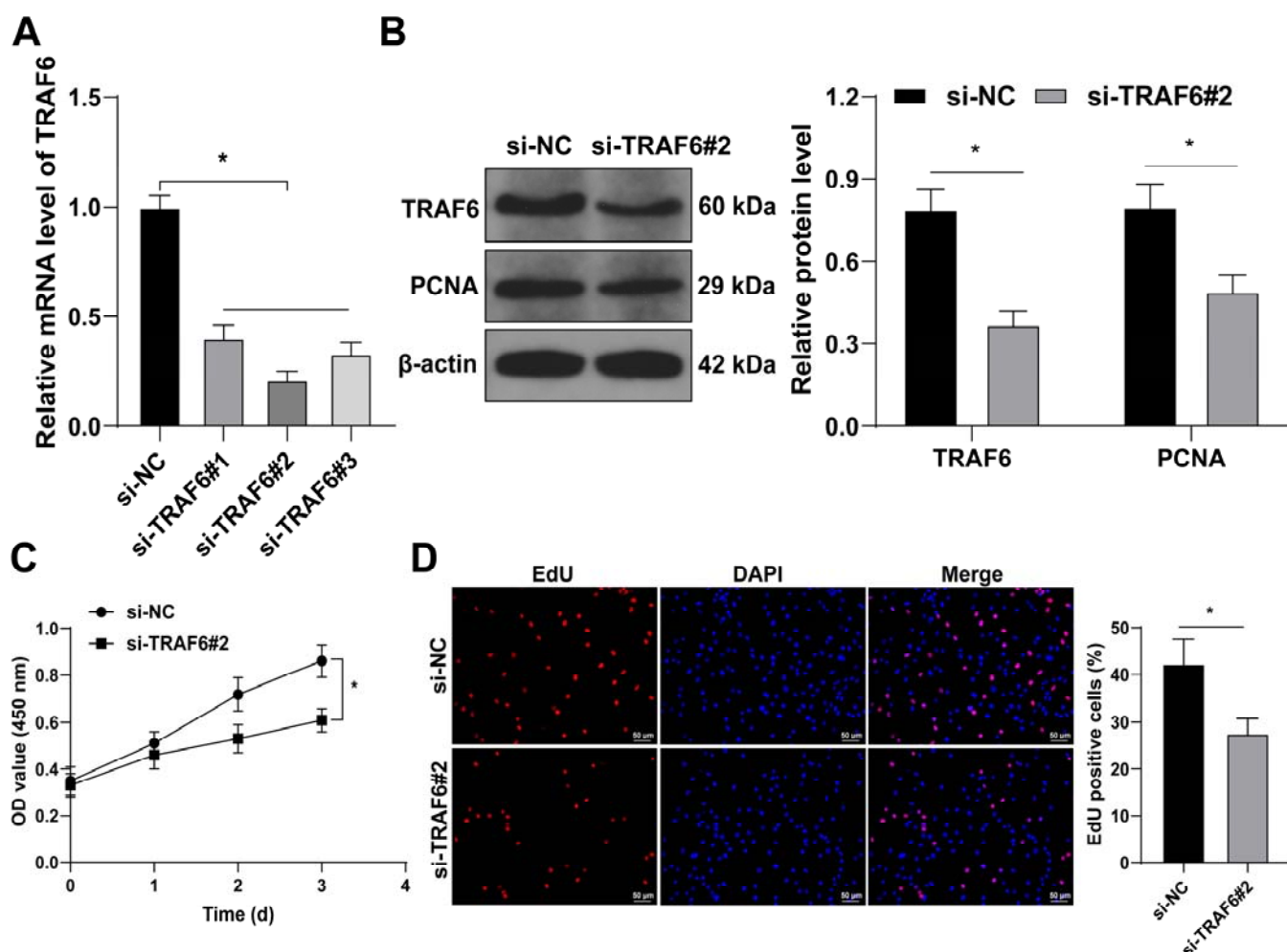


Figure 2. TRAF6 downregulation suppresses malignant proliferation of HANK1 cells. HANK1 cells were transfected with TRAF6 siRNAs (si-TRAF6#1, si-TRAF6#2, and si-TRAF6#3), with si-NC as the negative control. **A:** Knockdown efficiency of TRAF6 siRNAs was determined by RT-qPCR; **B:** Protein levels of TRAF6 and PCNA were determined by Western blot analysis; **C-D:** Cell proliferation was assessed by the CCK-8 method (**C**) and EdU staining (**D**). $n = 3$. Data were presented as mean $\pm$ standard deviation. Pairwise comparisons in panel **D** were analyzed using the *t*-test, and multi-group comparisons in panels **B** and **C** were analyzed using two-way ANOVA, followed by Tukey's multiple comparison test. * $P < 0.05$.

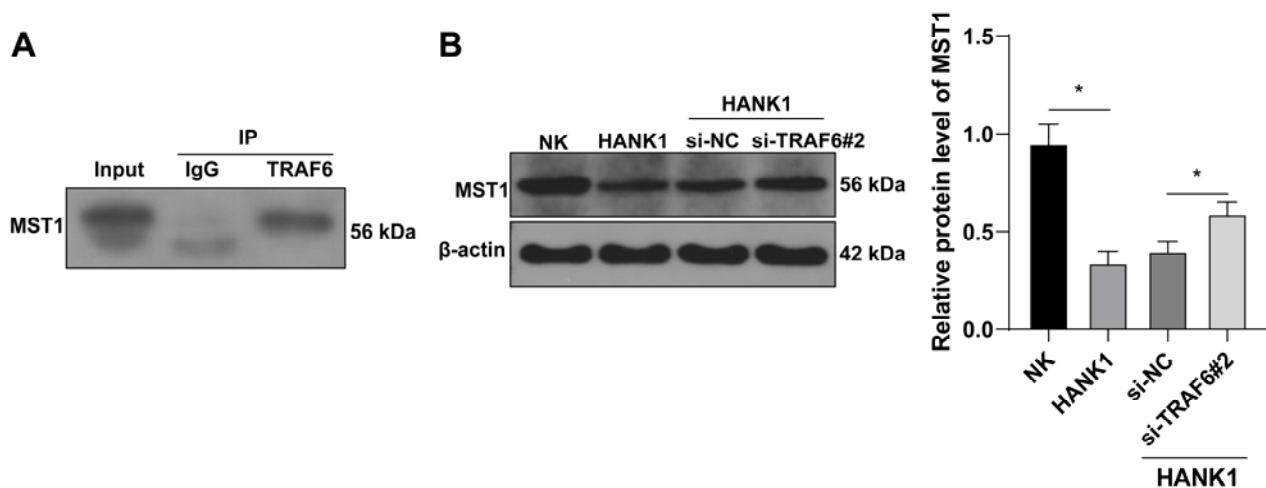


Figure 3. TRAF6 binds to MST1 to downregulate MST1 protein levels. **A:** Binding of TRAF6 to MST1 in HANK1 cells was tested by the Co-IP assay, with IgG used as the negative control; **B:** Protein levels of MST1 were determined by Western blot analysis, with NK cells or si-NC as the negative control. $n = 3$. Data were presented as mean $\pm$ standard deviation. Multi-group comparisons in panel **B** were analyzed using one-way ANOVA, followed by Tukey's multiple comparison test. * $P < 0.05$.

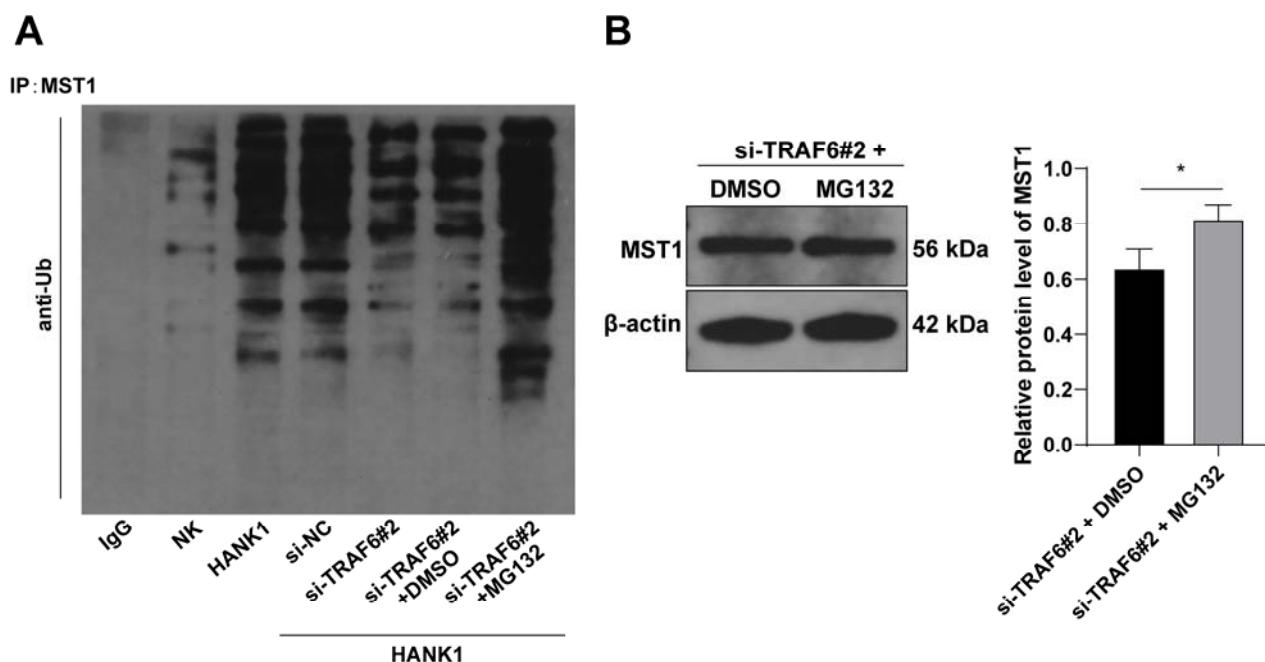


Figure 4. TRAF6 binds to MST1 to promote the ubiquitination-mediated degradation of MST1 and decrease MST1 protein levels. **A:** Ubiquitination levels of MST1 in cells were determined by the ubiquitination assay, with IgG used as the negative control; HANK1 cells in the si-TRAF6#2 group were treated with the proteasome inhibitor MG132, with the treatment of dimethylsulfoxide (DMSO) as the negative control. **B:** Protein levels of MST1 were determined by Western blot analysis. $n = 3$. Data were presented as mean $\pm$ standard deviation. Pairwise comparisons in panel **B** were analyzed using the t test. * $P < 0.05$.

TRAF6 binds to MST1 to downregulate MST1 protein levels. It has been demonstrated that TRAF6 binds to MST1 to promote the ubiquitination-mediated degradation of MST1,²³ and MST1 is weakly expressed in NKTL.²⁴ Therefore, the Co-IP assay was performed in HANK1 cells. The findings revealed the binding of TRAF6 to MST1 (**Figure 3A**). Findings of Western blot assay showed that the protein levels of MST1 were downregulated in HANK1 cells compared with normal NK cells and were increased after knockdown of TRAF6 ($P < 0.05$, **Figure 3B**). Above all, these findings

suggested that TRAF6 bound to MST1 to downregulate MST1 protein levels.

TRAF6 binds to MST1 to promote the ubiquitination-mediated degradation of MST1 and decrease MST1 protein levels. To confirm whether TRAF6 regulates MST1 via ubiquitination modification, the ubiquitination levels of MST1 in cells were determined and found to be markedly elevated in HANK1 cells and decreased as a result of the knockdown of TRAF6 (**Figure 4A**). Next, HANK1 cells were treated with the proteasome inhibitor MG132 to accumulate the ubiquitination levels of MST1

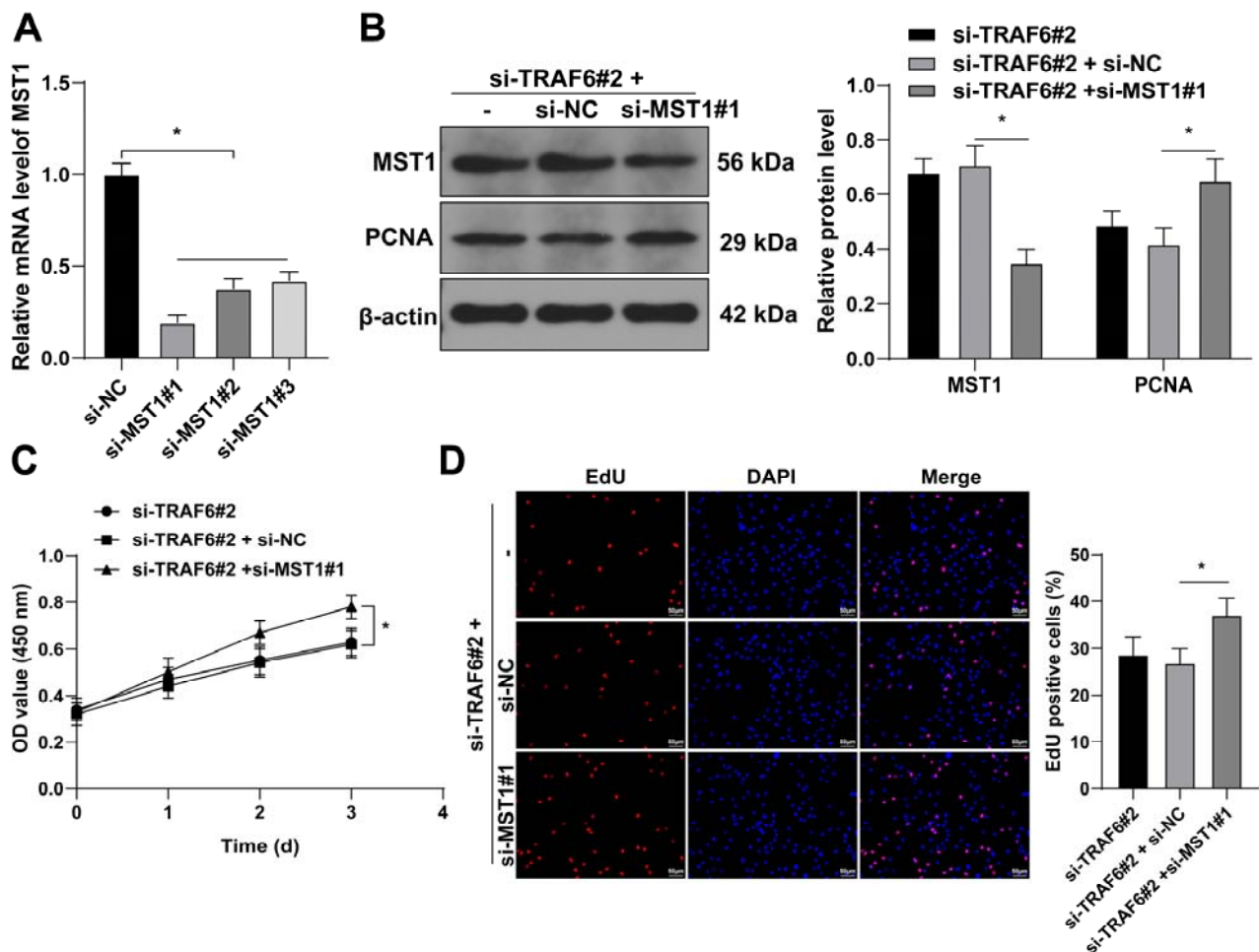


Figure 5. MST1 downregulation neutralizes the inhibition of silencing TRAF6 on malignant proliferation of HANK1 cells. HANK1 cells were transfected with MST1 siRNAs (si-MST1#1, si-MST1#2, si-MST1#3), with si-NC as the negative control. **A:** Knockdown efficiency of MST1 siRNAs was determined by RT-qPCR; Cells were co-treated with si-TRAF6#2. **B:** Protein levels of MST1 and PCNA were determined by Western blot analysis; **C-D:** Cell proliferation was assessed by the CCK-8 method (**C**) and EdU staining (**D**). $n = 3$. Data were presented as mean $\pm$ standard deviation. Multi-group comparisons in panels **A** and **D** were analyzed using one-way ANOVA and in panels **B-C** were analyzed using two-way ANOVA, followed by Tukey's multiple comparison test. * $P < 0.05$.

in cells (**Figure 4A**), upon which the protein levels of MST1 were further augmented ($P < 0.05$, **Figure 4B**). These findings suggested that TRAF6 binds to MST1 to promote the ubiquitination-mediated degradation of MST1 and decrease MST1 protein levels.

MST1 downregulation neutralizes the inhibition of silencing TRAF6 on the malignant proliferation of HANK1 cells. At last, HANK1 cells were transfected with MST1 siRNAs (si-MST1#1, si-MST1#2, si-MST1#3) to downregulate MST1 expression ($P < 0.05$, **Figure 5A- B**), and si-MST1#1 was found to have the highest knockdown efficiency and was selected for combined treatment with si-TRAF6#2. Our results showed that after knockdown of MST1, protein levels of PCNA increased ($P < 0.05$, **Figure 5B**), cell potential for proliferation was strengthened ($P < 0.05$, **Figure 5C**), and the number of EdU-positive cells was augmented ($P < 0.05$, **Figure 5D**). These findings suggested that MST1 downregulation neutralized the inhibition of silencing TRAF6 on malignant proliferation of HANK1 cells

Discussion. NKTL is a highly aggressive malignancy, with few therapies effective in prolonging the survival time of affected patients.²⁶ Exploring epigenetic aberrations in NKTL helps identify effective targets for the treatment of NKTL.²⁶ TRAF6 has been demonstrated to play a role in the epigenetic regulation in various cancers.^{10,11} However, relevant data on the molecular mechanism of TRAF6 in NKTL are largely limited. In the current study, our findings suggested that TRAF6 promoted the ubiquitination-mediated degradation of MST1 to facilitate malignant proliferation of HANK1 cells. Our study is the first to report the role of the TRAF6/MST1 axis in HANK1 cells, providing a new theoretical basis for the diagnosis and treatment of NKTL.

TRAF6 has been identified as a positive regulator of lymphoma biology.¹⁶ As an adaptor molecule, TRAF6 plays a prominent role in signal transduction of the NF- κ B signaling, leading to lymphomagenesis.²⁷ Notably, the TRAF6/NF- κ B pathway forms a negative feedback

loop with miR-146a to regulate apoptosis and chemosensitivity in NKTL.¹⁴ PCNA is a well-documented factor that is indicative of cancer cell proliferation.²⁸ In our experiments, TRAF6 was upregulated in HANK1 cells, and silencing TRAF6 repressed the malignant proliferation of HANK1 cells along with decreased protein levels of PCNA. Consistently, former studies have elucidated that TRAF6 interacts with LIM domain-containing protein 1 to prevent DNA damage and apoptosis and promote autophagy in EBV-associated lymphomas^{29,30} and TRAF6 is involved in lncRNA NORAD-mediated oncogenic competitive endogenous RNA network to promote cell proliferation and inhibit cell cycle arrest and apoptosis in diffuse large B cell lymphoma (DLBCL).¹⁷ Besides, inhibition of TRAF6 displays therapeutic implications in lymphomas. For instance, TRAF6 depletion caused cell cycle arrest at the G0/G1 phase and cell death of DLBCL.³¹ High expression of TRAF6 correlates with poor prognosis and silencing of TRAF6 increases the sensitivity of DLBCL cells to doxorubicin.¹⁶ Collectively, our findings and existing evidence both support the prominent expression of TRAF6 in HANK1 cells and its promotive role in the malignant proliferation of HANK1 cells.

With Lys of MST1 as a site of TRAF6-mediated ubiquitination,³² TRAF6 has been reported to catalyze the ubiquitination of MST1 to repress its expression.²³ A pioneering study has highlighted the correlation between the MST1 mutation and the onset of NKTL.³³ Another study has also reported the downregulation of MST1 in NKTL tissues and the promotive role of MST1 knockdown in NKTL cell growth.²⁴ Besides, MST1 deficiency facilitates lymphoma development by causing chromosomal instability.³⁴ In accordance, our experiments uncovered reduced protein levels and increased ubiquitination levels of MST1 in HANK1 cells, and these trends were reversed by TRAF6 downregulation or MG132 treatment, suggesting that TRAF6 bound to MST1 to promote the ubiquitination-mediated degradation of MST1 and decrease MST1 protein levels in HANK1 cells. Furthermore, silencing MST1 decreased protein levels of PCNA and enhanced malignant proliferation of HANK1 cells in the si-TRAF6 group. Supporting our findings, Hippo pathways, including MST1, are found to be associated with the progression-free survival of B-cell lymphoma.³⁵ Moreover, as a tumor suppressor in the Hippo pathways, MST1 can be independent of or involved in the Hippo signaling pathway to inhibit the progression of many cancer types.^{36,37} For instance, MST1 triggers reactive

oxygen species-mediated pyroptosis to slow down the progression of pancreatic cancer;³⁸ MST1 activates the Hippo signaling pathway to enhance the malignant behaviors of breast cancer cells.³⁹ Above all, we are the first to unveil that TRAF6 promoted malignant proliferation of HANK1 cells via inducing ubiquitination of MST1, which underpinned the novelty of our study.

Conclusions. Our study initially demonstrated the connection of the TRAF6/MST1 signaling in malignant proliferation of HANK1 cells and provided a novel insight into the application of targeted therapy for NKTL treatment. The aberrant activation of TRAF6 in NKTL may serve as a molecular feature distinguishing it from other lymphoma subtypes. The TRAF6/MST1 signaling may target specific other signaling networks (including MST1) in the tumor microenvironment, thereby addressing the gap in resistance to existing therapies (e.g., PD-1 inhibitors) and exhibiting potential synergistic effects, especially in relapsed/refractory cases. In future studies, we will supplement data on long-term survival rates and evaluate the impact of targeted therapy on immune reconstitution. However, our current investigation of TRAF6 functionality and downstream mechanism was limited to HANK1 cells; the roles of TRAF6 in other cellular processes of NKTL cells, such as invasion and migration, remain unknown; the role of TRAF6 in NKTL was not validated in animal models; the function of complete TRAF6 knockout on NKTL cells requires further verification; phosphorylation plays an important role in ubiquitination modification, however, the effect of TRAF6 on MST1 phosphorylation cannot be verified under our current conditions; other downstream mechanisms of TRAF6 in NKTL, except MST1, remain to be explored. In the next step, we shall validate other mechanisms of TRAF6 using more NKTL cell lines, employ various experimental approaches to explore the novel downstream mechanisms of TRAF6, conduct preliminary experiments to verify the effect of TRAF6 on MST1 phosphorylation, and comprehensively investigate the impact of TRAF6 on the malignant phenotype of NKTL, providing updated theoretical knowledge for NKTL treatment.

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