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ARTICLE

TRIM25 activates AKT/mTOR by inhibiting PTEN via K63-linked polyubiquitination in non-small cell lung cancer

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The PTEN/AKT/mTOR signaling pathway is frequently dysregulated in non-small cell lung cancer (NSCLC), but the mechanisms are not well-understood. The present study found that the ubiquitin ligase TRIM25 is highly expressed in NSCLC tissues and promotes NSCLC cell survival and tumor growth. Mechanistic studies revealed that TRIM25 binds to PTEN and mediates its K63-linked ubiquitination at K266. This modification prevents the plasma membrane translocation of PTEN and reduces its phosphatase activity therefore accumulating PI(3,4,5)P3. TRIM25 thus activates the AKT/mTOR signaling. Moreover, we found that the antibacterial nitroxoline can activate PTEN by reducing its K63-linked polyubiquitination and sensitizes NSCLC to cisplatin-induced apoptosis. This study thus identified a novel modulation on the PTEN signaling pathway by TRIM25 and provides a potential target for NSCLC treatment.

Keywords: PTEN; TRIM25; K63-linked polyubiquitination; non-small cell lung cancer

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INTRODUCTION

Non-small cell lung cancer (NSCLC) is a type of rapidly progressive malignancy of the lung and is the major cause of cancer-related death in China and worldwide. Advances in cancer biology have demonstrated that receptor tyrosine kinases (RTKs), such as epidermal growth factor receptors and vascular endothelial growth factor receptors, play critical roles in lung cancer development and progression. Notably, these RTK signals converge to activate the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/AKT/mTOR pathway [1]. Once activated, PI3K catalyzes the synthesis of PI(3,4,5)P3 from PI(4,5)P2, and the resultant PI(3,4,5)P3 then acts as a second messenger that leads to activation of the AKT and mTOR kinases, therefore promoting NSCLC cell proliferation, survival, invasion, and intracellular trafficking [1]. Notably, PI(3,4,5)P3 can be hydrolyzed to PI(4,5)P2 by a specific phosphatase called phosphatase and tensin homolog (PTEN). PTEN therefore antagonizes the activation of AKT and suppresses the PI3K/AKT/mTOR signaling pathway. However, PTEN is frequently inactivated in NSCLC cells by genetic events (such as loss of heterozygosity and/or gene mutation) [1] and post-translational modifications (such as phosphorylation and ubiquitination) [2]. Ubiquitination is a biological process that involves the addition of ubiquitin molecules to lysine (K) residues of target proteins under the direction of a well-ordered enzyme cascade that includes ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s) [3]. Notably, protein ubiquitination is a dynamic process, and ubiquitin-specific proteases can hydrolyze ubiquitin molecules from their substrates [4]. PTEN undergoes degradation by the proteasome after K48-linked polyubiquitination mediated by the ubiquitin ligase NEDD4-1 [5]. Moreover, NEDD4-1 mediates PTEN monoubiquitination, which results in its nuclear translocation [6]. In the present study, we found that the E3 tripartite motif-containing 25 (TRIM25) interacts with PTEN and mediates its K63-linked polyubiquitination, thereby activating the AKT/mTOR signaling pathway. Moreover, we found that inhibition of TRIM25-mediated PTEN ubiquitination can sensitize NSCLC to chemotherapy.

MATERIALS AND METHODS

Cell culture

Human NSCLC cell lines (H1299, H460, and A549) were purchased from Procell Life Science & Technology Co., Ltd. (Wuhan, China) and were cultured in RPMI-1640 medium. All media were supplemented with 10% fetal bovine serum (ExCell Bio, Inc., Shanghai, China), glutamine, and antibiotics.

Plasmids and antibodies

TRIM25 and PTEN were cloned as described previously [7, 8]. The K13R, K289R, K254R, and K266R PTEN mutants were constructed by using a site-directed mutagenesis kit (TransGen, Beijing, China) with specific primers for PTEN K13R (5′-AGCAGAAACagaAGGAGATAT-3′ and 5′-ATATCTCCTtctGTTTCTGCT-3′), PTEN K289R (5′-ACCTCAGAAagaGTAGAAAAT-3′ and 5′-ATTTTCTACtctTTCTGAGGT-3′), PTEN K254R

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(5′-TGATATCagaGTAGAGTTCTTCCACAAACA-3′ and 5′-ACTCTACtctGATATCACCACACACAGGTA-3′), and PTEN K266R (5′-AAGATGCTAagaAAGGACAAA-3′ and 5′-TTTGTCCTTtctTAGCATCTT-3′). The truncated fragments of TRIM25 were subcloned by polymerase chain reaction (PCR). Monoclonal anti-HA, anti-Myc, and anti-Flag antibodies were obtained from MBL (Tokyo, Nagoya, Japan). The antibody against GAPDH was purchased from Proteintech Group, Inc. (Wuhan, Hubei, China). Antibodies against AKT, p-AKT, mTOR, p-mTOR, P70S6K, p-P70S6K, PTEN, TRIM25, PARP, p21, p53, IκBα, and caveolin were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). An antibody against Ub was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HRP-labeled goat anti-mouse and goat anti-rabbit IgG (H + L) antibodies were purchased from Beyotime Institute of Biotechnology (Nantong, Jiangsu, China).

Gene transfection

One day before transfection, HEK293T cells were seeded in six-well plates. At 50% confluence, cells were subjected to gene delivery using polyethylenimine (PEI)-mediated transfection as described previously [9]. NSCLC cell lines were transfected with the indicated plasmids or small interfering RNAs (siRNAs) using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Isolation of the cytosol and membrane fractions

Membrane proteins were extracted with a Membrane and Cytosol Protein Extraction Kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions.

Primary NSCLC tissues

NSCLC patients were diagnosed by pathological examination and CT imaging at the First Affiliated Hospital of Soochow University. All patients were provided with written notice for use of their tissue specimens for research purposes, and an informed consent form was signed by each patient before surgery. After NSCLC tissues were obtained, the para-cancerous tissues were isolated from the tumor tissues. All samples were then frozen by dipping in liquid nitrogen for further use. This study was approved by the Review Board and Ethical Committee of Soochow University.

Immunoblotting (IB)

After transfection with the appropriate plasmids, cells were lysed on ice in lysis buffer as described previously [9]. After clarification by high-speed centrifugation at 4 °C, protein concentrations were determined by a BCA assay (Beyotime). Equal amounts of protein (30 μg) were fractionated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore®). The membranes were probed with the appropriate antibodies as described previously [9].

Immunoprecipitation (IP)

After transfection with the appropriate plasmids for 48 h, cells were harvested to prepare whole cell lysates, and the IP assay was performed as described previously [9]. The precipitated beads were then boiled with 2 × SDS loading buffer for IB.

Immunofluorescence assay (IF)

The IF assay was performed as described previously [9]. Briefly, cells were replated on coverslips 24 h before being washed with ice-cold PBS. After being fixed in 4% paraformaldehyde and permeabilized with 0.02% Triton X-100, cells were sequentially incubated with antibodies specific for PTEN and TRIM25. Cells were then washed and incubated with a secondary antibody mixture containing goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC, Beyotime) and goat anti-rabbit IgG conjugated to cyanine (Cy)3 (Beyotime) at room temperature for 3 h. Images were acquired with a laser scanning confocal microscope.

Gene knockdown with siRNA and short hairpin RNA (shRNA)

Specific siRNAs obtained from RiboBio (Guangzhou, Guangdong, China) were transfected into HEK293T cells using PEI as the gene carrier. Forty-eight hours later, cells were prepared for IB to evaluate the knockdown efficiency. The siRNA with the highest knockdown efficiency was then selected for further studies. The lentiviral shRNA plasmid hU6-MCS-CBh-gcGFP-IRES-puro targeting human PTEN and containing the puromycin resistance gene was purchased from GeneChem Gene Technologies (Shanghai, China).

Cycloheximide (CHX) chase assay

After transfection with plasmids of interest for 36 h, cells were treated with CHX (100 μg/mL, Beyotime) for 0–12 h before lysis. Proteins were then separated by SDS-PAGE, and IB was performed with specific antibodies as described previously [10].

Cell viability, proliferation, and apoptosis assays

NSCLC cell viability was measured by an MTT assay as described previously. To evaluate apoptosis, treated cells were stained with Annexin V-FITC and propidium iodide (PI) according to the manufacturer's instructions (MultiSciences Biotech Co., Ltd., Hangzhou, China). Stained cells were analyzed with a flow cytometer (FACSCalibur®, Becton Dickinson).

Reverse-transcription PCR

Total RNA was extracted using TRIzol® (TransGen, Beijing, China). RNA (2.5 μg) was reverse-transcribed using EasyScipt® First-strand cDNA Synthesis SuperMix (TransGen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. PCR amplification was performed using the following primers: for TRIM25, 5′-CCACCAGAGCACCATAGACC-3′ (forward) and 5′ -CTGGGTAAGGCAGGGACA-3′ (reverse); for GAPDH, 5′-AATCCCATCACCATCTTCC-3′ (forward) and 5′-CATCACGCCACAGTTTCC-3′ (reverse). The PCR products were visualized by GoldView® staining (TransGen) after electrophoresis on 2% agarose gels.

PI(3,4,5)P3 assay

The PI(3,4,5)P3 concentration was determined by using a PI(3,4,5)P3 kit provided by Elabscience Biotechnology Co., Ltd. (Wuhan, Hubei, China). Specifically, PI(3,4,5)P3 was released by repeated freezing and thawing of NSCLC cells prior to high-speed centrifugation to remove cell debris. To measure the concentration of PI(3,4,5)P3, the supernatants (10 μL) of each sample were mixed with 40 μL of dilution buffer before being added to an ELISA plate pretreated with an anti-PI(3,4,5)P3 antibody. The reaction was then allowed to proceed for 30 min at 37 °C prior to incubation with an HRP-conjugated reagent for 30 min. After gentle washing, chromogen solutions A and B were added to each well and incubated in the dark for 15 min at 37 °C before the stop solution was added. The absorbance was then read at 450 nm. The concentration of PI(3,4,5)P3 was then determined by scaling to the standard curve.

In vivo xenograft models

H1299 cells stably expressing TRIM25, A549 cells transfected with siTRIM25, and their control counterparts were injected subcutaneously into the flanks of severe combined immunodeficient (SCID mice (female, 5–6 weeks old, SLAC Laboratory Animal Co. Ltd., Shanghai, China) at a density of 2×10^6 cells per injection ($n = 5$). Tumor sizes and body weights were monitored every other day. At the end of the in vivo experiments, mice were sacrificed, and tumor tissues were dissected, weighed, and frozen by dipping in liquid nitrogen. Proteins from the tumor tissues were subjected to IB. This animal experiment was approved by the Review Board for Animal Welfare and Ethics of Soochow University.

Statistics
Statistical differences between the control and the experimental groups were analyzed by Student's *t* test.

RESULTS

TRIM25 is highly expressed in NSCLC tissues and negatively associated with poor outcomes of NSCLC patients

To examine the potential role of TRIM25 in NSCLC, we first evaluated its expression profile in primary NSCLC tissues and paired para-cancerous tissues. The results showed that TRIM25 was overexpressed in NSCLC tissues at both the protein and mRNA levels (representative data are shown in Fig. 1a, b). TRIM25 was significantly increased in NSCLC tissues compared with the adjacent normal tissues (Fig. 1c). This finding was consistent with the analysis of the TCGA Lung 2 cancer dataset deposited in Oncomine (https://www.oncomine.org). The TCGA Lung 2 dataset is composed of DNA microarray analysis data from 729 NSCLC samples and paired normal tissues, in which the TRIM25 gene copy number was significantly increased in NSCLC tissues compared with normal control tissues (Fig. 1d). Next, we evaluated the correlation of the expression level of TRIM25 and the overall survival rate of NSCLC patients based on two independent datasets (GSE14814 [11] and GSE19188 [12]) using the PROGgeneV2 platform (http://watson.compbio.iupui.edu/chirayu/proggene/database/?url=proggene). The higher the TRIM25 expression level, the lower was the overall survival rate of NSCLC patients (Fig. 1e). Therefore, high expression of TRIM25 in NSCLC might be a novel biomarker that predicts poor outcomes of NSCLC patients.

TRIM25 promotes NSCLC cell survival and xenograft growth

We next sought to determine whether TRIM25 contributes to NSCLC cell proliferation and survival. To this end, TRIM25 was overexpressed in A549 and H1299 cells, and an MTT assay was conducted. The results showed that TRIM25 markedly increased cell viability in both NSCLC cell lines (Fig. 1f, g). In line with this finding, knockout of TRIM25 decreased cell viability (Fig. 1h, i). Furthermore, NSCLC xenografts derived from H1299 cells stably overexpressing TRIM25 grew at a more rapid rate than those derived from the parental cells (Fig. 1j). To confirm this finding, TRIM25 was knocked down in A549 cells, and a xenograft assay was conducted. The results showed that knockdown of TRIM25 significantly decreased the growth rate of the xenografts (Fig. 1k), further indicating that TRIM25 promotes NSCLC tumor growth. Moreover, TRIM25 confers chemoresistance on NSCLC cells. We found that ectopic expression of TRIM25 strikingly reduced CDDP-induced H1299 cell apoptosis; in contrast, when TRIM25 was knocked down, the sensitivity of A549 cells to CDDP was restored (data not shown). Taken together, these results indicate that TRIM25 promotes cell proliferation, tumor growth, and chemoresistance in NSCLC. Understanding the role and mechanism of TRIM25 in NSCLC might facilitate the treatment of NSCLC patients.

TRIM25 interacts with PTEN via its B-box, coiled-coil (CC), and SPRY domains

Considering that TRIM25 is an oncogenic ubiquitin ligase, we sought to determine whether it can mediate the polyubiquitination of any tumor suppressor protein(s), thus inhibiting its function and promoting NSCLC progression. To this end, we evaluated the interaction of TRIM25 with typical tumor suppressor proteins, including p21, p53, PTEN, and IκBα. PTEN and p53 but not the other proteins examined were found in the TRIM25 coimmunoprecipitates (Fig. 2a), indicating that TRIM25 interacted with PTEN and p53. Because p53 has been demonstrated to interact with and modulate TRIM25 [13], PTEN was selected for further studies. To verify the interaction between TRIM25 and PTEN, TRIM25 was transfected into NSCLC cell lines, and the results of subsequent IP/IB showed that TRIM25 bound to PTEN (Fig. 2b, c). Moreover, an endogenous interaction between TRIM25 and PTEN was found in the NSCLC cell lines A549 and H1299 (Fig. 2d). To identify the domain in TRIM25 critical for its interaction with PTEN, TRIM25 truncation mutants were constructed based on its specific domains, and these mutants were cotransfected with PTEN. The IP/IB results showed that the B-box, CC, and SPRY domains were present in the complex co-immunoprecipitated with PTEN (Fig. 2e), suggesting that these domains but not the RING domain might mediate the interaction of TRIM25 with PTEN. The interaction between TRIM25 and PTEN was also confirmed by the IF assay. As shown in Fig. 2f, TRIM25 was mainly localized in both the plasma membrane and cytosol, while PTEN was localized in the plasma membrane, cytosol, and nucleus. The merged image indicates that both proteins can be colocalized in the cytosol and at the plasma membrane.

TRIM25 mediates K63-linked polyubiquitination of PTEN at K266

Given that TRIM25 is a ubiquitin ligase, we sought to determine whether TRIM25 can mediate PTEN ubiquitination. To this end, TRIM25 and separate plasmids expressing ubiquitin with a single available lysine residue were cotransfected into HEK293T cells, and IP/IB were then conducted. As shown in Fig. 3a, TRIM25 promoted polyubiquitination in the presence of the Ub-K63 plasmid. To confirm this finding, WT-, K48-, or K63-Ub plasmids were further cotransfected with TRIM25 and PTEN. Subsequent IP/IB again showed that TRIM25 significantly promoted K63- but not K48-linked ubiquitination of PTEN (Fig. 3b). To confirm this finding, we next performed a PTEN ubiquitination assay by co-transfecting the K63R or K48R Ub mutant with PTEN and TRIM25. Consistent with the above results, when K63 in Ub was mutated to R (K63R), the ubiquitination level of PTEN was not markedly increased by TRIM25; however, when the K48R mutant, which contains an intact K63 residue, was introduced, PTEN ubiquitination was significantly increased by TRIM25 (Fig. 3c). Furthermore, similar findings were obtained in A549 and H1299 cells in the presence of TRIM25 (Fig. 3d). In agreement with these findings, knockdown of TRIM25 robustly decreased endogenous PTEN ubiquitination levels (Fig. 3e). Therefore, TRIM25 promotes K63-linked polyubiquitination of PTEN.

There are 34 lysine residues in PTEN, among which K13 and K289 have been reported to be the ubiquitin acceptor sites for monoubiquitination or polyubiquitination mediated by NEDD4-1 [5, 14]. To determine whether TRIM25 also mediates PTEN ubiquitination at these two lysine residues, we constructed K13R and K289R PTEN mutants and evaluated the effects of these mutations by cotransfection and IP/IB. The results showed that neither of these two mutations abolished PTEN polyubiquitination in the presence of TRIM25 (Fig. 3f), suggesting that other sites but not K13 and K289 might act as ubiquitin acceptors in TRIM25-mediated ubiquitination of PTEN. By reviewing the literature, we found that K254 and K266 are critical in maintaining PTEN activity [15]. To determine the roles of these sites in TRIM25-mediated PTEN polyubiquitination, we constructed K254R, K266R, and K254R/K266R PTEN mutants. These mutants were transfected with wild-type PTEN into H1299 cells stably expressing TRIM25. The IP/IB results showed that PTEN K266R but not PTEN K254R lost the capacity for TRIM25-mediated ubiquitination (Fig. 3g). In line with this finding, the PTEN K254R/K266R double mutant could not be ubiquitinated by TRIM25 (Fig. 3g). Therefore, TRIM25 mediated K63-linked polyubiquitination of PTEN at K266. Because K63-linked ubiquitination is not associated with protein stability, the half-life of PTEN was measured in the presence of TRIM25 and the protein synthesis inhibitor CHX. As expected, when de novo protein synthesis was blocked by CHX, both the expression and knockdown of TRIM25 failed to alter PTEN stability (Fig. 3h, i). Therefore, TRIM25 modifies PTEN via K63-linked polyubiquitination at K266 without affecting its protein level.

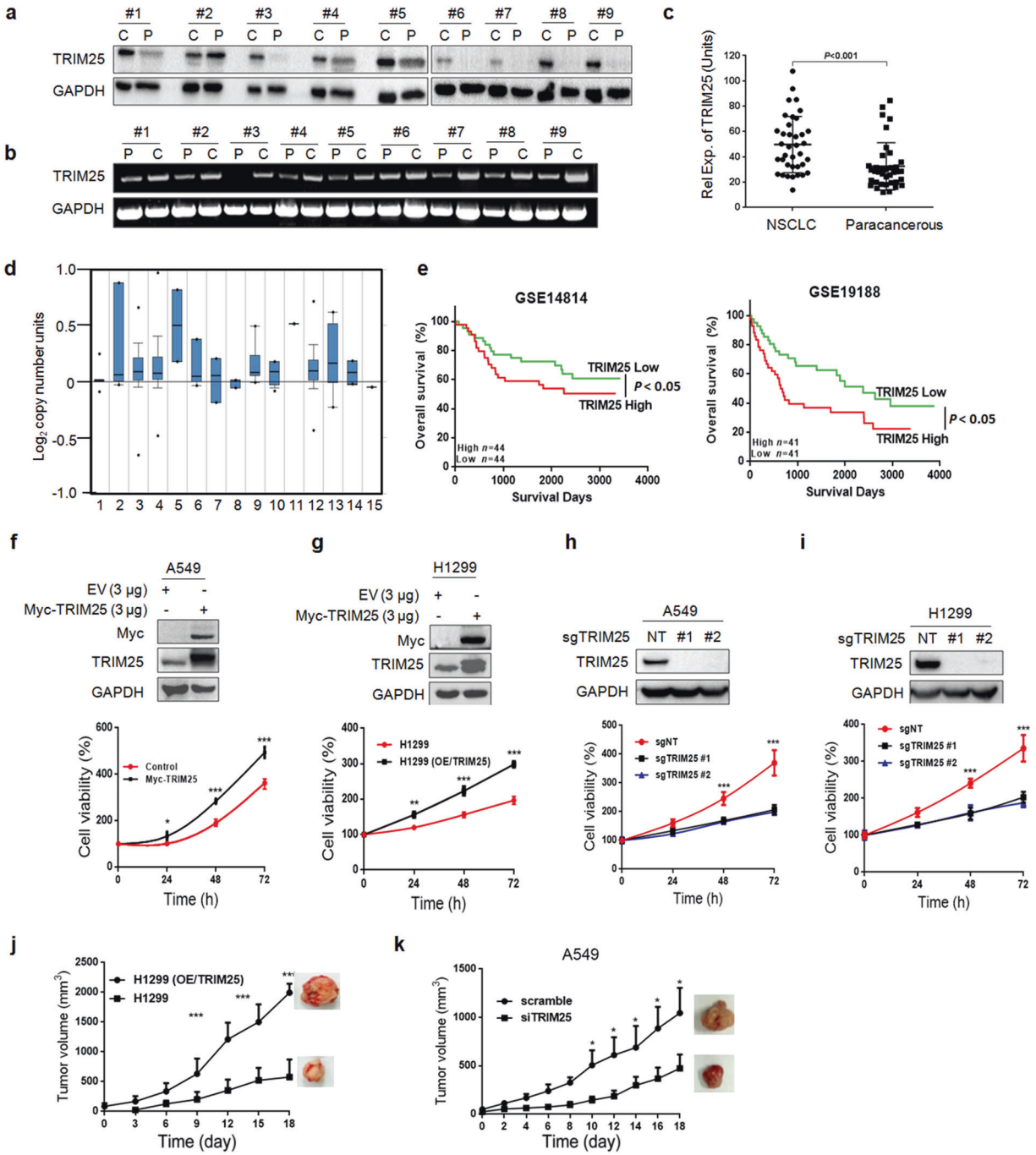

Fig. 1 TRIM25 is overexpressed in NSCLC cells and is negatively associated with the overall survival of NSCLC patients. The expression pattern of TRIM25 in NSCLC tumor (C) and para-cancerous (P) tissues was analyzed by IB (**a**) and RT-PCR (**b**). **c** Statistical analysis of TRIM25 expression in NSCLC tissues and adjacent normal counterparts ($n = 37$). **d** TRIM25 expression in various types of lung cancer based on a dataset from Oncomine (www.oncomine.org). (1) Normal lung tissues ($n = 810$); (2) acinar lung adenocarcinoma ($n = 6$); (3) lung adenocarcinoma (261); (4) lung adenocarcinoma, mixed subtype ($n = 67$); (5) lung clear cell adenocarcinoma ($n = 2$); (6) lung mucinous adenocarcinoma ($n = 6$); (7) micropapillary lung adenocarcinoma ($n = 3$); (8) mucinous bronchioloalveolar carcinoma ($n = 3$); (9) nonmucinous bronchioloalveolar carcinoma ($n = 9$); (10) papillary lung adenocarcinoma ($n = 10$); (11) solid lung adenocarcinoma ($n = 1$); (12) squamous cell lung carcinoma ($n = 348$); (13) squamous cell lung carcinoma, basaloid variant ($n = 8$); (14) squamous cell lung carcinoma, papillary variant ($n = 2$); (15) squamous cell lung carcinoma, small cell variant ($n = 1$). **e** Correlation of TRIM25 expression and overall survival of NSCLC patients from two independent datasets, GSE14814 and GSE19188, retrieved from http://watson.compbio.iupui.edu/chirayu/proggene/database/index.php. A549 (**f**) and H1299 (**g**) cells transfected with the Myc-TRIM25 plasmid were plated in 96-well plates for the indicated periods prior to an MTT assay. TRIM25 was knocked out by siRNA in A549 (**h**) and H1299 (**i**) cells, and an MTT assay was conducted. **j** H1299 cells expressing TRIM25 or empty vector were subcutaneously injected into the right flanks of nude mice, and tumor growth was monitored. **k** TRIM25 was knocked down in A549 cells, and the resultant cells were then injected s.c. into nude mice. Tumor growth was monitored every other day.

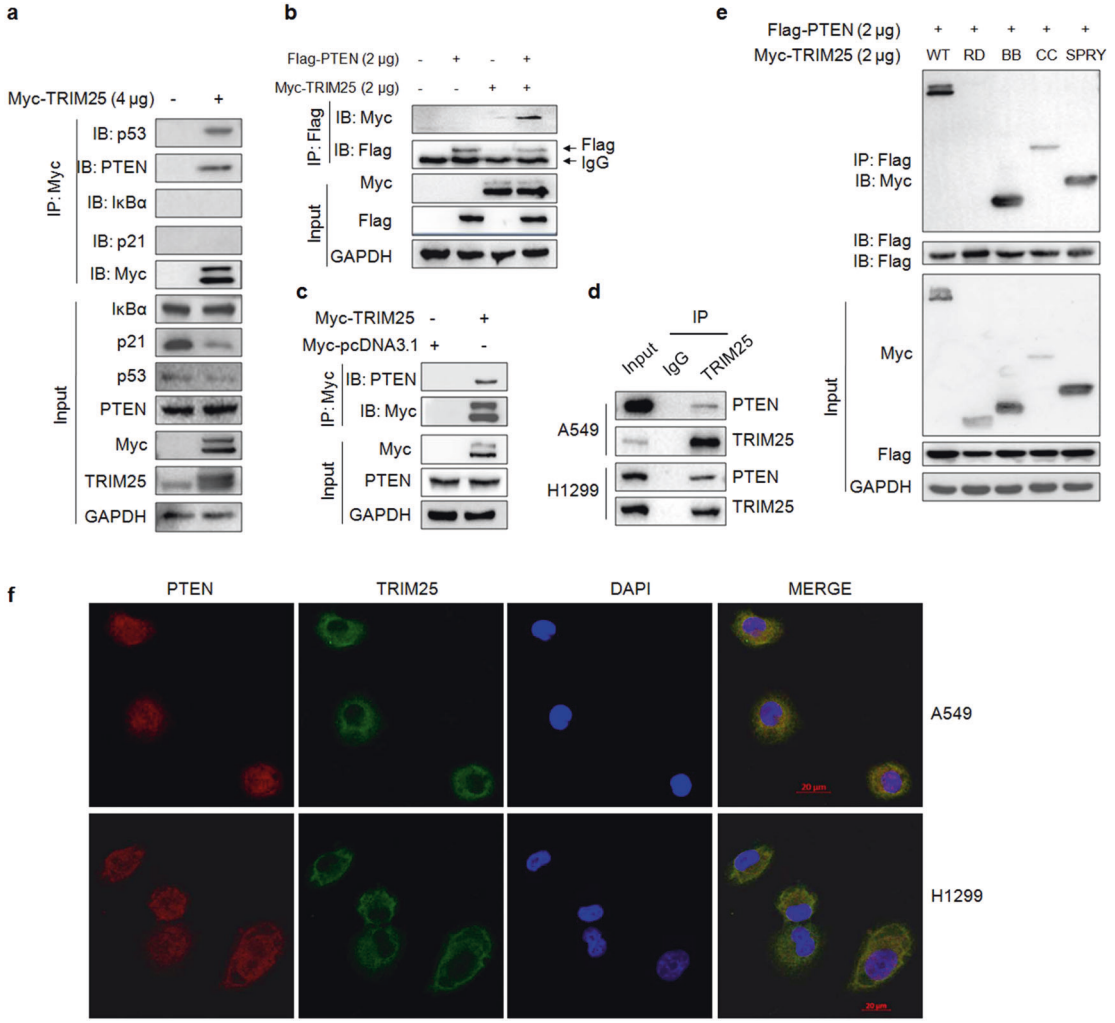

Fig. 2 TRIM25 interacts with PTEN. a HEK293T cells were transfected with the Myc-TRIM25 plasmid for 48 h prior to cell lysate preparation and immunoprecipitation (IP) with an anti-Myc antibody. The precipitates were then subjected to immunoblotting (IB) for the indicated proteins. **b** H1299 cells were cotransfected with Flag-PTEN and Myc-TRIM25 plasmids, and 48 h later, cell lysates were prepared for IP/IB. **c** A549 cells were transfected with Myc-TRIM25 plasmids for 48 h prior to IP/IB. **d** A549 and H1299 cells were subjected to IP with an anti-TRIM25 antibody followed by IB for PTEN. **e** HEK293T cells were cotransfected with Flag-PTEN and TRIM25 truncation mutant plasmids for 48 h prior to cell lysate preparation and IP/IB. **f** A549 and H1299 cells were stained with specific antibodies prior to immunofluorescence analysis.

TRIM25 activates the AKT/mTOR signaling pathway by inhibiting the phosphatase activity of PTEN

The above studies showed that TRIM25 mediates K63-linked polyubiquitination of PTEN but does not alter its stability; therefore, we sought to determine whether this modification can modulate PTEN activity. Given that PTEN is a negative regulator of the AKT/mTOR signaling pathway, we first evaluated the effects of TRIM25 on the activation of AKT and mTOR. The results showed that when TRIM25 was introduced, the phosphorylation levels of both AKT and mTOR were drastically increased, but their total protein levels remained unchanged (Fig. 4a). In line with this finding, knockout of TRIM25 markedly reduced the phosphorylation levels of both AKT and mTOR (Fig. 4b), suggesting that TRIM25 activates AKT and mTOR. Notably, sgTRIM25 had no effects on PTEN stability (Fig. 4b). Moreover, we found that mTOR activation was consistent with TRIM25 expression in primary NSCLC specimens (Fig. 4c). We further measured the activation levels of AKT and mTOR in NSCLC xenografts established as described in Fig. 1j, k. The results showed that the phosphorylation levels of both AKT and mTOR were markedly reduced in TRIM25 knockdown A549-derived xenografts (Fig. 4d, e). In contrast, AKT and mTOR phosphorylation was significantly induced in xenografts derived from cells overexpressing TRIM25 (Fig. 4d, e). Therefore, TRIM25 activates the AKT/mTOR signaling pathway. To determine whether PTEN is essential for the role of TRIM25 in modulating AKT/mTOR activation, PTEN was knocked down in both A549 and H1299 cells; these cells were then transfected with TRIM25, and AKT/mTOR signaling was analyzed. The IB results showed that TRIM25 markedly increased the phosphorylation levels of both AKT and mTOR in the mock-transfected cells; in contrast, when PTEN was knocked down, AKT and mTOR could not be markedly activated by TRIM25 (Fig. 4f, g). Therefore, PTEN is required for TRIM25 to activate AKT/mTOR. Moreover, binding to the plasma membrane is a critical step in PTEN-mediated suppression of the PI3K/AKT/mTOR signaling pathway [16]. K266 is important for PTEN to be localized to the plasma membrane and perform its function [15]; moreover, K266 is the acceptor site for PTEN K63-linked polyubiquitination (Fig. 3g). Thus, we sought to determine whether TRIM25-mediated ubiquitination of PTEN disrupts its plasma membrane localization. To this end, we performed IB after isolation of the cytosolic and plasma membrane fractions. The results showed that PTEN was markedly reduced in the membrane fraction by transfection with TRIM25 alone or cotransfection with K63-Ub (Fig. 4h–k),

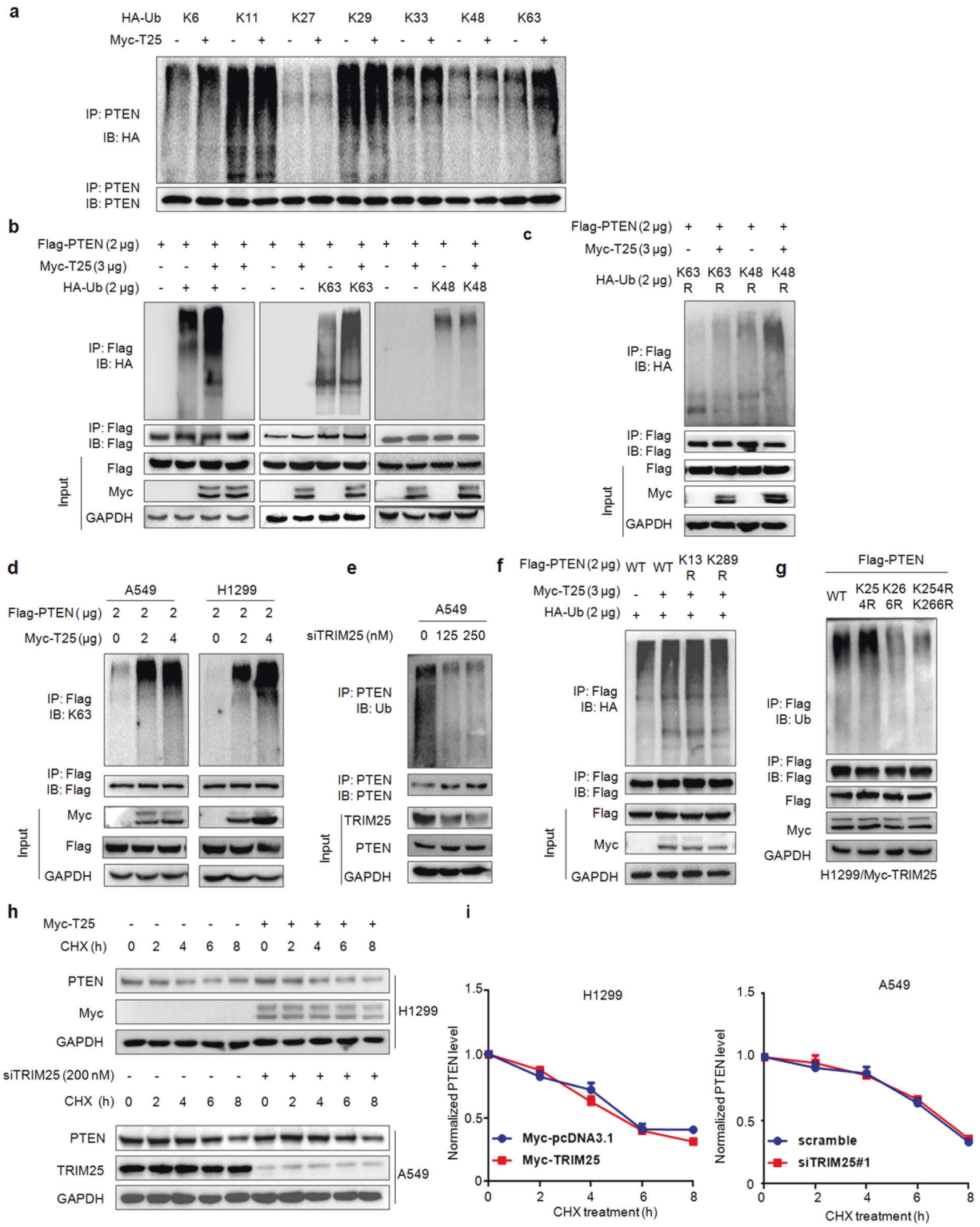

Fig. 3 TRIM25 mediates K63-linked polyubiquitination of PTEN at K266. a HEK293T cells were cotransfected with individual Ub mutants with or without TRIM25 for 48 h, and IP/IB were then conducted as indicated. **b** HEK293T cells were cotransfected with Flag-PTEN, Myc-TRIM25 and HA-Ub, K63-Ub or K48-Ub for 48 h, and IP/IB were then conducted. **c** HEK293T cells were cotransfected with Flag-PTEN and HA-K63R-Ub or HA-K48R-Ub plasmids in the presence or absence of TRIM25 for 48 h, and IP/IB were then conducted. **d** A549 and H1299 cells were cotransfected with Flag-PTEN and TRIM25 plasmids for 48 h, and IP/IB were then conducted. **e** TRIM25 was knocked down in A549 cells for 48 h, and IP/IB were then conducted using specific antibodies as indicated. **f** Plasmids expressing PTEN with mutation of specific lysine residues were cotransfected into A549 cells with TRIM25 and Ub plasmids, and IP/IB were then conducted as indicated. **g** TRIM25-expressing H1299 cells were cotransfected with PTEN mutants, and IP/IB were then conducted. **h** H1299 cells were transfected with TRIM25 for 24 h, while TRIM25 was knocked down in A549 cells for 48 h. All cells were then treated with cycloheximide (CHX) for the indicated periods and subjected to IB. **i** PTEN stability after normalization to GAPDH based on the results in **h**. Myc-T25: Myc-TRIM25.

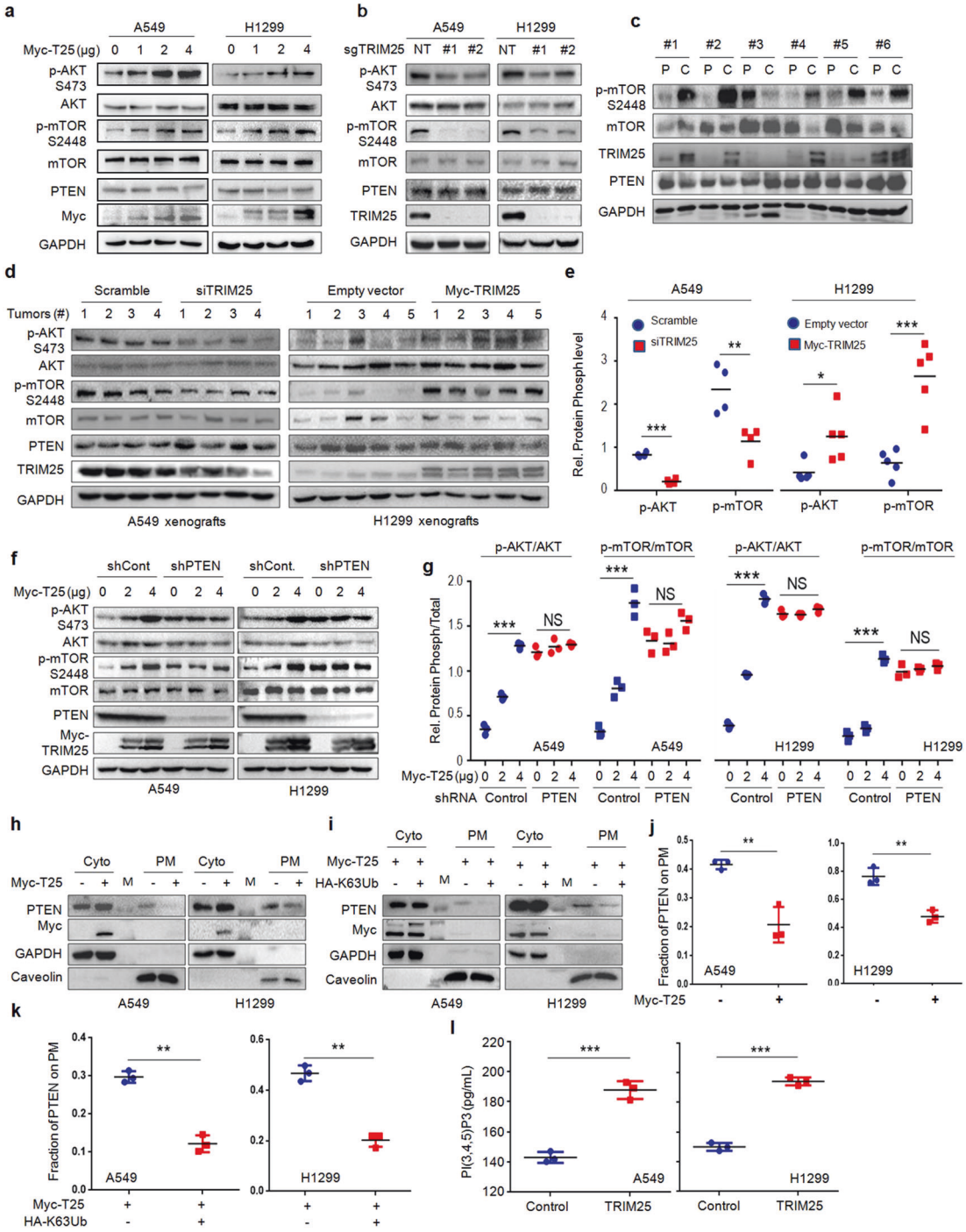

Fig. 4 TRIM25 activates the AKT/mTOR signaling pathway by inhibiting the membrane localization and activity of PTEN. a NSCLC cell lines were transfected with TRIM25 plasmids for 48 h prior to cell lysate preparation and IB for proteins as indicated. **b** TRIM25 was knocked out by siRNA, and IB was then performed using the indicated antibodies. **c** mTOR, PTEN, and TRIM25 expression levels were measured in primary NSCLC tumor (C) and paired para-cancerous (P) tissues. **d** AKT and mTOR activation was measured in NSCLC xenografts derived from A549 cells with TRIM25 knockdown or H1299 cells stably expressing TRIM25. **e** Statistical analysis of AKT and mTOR activation based on the results in **d**. **f** PTEN-knockdown NSCLC cells were transfected with TRIM25 for 48 h prior to cell lysate preparation and analysis of AKT and mTOR activation by IB. **g** Statistical analysis of AKT and mTOR activation levels based on three independent assays, as shown in **f**. **h** NSCLC cells were transfected with TRIM25 for 48 h prior to isolation of the membrane fraction from the cytosolic fraction, and IB was then performed as indicated. **i** NSCLC cells were cotransfected with K63-Ub and TRIM25 for 48 h prior to isolation of the membrane fraction from the cytosolic fraction, and IB was then performed as indicated. M protein marker. **j**, **k** Statistical analyses of PTEN distribution at the plasma membrane based on three independent assays, as shown in **h** and **i**. **l** Myc-TRIM25 plasmids were transfected into A549 and H1299 cells for 48 h prior to measurement of the PI(3,4,5)P3 concentration. Myc-T25: Myc-TRIM25.

suggesting that TRIM25-mediated K63 ubiquitination prevents PTEN from localizing to the plasma membrane.

Since translocation to the plasma membrane is required for PTEN to hydrolyze PI(3,4,5)P3 to PI(4,5)P2, thus inhibiting AKT/mTOR signaling, we sought to determine whether PTEN is inactivated after failure of plasma membrane translocation due to K63-linked polyubiquitination. To this end, we measured the concentration of PI(3,4,5)P3 in NSCLC cells in the presence of TRIM25. The results showed that overexpression of TRIM25 resulted in a significant increase in PI(3,4,5)P3 in both A549 and H1299 cells (Fig. 4l). This finding confirmed the hypothesis that TRIM25 suppresses PTEN activity by K63-linked polyubiquitination, leading to PI(3,4,5)P3 accumulation and activation of AKT/mTOR signaling.

Suppression of PTEN K63-linked polyubiquitination enhances NSCLC chemosensitivity

Given the role of TRIM25 in NSCLC cell proliferation and survival via suppression of PTEN/AKT/mTOR signaling, we sought to determine whether inhibition of TRIM25-activated AKT/mTOR signaling induces NSCLC cell apoptosis. Although no direct inhibitors of TRIM25 have been reported, our previous study found that antibacterial agent nitroxoline (NXQ) was able to induce myeloma cell apoptosis by downregulating TRIM25 [7]. To determine whether NXQ also targets the TRIM25/PTEN pathway and induces apoptosis in NSCLC cells, we examined the effects of NXQ on PTEN polyubiquitination. As shown in Fig. 5a, NXQ indeed decreased PTEN polyubiquitination without altering its protein level. Moreover, NXQ prevented K63-linked ubiquitination of PTEN in NSCLC cells (Fig. 5b). To determine whether NXQ affects AKT/mTOR signaling activation in NSCLC cells, NSCLC cell lines (A549 and H1299) and a myeloma cell line (OCI-MY5) were treated with NXQ for 24 h, and the subsequent assay showed that NXQ suppressed the phosphorylation of both AKT and mTOR (Fig. 5c). Notably, NXQ downregulated TRIM25 in MM but not NSCLC cells, consistent with a previous report [7]. These results suggested that NXQ probably inhibits the polyubiquitination of PTEN and then restores its activity. Given that the interaction between TRIM25 and PTEN is critical for TRIM25-mediated polyubiquitination of PTEN, we sought to determine whether NXQ interferes with the binding of TRIM25 to PTEN. To verify this hypothesis, A459 and H1299 cells were treated with increasing concentrations of NXQ, and cell lysates were then prepared for IP with an anti-TRIM25 antibody and IB for PTEN. As shown in Fig. 5d, the IP/IB results showed that the abundance of PTEN decreased as the concentration of NXQ increased, suggesting that NXQ impaired the interaction of TRIM25 and PTEN. We therefore sought to determine whether combination treatment with NXQ and CDDP can enhance the inhibition of AKT/mTOR signaling. To this end, A549 and H1299 cells were treated with NXQ and CDDP alone or in combination for 24 h, and the resultant cell lysates were subjected to IB. The results showed that although both AKT and mTOR were slightly inhibited by either compound alone, the combination treatment significantly suppressed AKT and mTOR activation (Fig. 5e). Subsequent IB of the same cell lysates revealed that with the combination treatment, TRIM25 was markedly downregulated, accompanied by pro-apoptotic PARP cleavage (Fig. 5f). Notably, when TRIM25 was introduced, PARP cleavage was abolished (Fig. 5g). These results suggested that NXQ enhanced the effects of CDDP in terms of TRIM25 downregulation and NSCLC cell apoptosis. Finally, apoptosis was further evaluated by flow cytometry with PI and Annexin V staining after combined treatment with NXQ and CDDP. As shown in Fig. 5h, combination treatment with NXQ and CDDP markedly increased NSCLC cell apoptosis compared with single-agent treatment. These results thus indicated that suppression of the TRIM25/PTEN/AKT/mTOR pathway could be a promising strategy for NSCLC treatment.

DISCUSSION

Ubiquitination is a process by which ubiquitin molecules are attached to substrate proteins; however, the specific type of ubiquitin chain linkage influences the fate of the target protein. Based on the length of the ubiquitin chain and the linking lysine residue on ubiquitin, ubiquitination can be classified as mono-ubiquitination, which modulates substrate function or subcellular location, K48-linked polyubiquitination, which leads to substrate degradation by the proteasome, and K63-linked polyubiquitination, which modifies substrate functions such as signal transduction and DNA repair [17]. Currently, all three modifications have been found on PTEN. Monoubiquitination leads to nuclear import of PTEN and maintains its activity in antagonizing AKT [14, 18], K48-linked polyubiquitination leads to the degradation and inactivation of PTEN [5], and K63-linked polyubiquitination alters the subcellular distribution and function of PTEN [16, 19]. Therefore, ubiquitination may regulate PTEN stability and functions in specific biological contexts. Interestingly, all three modifications of PTEN are mediated by the same ubiquitin ligase, NEDD4 [5, 14, 16]. In the present study, we identified TRIM25 as a novel E3 that promotes K63-linked ubiquitination and activity of PTEN.

TRIM25, also called estrogen-responsive finger protein, has been reported in various cancer tissues, including those of breast, liver, and prostate cancers, by mediating K48-linked ubiquitination and degradation of 14-3-3σ [20], ERG [21], and metastasis-associated 1 protein [22], respectively. The present study adds PTEN as a novel substrate of TRIM25 by showing that TRIM25 interacts with PTEN and mediates its K63-linked polyubiquitination. Moreover, we found that TRIM25 is different from NEDD4 in terms of PTEN ubiquitination. First, the ubiquitin acceptor sites in PTEN for NEDD4-mediated ubiquitination are K13 and K289 [5, 14]. However, these two sites do not respond to TRIM25, because PTEN with the K13R or K289R mutation still undergoes K63-linked ubiquitination. In contrast, K266, a previously reported SUMOylation site, is essential for K63-linked ubiquitination of PTEN mediated by TRIM25, because PTEN K266R cannot be ubiquitinated by TRIM25. Second, TRIM25-mediated K63-linked ubiquitination of PTEN leads to reduced distribution of PTEN at the plasma membrane. PTEN can be localized to the cytosol, nucleus, and plasma membrane. Monoubiquitination promotes nuclear import of PTEN and maintains its tumor suppressor activity by preventing its degradation [14]. PTEN at the plasma membrane is more catalytically active than PTEN at other sites because the N-terminal domain of PTEN can bind specifically to PI(4,5)P2, resulting in its plasma membrane docking and activation [23]. K266 is a SUMOylation site in PTEN, and this modification facilitates the plasma membrane translocation of PTEN [24]. In the present study, we found that TRIM25-mediated K63-linked ubiquitination leads to decreased plasma membrane localization, probably due to ubiquitination at K266. One of the possible mechanisms is that K63-linked polyubiquitination at K266 abolishes the SUMOylation of PTEN. If it cannot access the plasma membrane, PTEN cannot hydrolyze PI(3,4,5)P3, which leads to AKT/mTOR activation [23] because PI(3,4,5)P3, as an essential second messenger, recruits AKT to the plasma membrane, where it is phosphorylated and activated by phosphoinositide-dependent kinase 1 and 2. Our finding is thus consistent with the hypothesis that TRIM25 activates AKT/mTOR by mediating K63-linked ubiquitination of PTEN and reducing its plasma membrane localization/retention.

Furthermore, high expression of TRIM25 confers chemoresistance on NSCLC cells and predicts poor clinical outcomes. In line with this finding, downregulation of TRIM25 restores PTEN activity and inactivates the AKT/mTOR pathway, therefore leading to NSCLC cell apoptosis. Moreover, we found that the antibacterial agent NXQ can prevent K63-linked ubiquitination of PTEN, therefore inhibiting the AKT/mTOR pathway and inducing NSCLC

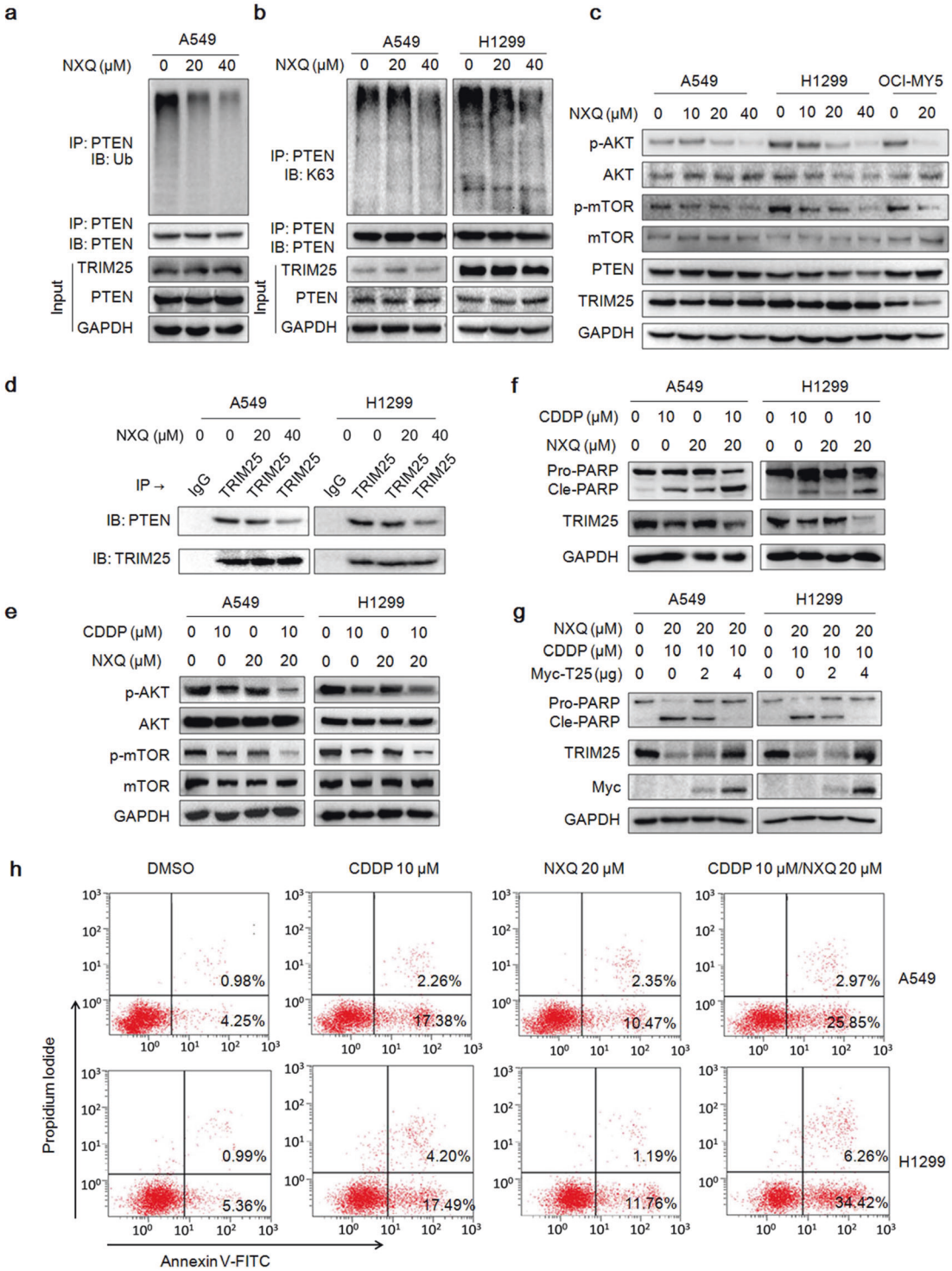

Fig. 5 Inhibition of the TRIM25/PTEN axis sensitizes NSCLC cells to cisplatin. a A549 cells were treated with nitroxoline (NXQ) overnight prior to IP/IB for PTEN ubiquitination. **b** A549 and H1299 cells were treated with NXQ overnight, and IP/IB were then conducted as indicated. **c** A549, H1299, and OCI-MY5 cells were treated with NXQ for 24 h, and AKT/mTOR activation and TRIM25 expression were then measured. **d** A549 and H1299 cells were treated with NXQ for 24 h, and IP with an antibody specific for TRIM25 and IB for PTEN or TRIM25 were then performed. **e** A549 and H1299 cells were treated with NXQ and/or CDDP for 24 h prior to measurement of AKT and mTOR activation. **f** A549 and H1299 cells were treated with NXQ and/or CDDP for 48 h prior to measurement of PARP and TRIM25 expression. **g** TRIM25 was overexpressed in A549 and H1299 cells for 48 h prior to CDDP and NXQ treatment. Cell lysates were subjected to IB. **h** A549 and H1299 cells were treated with NXQ and/or CDDP for 24 h prior to Annexin V/PI staining and flow cytometric analysis. Myc-T25: Myc-TRIM25.

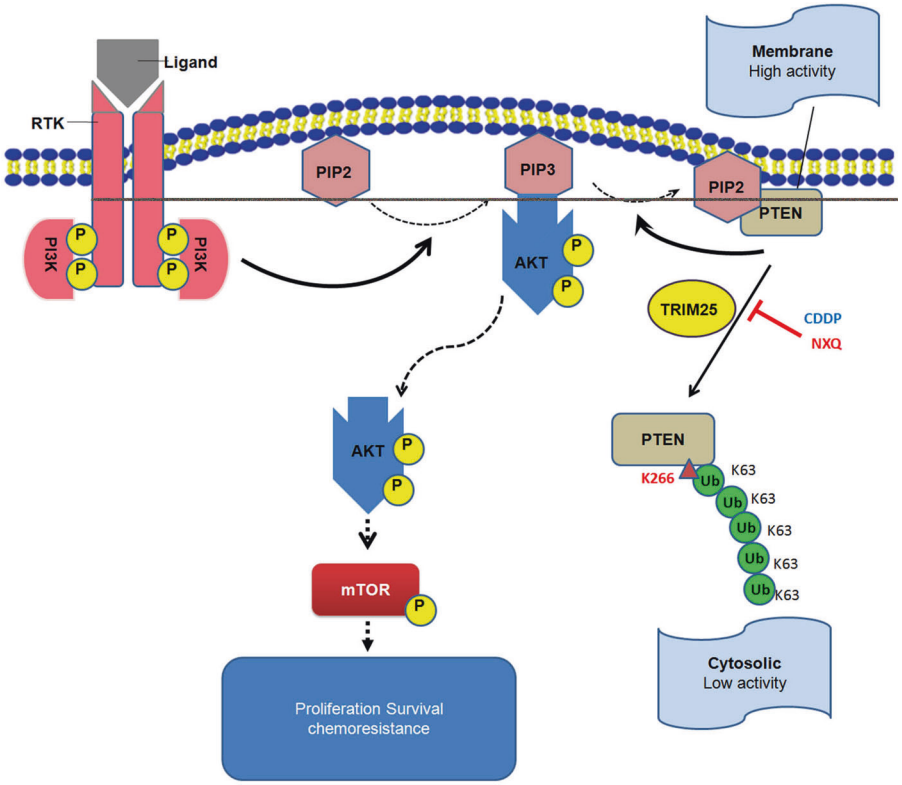

Fig. 6 The working model of the link between TRIM25 and the PTEN/AKT/mTOR signaling pathway. Upon stimulation of receptor tyrosine kinases (RTKs) by their specific ligands, PI3K catalyzes the production of PIP3, which recruits AKT to the plasma membrane, where AKT is activated. PTEN binds to PIP2 and is translocated to the plasma membrane, where it hydrolyzes PIP3 to PIP2, therefore inhibiting AKT/mTOR signaling activation. TRIM25-mediated K63-linked polyubiquitination retains PTEN in the cytosol, where PTEN is less active. Therefore, TRIM25 promotes K63-linked polyubiquitination of PTEN and NSCLC cell proliferation and survival. Small molecules such as nitroxoline (NXQ) can inhibit K63-linked ubiquitination of PTEN, therefore suppressing AKT/mTOR signaling and sensitizing NSCLC cells to anti-cancer drugs such as CDDP.

cell apoptosis. This finding demonstrates that targeting the TRIM25/PTEN/AKT/mTOR signaling pathway is a promising strategy to overcome chemoresistance in NSCLC. Given its long history of clinical application and proven safety, NXQ could be examined for use in combination therapies for NSCLC patients.

CONCLUSIONS

Collectively, the present study demonstrates for the first time that TRIM25 modulates K63-linked ubiquitination of PTEN, therefore inhibiting its plasma membrane distribution and phosphatase activity and altering AKT/mTOR signaling (Fig. 6). This novel mechanism mediating the PTEN/AKT/mTOR signaling pathway also highlights a novel potential target for the treatment of NSCLC patients.

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AUTHOR CONTRIBUTIONS

Conception and design: XLM, JZ, and YYZ. Development of methods: XLM, YMH, XMZ, SYJ, ZBZ, and BYC. Acquisition of data: XLM, XMZ, YMH. Analysis and interpretation of data: XLM, YYZ, YMH, and JBL. Writing and composition: XLM and YMH. Administration, technical, and material support: XLM and YYZ.

ADDITIONAL INFORMATION

Conflict of interest: The authors declare no competing interests.

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