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Original Article

TNIP1 Knockdown Induces the Growth Arrest and Apoptosis of Breast Cancer Cells by Activating the NF-κB Pathway

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Abstract

Background and objectives: Breast cancer is one of the leading causes of mortality among women worldwide. Tumor necrosis factor α-induced protein 3-interacting protein 1 (TNIP1) is a ubiquitin-binding protein that is widely expressed, but its function in breast cancer cells remains unknown. This study aimed to elucidate the molecular mechanism of TNIP1 regulation in the proliferation and apoptosis of breast cancer cells.

Methods: A colony formation assay was conducted on MCF-7 and T47D cells stably transfected with TNIP1/cyclin G1 (CCNG1) short hairpin RNAs. Quantitative polymerase chain reaction was performed to assess the relative abundances of TNIP1, CCNG1, and cyclin D1 (CCND1) messenger RNAs. Immunoprecipitation and immunoblotting were used to detect the expression of TNIP1, CCNG1, CCND1, and related proteins. A dual-luciferase reporter assay was employed to explore the molecular mechanism of TNIP1 in signal transduction. Caspase activity in MCF-7 and T47D cells transfected with TNIP1 short hairpin RNAs was measured using the Caspase-Glo 3/7 assay.

Results: Ablation of TNIP1 induced growth arrest in breast cancer cells. TNIP1 directly interacted with CCNG1, and TNIP1 knockdown increased the ubiquitination of CCNG1. CCNG1 knockdown also induced growth arrest in MCF-7 and T47D cells. Furthermore, TNIP1 knockdown activated the NF-κB pathway and induced apoptosis in these cells.

Conclusions: TNIP1 regulates the proliferation and apoptosis of breast cancer cells, suggesting that TNIP1 may serve as a potential biomarker for breast cancer.

Introduction

Breast cancer is one of the leading causes of mortality among women worldwide, accounting for 29% of newly diagnosed cases and 14% of estimated deaths.[1,2] Despite significant advancements in breast cancer diagnosis and therapy over the past decades, long-term clinical prognosis and mortality rates remain unsatisfactory.[3] The uncontrolled proliferation of tumor cells, including those in breast cancer, presents a major challenge in cancer treatment. Genes that regulate cell proliferation are therefore potential therapeutic targets.

Tumor necrosis factor α-induced protein 3-interacting protein 1 (TNIP1) is a retinoic acid receptor corepressor and A20-binding inhibitor of NF-κB, found in both nuclear and cytoplasmic locations. It belongs to the TNIP family, which includes TNIP1, TNIP2, and TNIP3.[4,5] TNIP1 interacts with the deubiquitylase A20 and inhibits NF-κB transcriptional activity, which is considered its most important function.[6–8] TNIP1 is associated with several autoimmune diseases and plays a critical role in regulating immunity and maintaining tissue homeostasis.[6,9,10] Mice lacking TNIP1 experience extensive tumor necrosis factor (TNF)-induced apoptosis in the liver, and few survive postnatally.[8] TNIP1 also acts as an inhibitory factor of NF-κB-mediated MHC-1 expression in neuroblastoma.[11] Additionally, TNIP1 promotes cell survival and is essential for development; its knockdown induces apoptosis in HEK293 and U-2 OS cells.[12] How-

Keywords: Breast cancer cells; Tumor necrosis factor α-induced protein 3-interacting protein 1/TNIP1; Cyclin G1/CCNG1; Proliferation; Apoptosis; NF-κB pathway.

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Table 1. Sequences of shRNAs for TNIP1 and CCNG1

shRNA	Target site sequence
TNIP1	
sh T1	GATGAGCAATGGCAACAAAG
sh T2	CCACCCAGAAGGCTTTCATTT
sh T3	GATGAGGAGAAGGCAAGAGAA
CCNG1	
sh C1	CCAAATGTTCAGAAGTTGAAA
sh C2	CTGGACAGATTCCTGTCTAAA
sh C3	CACACGATAATGGCCTCAGAA

CCNG1, cyclin G1; shRNA, short hairpin RNA; TNIP1, tumor necrosis factor α-induced protein 3-interacting protein 1.

ever, the function of TNIP1 in breast cancer cells remains unknown.

Cyclin G1 (CCNG1) was primarily identified as a novel member of the cyclin family with homology to C-SRC and was first recognized as a p53-regulated transcript induced by DNA damage.[13,14] CCNG1 acts as a cell cycle regulator in human tumor cells, such as cervical carcinoma, hepatocellular carcinoma, breast cancer, and lung carcinoma.[15–18] Moreover, CCNG1 may serve as a promising biomarker and contribute to recurrence and chemoresistance in hepatocellular carcinoma.[16] However, its role in breast cancer remains to be further elucidated.

In this study, we investigated the functional roles of TNIP1 and CCNG1 in regulating the proliferation and apoptosis of breast cancer cells. We also explored the potential molecular mechanisms involved, with the aim of providing novel insights into TNIP1's role in breast cancer development.

Materials and methods

Plasmids construction

The short hairpin RNAs (shRNAs) for TNIP1 and CCNG1, inserted into pLKO.1 plasmids, were purchased from SIGMA–ALDRICH (Merck, Germany), and their specific sequences are listed in Table 1. The plasmids pET28a-CCNG1-His6, pGEX4T-1-GST-TNIP1, pGEX4T-1-GST-TNIP1(1-50aa), pGEX4T-1-GST-TNIP1(51-343aa), pGEX4T-1-GST-TNIP1(344-636aa), psPAX2, pMD2.G, pGL3-NK-κB-luc, and pRL-TK were purchased from GeneChem Co., Ltd. (Shanghai, China).

Cell culturing, lentiviral particles producing and infecting

The human breast cancer cell lines MCF-7 and T47D, along with the human embryonic kidney cell line HEK293T, were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). MCF-7 and HEK293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 U/mL penicillin and 100 mg/mL streptomycin (from Gibco, USA) in a 37°C humidified atmosphere with 5% CO_2. T47D cells were cultured in 1640 supplemented with 10% fetal bovine serum. The pLKO.1 shRNAs, psPAX2, and pMD2.G (in a ratio of 4:3:1) were co-transfected into HEK293T cells using Lipofectamine 2000 (Life Technologies, USA) according to the manufacturer's instructions. The media containing lentiviral particles were harvested 48 h later, diluted with fresh culture medium, and added to MCF-7 cells that were approximately 80–90% confluent. The cells were screened with puromycin (3 μg/mL) to establish stable cell lines. The pGL3-NK-κB-luc and pRL-TK plasmids were transfected into MCF-7 cells by the electroporation method as previously described.[19]

Cell proliferation assay

A total of 2,500 MCF-7 or T47D cells stably transfected with TNIP1/CCNG1 shRNAs were seeded into a 96-well plate. The time point 0 h was defined as 6 h after cell seeding. At 0, 24, and 48 h, the cells were incubated with 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (C0009, Beyotime Biotechnology, China) for 4 h at 37°C. The resulting formazan was dissolved in dimethyl sulfoxide and quantified spectrophotometrically at a wavelength of 570 nm using a microplate reader (Bio-Rad, USA). The experiments were conducted in six replicates and repeated three times.

Colony formation assay

A total of 1,000 MCF-7 or T47D cells stably transfected with TNIP1/CCNG1 shRNAs were seeded into a six-well plate. After seven days, the plates were fixed with 4% paraformaldehyde (Sigma, Germany) and stained with 0.1% crystal violet (C0121, Beyotime Biotechnology, China). The colony number was then counted and calculated.

Quantitative reverse transcription polymerase chain reaction

Total RNAs were extracted from cells using a total RNA kit (Tiangen, China, Cat. No. DP304-02). Complementary DNA was synthesized using ReverTra Ace qPCR RT Master Mix (Toyobo, Japan). Quantitative PCR assays were performed to assess the relative abundances of TNIP1, CCNG1, and cyclin D1 (CCND1) messenger RNAs using specific primers (Table 2) and stained with SYBR Green (Toyobo, Japan) on an ABI 7500 Fast Real-Time PCR System (ABI, USA). The relative abundances of TNIP1, CCNG1, and CCND1 were normalized to that of the *GAPDH* gene using the $^{\Delta\Delta}$Ct method.[20] All data were obtained from three independent experiments.

Table 2. Sequences of the primers used in qRT-PCR

	Primers for qRT-PCR	
Target gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
GAPDH	GAGTCAACGGATTTGGTCGTATTG	ATTTGCCATGGGTGGAATCATATTG
TNIP1	AGCTTTTGAGCGCCTAGTGA	CTAGCTCCTCTGCCTTCTGC
CCNG1	TCTGACCTTCTGGCAAGAGC	ATGCTTCAATTGCCGTGCAG
CCND1	GAAGGAGACCATCCCCCTGA	GAAATCGTGCGGGGTCATTG

CCND1, cyclin D1; CCNG1, cyclin G1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; qRT-PCR, quantitative real-time polymerase chain reaction; TNIP1, tumor necrosis factor α-induced protein 3-interacting protein 1.

Co-immunoprecipitation, immunoprecipitation, and immunoblotting

For co-immunoprecipitation, MCF-7 cells were lysed in CO-IP buffer (50 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, 1% NP-40) supplemented with a protease inhibitor cocktail (Roche, Switzerland). The cell lysates were then incubated with TNIP1 antibody (1:100, 15104-1-AP, Proteintech, USA) and Protein G agarose beads (Merck Millipore, Germany) overnight at 4°C. For immunoprecipitation, cells were lysed in IP buffer (50 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, 0.1% sodium dodecyl sulfate, 1% NP-40) supplemented with a protease inhibitor cocktail. The cell lysates were incubated with P53 antibody and Protein G agarose beads overnight at 4°C, similar to the co-immunoprecipitation procedure.[21] The immunoprecipitants were enriched and denatured at 100°C for 10 m in 2× sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. The inputs, immunoprecipitants, and other cell lysates were then subjected to SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Bio-Rad, USA). The membrane was incubated with appropriate antibodies against TNIP1 (1:1,000, 15104-1-AP, Proteintech, USA), CCNG1 (1:2,000, 10897-1-AP, Proteintech, USA), CCND1 (1:1,000, 60186-1-Ig, Proteintech, USA), His-Tag (1:1,000, 66005-1-Ig, Proteintech, USA), GST-Tag (1:1,000, 10000-0-AP, Proteintech, USA), Ubiquitin (sc-47721, Santa Cruz, USA), Caspase 3 (1:1,000, 19677-1-AP, Proteintech, USA), Cleaved Caspase 3 (1:1,000, 9664, Cell Signaling Technology, USA), and GAPDH (1:5,000, 60004-1-Ig, Proteintech, USA). Secondary antibodies were labeled with horseradish peroxidase, and the signals were visualized using the Tanon 5200 Imaging System (Tanon, China).

Expression and purification of recombinant proteins

pGEX4T-1-GST-TNIP1 (full-length and truncations) and pET28a-CCNG1-His6 plasmids were expressed in BL21 E. coli cells. After IPTG (Sangon, China) induction, cells were pelleted, lysed in phosphate buffered saline (PBS) buffer, and incubated with glutathione or Ni^{2+}TA beads (GE, USA) to enrich the respective proteins. The proteins were then eluted with 25 mM L-glutathione reduced or 1 M imidazole dissolved in PBS buffer, followed by dialysis in PBS buffer supplemented with 20% glycerol before aliquotted and preserved at −80°C as previously reported.[22]

GST pull-down assay

Purified GST-TNIP1 (20 μg), GST-TNIP1 (1-50aa), GST-TNIP1 (51-343aa), GST-TNIP1 (344-636aa), and CCNG1-His6 (20 μg) were incubated with Glutathione Sepharose 4B at 4°C overnight in 500 μL of pull-down buffer (20 mM Tris-Cl, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.5% (v/v) NP-40, and 10 μg/mL BSA, pH 7.5). The beads were then pelleted and washed three times with the pull-down buffer. The recovered beads were denatured at 100°C for 10 m in 2× SDS-PAGE loading buffer and subjected to immunoblotting analysis.

Luciferase reporter assays

MCF-7 or T47D cells stably transfected with the TNIP1 shRNAs (Scramble, T1, T3) were seeded at 0.5×10 cells per well in 24-well plates. After overnight culture, the cells were co-transfected with pGL3-NK-κB-luc and pRL-TK plasmids by the electroporation method. Forty-eight hours after transfection, the cells were harvested, lysed with 5× passive buffer, and subjected to a Dual-Luciferase Reporter assay according to the manufacturer's instruction (E2920, Promega, USA).

Caspase-Glo assay

MCF-7 or T47D cells stably transfected with the TNIP1 shRNAs (Scramble, T1, T3) were seeded at 0.5×10^5 cells per well in 24-well plates. Caspase activity was measured 48 h later using the Caspase-Glo 3/7 assay according to the manufacturer's protocol (G8090, Promega, USA).

Statistical analysis

All experiments were performed in triplicate, and values are presented as mean ± standard deviation. One-way analysis of variance was conducted using GraphPad Prism (GraphPad Software, USA). A P-value < 0.05 was considered statistically significant, while a P-value < 0.01 was considered highly significant.

Results

TNIP1 knockdown induced growth arrest in breast cancer cells

To explore the effect of TNIP1 on the proliferation of breast cancer cells, three shRNAs targeting TNIP1 were designed and packaged as lentiviral particles to infect MCF-7 and T47D cells. Stable cell lines were established through puromycin selection. Immunoblotting and real-time PCR analysis indicated that the protein and mRNA levels of TNIP1 were significantly reduced in cells stably transfected with sh T1 and sh T3, and these cells were selected for further study (Fig. 1a and b). Cell viability of MCF-7 cells expressing TNIP1 shRNAs (Scramble, T1, T3) was assessed by MTT assay at 24 h and 48 h post-culture, revealing a marked growth arrest in TNIP1 knockdown cells compared to the control group (Scramble) (Fig. 1c). Subsequently, a colony formation assay demonstrated that the colony number in the TNIP1 knockdown groups decreased significantly compared to the control groups (Fig. 1d). Similar results were obtained in the T47D breast cancer cell line (Fig. 2a–d). These findings suggest that TNIP1 knockdown induces growth arrest in breast cancer cells.

TNIP1 knockdown reduced the protein level of CCNG1

Immunoblotting analysis indicated that CCNG1, rather than CCND1, decreased significantly in TNIP1 knockdown cells compared to the control group (Fig. 3a). To investigate whether the decrease in CCNG1 was due to transcriptional or post-translational alterations, gene expression levels of CCNG1 and CCND1 were assessed by quantitative PCR, revealing no significant changes in mRNA levels (Fig. 3b). This suggests that the change in CCNG1 protein levels under TNIP1 knockdown conditions may occur at the post-translational level.

TNIP1 directly interacted with CCNG1, and TNIP1 knockdown increased the ubiquitination of CCNG1

Immunoprecipitation analyses showed that endogenous TNIP1 could form a complex with endogenous CCNG1 in MCF-7 cells (Fig. 3c). We then tested whether TNIP1 could directly interact with CCNG1 *in vitro*. Recombinant GST-tagged TNIP1 and His6-tagged CCNG1 were purified, and GST pull-down assays were performed. The results demonstrated that TNIP1 and CCNG1 could form a complex *in vitro* (Fig. 3d). To map the interaction region of TNIP1 with CCNG1, three GST-tagged TNIP1 truncations were subjected to GST pull-down assays. The results indicated that CCNG1 directly interacted with the 51-343 amino acid region of TNIP1. Further experimental results showed that TNIP1 knockdown could increase the ubiquitination of CCNG1 in MCF-7 cells (Fig. 3e).

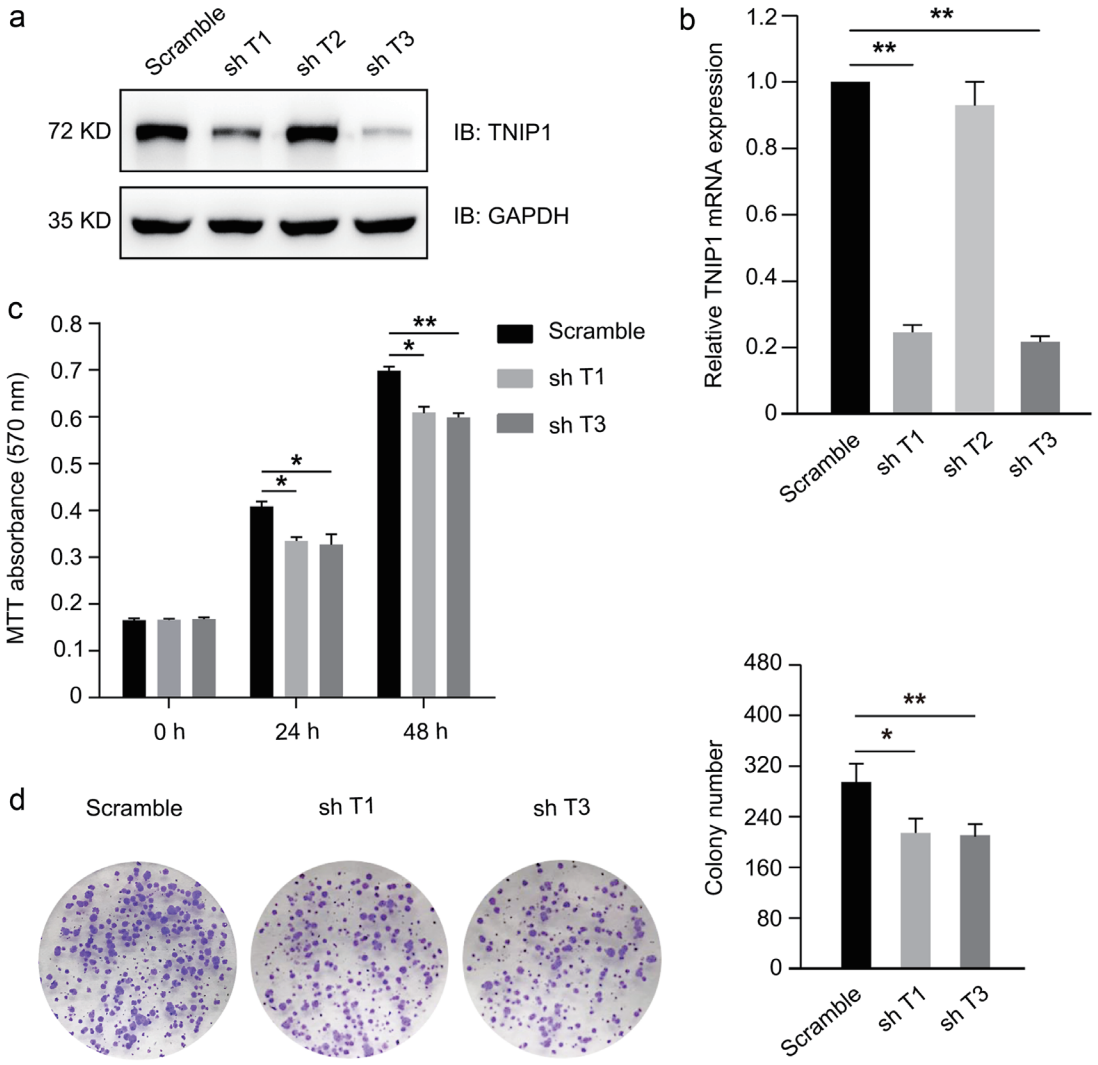

Fig. 1. TNIP1 knockdown induced growth arrest in MCF-7 cells. (a, b) The knockdown efficiency of shRNAs for TNIP1 was detected by immunoblotting and quantitative polymerase chain reaction (qPCR). MCF-7 cells were infected with shRNAs (Scramble, T1, T2, T3) lentivirus targeting TNIP1 and screened with puromycin to establish stable cell lines. Data are expressed as mean ± SD and analyzed using one-way ANOVA. $^{**}P < 0.01$, significant statistical difference, three independent experiments. (c) TNIP1 knockdown inhibited MCF-7 cell proliferation. MCF-7 cells that stably expressed TNIP1 shRNAs (Scramble, T1, T3) were seeded into 96-well plates and assessed by MTT assay at the indicated time points. Six hours after cell seeding were taken as 0 h time point. Data are expressed as mean ± SD and analyzed using one-way ANOVA with Tukey's post hoc test. $^{*}P < 0.05$, statistical difference; $^{**}P < 0.01$, significant statistical difference, three independent experiments. (d) TNIP1 knockdown inhibited colony formation in MCF-7 cells. One thousand MCF-7 cells that stably expressed TNIP1 shRNAs (Scramble, T1, T3) were seeded into six-well plates. Colonies were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet seven days later. The colony numbers were counted and calculated, with three samples in each group. Data are expressed as mean ± SD and analyzed using one-way ANOVA. $^{*}P < 0.05$, statistical difference; $^{**}P < 0.01$, significant statistical difference, three independent experiments. ANOVA, analysis of variance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mRNA, messenger RNA; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SD, standard deviation; shRNA, short hairpin RNA; TNIP1, tumor necrosis factor α-induced protein 3-interacting protein 1.

CCNG1 knockdown induced growth arrest in MCF-7 cells

To explore the effect of TNIP1 on the proliferation of MCF-7 cells, three shRNAs targeting CCNG1 were designed, and stable cell lines were constructed as described above. Immunoblotting analysis indicated that the protein level of CCNG1 was significantly reduced in cells stably transfected with sh C2 and sh C3 compared to the scramble group (Fig. 4a), and these cells were selected for further study. Cell viability of MCF-7 cells expressing CCNG1 shRNAs (Scramble, C2, C3) was assessed by MTT assay at indicated time points, showing clear growth arrest in CCNG1 knockdown cells compared to the control group (Fig. 4b). The colony formation assay indicated that the colony number in the CCNG1 knockdown groups decreased significantly compared to the control group (Fig. 4c). These results suggest that CCNG1 knockdown induces growth arrest in MCF-7 cells.

TNIP1 knockdown activated the NF-κB pathway and induced apoptosis in MCF-7 cells

To explore the effect of TNIP1 on NF-κB pathway activity, pGL3-NF-κB-luc and pRL-TK plasmids were co-transfected into MCF-7

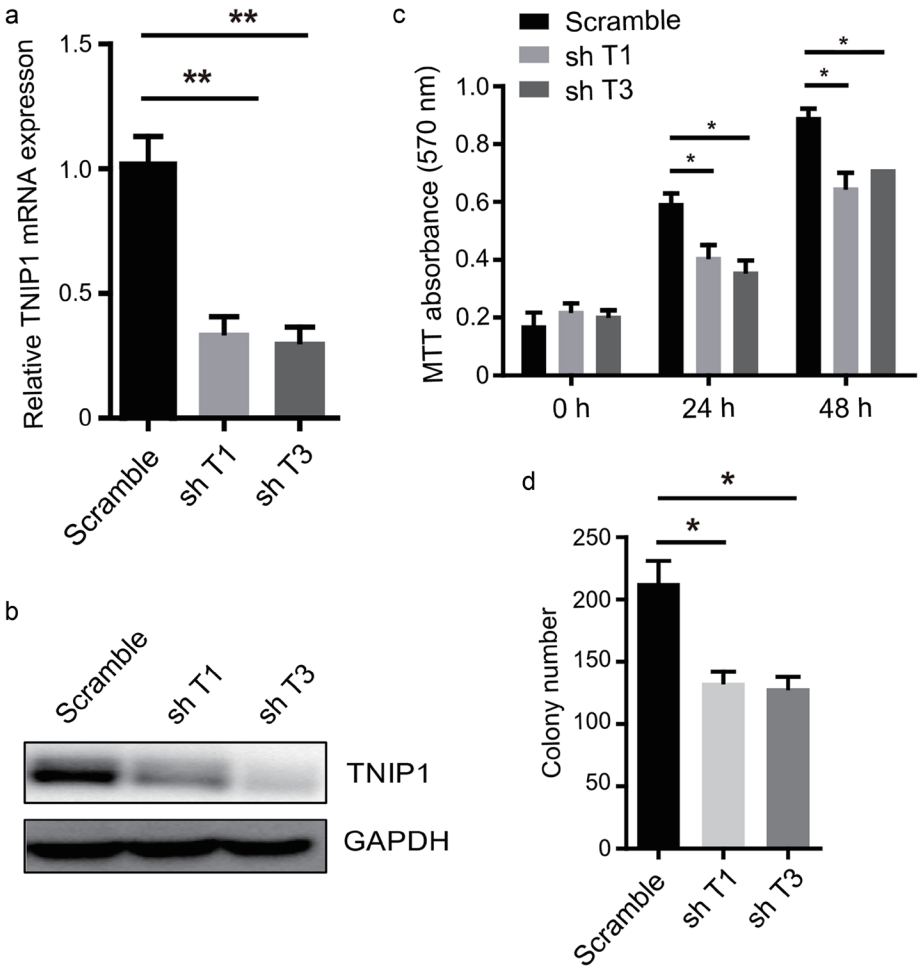

Fig. 2. TNIP1 knockdown induced growth arrest in T47D cells. (a, b) The knockdown efficiency of shRNAs for TNIP1 was confirmed by immunoblotting and quantitative polymerase chain reaction (qPCR) in T47D cells. $^{**}P < 0.01$. (c) TNIP1 knockdown inhibited the proliferation of T47D cells. T47D cells that stably expressed TNIP1 shRNAs (Scramble, T1, T3) were seeded into 96-well plates and assessed by MTT assay at the indicated time points. $^{*}P < 0.05$. (d) TNIP1 knockdown inhibited colony formation in T47D cells. $^{*}P < 0.05$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mRNA, messenger RNA; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; shRNA, short hairpin RNA; TNIP1, tumor necrosis factor α-induced protein 3-interacting protein 1.

cells stably expressing TNIP1 shRNAs (Scramble, T1, T3) using the electroporation method. NF-κB luciferase activity was significantly increased in TNIP1 knockdown groups (sh T1 and sh T2) compared to the control group (Scramble) (Fig. 5a). Mice lacking TNIP1 exhibit extensive TNF-induced apoptosis in the liver, and few survive postnatally.[8] To test the effect of TNIP1 on apoptosis, the protein levels of Caspase 3 and cleaved Caspase 3 were detected in MCF-7 cells stably expressing TNIP1 shRNAs (Scramble, T1, T3) via immunoblotting, revealing a significant increase in cleaved Caspase 3 in TNIP1 knockdown groups (Fig. 5b). Further Caspase-Glo assays indicated that TNIP1 knockdown significantly increased CASP-3/7 activity (Fig. 5c). Together, these results suggest that TNIP1 knockdown contributes to apoptosis in breast cancer cells.

Discussion

In this study, we first demonstrated that the ablation of TNIP1 could induce growth arrest in breast cancer cells. Further investigation indicated that TNIP1 directly interacted with CCNG1 and that TNIP1 knockdown increased the ubiquitination of CCNG1. We also found that CCNG1 knockdown led to growth arrest in MCF-7 cells. Additionally, our study demonstrated that TNIP1 knockdown could activate the NF-κB pathway and induce apoptosis in MCF-7 cells.

Breast cancer is one of the leading causes of death among women worldwide. Various anti-cancer strategies have been developed and are used in clinical treatment, including chemotherapy, immunotherapy, and targeted therapy.[1,2] Compared to other strategies, targeted therapy is widely used in cancer treatment due to its minimal side effects. There is an urgent need for new targets in breast cancer treatment. Although TNIP1 plays an important role in various cancers, its expression and function in breast cancer have not been well demonstrated.[23]

It has been reported that TNIP1 can promote cell survival and is associated with increased cell proliferation.[12] This aligns with our findings that TNIP1 knockdown induces growth arrest in MCF-7 cells. TNIP1 is a widely expressed ubiquitin-binding protein that interacts with the deubiquitylase A20, regulating A20/TNFAIP3-mediated deubiquitination of the Inhibitor of Nuclear Factor Kap-

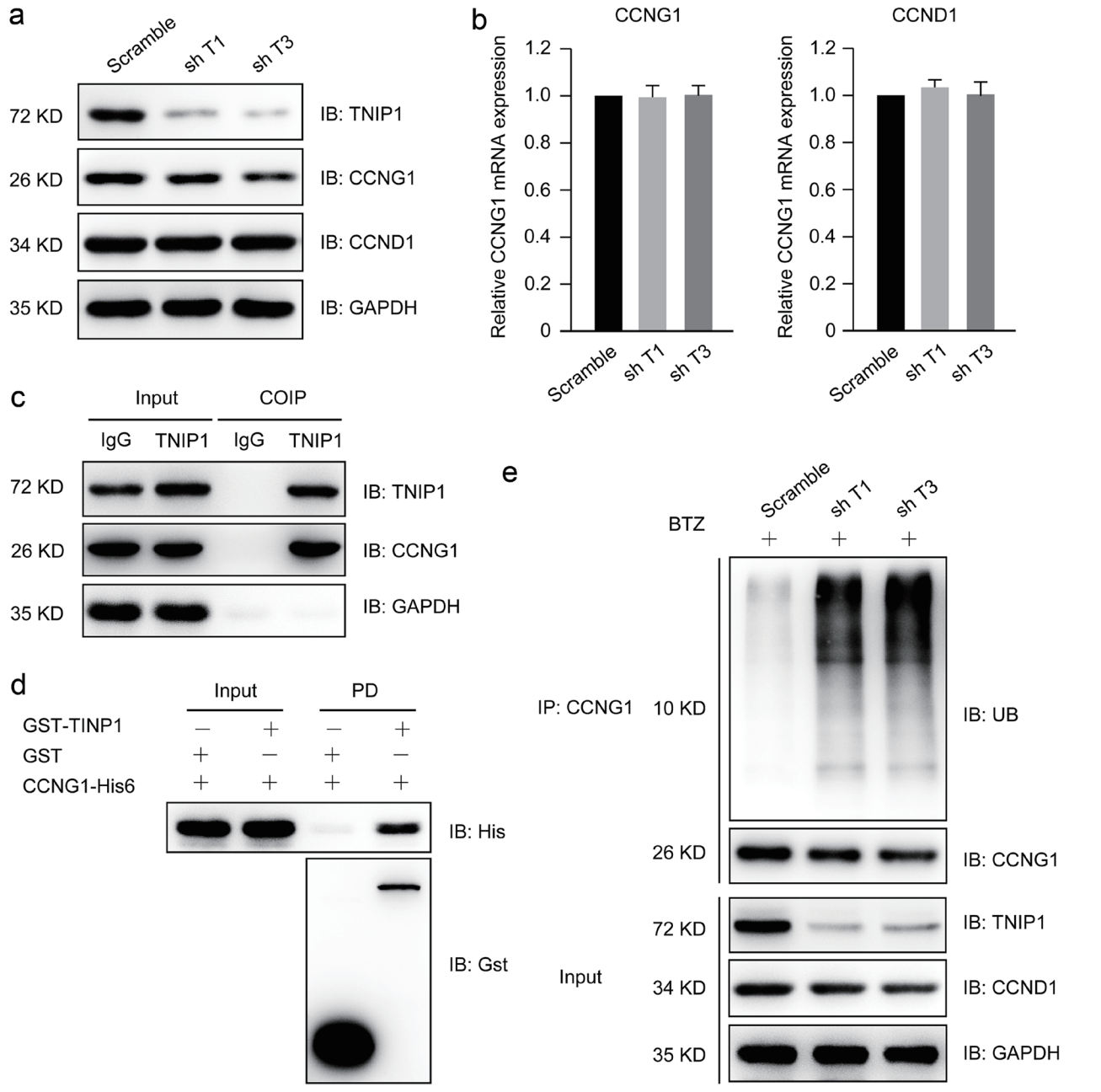

Fig. 3. TNIP1 knockdown reduced the protein level of CCNG1, and TNIP1 directly interacted with CCNG1. (a, b) The protein and mRNA levels of CCNG1 and CCND1 in MCF-7 cells that stably expressed TNIP1 shRNAs (Scramble, T1, T3) were detected by immunoblotting and quantitative polymerase chain reaction (qPCR). Data are presented as mean ± SD and analyzed using one-way ANOVA. **P* < 0.05, statistical difference; ***P* < 0.01, significant statistical difference, three independent experiments. (c) Endogenous TNIP1 and CCNG1 formed a complex in MCF-7 cells. Cell lysates of MCF-7 cells were immunoprecipitated with anti-TNIP1 antibody and subjected to immunoblotting analysis. COIP, co-immunoprecipitation. (d) TNIP1 interacted with CCNG1 *in vitro*. Recombinant GST-tagged TNIP1 and His6-tagged CCNG1 were purified, and GST pull-down assays were performed, followed by immunoblotting analysis. PD, GST pull-down. (e) TNIP1 knockdown increased the ubiquitination of CCNG1 *in vivo.* MCF-7 cells that stably expressed TNIP1 shRNAs (Scramble, T1, T3) were treated with BTZ (bortezomib) for 8 h, then lysed in RIPA buffer, immunoprecipitated with anti-CCNG1 antibody, and subjected to immunoblotting analysis using the indicated antibodies. ANOVA, analysis of variance; CCND1, cyclin D1; CCNG1, cyclin G1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; mRNA, messenger RNA; RIPA, radio-immunoprecipitation assay; SD, standard deviation; shRNA, short hairpin RNA; TNIP1, tumor necrosis factor α-induced protein 3-interacting protein 1.

pa-B Kinase Gamma.[8] To some extent, the increase in CCNG1 ubiquitination dependent on TNIP1 knockdown may be caused by this mechanism. CCNG1 may serve as a substrate for the deubiquitylase A20, although this requires further experimental verification. While TNIP1 interacts with A20 and shares a common role in repressing NF-κB activity, it can also function independently

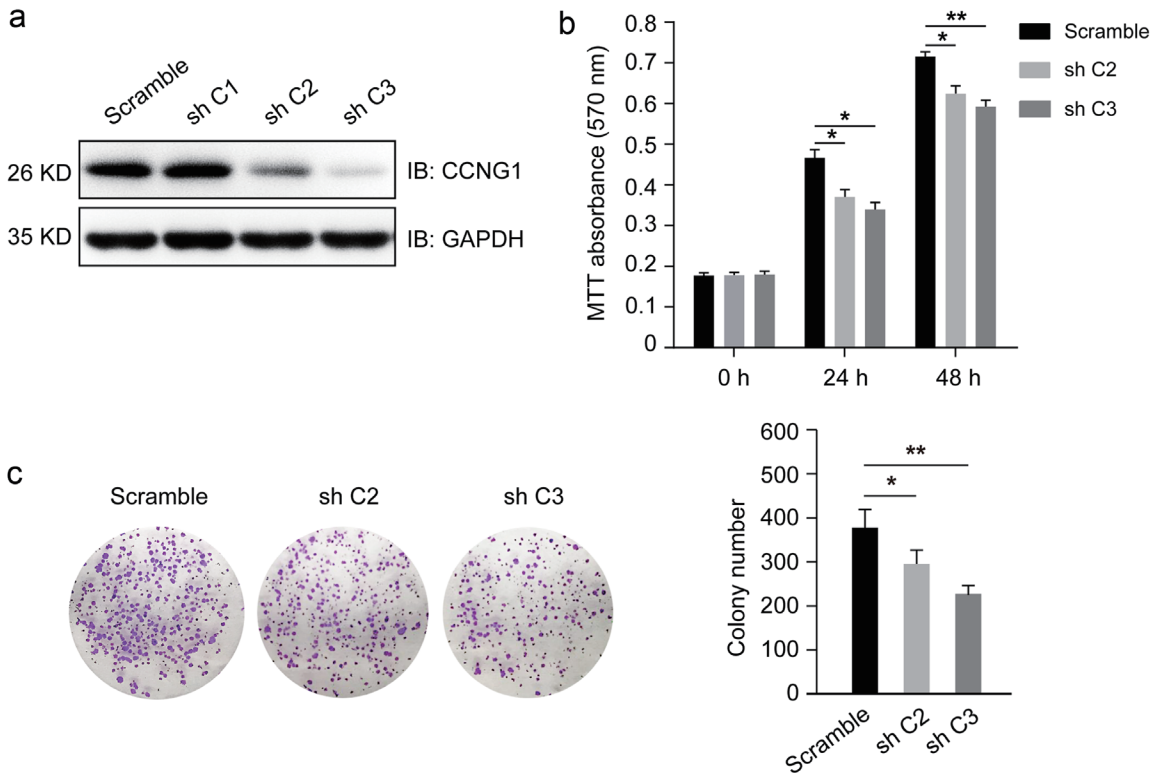

Fig. 4. CCNG1 knockdown induced growth arrest in MCF-7 cells. (a) The knockdown efficiency of shRNAs for CCNG1 was detected by immunoblotting. MCF-7 cells were infected with shRNAs (Scramble, C1, C2, C3) lentivirus targeting CCNG1 and screened with puromycin to establish stable cell lines. (b) CCNG1 knockdown inhibited the proliferation of MCF-7 cells. MCF-7 cells that stably expressed CCNG1 shRNAs (Scramble, C2, C3) were seeded into 96-well plates and assessed by MTT assay at the indicated time points. Six hours after cell inoculation were taken as 0 h time point. Data are expressed as mean ± SD and analyzed using one-way ANOVA. **P* < 0.05, significant difference, ***P* < 0.01, significant statistical difference, three independent experiments. (c) CCNG1 knockdown inhibited colony formation in MCF-7 cells. MCF-7 cells that stably expressed CCNG1 shRNAs (Scramble, C2, C3) were seeded into six-well plates, colonies were fixed with 4% paraformaldehyde, and stained with 0.1% crystal violet seven days later. The colony numbers were counted and calculated, with three samples in each group. Data are expressed as mean ± SD and analyzed using one-way ANOVA. **P* < 0.05, statistical difference; ***P* < 0.01, significant statistical difference, three independent experiments. ANOVA, analysis of variance; CCNG1, cyclin G1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SD, standard deviation; shRNA, short hairpin RNA.

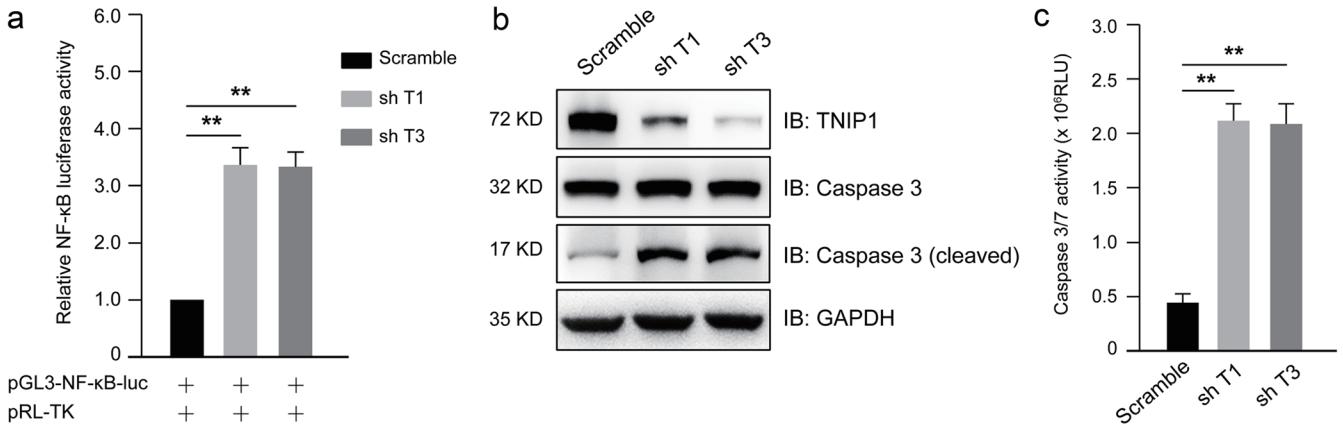

Fig. 5. TNIP1 knockdown activated the NF-κB pathway and suppressed breast cancer cell growth. (a) The NF-κB luciferase activity was detected in MCF-7 cells that stably expressed TNIP1 shRNAs (Scramble, T1, T3). The pGL3-NK-κB-luc and pRL-TK plasmids were introduced into MCF-7 cells by electroporation. Firefly luciferase activity was normalized to that of Renilla luciferase. **P* < 0.05, statistical difference; ***P* < 0.01, significant statistical difference, three independent experiments. (b) The protein levels of Caspase 3 and cleaved Caspase 3 were detected in MCF-7 cells that stably expressed TNIP1 shRNAs (Scramble, T1, T3) by immunoblotting. (c) The Caspase 3/7 activity was detected in MCF-7 cells that stably expressed TNIP1 shRNAs (Scramble, T1, T3) using the Caspase-Glo 3/7 assay (Promega). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; pRL-TK, renilla luciferase-thymidine kinase plasmid; shRNA, short hairpin RNA; TNIP1, tumor necrosis factor α-induced protein 3-interacting protein 1.

of A20.[24–26] Mice lacking TNIP1 exhibit extensive TNF-induced apoptosis in the liver, with few survive postnatally.[8] The ablation of TNIP1 could activate the NF-κB pathway, increase CASP-3/7 activity, and enhance cleavage of Caspase 3 in MCF-7 cells, consistent with previous reports.

CCNG1 has been proposed to participate in p53-dependent G1/S and G2 checkpoints and may function as an oncogenic protein in the initiation and metastasis of ovarian carcinoma.[27] Thus, CCNG1 may act as a potential target for cancer therapy. In our study, TNIP1 knockdown reduced CCNG1 protein expression rather than CCND1 protein expression, indicating that the TNIP1-CCNG1 axis plays an important role in breast cancer cell proliferation. Altogether, we speculate that TNIP1 may serve as a pivotal marker for breast cancer.

Due to limitations in research time and conditions, we only explored the effect and mechanism of the TNIP1 gene on breast cancer cells *in vitro*. An ongoing effort in our research is to test the functions of TNIP1 in animal models and patient samples. We will also conduct histological analyses on xenograft tumors in future studies, including immunohistochemical analyses of proliferation and apoptosis markers. Further summarization of the corresponding working model based on the experimental results will enhance our understanding of TNIP1's role in breast cancer development.

Conclusions

We demonstrate that TNIP1 knockdown leads to growth arrest in breast cancer cells through interacting with CCNG1 and promoting its ubiquitination. This mechanism reveals a novel pathway by which TNIP1 regulates cell proliferation in breast cancer. Our findings also reveal that TNIP1 knockdown not only affects CCNG1 levels but also activates the NF-κB signaling pathway, leading to increased apoptosis in MCF-7 cells. Overall, our study highlights TNIP1 as a crucial marker and suggests its potential as a target for breast cancer therapies.

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None.

Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Study conception and design (ZZL), experiment performance, data collection and analysis (QHL, SPC, YBZ), and manuscript writing (QHL, ZS). All authors reviewed and edited the manuscript.

Ethical statement

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Shenyang Grenst Biotechnology Co., Ltd. (Protocol Number: CR 2204012). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Data sharing statement

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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