

# TRIM27 Functions as a Novel Oncogene in Non-Triple-Negative Breast Cancer by Blocking Cellular Senescence through p21 Ubiquitination

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**In the current study, we aimed to explore the correlation between TRIM27 and breast cancer prognosis, as well as the functions of TRIM27 in breast cancer and their underlying mechanisms. Bioinformatics analyses were used to examine the correlation between TRIM27 and breast cancer prognosis. Moreover, TRIM27 knockdown and overexpression in breast cancer cells were performed to investigate its functions in breast cancer. Tamoxifen (TAM) was applied to evaluate the influence of TRIM27 on chemoresistance of breast cancer cells, while co-immunoprecipitation (coIP) was performed to identify the E3 ubiquitin ligase capability of TRIM27. High expression of TRIM27 was found in non-triple-negative breast cancer (non-TNBC) tumor tissues and was positively correlated with the mortality of non-TNBC patients. Moreover, TRIM27 could suppress non-TNBC cell apoptosis and senescence, promote cell viability and tumor growth, counteract the anti-cancer effects of TAM, and mediate ubiquitination of p21. In addition, EP300 could enhance the expression of TRIM27 and its transcription promoter H3K27ac. TRIM27, through ubiquitination of p21, might serve as a prognostic biomarker for non-TNBC prognosis. TRIM27 functions as a novel oncogene in non-TNBC cellular processes, especially suppressing cell senescence and interfering with non-TNBC chemoresistance.**

## INTRODUCTION

Breast cancer is the most common type of female cancer, with an incidence of 250,000–300,000 cases annually worldwide.<sup>1</sup> Because of its high heterogeneity, breast cancer is one of the primary causes of cancer-related deaths worldwide and is frequently associated with poor prognosis.<sup>2,3</sup> Depending on the presence/absence of three biomarkers, estrogen receptor (ER), human epidermal growth factor-2 receptor (HER2), and progesterone receptor (PR), breast cancer has been categorized into triple-negative breast cancer (TNBC) and non-TNBC.<sup>4,5</sup> Non-TNBC accounts for nearly 80% of all breast cancer diagnoses, although it has better overall survival and prognosis.<sup>6,7</sup> Several studies have demonstrated that TNBC is associated with a higher risk of recurrence, earlier propensity to metastasis, and more aggressive clinicopathological characteristics.<sup>4,6,7</sup> However, the chemosensitivity of both TNBC and non-TNBC are low due to the presence of persistent chemoresistant cancer stem cells.<sup>8,9</sup>

In response to stresses, for example anti-cancer drug treatment, cancer cells can undergo steady growth arrest, with irreversible DNA damage, limited viability, and sustained energy metabolism, the cellular state of which is called senescence.<sup>10,11</sup> Indeed, the induction of cellular senescence has critical roles in therapeutic intervention and suppression of various human cancers.<sup>11,12</sup> Normally, cell senescence is accompanied by the upregulation of an important cyclin-dependent kinase inhibitor, p21,<sup>13,14</sup> which further dephosphorylates pRb,<sup>15</sup> and together induces cell cycle arrest at the G1 or G2/M phase.<sup>16,17</sup> In contrast, the balance between cell proliferation and programmed death is decisive for normal cell growth or tumorigenesis,<sup>18</sup> and the majority of chemotherapies for breast cancer are targeted to induce tumor cell apoptosis.<sup>19,20</sup> However, breast cancer cells retain overexpression of the anti-apoptotic protein, B cell lymphoma 2, which represses caspase activation and contributes to chemoresistance.<sup>21</sup>

Tripartite motif-containing (TRIM) proteins are involved in regulating an assortment of important biological processes, especially cell differentiation, proliferation, and apoptosis.<sup>22,23</sup> Several TRIM proteins possess E3 ubiquitin ligase capability, such as TRIM27, which belongs to the superfamily of zinc finger proteins.<sup>24,25</sup> TRIM27 has been previously reported as a transcriptional repressor for suppressing cell senescence and has roles in the development of cancer.<sup>26</sup> Furthermore, TRIM27 is active in modulating cellular processes, and its high expression in cancer cells is connected with cell cycle dysregulation, as well as tumor cell proliferation and migration.<sup>26</sup> Dysregulation of TRIM27 can lead to oncogenesis and chemoresistance of multiple types of cancers, including breast, lung, and ovarian cancers.<sup>27,28</sup> Therefore, TRIM27 has the potential to serve as a prognostic biomarker for cancer patients. However,

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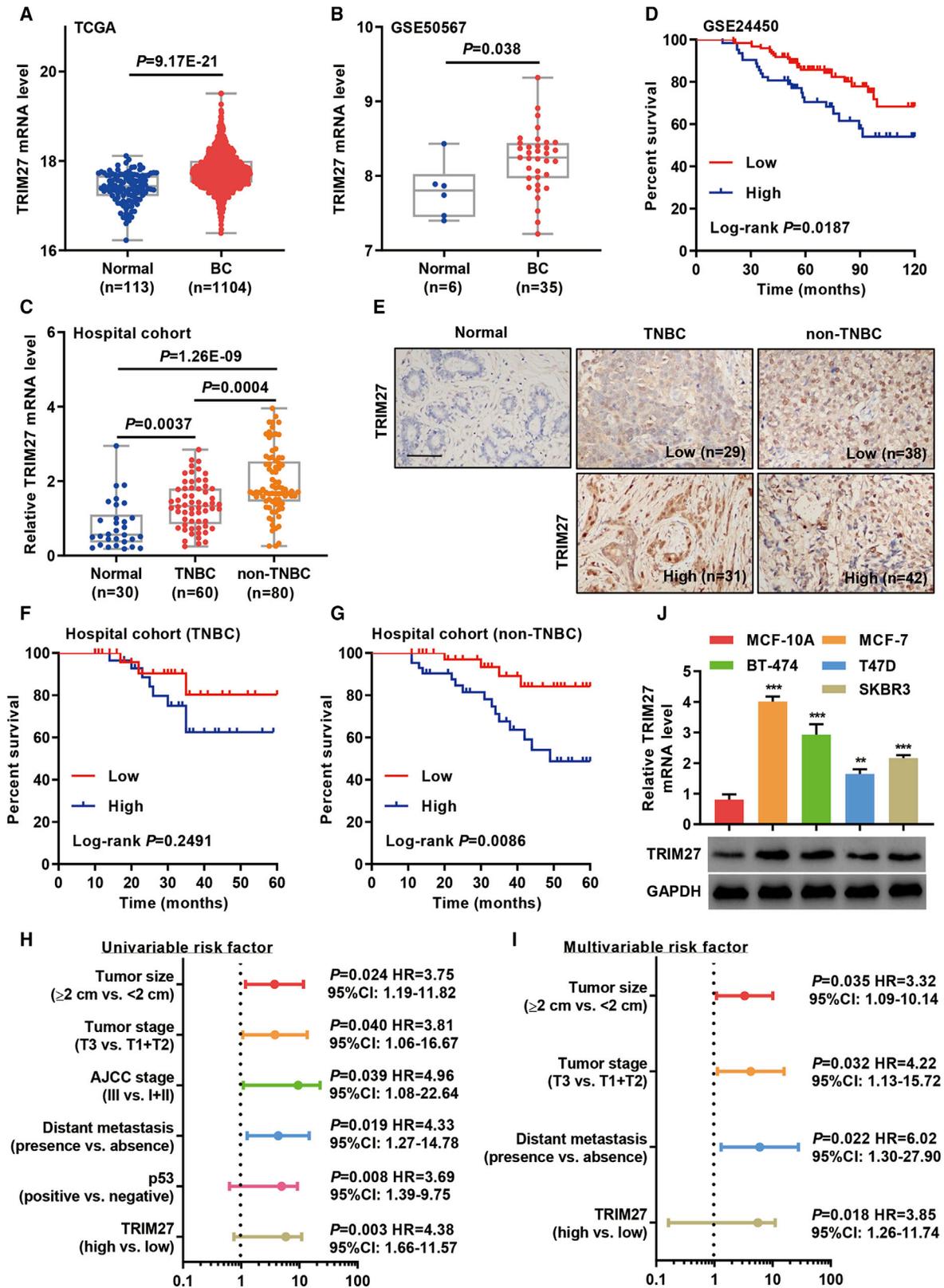
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the comprehensive connection between TRIM27 and breast cancer prognosis, as well as its detailed functions in breast cancer, remain unclear.

In the current study, we sought to investigate the relationship between TRIM27 and breast cancer prognosis, as well as the functions of TRIM27 in breast cancer (especially apoptosis and senescence), and their underlying mechanisms. We show that the expression of TRIM27 is relatively high in breast cancer cells, particularly non-TNBC. Furthermore, TRIM27 expression was found to be negatively correlated with the survival probability of non-TNBC patients. Moreover, overexpression or knockdown of TRIM27 in non-TNBC cells demonstrated that TRIM27 could regulate the cell cycle, promote cell viability and tumor growth, suppress apoptosis and senescence, and reduce the chemosensitivity of non-TNBC. Additionally, TRIM27 is ubiquitinated by p21 while its promoter is enhanced by p300.

## RESULTS

### TRIM27 Expression Correlates with Non-TNBC

In order to investigate the relationship between TRIM27 and breast cancer, we first collected breast cancer patient data from both online databases and the hospital cohort for bioinformatics analysis. *TRIM27* mRNA expression was significantly higher in breast cancer tumor tissues compared to that in normal tissues, based on both The Cancer Genome Atlas (TCGA) (fold change = 1.021;  $p = 9.17E-21$ ; Figure 1A) and Gene Expression Omnibus (GEO: GSE50567) (fold change = 1.052;  $p = 0.038$ ; Figure 1B). Similarly, based on hospital patient samples, the relative transcriptional level of *TRIM27* was found to be higher in both non-TNBC (fold change = 2.388;  $p = 0.0037$ ) and TNBC (fold change = 1.666;  $p = 1.26E-09$ ) tissues than in normal tissues (Figure 1C). Moreover, a significant ( $p = 0.0187$ ) correlation was observed between a high/low *TRIM27* mRNA level and survival probability in breast cancer samples from GSE24450 (Figure 1D).

We further divided breast cancer patients into high- and low-TRIM27 protein expression groups by immunohistochemistry (IHC) staining (Figure 1E). TRIM27 protein expression was not associated with the survival rate of TNBC patients ( $p = 0.2491$ ) (Figure 1F), whereas it was significantly ( $p = 0.0086$ ) correlated with the survival of non-TNBC patients (Figure 1G). Therefore, we focused on non-TNBC for the remainder of the study. We also observed that TRIM27 expression was significantly ( $p < 0.05$ ) associated with several clinical parameters in non-TNBC patients, including tumor size, histologic

grade, American Joint Committee on Cancer (AJCC) stage, tumor stage, and HER2, ER, and PR status (Table 1). Additionally, through univariate analysis, we identified that tumor size, tumor stage, AJCC stage, distant metastasis, p53 expression, and TRIM27 expression were all significantly ( $p < 0.05$ ) correlated with non-TNBC (Figure 1H). However, only tumor size, tumor stage, distant metastasis, and TRIM27 expression were independent and significant ( $p < 0.05$ ) risk factors for non-TNBC, based on multivariate analysis (Figure 1I).

### TRIM27 Influences Viability, Cell Cycle, and Tamoxifen (TAM) Sensitivity of Non-TNBC Cells

The mRNA and protein expression of TRIM27 in the human breast epithelial cell line MCF-10A and non-TNBC cell lines, including MCF-7, BT-474, T47D, and SKBR3, are shown in Figure 1J. Based on their high expression of TRIM27, we further selected MCF-7 and BT-474 cells ( $p < 0.001$ ) for TRIM27 knockdown ( $p < 0.001$ ; Figures 2A and 2B), while overexpression ( $p < 0.001$ ) was conducted in T47D cells, which had lower ( $p < 0.01$ ) TRIM27 expression (Figure 2C).

We observed that TRIM27 knockdown in both MCF-7 (Figure 2D) and BT-474 (Figure 2E) cells significantly ( $p < 0.001$ ) reduced their viability compared to control cells, while TRIM27 overexpression in T47D cells significantly ( $p < 0.001$ ) improved their viability (Figure 2F). Moreover, TRIM27 knockdown in both MCF-7 (Figure 2G) and BT-474 (Figure 2H) cells significantly increased the number of cells in G0–G1 phase ( $p < 0.001$ ) but decreased the number of cells in S ( $p < 0.01$ ) and G2–M ( $p < 0.001$ ) phases. On the contrary, TRIM27 overexpression in T47D cells significantly decreased the number of cells in G0–G1 phase ( $p < 0.001$ ) but increased the number of cells in S ( $p < 0.05$ ) and G2–M ( $p < 0.001$ ) phases (Figure 2I).

When treated with TAM, apoptosis was induced in MCF-7 and T47D in a dose-dependent manner (Figures 3A and 3B). However, TRIM27 knockdown in TAM-treated MCF-7 cells significantly ( $p < 0.001$ ) stimulated apoptosis (Figure 3A), while TRIM27 overexpression in TAM-treated T47D cells significantly ( $p < 0.05$ ) prevented the apoptotic effect of TAM (Figure 3B). In addition, based on analysis of senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal) staining, TAM-induced cell senescence in MCF-7 and BT-474 cells was stimulated by TRIM27 knockdown, but it was suppressed by TRIM27 overexpression in TAM-treated T47D cells (Figure 3C). The  $\beta$ -galactosidase protein and cleaved caspase-3 levels were upregulated by TRIM27 knockdown and downregulated by TRIM27 overexpression (Figure 3D).

### Figure 1. TRIM27 Is Overexpressed in Non-TNBC and Correlates with Prognosis of Non-TNBC Patients

(A and B) The expression levels of *TRIM27* were examined in tumor and normal tissues in breast cancer patients from (A) TCGA and (B) GSE50567 samples. (C) The expression levels of *TRIM27* were examined in normal, non-TNBC, and TNBC breast tissues from the hospital cohort. (D) Kaplan-Meier analysis of overall survival rates of patients with breast cancer from GSE24450 samples, based on *TRIM27* expression levels. (E) IHC for TRIM27 in normal, non-TNBC, and TNBC breast tissues from the hospital cohort (scale bar, 100  $\mu$ m). (F and G) Kaplan-Meier analysis for overall survival rates of (F) TNBC and (G) non-TNBC patients, based on the expression of TRIM27. (H and I) Univariate (H) and multivariate (I) analyses were performed based on the hospital cohort. Bars indicate 95% confidence intervals. (J) The expression levels of TRIM27 were examined in the human breast epithelial cell line MCF-10A and various non-TNBC cell lines. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , compared to the normal or MCF-10A group.

**Table 1. Relationship between TRIM27 Expression and Clinical Parameters in Non-TNBC Patients**

Clinical Parameter	Number of Cases	TRIM27 Expression		p Value
		High (%)	Low (%)	
<b>Age (Years)</b>				
≥ 50	32	14 (43.8)	18 (56.2)	0.2007
<50	48	28 (58.3)	20 (41.7)	
<b>Tumor Size</b>				
≥ 2 cm	56	23 (41.1)	33 (58.9)	0.0029
<2 cm	24	19 (79.2)	5 (20.8)	
<b>Histopathology</b>				
Ductal	55	32 (58.2)	23 (41.8)	0.2367
Lobular	25	18 (72.0)	7 (28.0)	
<b>Histologic Grade</b>				
1	13	3 (23.1)	10 (76.9)	0.0317
2	44	28 (63.6)	16 (36.4)	
3	23	11 (47.8)	12 (52.2)	
<b>AJCC Stage</b>				
I	14	3 (21.4)	11 (78.6)	0.0343
II	47	27 (57.4)	20 (42.6)	
III	19	12 (63.2)	7 (36.8)	
<b>Tumor Stage</b>				
T1	11	3 (27.3)	8 (72.7)	0.0076
T2	48	32 (66.7)	16 (33.3)	
T3	21	7 (33.3)	14 (66.7)	
<b>Distant Metastasis</b>				
Presence	19	8 (42.1)	11 (57.9)	0.2988
Absence	61	34 (55.7)	27 (44.3)	
<b>ER Status</b>				
Positive	51	32 (62.7)	19 (37.3)	0.0150
Negative	29	10 (34.5)	19 (65.5)	
<b>PR Status</b>				
Positive	54	35 (64.8)	19 (35.2)	0.0015
Negative	26	7 (26.9)	19 (73.1)	
<b>HER2 Status</b>				
Positive	22	16 (72.7)	6 (27.3)	0.0257
Negative	58	26 (44.8)	32 (55.2)	
<b>p53</b>				
Positive	47	28 (59.6)	19 (40.4)	0.1305
Negative	33	14 (42.4)	19 (57.6)	

Differences between groups were determined by the Chi-square test.

**TRIM27 Promotes Non-TNBC Tumor Growth and Interferes with TAM Sensitivity in Mice**

To evaluate the *in vivo* functions of TRIM27 in non-TNBC, we injected nude mice with either MCF-7 cells with/without TRIM27 knockdown or T47D cells with/without TRIM27 overexpression.

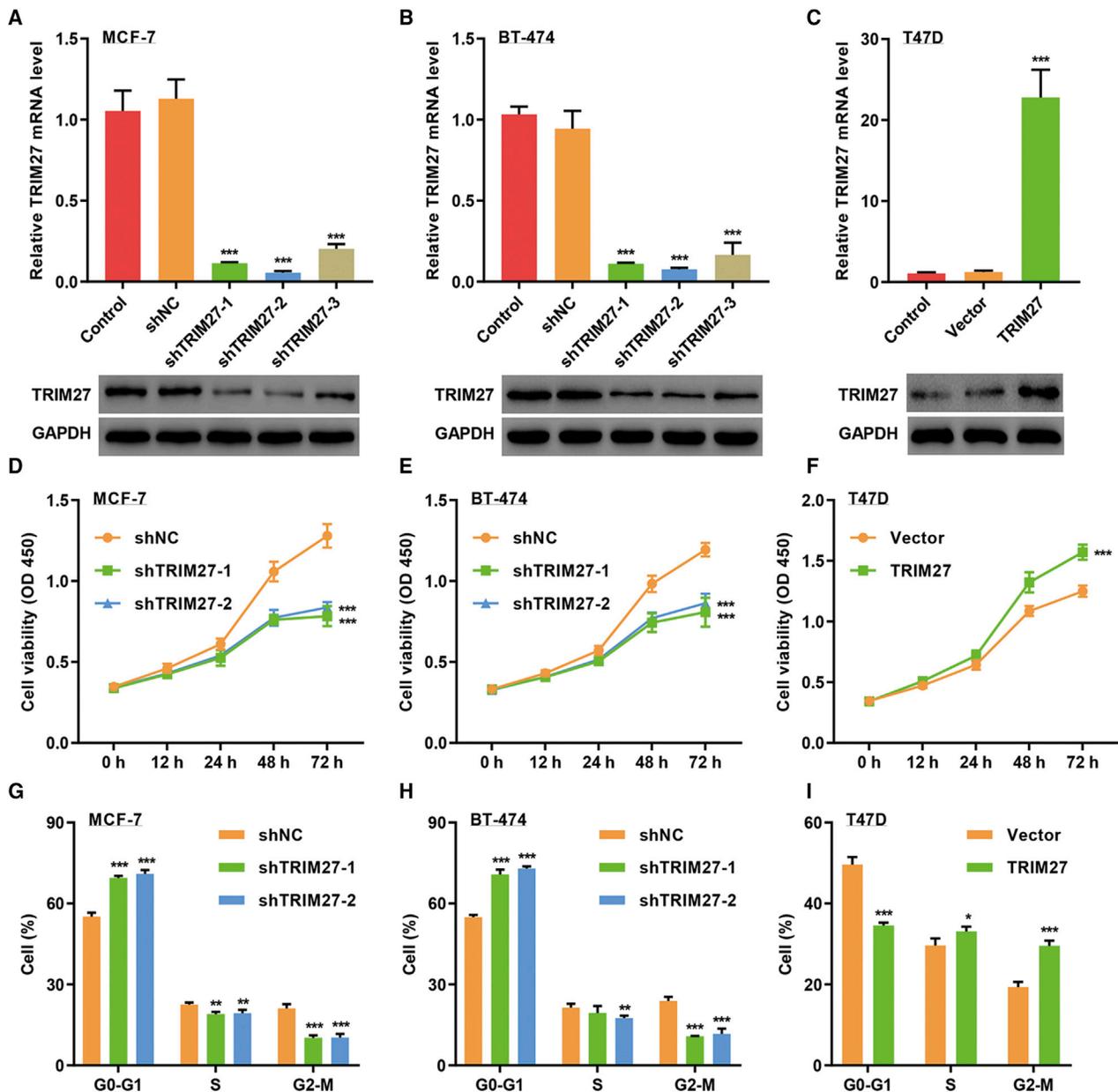
On the 33<sup>rd</sup> day after injection, the mice injected with MCF-7 cells with knockdown of TRIM27 developed substantially smaller tumors, in terms of both volume ( $p < 0.001$ ; Figure 4A) and weight ( $p < 0.01$ ; Figure 4B), lower Ki67 expression in tumor cells (Figure 4C), and upregulated cellular protein levels of TRIM27 (Figure 4D) compared to those in the control mice. In contrast, compared to the control mice, the mice injected with T47D cells with overexpression of TRIM27 developed substantially larger tumors ( $p < 0.001$ ; Figures 4E and 4F), lower tumor cell apoptosis ( $p < 0.001$ ; Figures 4G and 4J), and decreased survival probability ( $p < 0.001$ ; Figure 4H).

Furthermore, TAM treatment could significantly ( $p < 0.05$ ) reduce the developed tumor volume (Figure 4F) and weight (Figures 4G and 4H), increase tumor cell apoptosis (Figures 4I and 4J), and increase the survival probability (Figure 4K) of mice. However, the mice injected with T47D cells with TRIM27 overexpression displayed reduced TAM sensitivity (Figures 4F–4K).

**TRIM27 Regulates Cell Apoptosis, Cell Senescence, and TAM Sensitivity in Non-TNBC through p21 Ubiquitination**

Proteomics analysis was performed to identify candidate proteins associated with TRIM27 in order to investigate the molecular mechanism through which TRIM27 exerts its function. Candidate proteins were identified by liquid chromatography/mass spectrometry (LC/MS) and are shown in Figure 5A and listed in Figure 5B. Among the identified interaction partners, p21, which has tumor suppressor functions in breast cancer and is associated with cell apoptosis and senescence, was selected for further study. Co-immunoprecipitation (coIP) was used to confirm the protein association between TRIM27 and p21 (Figure 5C). The protein, but not the mRNA level, of p21 was downregulated in T47D cells with TRIM27 overexpression, compared to that in the control cells, whereas MG132 treatment rescued p21 back to its normal protein level in the control cells (Figure 5D). The ubiquitination of p21 was also enhanced by the overexpression of TRIM27 in T47D cells (Figure 5E). *In vitro* ubiquitination of purified p21 by TRIM27 was also observed (Figure 5F). These results indicate that TRIM27 may regulate p21 expression by promoting ubiquitination of p21.

We next induced p21 knockdown in MCF-7 cells (Figure 6A) to further explore the underlying mechanism of the functions of TRIM27 on non-TNBC. Knockdown of p21 in MCF-7 cells could suppress TAM-induced cell apoptosis ( $p < 0.001$ ; Figure 6B) and senescence (Figure 6C), as well as downregulate the induced protein levels of  $\beta$ -galactosidase (Figure 6D). Furthermore, p21 overexpression in T47D cells could further enhance TAM-induced cell apoptosis ( $p < 0.001$ ; Figures 6E and 6F), cell senescence (Figure 6G), and cellular protein levels of  $\beta$ -galactosidase (Figure 6H). Meanwhile, simultaneous knockdown of p21 and TRIM27 in MCF-7 cells or their simultaneous overexpression in T47D cells both counteracted the effects of single TRIM27 knockdown or overexpression, respectively, on non-TNBC cells (Figures 6B–6D and 6F–6H).



**Figure 2. TRIM27 Regulates the Cell Cycle and Cell Viability in Non-TNBC Cell Lines**

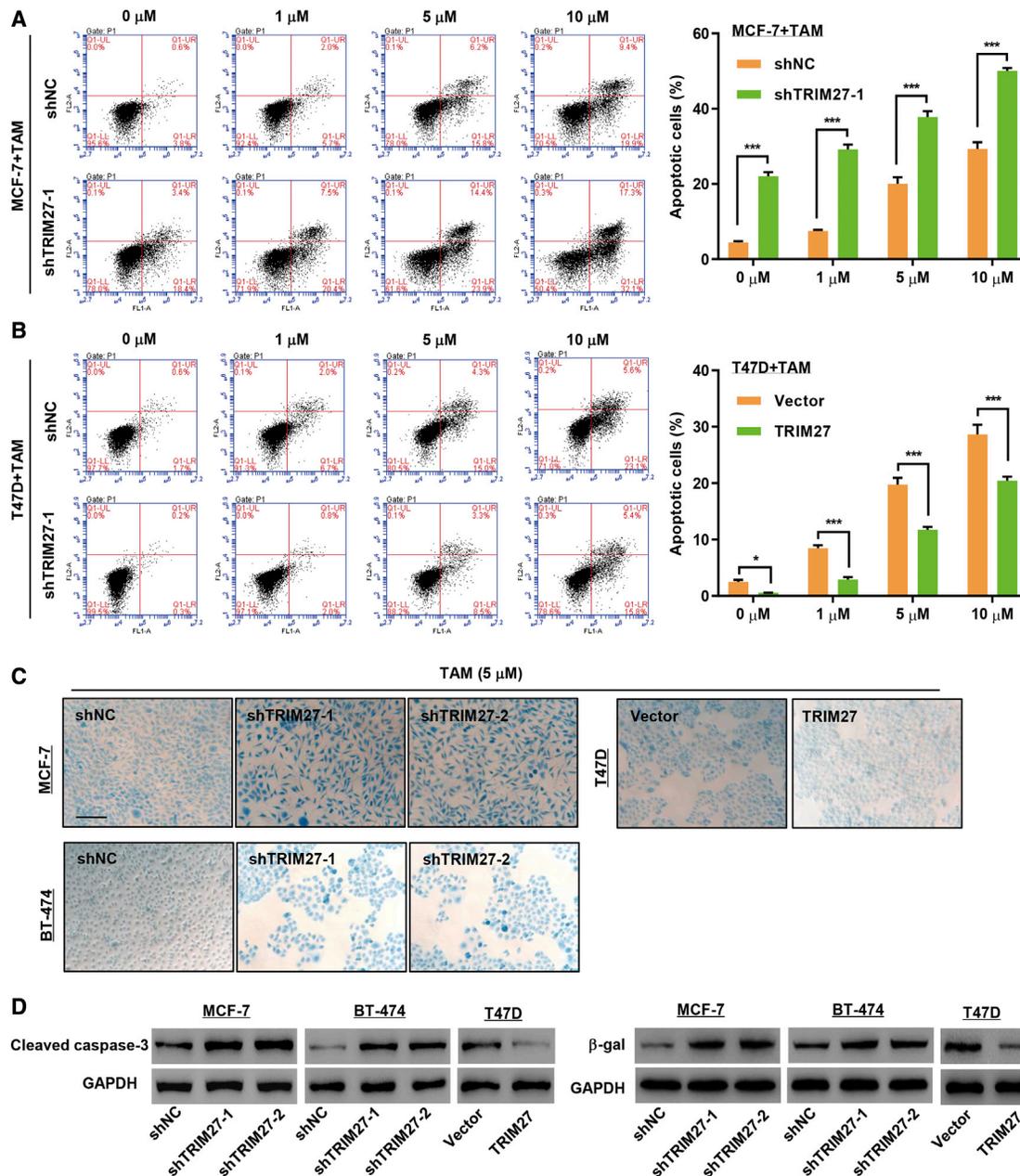
(A–C) TRIM27 mRNA and protein expression levels in (A) MCF-7, (B) BT-474, and (C) T47D cells transduced with the indicated vectors. (D–F) Cell viability was measured in (D) MCF-7, (E) BT-474, and (F) T47D cells transduced with the indicated vectors. (G–I) cell cycle were measured in (G) MCF-7, (H) BT-474, and (I) T47D cells transduced with the indicated vectors. \*\*\* $p < 0.001$ , compared to shNC or vector group.

#### Transcription of the TRIM27 Promoter H3K27ac Is Enhanced by p300 through Acetylation

Analysis using University of California, Santa Cruz (UCSC) Genome Browser (<http://genome.ucsc.edu/>) revealed that the promoter region of TRIM27 had a high density of H3K27ac. At the same time, based on data from GeneCards (<https://www.genecards.org>), the promoter element GH16]028921 in the TRIM27 promoter region contained 346 binding sites for transcriptional factors, including EP300

(p300). Therefore, we speculated that p300 could enhance the upregulation of TRIM27 expression by activating H3K27ac.

To investigate this further, we treated non-TNBC cells with a histone acetyltransferase p300 inhibitor C646. Based on the Renilla luciferase activity, the TRIM27 promoter activity in MCF-7 and BT-474 cells were substantially ( $p < 0.001$ ) suppressed by C646 (Figure 7A). Similarly, the relative mRNA levels of TRIM27 in both cell lines were also



**Figure 3. TRIM27 Regulates TAM-Induced Cell Apoptosis and Senescence in Non-TNBC Cell Lines**

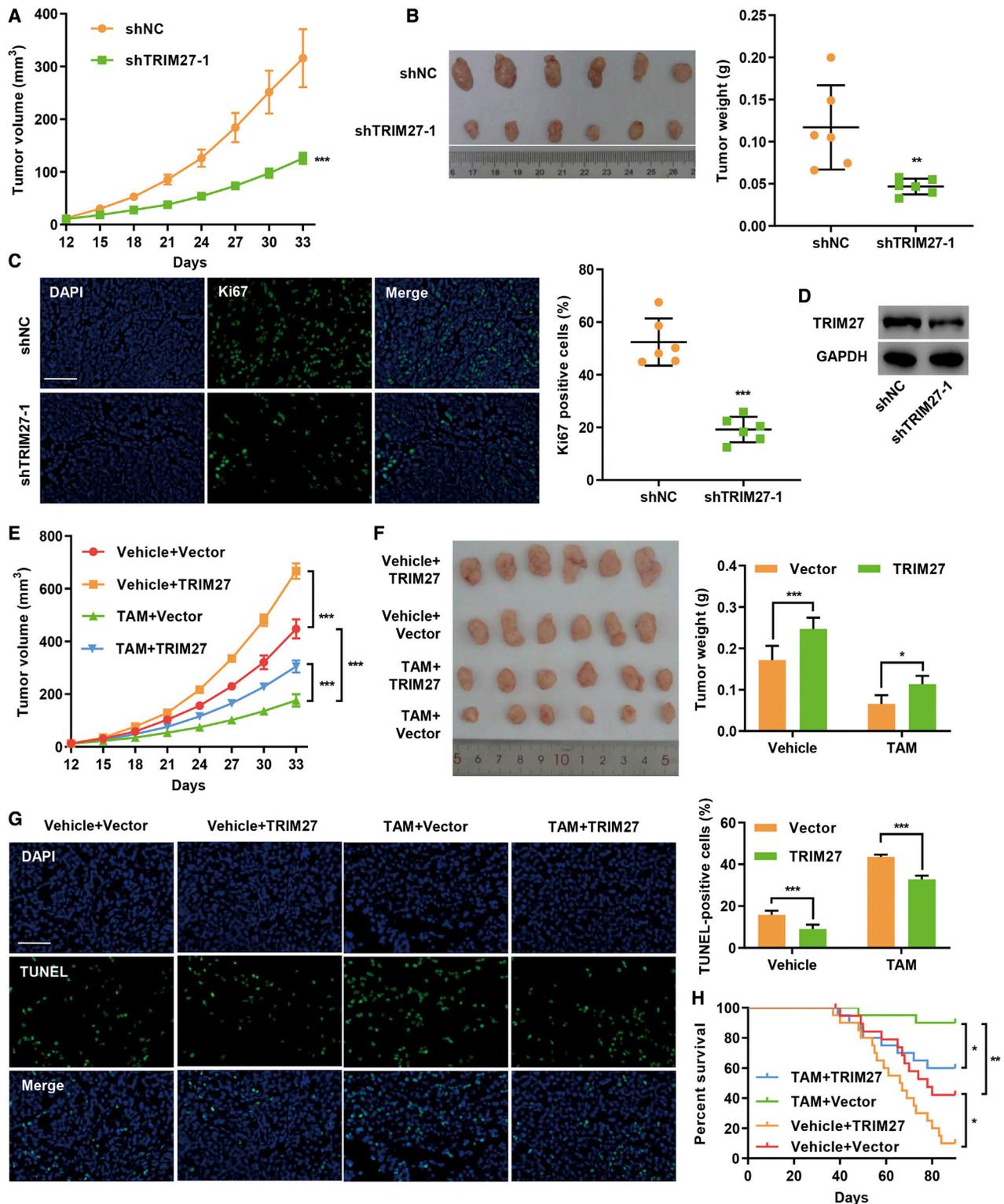
MCF-7 and BT-474 cells were transduced with pLKO.1-TRIM27 shRNA or shNC, and T47D cells were transduced with the lentivirus containing TRIM27 expression vector (TRIM27) or blank vector alone (Vector). (A and B) Cell apoptosis was measured in (A) MCF-7 and (B) T47D cells transduced with the indicated vectors following 48 h TAM (0, 1, 5, or 10 μM) treatment. (C) SA-β-gal staining of cells with 48 h 5 μM TAM treatment (scale bar, 100 μm). (D) Protein levels of β-gal and cleaved caspase-3 in MCF-7, BT-474, and T47D cells transduced with indicated vectors measured by WB. \* $p < 0.05$ , \*\*\* $p < 0.001$ .

substantially ( $p < 0.001$ ) downregulated by C646 (Figure 7B). Moreover, C646 induced the downregulation of TRIM27 and H3K27ac protein levels in MCF-7 and BT-474 cells (Figures 7C and 7D).

## DISCUSSION

TRIM proteins, which contain a RING domain, are active in a wide range of biological and physiological activities related to innate

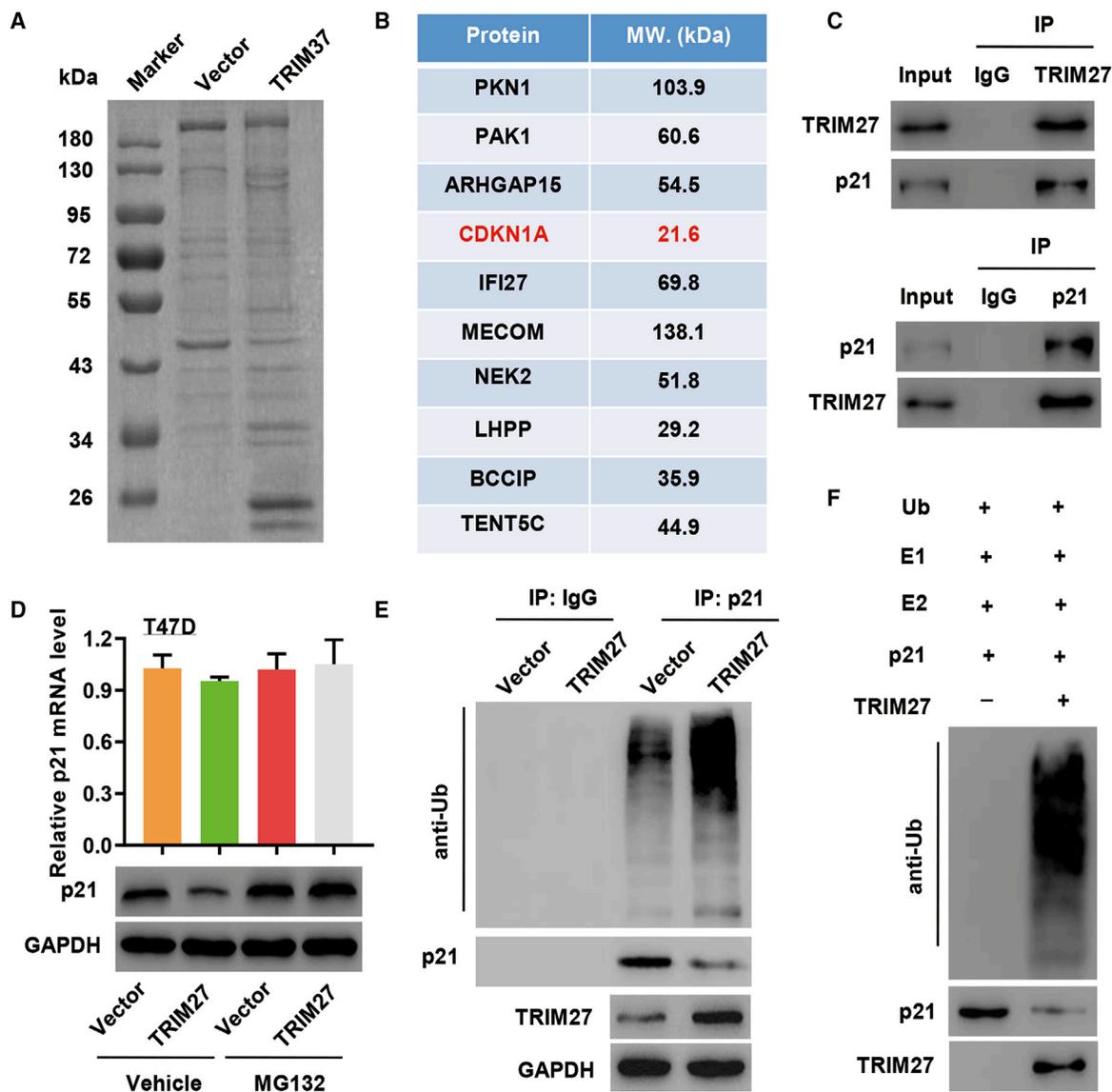
immunity, neurological and genetic disorders, and human cancers.<sup>29</sup> In the present study, we concentrated on TRIM27, based on its E3 ubiquitin ligase property in catalyzing the auto-ubiquitination of interactive proteins and its roles in regulating the cell cycle.<sup>24,26</sup> By analyzing data from online databases, we observed high expression of TRIM27 in breast cancer, as well as its relationship with mortality. By separating breast cancer samples into TNBC and non-TNBC, we



**Figure 4. TRIM27 Regulates Tumor Growth and Chemosensitivity of Non-TNBC Cells to TAM *In Vivo***

Nude mice were subcutaneously injected with MCF-7 cells with shTRIM27-1 or shNC transduction at the right armpit. (A–E) At the 33<sup>rd</sup> day post-injection, (A) tumor volume and (B) weight, (C) xenograft tumors with Ki67 immunofluorescence staining, and (D) the TRIM27 protein levels were measured in xenograft tumors (n = 6 per group). Mice

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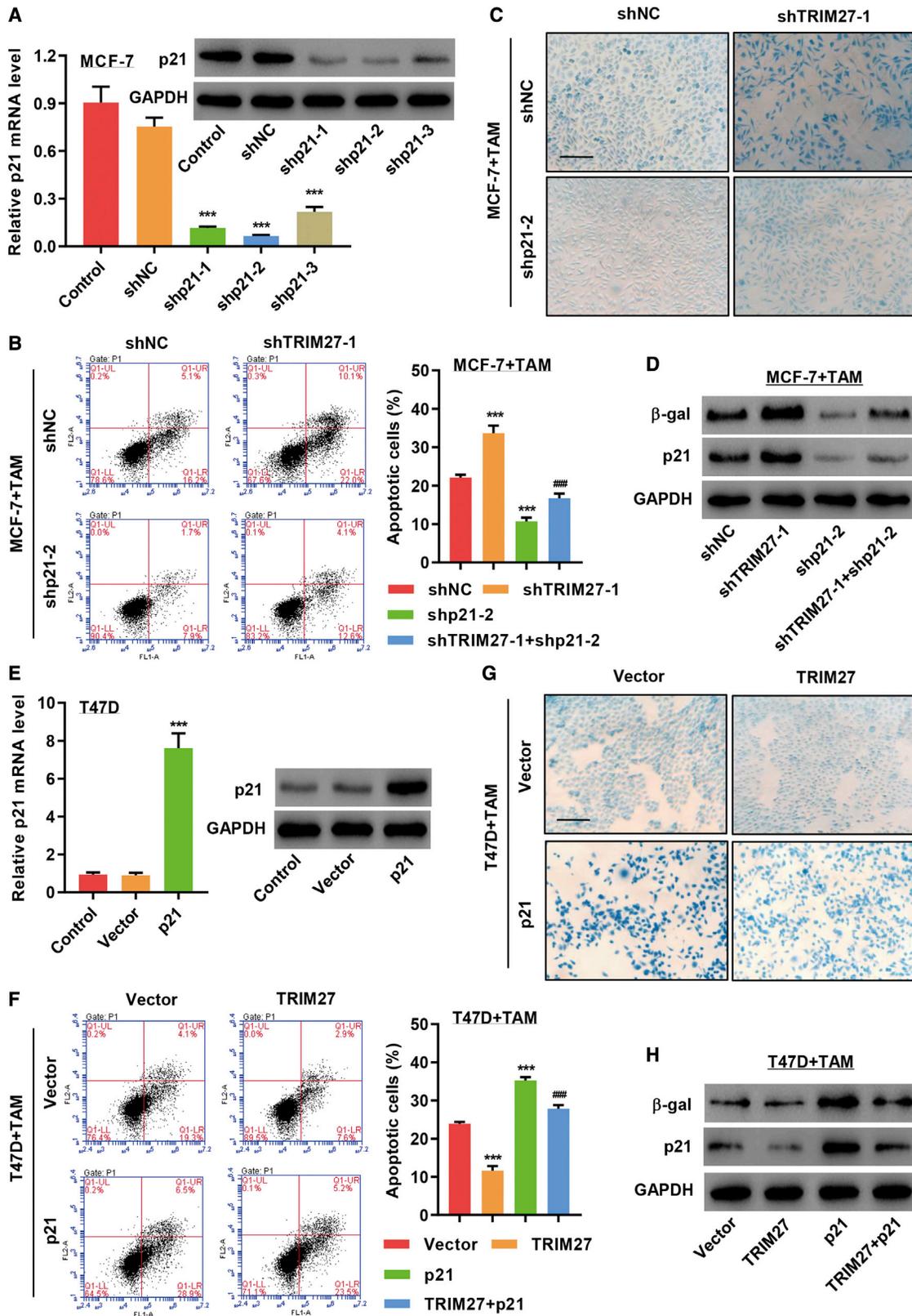
**Figure 5. TRIM27 Induces Ubiquitination of p21**

(A) Purification of the TRIM27 complex was carried out according to the procedure outlined in the [Materials and Methods](#). Proteins were separated on SDS-PAGE and stained with Coomassie blue. (B) List of TRIM27-associated proteins identified by MS analysis. (C) The association between TRIM27 and p21 in T47D cells was examined by coIP. (D) The protein levels of p21 in T47D cells transduced with the lentivirus containing TRIM27 expression vector (TRIM27) or blank vector alone (Vector) treated with 10  $\mu$ M MG132 or control (Vehicle) were measured by quantitative real-time PCR and WB. (E) Effect of TRIM27 overexpression on p21 ubiquitination in T47D cells. (F) *In vitro* ubiquitination assay for purified p21 by TRIM27.

further detected that the expression of TRIM27 was even higher in non-TNBC tumor tissues. Moreover, we found that the protein expression of TRIM27 was negatively correlated with the survival rate of non-TNBC patients, indicating that TRIM27 could indicate

non-TNBC tumor recurrence after related therapies. This finding is in accordance with previous studies, in which similar correlations between TRIM27 and prognostic conditions in human cancers were also identified.<sup>26,27</sup> Moreover, we detected a close relationship

injected with T47D cells transduced with the lentivirus containing TRIM27 expression vector (TRIM27) or blank vector alone (Vector) were treated with TAM (10 mg/kg) or control (Vehicle) for 33 days. (E–G) Tumor volume (E), weight (F), and xenograft tumors (G) with TUNEL staining were visualized and quantified (n = 6 per group). (H) Survival rate of mice injected with T47D cells transduced with the lentivirus containing TRIM27 expression vector (TRIM27) or blank vector alone (Vector) with (TAM) or without (Vehicle) treatment (n = 20 per group) (scale bar, 50  $\mu$ m). \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.



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between TRIM27 expression and multiple non-TNBC clinicopathological parameters, and, relying on multivariate analysis, we revealed the independency of TRIM27 expression as a risk factor for non-TNBC. Taken together, these results suggest that TRIM27 possesses great value in predicting the prognosis of non-TNBC patients and improving their clinical management.

TRIM27 has been reported to be capable of influencing multiple crucial cell activities, especially cell proliferation and tumorigenesis.<sup>30,31</sup> In line with this, we found that TRIM27 knockdown suppresses non-TNBC cell viability and tumor growth in mice, whereas its overexpression promoted cell viability and tumor growth; these findings demonstrate the roles of TRIM27 in stimulating tumor cell proliferation *in vitro* and breast tumor development *in vivo*. It has also been shown that TRIM27 participates in cell cycle regulation through controlling the oscillating capabilities of cyclin-dependent kinases, as well as related co-activators, thus regulating the transition phases and functional elements of mitotic processes.<sup>32,33</sup> In the current study, we also detected that TRIM27 could affect the non-TNBC cell cycle by favoring the S and G2–M phases over the G0–G1 phase. These findings again suggest that high TRIM27 expression is one of the biomarkers indicating the development of non-TNBC and its recurrence after treatment.

Programmed cell death is one of the positive responses to tumor chemotherapy, in which anti-apoptotic factors are antagonized and caspases are directly activated.<sup>34,35</sup> TRIM27 has been shown to be associated with cell apoptosis, dependent on its regulatory functions in multiple inflammatory cytokines and signaling pathways.<sup>31,36,37</sup> In accordance with these studies, we have also observed that TRIM27 could repress the apoptosis of non-TNBC cells both *in vitro* and *in vivo*. The anti-breast cancer drug TAM functions by blocking estrogen from binding with its receptor<sup>38</sup> but also induces cellular senescence in an estrogen-independent manner,<sup>39,40</sup> which can be detected by the common biomarker SA- $\beta$ -gal.<sup>41</sup> In the current study, TRIM27 knockdown was shown to stimulate TAM-induced cell senescence of non-TNBC, while TRIM27 overexpression demonstrated an inverse effect. The counteractive functions of TRIM27 on TAM-induced promotion of apoptosis and senescence demonstrate that TRIM27 interferes with non-TNBC cells and reduces their chemosensitivity toward TAM. In fact, the influences of TRIM27 on tumor chemoresistance have been reported previously, attributed to its modulation of p53 degradation, as well as via p53-independent pathways.<sup>42,43</sup>

We demonstrated that the cellular p21 levels were influenced by TRIM27, which indicates that TRIM27 might participate in the regu-

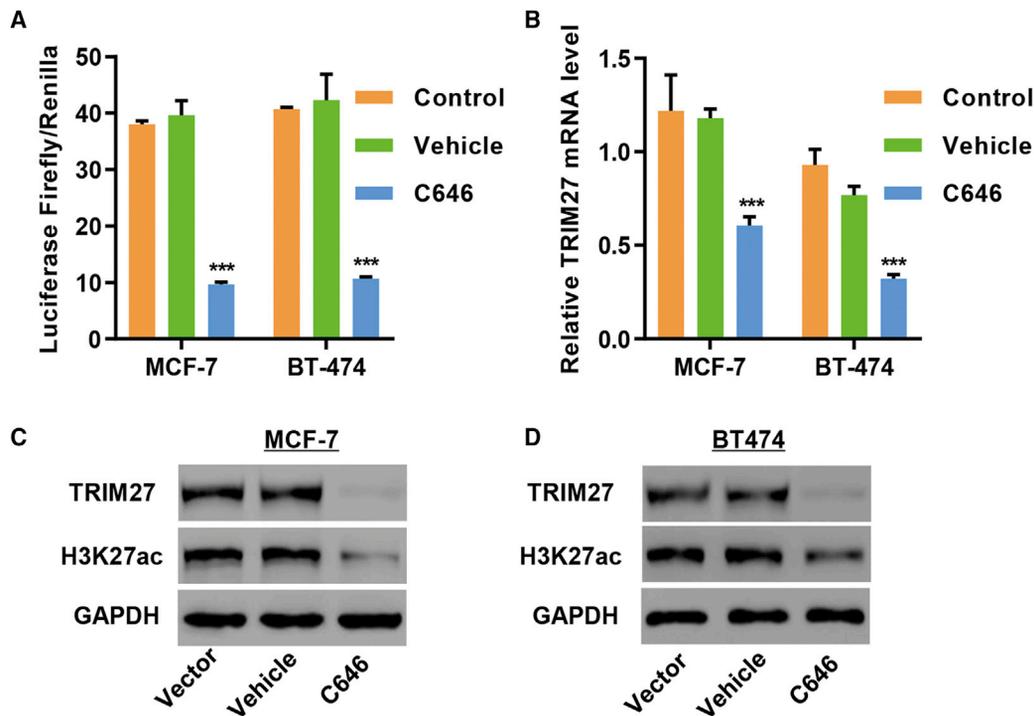
lation of p21, thereby further modulating the cell cycle and relevant biological processes. Indeed, p21, in further preventing the phosphorylation of pRb, is able to downregulate proliferation-related gene expression as well as facilitate cell senescence and apoptosis.<sup>13,44</sup> As a consequence of its systemic regulation by signaling factors, p21 can activate cell cycle checkpoints, while its cellular level is also controlled by ubiquitination and proteasomal degradation mediated by several E3 ligases.<sup>45,46</sup> Accordingly, in the current study, we observed the association between TRIM27 and p21 and further confirmed that TRIM27 could ubiquitinate p21. Furthermore, additional p21 knockdown/overexpression counteracted the effects introduced by TRIM27 knockdown/overexpression in non-TNBC cells, and vice versa, which further validates the dynamic interaction between these two critical proteins. Taken together, these results imply that TRIM27 suppresses cell apoptosis, cell senescence, and TAM sensitivity of non-TNBC cells through mediating the ubiquitination of p21.

EP300 is an important histone acetyltransferase that regulates gene transcription through chromatin remodeling.<sup>47,48</sup> The functions of EP300 in human cancer are mainly based on its abilities to modulate cell differentiation and proliferation.<sup>49</sup> We predicted that EP300 could enhance TRIM27 elevation, dependent on the identified EP300 transcriptional binding site in the TRIM27 promoter region, by analyzing online databases. This was further confirmed by the findings that the EP300 inhibitor C646 could suppress the cellular levels of both TRIM27 and its promoter H3K27ac. In fact, EP300 serves as a transcriptional coactivator in binding and activating transcription factors or transcription machinery.<sup>50</sup> However, the detailed molecular mechanism of the interactions between EP300 and H3K27ac remains to be examined in future studies.

In conclusion, this study systematically illustrates the biological functions of TRIM27 in breast cancer. We demonstrate high expression of TRIM27 in non-TNBC and suggest that TRIM27 might be developed as a prognostic biomarker for non-TNBC. Moreover, TRIM27 was shown to promote non-TNBC as a result of increasing cellular viability, enhancing tumor growth, suppressing cell apoptosis and senescence, and increasing chemoresistance. The role of TRIM27 in the regulation of non-TNBC cellular processes was found to be reliant on its capability to ubiquitinate p21. These findings suggest that TRIM27 is a novel oncogene endorsing non-TNBC, which could represent a valuable resource for improving the clinical management of non-TNBC and the development of therapeutics. In addition, the transcription of TRIM27 may be activated by EP300, which represents another avenue that could be explored in future molecular studies.

#### Figure 6. TRIM27 Regulates Cell Apoptosis and Senescence Induced by TAM in Non-TNBC Cells through p21

(A) The mRNA levels of *p21* were measured in MCF-7 cells transduced with pLKO.1-p21 shRNA (shp21-1, -2, and -3) or shNC. (B–D) Cell apoptosis (B), SA- $\beta$ -gal staining (C), and protein levels (D) of  $\beta$ -gal and p21 in MCF-7 cells transduced with indicated vectors and treated with 5  $\mu$ M TAM for 48 h (scale bar, 100  $\mu$ m). (E) The mRNA levels of *p21* were measured in T47D cells transduced with the lentivirus containing p21 expression vector (p21) or blank vector alone (Vector). (F–H) Cell apoptosis (F), SA- $\beta$ -gal staining (G), and protein levels (H) of  $\beta$ -gal and p21 in T47D cells transduced with indicated vectors and treated with 5  $\mu$ M TAM for 48 h (scale bar, 100  $\mu$ m). \*\*\**p* < 0.001, compared to shNC or vector group. ####*p* < 0.001, compared to shTRIM27-1 or TRIM27 group.



**Figure 7. Histone Acetyltransferase p300 Promotes the Transcription of the TRIM27 Promoter through H3K27 Acetylation**

(A–D) MCF-7 and BT-474 cells were treated with 20  $\mu$ M histone acetyltransferase p300 inhibitor (C646) or vehicle, and (A) the activity of TRIM27 promoter, (B) mRNA, and protein levels of (C) TRIM27 and (D) H3K27 acetylation (H3K27ac) were measured. \*\*\* $p < 0.001$ , compared to the vehicle group.

## MATERIALS AND METHODS

### Patient Cohort and Corresponding Tissue Specimens

The present study was approved by the Ethics Committee of Shanghai Eighth People Hospital. All study participants provided written informed consent. The hospital cohort included 60 TNBC and 80 non-TNBC patients treated between 2012 and 2015. Clinical and prognostic information was collected, as well as tumor and non-tumorous tissue specimens. None of the included patients received hormone therapy, radiotherapy, or neo-adjuvant chemotherapy prior to the surgery. Specimens acquired from the hospital cohort were paraffin-embedded and subjected to IHC staining with anti-TRIM27 antibody (1:100 dilution). The expression levels of proteins were scored based on visual grading of the staining intensity. A scale of 0–4 was used for the grading, with <5% staining scored as 0, 5%–25% staining scored as 1, 25%–50% staining scored as 2, 50%–75% staining scored as 3, and >75% staining scored as 4. The TRIM27 high-expression group was categorized by scores  $\geq 2$ , while the TRIM27 low-expression group was categorized by scores <2.

### Bioinformatics Analysis

Data on TRIM27 expression were downloaded from breast cancer projects of the GEO (GEO: GSE50567) and TCGA databases. Survival data were downloaded from the GEO (GEO: GSE24450). Kaplan-Meier survival analysis combined with log-rank test was performed to examine the overall survival of patients.

### Cell Culture

The normal human breast epithelial cell line MCF-10A and the non-TNBC cell lines MCF-7, BT-474, T47D, and SKBR3 were all obtained from the American Type Culture Collection (ATCC, USA). All cell lines were maintained in RPMI-1640 medium and 10% FBS at 37°C with proper humidity.

### Plasmid Construction and Gene Knockdown

The RNA interference sequence (short hairpin RNA [shRNA]) for the human TRIM27 gene (#1 CCCAGTTCTCTGCAACAT; #2 CCCAGTTCTCTGCAACAT; #3 GGATTCTGGGCAGTGCTT), human CDKN1A (p21) gene (#1 CCGCGACTGTGATGCGCTA; #2 CGACTTTGTCACCGAGACA; #3 GACCTGTCAGTCTTGTA), or scramble control was cloned into pLKO.1. The complete human TRIM27 or p21 gene was inserted into a pLVX-Puro vector (Addgene, USA) for TRIM27 or p21 overexpression, respectively. Lentivirus vector transfection was accomplished by Lipofectamine 2000 based on the manufacturer's protocol (Invitrogen, USA); 48 h post-transfection, the collected plasmids were further transduced into non-TNBC cells.

pLKO.1-TRIM27 shRNA (shTRIM27) or pLKO.1-scramble shRNA (shNC) was transduced into MCF-7 and BT-474 cells. A lentivirus containing TRIM27 expression vector (TRIM27) or blank vector (Vector) was transduced into T47D cells.

### Cell Viability Assay

The Cell Counting Kit-8 (CCK-8; SAB, USA) was used for the estimation and measurement of cell viability according to the manufacturer's instructions. The 450 nm optical density in each well was determined using a multi-plate reader DNM-9602 (Perlong Medical, China).

### Analyses of Cell Cycle and Cell Apoptosis

For the cell cycle assay, cells were centrifuged for 5 min at  $1,000 \times g$ , fixed with 700  $\mu$ L absolute ethyl alcohol on ice, incubated with 0.1 mg RNase A (100  $\mu$ L) for 30 min in the dark, and stained with 20  $\mu$ g propidium iodide (PI; Biovision, USA) for 10 min. For the cell apoptosis assay, cells were stained with Annexin V- fluorescein isothiocyanate (FITC) and PI. The following analyses were performed using a FACS-can flow cytometer and Cell Quest software (Becton Dickinson, USA).

### SA- $\beta$ -Gal Staining

Cells were washed three times with phosphate buffered saline, followed by fresh SA- $\beta$ -gal staining in accordance with the manufacturer's protocol (Beyotime Biotechnology, China). A microscope was used for visualization of the stained cells.

### RNA Isolation and Quantitative Real-Time PCR

Total RNA was extracted by Trizol reagent (Invitrogen, USA), and first-strand cDNA was reversely transcribed using the Reverse Transcription System Kit (Takara, Dalian, China). Quantitative real-time PCR was performed with a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA) using SYBR Green Quantitative Real-Time PCR Master Mix (Thermo Scientific, USA). The primers used were as follows: *TRIM27* forward: 5'-GGACCTGCCTGACAACCCC-3', *TRIM27* reverse: 5'-TCCAAGAAAATCCCCACCC-3'; *p21* forward: 5'-GCCCCGTGAGCGATGGAAC-3', *p21* reverse: 5'-CCCCGTGG GAAGGTAGAGC-3'; and *GAPDH* forward: 5'-AATCCCATCAC CATCTTC-3', *GAPDH* reverse: 5'-AGGCTGTTGTCATACTTC-3'. *GAPDH* was utilized as the housekeeping gene, and each assay was performed in triplicate.

### Western Blotting (WB) Analysis

The harvested cells were lysed and centrifuged for supernatant collection. Then, 25  $\mu$ g of protein was loaded into each well of a 10% or 15% SDS-PAGE gel. The separated proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane, followed by 1 h room temperature blocking with 5% non-fat milk. The membrane was then incubated with primary antibody (*TRIM27*: Abcam, USA, ab154931; *p16*: Abcam, ab51243; cleaved caspase-3: Abcam, ab2302;  $\beta$ -gal: Santa Cruz Biotechnology, sc-377257; *p53*: Santa Cruz Biotechnology, sc-393031; *p21*: Santa Cruz Biotechnology, sc-6246; H3K27ac: Abcam, ab4729; *GAPDH*: Cell Signaling Technology, #5174) overnight at 4°C. The membrane was incubated with horseradish peroxidase (HRP)-labeled secondary antibody (Beyotime Biotechnology: A0208 or A0216) for 1 h at 37°C. The proteins were visualized by exposing the membrane to a chemiluminescence detection kit (ECL, Millipore, USA) for image scanning. *GAPDH* was utilized as the loading control.

### IP and LC/MS

A pCMV-Tag2-TIRM27 or empty pCMV-Tag2 vector was transfected into 293T cells, and cells were harvested in radioimmunoprecipitation assay (RIPA) buffer after 48 h. The lysate was pre-cleared for 2 h with protein A/G beads (Santa Cruz Biotechnology, sc-2003) at 4°C and immunoprecipitated with anti-FLAG beads (Sigma-Aldrich; M8823) overnight at 4°C. The bound proteins were eluted with FLAG peptide (Sigma-Aldrich). The proteins were resolved in SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue. The differentially presented bands were excised and digested with trypsin, and the proteins were identified by LC/MS.

### CoIP and Ubiquitination Assays

Cell lysates were obtained through RIPA lysis buffer, incubated with anti-TRIM27 (Proteintech, 12205-1-AP), anti-p21 (Abcam, ab109520), or normal IgG (Santa Cruz Biotechnology, sc-2027) antibody, followed by incubation with protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology, sc-2003) for 2 h at 4°C. The immunocomplexes were then reacted with anti-p21 (Santa Cruz Biotechnology, sc-6246), anti-TRIM27 (Abcam, ab154931), or anti-ubiquitin (Abcam, ab7780) antibodies.

For the *in vitro* ubiquitination assay, TRIM27 and p21 were individually and separately expressed and purified from HEK293T cells, as previously described.<sup>29</sup> The ubiquitination reaction was performed with E1, E2 (UBCH5A), Ub, and ubiquitin reaction buffer at 30°C for 1 h, stopped with  $2 \times$  SDS loading buffer, and heated at 95°C for 10 min. The immunocomplexes were immediately separated by SDS-PAGE and blotted with the indicated antibodies.

### Luciferase Assay

Cells were transfected with a pGL3-basic plasmid containing the 5'-promoter region of *TRIM27* in the presence of 20  $\mu$ M C646, an inhibitor of the histone acetyltransferase p300, or the control (vehicle), along with 100 ng Renilla luciferase (phRL) by Lipofectamine 2000 (Invitrogen) at 37°C for 6 h according to the manufacturer's protocol. Then, at 24 h post-transfection, the cells were collected for luciferase activity detection on the Dual-Luciferase reporter system (Promega, USA). A Monolight 3010 luminometer (BD Biosciences) was used to measure luciferase activity. The efficiency of transfection was normalized by the luciferase activity of Renilla.

### Xenograft Study

Six-week-old male nude mice were randomly grouped ( $n = 6$  per group) and subcutaneously injected with MCF-7 cells stably transduced with shTRIM27 or shNC at the right armpit. On the 33<sup>rd</sup> day post-inoculation, the developed tumors were collected for photographing, weighing, Ki67 immunofluorescence staining using anti-Ki67 antibody (Abcam, ab15580), and WB analysis. Meanwhile, the mice in other groups were subcutaneously injected with T47D cells stably transduced with TRIM27 or vector at the right armpit. Mice were injection with TAM (10 mg/kg) or vehicle every day. The mice were sacrificed 33 days after TAM injection, and their tumors were collected, photographed, weighed, and stained by terminal

deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). A total of 80 mice were collected for survival analysis (n = 20 per group). All animal experiments were approved by the Ethics Committee of Shanghai Eighth People Hospital.

### Statistical Analyses

Data based on at least three independent experiments performed in triplicate are presented as mean  $\pm$  standard deviation (SD). All statistical analysis was performed using GraphPad Prism 8.0.2 (GraphPad Software, USA). The nonparametric Mann-Whitney test or analysis of variance was performed to compare different experimental groups. A p value <0.05 was considered to indicate statistical significance. The overall survival of patients was calculated by Kaplan-Meier analysis and Cox's proportional hazards regression model. The differences among groups were analyzed by log-rank test.

### AUTHOR CONTRIBUTIONS

L.X., X.T., and Y.Y. designed the study. X.T., K.W., and X.H. performed the experiments. K.W., X.H., and J.H. collected the data. X.H., Y.Y., and J.H. analyzed and interpreted the data. L.X., Y.Y., and J.H. prepared the manuscript. All authors read and approved the final manuscript.

### CONFLICTS OF INTEREST

The authors declare no competing interests.

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