

**ORIGINAL ARTICLE**

# Tumor necrosis factor receptor-associated factor 6 contributes to malignant behavior of human cancers through promoting AKT ubiquitination and phosphorylation

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Tumor necrosis factor receptor-associated factor 6 (TRAF6) has been found to be involved in carcinogenesis in multiple cancers. However, the precise role of TRAF6 in cancer has not been extensively investigated and remains largely unknown. In this study, we aimed to investigate the biological function of TRAF6 and its underlying molecular mechanisms in cancer. A positive correlation between poor tumor differentiation and TRAF6 expression status was observed in both oral cancer and breast cancer. Overexpression of TRAF6 promoted proliferation, migration, and G<sub>0</sub>/G<sub>1</sub> to S phase transition in tumor cells. Tumor necrosis factor receptor-associated factor 6-mediated AKT ubiquitination and subsequent phosphorylation played an essential role in the control of tumor cell malignant behavior. In vivo treatment with TRAF6, but not the E3 ligase deficient TRAF6 mutant, facilitated tumor growth. Our findings indicate that TRAF6 contributes to malignant behavior of human cancers through promoting AKT ubiquitination and phosphorylation. Therefore, TRAF6 could serve as a therapeutic target in cancers.

**KEYWORDS**

cell cycle, migration, phosphorylation, proliferation, ubiquitination

## 1 | INTRODUCTION

The tumor necrosis factor receptor-associated factors (TRAFs) comprise signal transducers of Toll-like receptor interleukin-1 family members, which trigger signaling transduction in innate immune responses. Tumor necrosis factor receptor-associated factor 6, a member of the TRAF family, has an E3 ubiquitin ligase activity that mediates lysine-63 (K63)-dependent ubiquitination. In recent years, TRAF6 has been suggested as an oncogene in various human cancer types. It was upregulated in several solid malignancies, such as pancreatic cancer,<sup>1</sup> colon cancer,<sup>2</sup> and gastric cancer,<sup>3</sup> and the overexpression of TRAF6 predicts a poor prognosis in patients with

glioma and gastric cancer.<sup>3,4</sup> Some evidence has shown that TRAF6 autoubiquitination-induced nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation might promote tumor growth. However, we previously reported that TRAF6 does not affect NF- $\kappa$ B signaling in cancer cells under normal growth conditions.<sup>5</sup> Therefore, the mechanism by which TRAF6 contributes to cancer development and progression is not clear.

In the present study, we found that the high expression of TRAF6 is associated with malignant behavior of human cancers, such as increased cell proliferation and migration and poor differentiation in both oral cancer and breast cancer. Tumor necrosis factor receptor-associated factor 6 induces AKT ubiquitination and activation, which, in turn, contributes to the proliferation and migration of cancer cells.

\*Shi and Liu contributed equally to this work.

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Taken together, these data suggest a crucial role of TRAF6-mediated AKT ubiquitination and phosphorylation in cancer progression.

## 2 | MATERIALS AND METHODS

### 2.1 | Patient specimens and immunohistochemical staining

Primary oral cancer tissues were collected from 53 patients who underwent surgical resection in the Department of Oral and Maxillofacial Surgery (Ninth People's Hospital, Shanghai, China). Among 53 cases, 40 patients had well and moderate differentiation by histology, whereas 13 patients had poor differentiation. Primary breast cancer tissue samples were collected from 54 patients at the Fudan University Shanghai Cancer Center (Shanghai, China). Of these 54 patients, 43 had well and moderate differentiation, whereas 11 patients had poor differentiation. Slides of the tissue sections (4  $\mu$ m) were incubated with primary Abs, washed, and reincubated with appropriate secondary Abs, adopting the method described previously.<sup>6</sup> The staining results were measured semiquantitatively using a computerized image analysis system as previously described.<sup>7</sup>

### 2.2 | Antibodies and reagents

The primary Abs used included: anti-TRAF6 Ab (Abcam), anti-TRAF2 Ab (Abcam, Cambridge, MA, USA), anti-AKT Ab (Cell Signaling Technology, Danvers, MA, USA), anti-phospho-AKT Ab (Thr308; Cell Signaling Technology), anti-I $\kappa$ B kinase (IKK) Ab (Cell Signaling Technology), anti-phospho-IKK $\alpha$ / $\beta$  Ab (Ser176/180; Cell Signaling Technology), anti-NF- $\kappa$ B p65 Ab (Cell Signaling Technology), anti-phospho-NF- $\kappa$ B p65 Ab (Ser536; Cell Signaling Technology), anti-I $\kappa$ B $\alpha$  Ab (Cell Signaling Technology), anti-phospho-I $\kappa$ B $\alpha$  Ab (Ser32; Cell Signaling Technology), anti-JNK Ab (Cell Signaling Technology), anti-phospho-JNK Ab (Thr183/Tyr185; Cell signaling), anti-p38 Ab (Cell Signaling Technology), anti-phospho-p38 Ab (Thr180/Tyr182; Cell Signaling Technology), anti-ERK Ab (Cell Signaling Technology), anti-phospho-ERK Ab (Thr202/Tyr204; Cell signaling), anti-ubiquitin K63 Ab (Cell signaling), and anti-GAPDH Ab (Cell signaling). The AKT inhibitor MK-2206, the IKK inhibitor IKK-16, and the proteasome inhibitor MG132 were purchased from Selleckchem (Houston, TX, USA).

### 2.3 | Cell culture

The human tongue squamous carcinoma cell lines SCC9 and HN12 were obtained from Professor Li Mao, University of Maryland Dental School (Baltimore, MD, USA), as described previously.<sup>8</sup> The human

breast cancer cell lines MCF-7 and MDA-MB-231 were purchased from ATCC (Manassas, VA, USA). The means of short tandem repeat profiling was used to test authenticity. The cells were cultured in high glucose DMEM or DMEM/Ham's F-12 (both from Gibco, Waltham, MA, USA) with 10% FBS (Gibco), penicillin (100 U/mL), and streptomycin (100  $\mu$ g/mL) and were maintained at 37°C in 5% CO<sub>2</sub>.

### 2.4 | Real-time quantitative PCR analysis

Total RNA from 4 cell lines was extracted using TRIzol reagent (Sigma, St Louis, MO, USA). cDNA was synthesized from 2  $\mu$ g total RNA using random hexamers. The mRNA expression of TRAF6 and TRAF2 was quantified by one-step SYBR Green-based real-time quantitative PCR. Expression of GAPDH mRNA served as the internal control for normalization. The primer sequences are provided as follows: TRAF6 forward 5'-ATGCGGCCATAGGTTCTGC-3', reverse 5'-TCCTCAAGATGTCTCAGTTCCAT-3'; TRAF2 forward 5'-TCCTGGAGTTGCTACAGC-3', reverse 5'-AGGCGGAGCACAGGTACTT-3'; and GAPDH forward 5'-GGACCTGACCTGCCGTCTAG-3', reverse 5'-GTAGCCCAGGATGCCCTTGA-3'.

### 2.5 | Immunoblotting analysis

For immunoblotting experiments, total protein (30  $\mu$ g) was separated on 10% SDS-PAGE gels and transferred to PVDF membranes (Merck Millipore, Bedford, MA, USA). The levels of protein expression were evaluated by western blot as previously described in detail.<sup>9</sup>

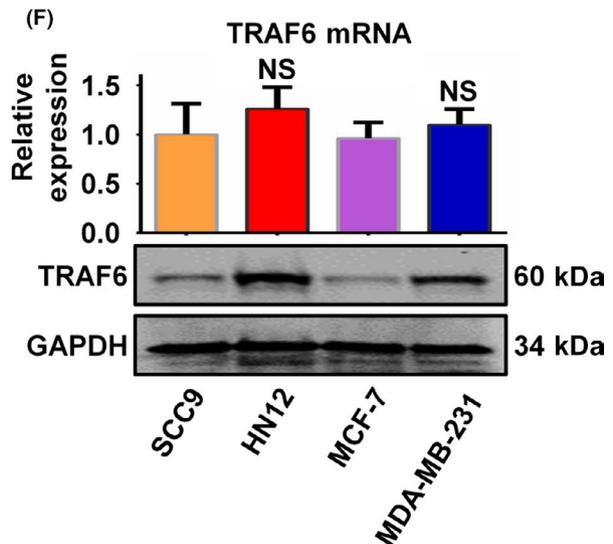
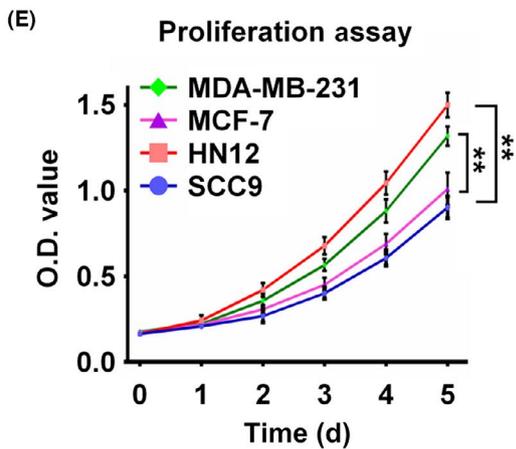
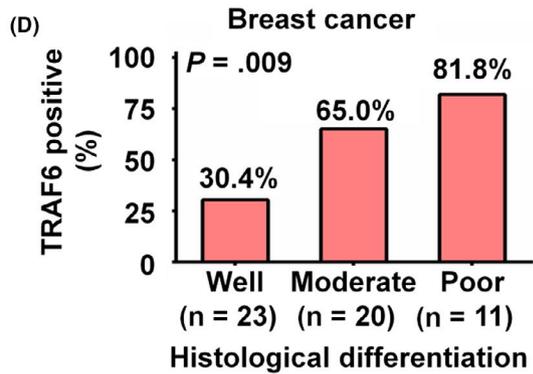
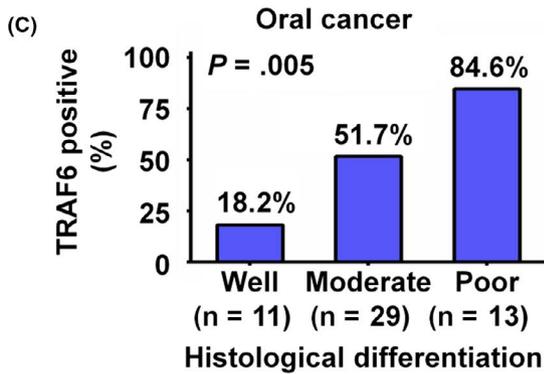
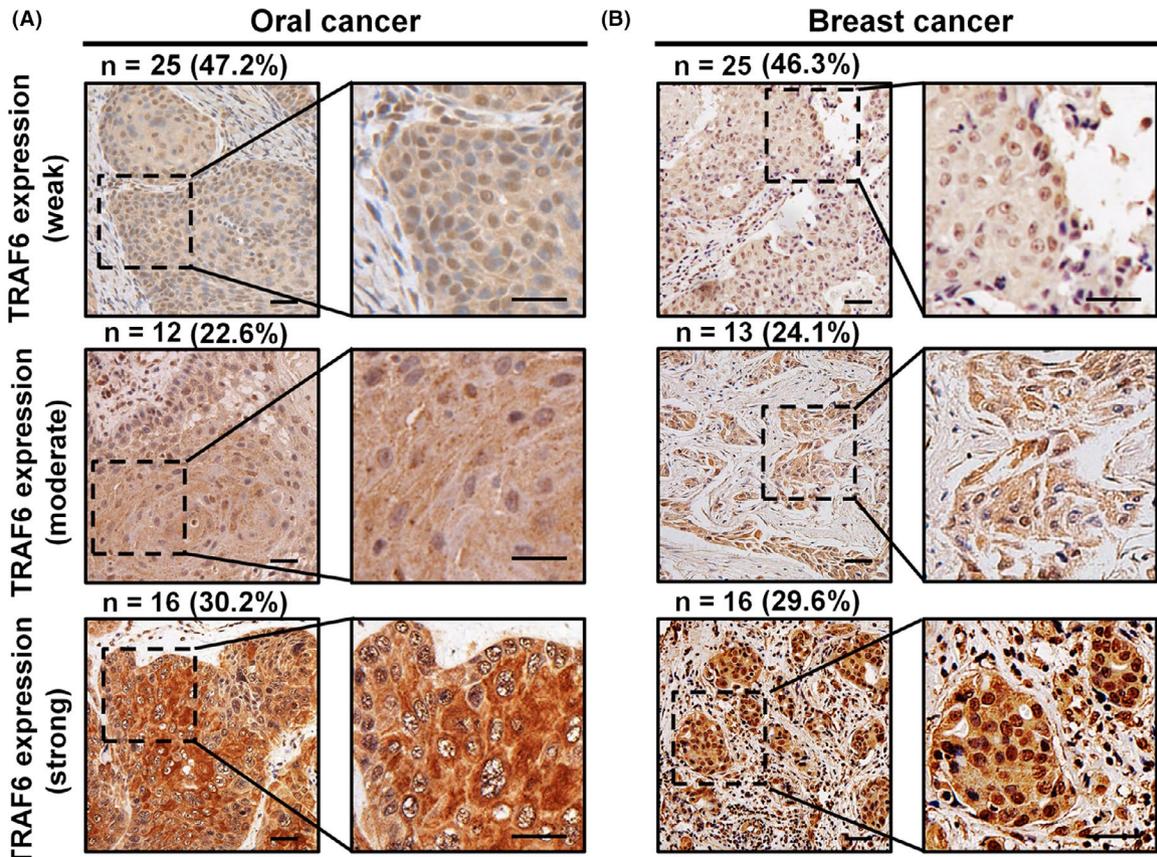
### 2.6 | Methylation analysis

Bisulfite sequencing was used to analyze the methylation of TRAF6 promoter region. The EZ DNA Methylation Kit (Zymo, Orange, CA, USA) was used to undertake bisulfite conversion, and the NEB PCR cloning kit (New England Biolabs, Ipswich, MA, USA), was used to clone PCR products and sequence individual clones.<sup>10</sup> The primer sequences of bisulfite sequencing were: forward, 5'-TGTATATAAAAGYGTAGTTGGGATT-3'; and reverse, 5'-AATTCRAAAAAATACCCCTAAC-3'.

### 2.7 | Plasmid transfection

The expression plasmids for WT TRAF6 and TRAF2 were purchased from Genechem (Zhangjiang, Shanghai, China), and an E3 ligase-deficient TRAF6 C70A mutant plasmid was produced as previously described.<sup>5</sup> Every clone used in this study was verified by sequencing. Lipofectamine 3000 reagent (Life Technologies, Carlsbad, CA, USA) was used to perform transfection of indicated cells. Stable clones

**FIGURE 1** Expression of tumor necrosis factor receptor-associated factor 6 (TRAF6) in oral cancer and breast cancer. A,B, Representative immunostaining of TRAF6 in primary oral tumors (n = 53) and breast tumors (n = 54) with different histological classifications. Scale line = 100  $\mu$ m. C,D, Positive correlation between poor tumor differentiation and TRAF6 expression status was observed in both oral cancer and breast cancer. ( $P = 0.005$  and  $P = 0.009$ , respectively;  $\chi^2$  test). E, Proliferative capacity of 4 cancer cell lines was measured by MTT assay. F, Endogenous mRNA and protein expression of TRAF6 in 4 cancer cell lines. \* $P < .05$ , \*\* $P < .01$ , Student's *t* test. O.D., optical density



Characteristic	No. of patients	Percentage	TRAF6 IHC positive (%)	P value <sup>a</sup>
<b>Oral cancer</b>				
Age (median, 64 y; range, 27-91 y)				
≤Median	27	50.9	51.9	0.884
>Median	26	49.1	53.9	
Gender				
Male	34	64.2	55.9	0.552
Female	19	35.8	47.4	
Metastasis				
Positive	28	52.8	64.3	0.077
Negative	25	47.2	40.0	
Pathologic tumor classification				
pG1	11	18.9	18.2	0.005
pG2	29	56.6	51.7	
pG3	13	24.5	84.6	
<b>Breast cancer</b>				
Age (median, 54 y; range, 33-77 y)				
≤Median	28	51.9	50.0	0.571
>Median	26	48.1	57.7	
Metastasis				
Positive	31	57.4	64.5	0.064
Negative	23	42.6	39.1	
Pathologic tumor classification				
pG1	23	44.4	30.4	0.009
pG2	20	37.1	65.0	
pG3	11	18.5	81.8	

<sup>a</sup> $\chi^2$  test.

IHC, immunohistochemical.

were selected using a culture medium with G418 sulfate (Sigma) at a density of 600  $\mu\text{g}/\text{mL}$ .

## 2.8 | RNA interference mediated gene silencing

The preparation of shRNA was undertaken using the pSuper.puro siRNA expression plasmid (Oligoengine, Seattle, WA, USA), as described previously.<sup>11</sup> The RNAi target sequence of TRAF6 was 5'-CCACGAAGAGAUAAUGGAU-3'. The shRNA constructs were then transfected with Lipofectamine into indicated cells. The transfected cells were selected with 1  $\mu\text{g}/\text{mL}$  puromycin for 2 weeks.

## 2.9 | Cell proliferation assay

All the cells were seeded in 96-well plates at a density of  $1 \times 10^3$ /well and counted every day for the next 5 days. At the same time each day, the cells were incubated with 10  $\mu\text{L}$  sterile MTT dye (5 mg/mL) at 37°C for 4 hours. After aspiration of the medium, the cells were lysed with DMSO. The absorbance at 490 nm in each well was recorded using a microplate reader.<sup>12</sup>

**TABLE 1** Tumor necrosis factor receptor-associated factor 6 (TRAF6) expression in specimens of oral cancer and breast cancer according to patient and tumor characteristics

## 2.10 | Colony formation assay

All the cells were seeded in 6-well plates at a density of 500 cells/well. After 14 days of incubation, the colonies were washed with PBS, fixed with 4% paraformaldehyde for 30 minutes, stained with 0.1% crystal violet, and counted.

## 2.11 | Cell migration assay

This assay used 24-well plates with 8- $\mu\text{m}$ -pore chambers (Millipore, Bedford, MA, USA). All the cells ( $3 \times 10^4$ ) were resuspended and then seeded in the upper chamber with 300  $\mu\text{L}$  serum-free medium, while the lower chamber contained 600  $\mu\text{L}$  medium with 10% FBS. After 24 hours of incubation at 37°C, the cells were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, and counted under a microscope at 100 $\times$  magnification.<sup>13</sup>

## 2.12 | Flow cytometry analysis of the cell cycle

The cells were cultured in serum-free medium for 24 hours and then cultured in medium with 10% FBS for 24 hours. The indicated cancer

cells were collected and fixed with precooling 75% ethanol at 4°C. The following day, the 75% ethanol was removed, and the cells were incubated in propidium iodide solution (100 µg/mL) at room temperature for 20 minutes in the dark. The cell cycle experiment was analyzed by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

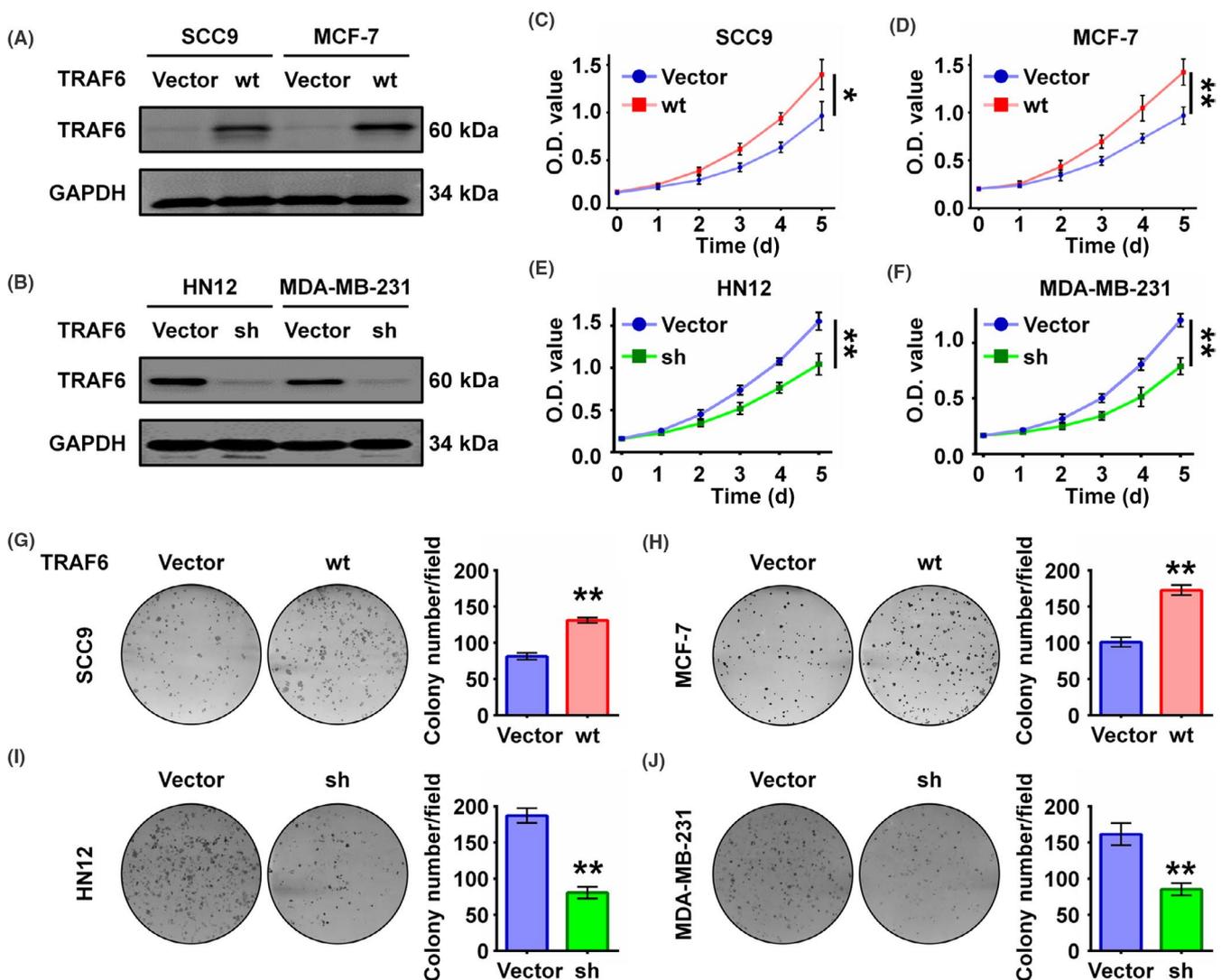
### 2.13 | Ubiquitination assay

To avoid degradation by proteasomes in the ubiquitination detection experiments, all the cells were treated with a proteasome inhibitor (5 µmol/L) as described previously.<sup>14</sup> All the cells from the indicated treatments were lysed in immunoprecipitated (IP) lysis buffer containing 1% SDS, phosphatase inhibitors, and a cocktail of proteases. The cell lysates were centrifuged at 10 000 g for 10 minutes to remove debris and diluted with IP lysis buffer. This experiment included a two-step IP protocol with overnight

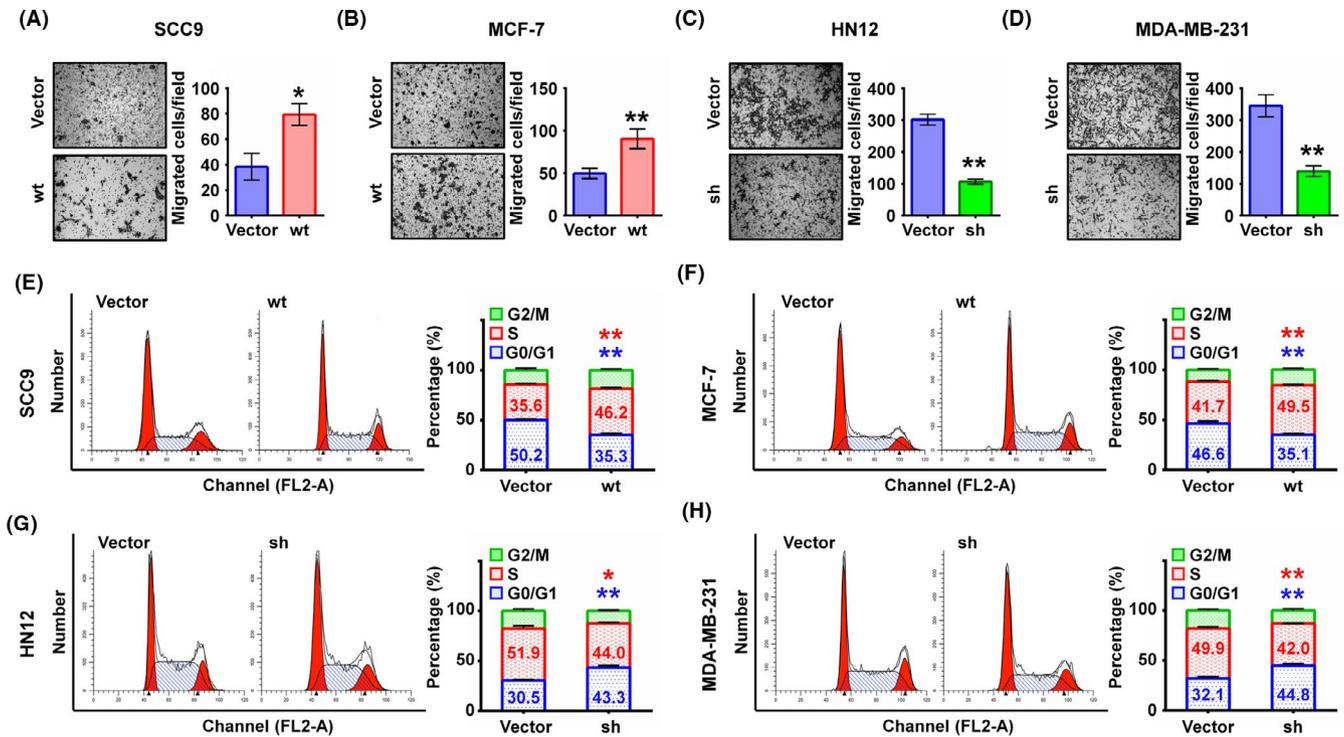
primary Ab incubation and subsequent 6 hours of incubation with protein A/G Sepharose beads at 4°C. The ubiquitin modification of precipitated proteins was examined by immunoblotting as described previously.<sup>15</sup>

### 2.14 | In vivo xenograft study

The xenograft study was approved by the Animal Ethics Committee of Shanghai Ninth People's Hospital. Female nude mice (4 weeks old) from Shanghai Laboratory Animal Center (Pudong, Shanghai, China) were used in this study. SCC9 and MCF-7 cells transfected with WT TRAF6 (wt) or corresponding E3 ligase-deficient TRAF6 C70A mutant (mut), and HN12 and MDA-MB-231 cells transfected with TRAF6-silencing shRNA (sh) were injected s.c. into the flank region ( $5 \times 10^6$  cells/mouse). After injection, tumor volume was calculated every 7 days using an electronic caliper with the following formula:



**FIGURE 2** Tumor necrosis factor receptor-associated factor 6 (TRAF6) overexpression promotes cancer cell proliferation. A,B, SCC9 and MCF-7 cells were transfected with TRAF6 wt or vector control. HN12 and MDA-MB-231 cells were transfected with TRAF6 shRNA (sh) or vector control. Immunoblotting shows the expression levels of TRAF6 in indicated cells. C-F, Proliferation assays following indicated treatments in SCC9, MCF-7, HN12, and MDA-MB-231 cells. G-J, Colony formation assay in 4 tumor cell lines that have received indicated treatments. Quantitative analysis of the colony formation assay was displayed. \* $P < .05$ , \*\* $P < .01$ , Student's *t* test. O.D., optical density



**FIGURE 3** Tumor necrosis factor receptor-associated factor 6 (TRAF6) overexpression promotes cancer cell migration and  $G_0/G_1$  to S phase transition. A,B, Migration assay and quantitative analysis of SCC9 and MCF-7 cells transfected with TRAF6 wt or vector control. C,D, Migration assay and quantitative analysis of HN12 and MDA-MB-231 cells transfected with TRAF6 shRNA (sh) or vector control. E-H, Cells following indicated treatments as described in A-D were subjected to flow cytometry analysis. Data represent mean  $\pm$  SD,  $n = 3$ . \* $P < .05$ , \*\* $P < .01$ , Student  $t$  test

tumor volume ( $\text{mm}^3$ ) =  $a \times b^2 \times 0.52$ .<sup>16</sup> After 4 weeks, all the mice were killed with 5% carbon monoxide, and the tumors were excised and then fixed with 10% formalin for 24 hours for further analysis.

## 2.15 | Statistical analysis

All statistical calculations were carried out using SPSS 19.0 statistical software (SPSS, Abbott Laboratories, Chicago, IL, USA). Unpaired  $t$  tests were used for continuous normally distributed data expressed as the means  $\pm$  SD between 2 unpaired groups, and a 1-way ANOVA test was adopted for multiple comparisons in multiple groups. The  $\chi^2$  test was used to compare TRAF6 immunohistochemical positivity among different groups. Spearman's correlation analysis was used to explore the relationships between expression levels of TRAF6 and phosphorylated AKT expression in cancer samples. A  $P$  value  $< .05$  was defined as significant.

## 3 | RESULTS

### 3.1 | Overexpression of TRAF6 is correlated with poor tumor differentiation and poor prognosis

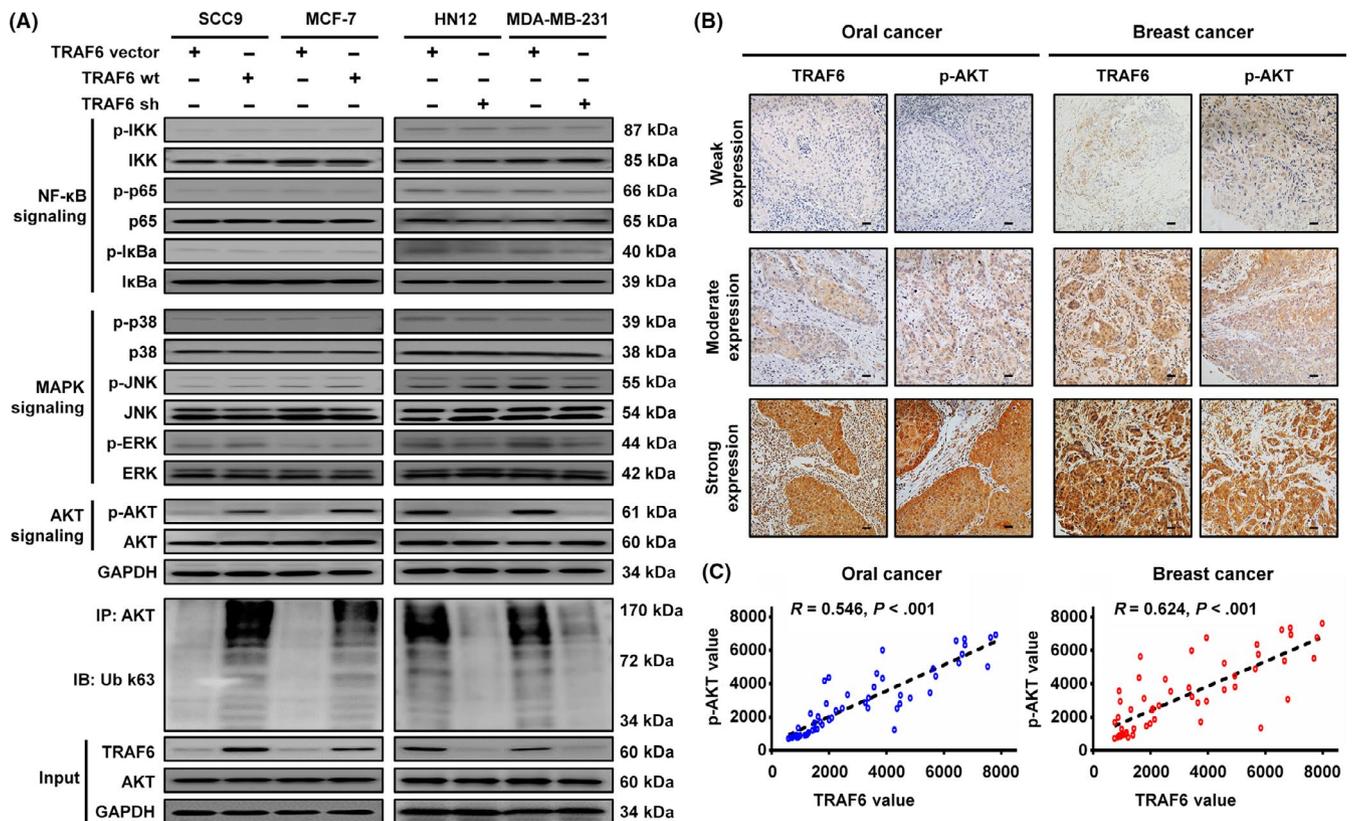
A total of 53 patients with oral cancer showed expression of TRAF6, with 16 (30.2%) samples showing a strong expression, 12 (22.6%) with moderate expression, and 25 (47.2%) with weak expression (Figure 1A). Similarly, 16 patients of breast cancer (29.6%) had

strong expression of TRAF6, 13 (24.1%) samples had moderate expression, and 25 (46.3%) samples were TRAF6-negative (Figure 1B). Clinicopathologic features according to TRAF6 status are summarized in Table 1. A positive correlation between poor tumor differentiation and TRAF6 expression status was observed (Figure 1C,D; oral cancer,  $P = 0.005$ ; breast cancer,  $P = 0.009$ ). Both oral cancer and breast cancer samples that were poorly differentiated (pG3) showed significantly stronger TRAF6 expression than those moderately (pG2) or well differentiated (pG1). There was no significant correlation between TRAF6 expression status and other patient characteristics.

We also present a survival analysis of TRAF6 using datasets from The Cancer Genome Atlas. It was found that high expression of TRAF6 is associated with poor recurrence-free survival in patients with head and neck cancer and poor overall survival in patients with breast cancer (Figure S1A,B). By contrast, other TRAFs (eg, TRAF2) show no association with prognosis (Figure S1C,D).

### 3.2 | Overexpression of TRAF6 promotes cancer cell proliferation, migration, and $G_0/G_1$ to S phase transition

To investigate the potential effect of TRAF6 on the pathogenesis of cancers, we measured the levels of TRAF6 in two oral cancer cell lines (SCC9 and HN12) and two breast cancer cell lines (MCF-7 and MDA-MB-231). The HN12 and MDA-MB-231 cells grew faster and



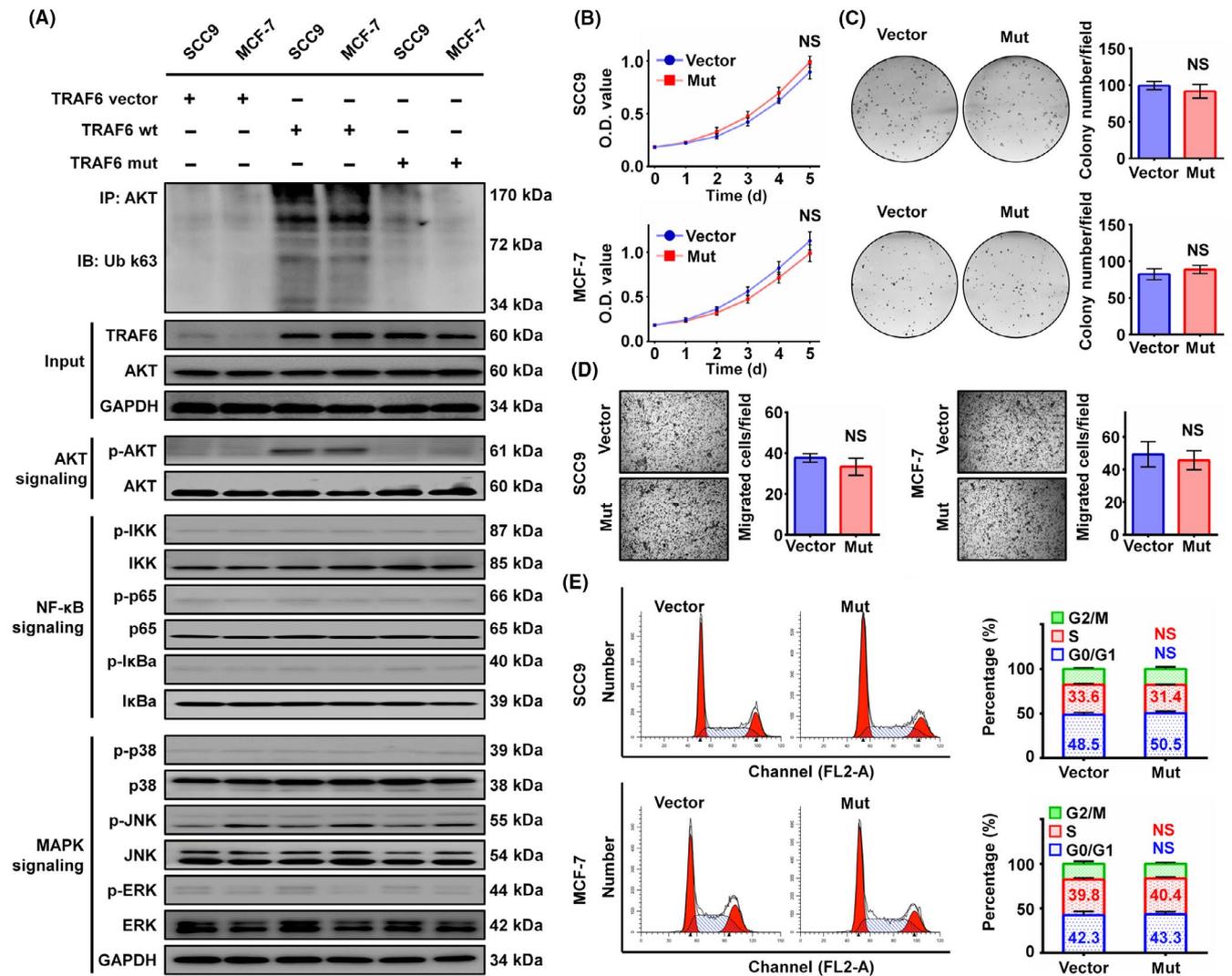
**FIGURE 4** Tumor necrosis factor receptor-associated factor 6 (TRAF6)-induced AKT ubiquitination and phosphorylation. A, SCC9 and MCF-7 cells transfected with TRAF6 wt increased phosphorylation (p-AKT) and ubiquitination of AKT (left). HN12 and MDA-MB-231 cells transfected with TRAF6 shRNA (sh) decreased phosphorylation and ubiquitination of AKT (right). TRAF6 expression did not affect the phosphorylation/activation of IκB kinase (p-IKK), IκBα (p-IκBα), p65 (p-p65), p38 (p-p38), JNK (p-JNK) and ERK (p-ERK). B, Representative immunostaining of phosphorylated AKT in primary oral tumors ( $n = 53$ ) and breast tumors ( $n = 54$ ) with different expression of TRAF6. Scale bars, 100  $\mu\text{m}$ . C, The correlation of TRAF6 expression and phosphorylated AKT expression in oral cancer (left) and breast cancer (right)

penetrated the filter more strongly than did SCC9 and MCF-7 cells (Figure 1E), indicating that the HN12 and MDA-MB-231 cells had a more malignant biological behavior than SCC9 and MCF-7 cells. The immunoblot analysis showed higher levels of TRAF6 protein expression in highly malignant (HN12 and MDA-MB-231) cells compared with less malignant (SCC9 and MCF-7) cells (Figure 1F). A similar TRAF6 mRNA level was observed in these 4 cell lines (Figure 1F). In addition, no obvious DNA methylation was found in the promoter sequences of TRAF6 (Figure S2). These results indicate that high expression of TRAF6 in tumor cells is not controlled at the transcriptional level. To test whether elevated TRAF6 expression might facilitate malignant cell behavior in cancer cells, we transfected SCC9 and MCF-7 cells with a TRAF6 expression plasmid and downregulated TRAF6 levels in HN12 and MDA-MB-231 cells by shRNA transfection (Figure 2A,B). The results indicated that the overexpression of TRAF6 enhanced both the proliferation and colony formation ability of SCC9 and MCF-7 cells, whereas the silencing of TRAF6 inhibited the growth and colony formation of HN12 and MDA-MB-231 cells (Figure 2C-J). In a migration assay, the overexpression of TRAF6 led to an increased number of migrated cells, whereas silencing TRAF6 suppressed the number of migrated cells (Figure 3A-D). The effect of TRAF6 on cell cycle progression was also determined by

flow cytometry. Ectopic expression of TRAF6 caused decreased  $G_0/G_1$ -phase cells and increased S-phase cells, indicating a marked  $G_0/G_1$  to S phase transition ( $P < 0.01$ ) (Figure 3E,F). In contrast, silencing TRAF6 in HN12 and MDA-MB-231 cells significantly suppressed the transition of cells from  $G_0/G_1$  to S phase ( $P < 0.01$ ) (Figure 3G,H). To exclude the possible effects of other TRAFs on tumor cells, we examined the effects of TRAF2 on cell growth, colony formation, and the cell cycle. Unlike TRAF6, TRAF2 did not influence the biological behavior of tumor cells (Figure S3A-E). These results indicate that TRAF6 could promote cancer cell proliferation, migration, and  $G_0/G_1$  to S phase transition of the cell cycle.

### 3.3 | Overexpression of TRAF6 induces AKT ubiquitination and activation

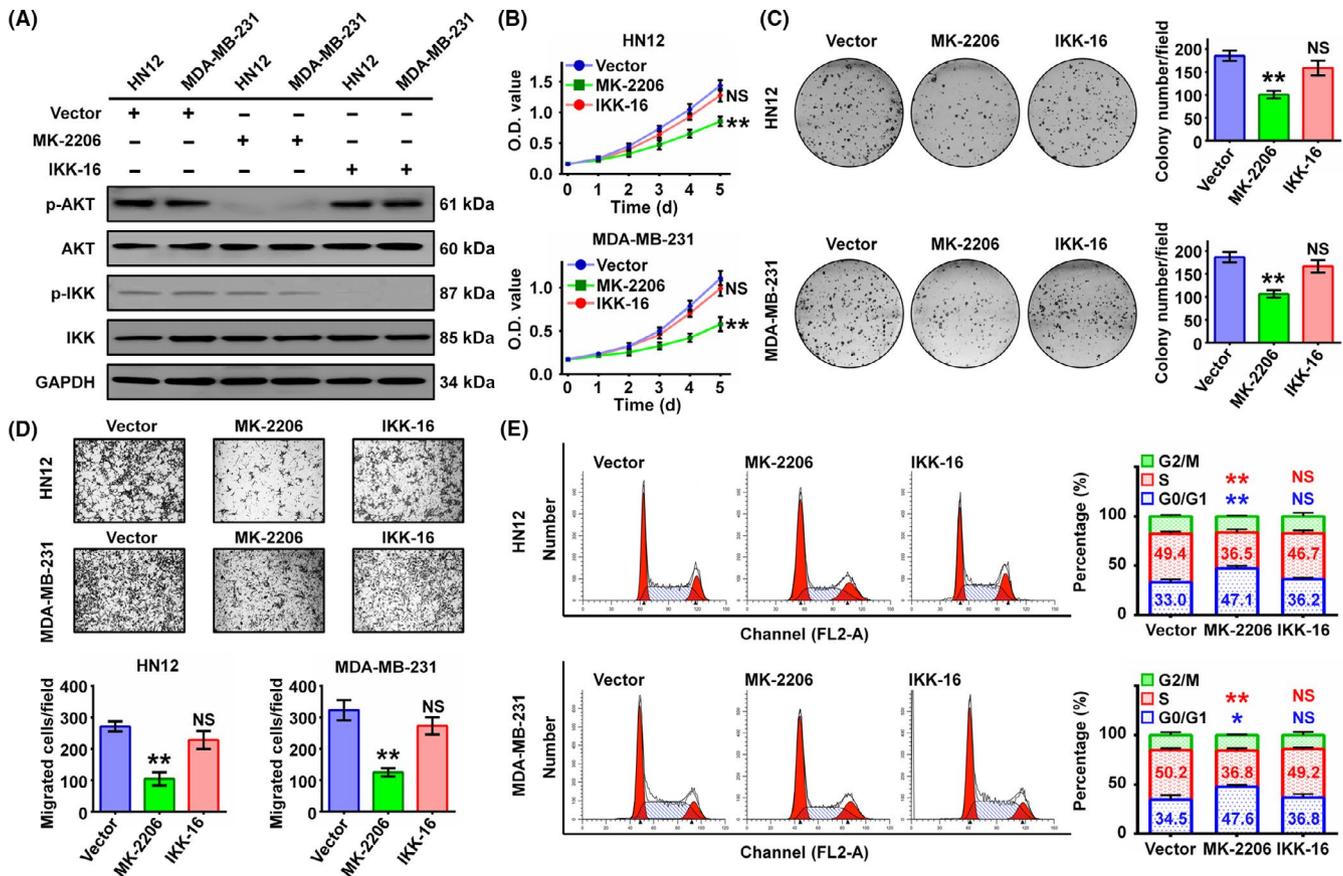
Given that TRAF6 could function as an E3 ubiquitin ligase as well as play crucial roles for post-translational activation of AKT in oncogenesis,<sup>17,18</sup> we sought to determine whether TRAF6 regulates tumor cell growth by affecting AKT activation and/or ubiquitination. The activation and ubiquitination of AKT was examined in both cells with high TRAF6 expression (HN12 and MDA-MB-231) and those with low TRAF6 expression (SCC9 and MCF-7). As



**FIGURE 5** E3 ligase activity-dependent tumor necrosis factor receptor-associated factor 6 (TRAF6)-mediated AKT ubiquitination/phosphorylation is crucial for its oncogenic function. A, E3 ligase-deficient TRAF6 mutant (mut) showed no effect on AKT ubiquitination/phosphorylation and has no influence on nuclear factor- $\kappa$ B (NF- $\kappa$ B) or MAPK signaling. B-E, TRAF6 mut was unable to promote cancer cell proliferation (B), colony formation (C), or migration (D) and showed no influence on the cell cycle (E). NS, not statistically significant

shown in Figure 4A, obvious activation and ubiquitination of AKT was observed in TRAF6 highly expressing cells compared with cells with low expression of TRAF6, suggesting that TRAF6 might activate AKT by promoting its ubiquitination. To test this notion, we evaluated the direct effect of TRAF6 on AKT ubiquitination. SCC9 and MCF-7 cells were transfected with TRAF6 wt or TRAF6 mut, and TRAF6 was silenced by shRNA transfection in HN12 and MDA-MB-231 cells. As shown in Figure 4A, the ectopic expression of TRAF6 wt significantly enhanced AKT ubiquitination in SCC9 and MCF-7 cells and TRAF6 silencing resulted in reduction of AKT ubiquitination in HN12 and MDA-MB-231 cells. These data indicated that TRAF6 can directly induce AKT ubiquitination in cancer cells. As previous studies reported that ubiquitination is a positive signal for AKT phosphorylation (activation),<sup>19</sup> we then assessed the effect of TRAF6 on AKT phosphorylation status. The results showed that AKT ubiquitination induced by TRAF6 is accompanied by obvious phosphorylation and that silencing TRAF6

significantly inhibited AKT activation (Figure 4A). This result was supported by the correlation analysis of phosphorylated AKT and TRAF6 expression in tumor specimens. A positive correlation between TRAF6 expression and elevated phosphorylated AKT level was observed in both oral cancer and breast cancer specimens (Figure 4B,C). Considering that TRAF6 might also affect other tumor-related signaling, we examined the influence of TRAF6 on NF- $\kappa$ B and MAPK signaling. As shown in Figure 4A, TRAF6 expression levels did not affect the activation of IKK, I $\kappa$ B $\alpha$ , p65, p38, JNK, or ERK. In addition, the effect of TRAF2 on these signaling pathways was also detected. No activation of these signaling pathways occurred in cells treated with TRAF2 (Figure S3F), indicating that TRAF6, but not TRAF2, affects the biological behavior of tumor cells through AKT signaling. Moreover, diminishing of the E3 ligase activity of TRAF6 significantly abrogated TRAF6-mediated AKT ubiquitination and activation (Figure 5A). Also, TRAF6 mut was unable to promote cancer cell proliferation, colony formation, or



**FIGURE 6** Inhibitor of AKT suppresses the malignant progression of cancer cells. A, Highly malignant HN12 and MDA-MB-231 cells were treated with AKT inhibitor (MK-2206, 5  $\mu$ M) or  $\text{I}\kappa$ B kinase (IKK) inhibitor (IKK-16, 5 mM) for 24 hours. B, Proliferation assays in HN12 and MDA-MB-231 cells that received indicated treatments. C, Colony formation assay following indicated treatments in 2 tumor cell lines and quantitative analysis. D, Migration assay and quantitative analysis of HN12 and MDA-MB-231 cells treated with MK-2206 or IKK-16. E, Cells following indicated treatments as described in (A) were subjected to flow cytometry analysis. Data represent mean  $\pm$  SD,  $n = 3$ . \*\* $P < .01$ , Student's  $t$  test. NS, not statistically significant

migration and showed no influence on the cell cycle (Figure 5B-E), supporting that E3 ligase activity of TRAF6 is critical during the malignant progression of tumors. To further illustrate that TRAF6 favors the malignant phenotype through activating AKT signaling, rather than other signaling pathways such as NF- $\kappa$ B, tumor cells were treated with either AKT inhibitor MK-2206 or NF- $\kappa$ B inhibitor IKK-16 to test whether the tumor malignant behavior can be reversed (Figure 6A). As expected, MK-2206, but not IKK-16, significantly suppressed the growth, colony formation, migration, and  $G_0/G_1$  to S phase transition in highly malignant HN12 and MDA-MB-231 cells (Figure 6B-E).

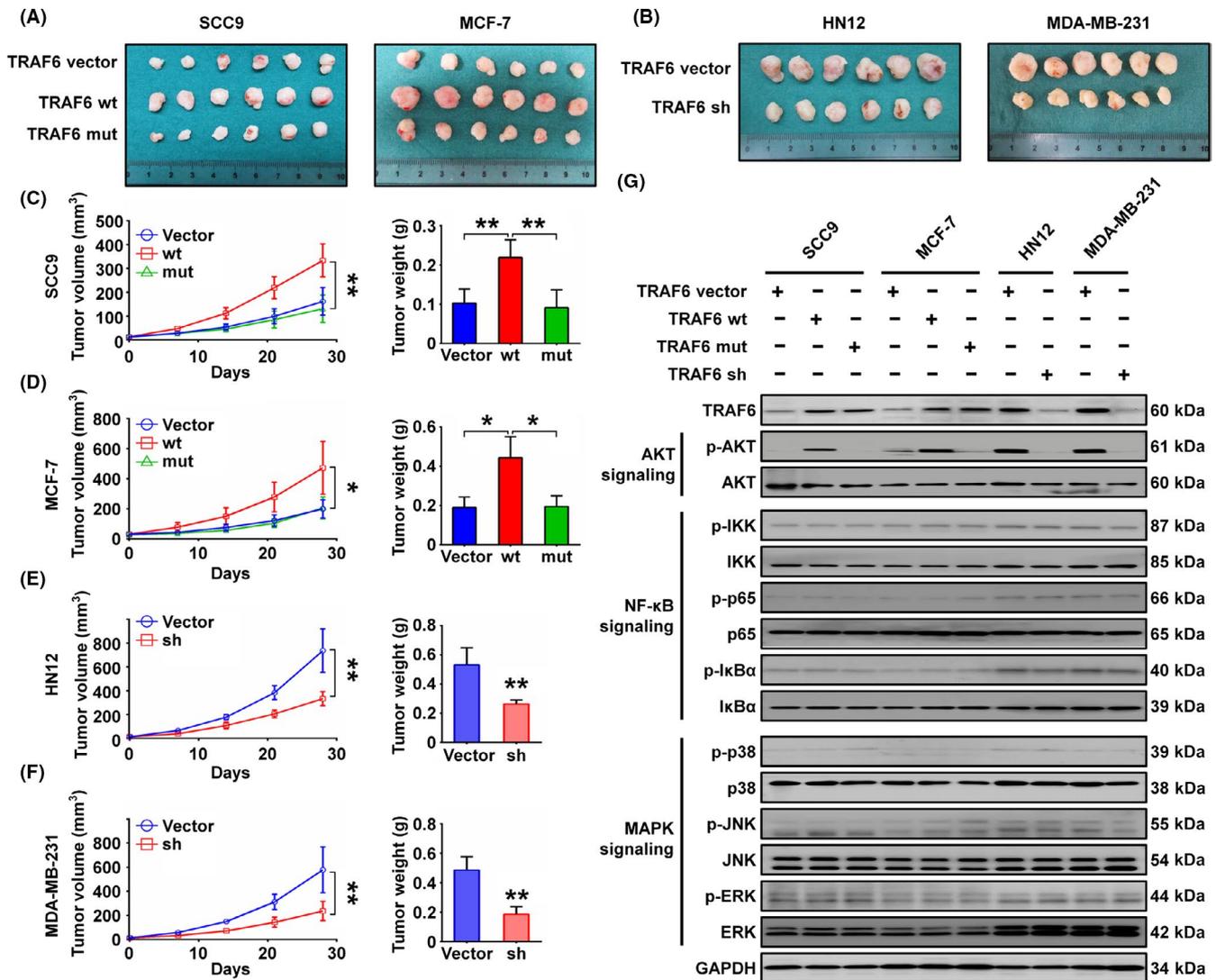
### 3.4 | Overexpression of TRAF6 contributes to cancer cell proliferation in vivo

We next investigated whether TRAF6-mediated AKT activation promotes tumor cell proliferation in vivo. It was found that cell lines with high expression of TRAF6 formed larger xenograft tumors in mice than cells expressing low levels of TRAF6. Ectopic expression of TRAF6 resulted in a 2.06-2.37 fold increase in tumor volume and a 2.13-2.34 fold increase in tumor weight in mice with SCC9 and MCF-7 cells. By

contrast, silencing TRAF6 significantly inhibited tumor growth in mice with HN12 and MDA-MB-231 cells. The TRAF6 mut showed no effect on tumor size (Figure 7A-F). Obvious AKT phosphorylation/activation, but not other signaling pathway activation, was observed in tumor samples from mice with TRAF6-overexpressing cells (Figure 7G), which is consistent with the results in vitro. These data provide in vivo evidence that TRAF6-mediated AKT activation contributes to cancer cell proliferation, and TRAF6 could serve as a therapeutic target.

## 4 | DISCUSSION

Recent studies indicate that TRAF6 is involved in cancer development.<sup>1,20,21</sup> It has also been reported that the amplification of the TRAF6 locus is a somatic and frequent event in several human cancer types.<sup>2,22</sup> Research findings showed that TRAF6 is important in the activation of the protein kinase complex IKK, which directly activates NF- $\kappa$ B and, in turn, inhibits apoptosis.<sup>23,24</sup> Therefore, TRAF6 is thought to function as a tumor activator by influencing apoptosis in cancer cells. However, our previous study showed that TRAF6 does not simply affect NF- $\kappa$ B signaling and apoptosis in cancer cells under



**FIGURE 7** Tumor necrosis factor receptor-associated factor 6 (TRAF6) contributes to tumor growth in vivo. A,B, Indicated treated cells were injected s.c. into immunocompromised mice ( $n = 6$ , each group). C-F, Growth curves of tumor volumes are shown for every group (left). Tumor weights were determined (right). G, Expression level of TRAF6 and the phosphorylation levels of AKT, IκB kinase (IKK), IκBα, p65, p38, JNK, and ERK were tested in tumor samples from each experimental group. Data represent mean  $\pm$  SD,  $n = 6$ , \* $P < .05$ , \*\* $P < .01$ , Student's  $t$  test. mut, mutant; sh, shRNA

normal growth conditions.<sup>5</sup> Thus, the precise role of TRAF6 in cancer has not been extensively investigated.

In this report, we showed that TRAF6 is upregulated in highly malignant tumor cells, and its expression is correlated with poor tumor differentiation in both oral cancer and breast cancer. In addition, TRAF6 has an important role in AKT signaling activation through promoting its ubiquitination, and facilitates cell proliferation, colony formation, migration, and G<sub>0</sub>/G<sub>1</sub> to S phase transition in cancer cells. Our data also showed marked in vivo antitumor activity of TRAF6 inhibition. These findings support the theory that high expression of TRAF6 is a sign of more aggressive tumor behavior, and TRAF6 functions as an oncogene.

It has recently been documented that TRAF6 exhibits E3 ligase activity, and it might catalyze substrate ubiquitination.<sup>25,26</sup> In an effort to identify the mechanism underlying TRAF6-induced malignant progression in cancer cells, we sought to explore whether

TRAF6 triggers cancer cell proliferation by affecting the ubiquitination of certain substrates. As a serine/threonine protein kinase, AKT plays a key role in multiple cancer processes.<sup>27,28</sup> Activated AKT could stimulate cancer cell proliferation and cell migration and influence cell cycle progression. Although the precise mechanism was unknown, it was reported that activation of AKT was often accompanied by TRAF6 overexpression in cancers.<sup>15,29</sup> Therefore, we speculated that TRAF6 might contribute to the malignant behavior of human cancers through affecting AKT ubiquitination. Our data showed that TRAF6 could efficiently catalyze the ubiquitination of AKT in cancer cells. As the intact RING domain of TRAF6 in conjunction with the E2 Ub-conjugating enzyme is necessary for its E3 ligase activity, an E3-ligase-deficient TRAF6 C70A mutant in which the highly critical Cys residue in its RING domain was mutated to Ala (TRAF6 C70A), was applied in our study to exclude a possible indirect effect of TRAF6 on AKT ubiquitination. In contrast to

TRAF6 wt, TRAF6 mut showed no influence on AKT ubiquitination, indicating that TRAF6 directly induced the ubiquitination of AKT.

The role of AKT signaling in cancer development has been well documented.<sup>30</sup> Aberrant activation of AKT signaling has been widely implicated in many cancers.<sup>27,28,30</sup> Although it is well known that AKT activity is regulated through phosphorylation, some other types of post-translational modifications, such as ubiquitination, SUMOylation, acetylation, and m6A mRNA methylation, have also been reported to promote AKT activity and function.<sup>31,32</sup> Recently, it was reported that AKT ubiquitination is correlated with its phosphorylation level, suggesting that ubiquitination represents a novel post-translational modification that plays a key role in AKT activation.<sup>33</sup> Consistent with these reports, our data indicated that, in addition to ubiquitination, the ectopic expression of TRAF6 wt but not TRAF6 E3-ligase-deficient mut could also significantly facilitate AKT phosphorylation. Moreover, the reconstitution of TRAF6 wt, but not TRAF6 mut, directly contributes to the proliferation, migration, and marked G<sub>0</sub>/G<sub>1</sub> to S phase transition in cancer cells. This result supports that AKT ubiquitination appears to be as equally important as AKT phosphorylation and highlighted the critical role of TRAF6-mediated AKT ubiquitination and subsequent phosphorylation in the malignant progression of cancer cells. However, AKT ubiquitination is not the only type of post-translational modification that could promote AKT phosphorylation/activation. Further studies are necessary to detect whether TRAF6 affects other types of post-translational modifications of AKT.

In summary, our findings indicate that TRAF6-mediated AKT ubiquitination and phosphorylation play important roles during the malignant progression of tumors. Our study also provides evidence that TRAF6 could be a potential therapeutic target in cancer.

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## DISCLOSURE

The authors have no conflict of interest.

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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