ORIGINAL ARTICLE

SUMOylation of TUFT1 is essential for gastric cancer progression through AKT/mTOR signaling pathway activation

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Abstract

Tuftelin (TUFT1) is highly expressed in various tumor types and promotes tumor growth and metastasis by activating AKT and other core signaling pathways. However, the effects of post-translational modifications of TUFT1 on its oncogenic function remain unexplored. In this study, we found that TUFT1 was SUMOylated at K79. SUMOylation deficiency significantly impaired the ability of TUFT1 to promote the proliferation, migration, and invasion of gastric cancer (GC) cells by blocking AKT/mTOR signaling pathway activation. SUMOylation of TUFT1 is mediated by the E3 SUMO ligase tripartite motif-containing protein 27 (TRIM27), and these two proteins regulate the malignant behavior of GC cells and AKT activation in the same pathway. TUFT1 binds to TRIM27 through its N-terminus, and decreased binding affinity of TUFT1 to TRIM27 significantly impairs its oncogenic effect. In addition, data collected from GC clinical samples indicated that the combined detection of TUFT1 and TRIM27 expression reflected tumor malignancy and patient survival with higher precision. In addition, we proved that SUMOylated TUFT1 is not only an upstream signal for AKT activation but also directly activates mTOR by forming a complex with Rab GTPase activating protein 1, which further inhibits Rab GTPases and promotes the perinuclear accumulation of mTORC1. Altogether, these data indicate that SUMOylated TUFT1 is the active form that affects GC progression through the AKT/mTOR signaling pathway and might be a promising therapeutic target or biomarker for GC progression.

KEYWORDS
AKT, gastric cancer, SUMOylation, TRIM27, TUFT1

1 | INTRODUCTION

Gastric cancer (GC) is the fifth most commonly diagnosed cancer and the fourth leading cause of cancer-related death worldwide, with over 1,000,000 new cases and 769,000 deaths in 2020.[1] The molecular mechanisms underlying GC carcinogenesis and progression are not fully understood, and more in-depth studies are required to explore new treatment strategies and therapeutic drugs.

SUMOylation is a reversible post-translational modification involved in various biological processes such as cell cycle regulation, DNA damage repair, and signal transduction.[2–4] SUMOylation follows a conserved catalytic cascade in which small ubiquitin-like

modifier (SUMO) proteins are covalently bound to lysine (K) residues, altering the stability, subcellular localization, or binding affinity of a wide range of proteins.[5,6] Several studies have shown that expression of the core SUMOylation machinery (E1 activating enzyme, E2 conjugating enzyme, several E3 ligases, and SUMO1/ sentrin specific peptidases) is enhanced in GC,[7–10] suggesting that SUMOylation is closely related to the survival and malignant behavior of GC cells. However, only a few transcription factors and receptors such as forkhead box protein C2 (FOXC2), N-myc downstream-regulated gene 2 (NDRG2), Sp1, CCAAT/enhancer-binding protein alpha (C/EBPα), insulin-like growth factor 1 receptor (IGF-1R), and death domain-associated protein 6 (DAXX) have been reported to be SUMOylated in GC.[10–15] Uncovering the roles of more SUMOylated proteins in GC will provide a promising perspective for investigating the molecular mechanism of tumorigenesis and progression, finding novel biomarkers with higher sensitivity, and contributing to the early diagnosis and targeted therapies of GC.

Tuftelin (TUFT1) was initially characterized as a protein involved in tooth enamel mineralization in vertebrates and subsequently found in nonmineralized tissues such as lung, stomach, and liver.[16,17] Oncological studies indicate that TUFT1 is highly expressed in various tumors, predicting worse clinical outcomes and poorer prognosis.[18–21] In GC, TUFT1 promotes tumor growth and metastasis by activating AKT and related downstream pathways.[22] Alternatively, TUFT1 directly modulates the activity of mTORC1 by regulating the intracellular lysosome localization and cellular trafficking of Rab GTPases.[19] In other cancers, TUFT1 promotes tumor growth, metastasis, and drug resistance by activating hypoxia inducible factor-1 (HIF-1)/Snail and Rab5/Ras-related C3 botulinum toxin substrate 1 (Rac1)/cadherin-associated protein, beta 1 (β-catenin) signaling pathways, or by upregulating long non-coding RNA differentiation antagonizing non-protein coding RNA (DANCR).[21,23–25] Therefore, TUFT1 is a potential biomarker and therapeutic target for various cancers; however, the effects of post-translational modification of TUFT1 on the regulation of its biological functions is yet to be explored.

Herein, we demonstrated for the first time that SUMOylation of TUFT1 at K79 is a precondition for its oncogenic effect. Loss of SUMOylation significantly blocked the ability of TUFT1 to promote GC cell proliferation, migration, invasion, and activation of the AKT/mTOR signaling pathway. We further demonstrated that SUMOylation of TUFT1 is mediated by the E3 SUMO ligase tripartite motif-containing protein 27 (TRIM27), and that the two proteins regulate the malignant behavior and AKT activation of GC cells in the same pathway. In addition, TUFT1 expression is associated with the clinical stage, lymph node metastasis rate, and overall survival (OS) of patients with GC only in the presence of SUMOylation. When TUFT1 was hypo-SUMOylated, there was no correlation between TUFT1 expression and the clinical parameters mentioned above. Therefore, SUMOylation of TUFT1 is a promising therapeutic target or biomarker for GC progression.

2 | MATERIAL AND METHODS

2.1 | Cell culture and transfection

GES-1 and MGC-803 cells were cultured in RPMI-1640 medium (BasalMedia) supplemented with 10% FBS (BasalMedia). AGS cells were cultured in Ham's F-12 medium (BasalMedia) supplemented with 10% FBS. HEK293T and HGC27 cells were cultured in DMEM medium (BasalMedia) supplemented with 10% FBS. All cells were kept at 37°C in a 5% CO_2 humidified incubator. JetPRIME® (Polyplus) was used to transfect cells according to the manufacturer's instructions.

2.2 | Generation of TUFT1 knockout GC cell lines

All the knockout cell lines were generated using the CRISPR/Cas9 approach.[26] To generate TUFT1 knockout GC cell lines, the target oligo 5′-CACCGGGAGTCCCATGATGGACATG-3′ was ligated into a lentiCRISPR v2 vector and transfected into the cells. Puromycin-resistant cells were selected and manually monocloned. The proliferated clones were subjected to immunoblot analysis and genome sequencing to verify successful knockout of TUFT1.

2.3 | Western blotting

Samples were separated using SDS-PAGE and transferred onto PVDF membranes (Millipore). The membranes were blocked with 4% nonfat milk and then probed with primary antibodies and HRP-conjugated secondary antibodies (Proteintech, 1:5000). The primary antibodies used are listed in Table S1.

2.4 | Immunoprecipitation assays

For immunoprecipitation, cells were lysed in lysis buffer (150 mM NaCl, 50 mM HEPES, 1 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, and protease inhibitors; pH 7.4). The supernatant of the cell lysates was mixed with Protein G-Sepharose beads and antibodies for 2 h at 4°C. The beads were further washed with lysis buffer and boiled at 100°C for 5 min in protein loading buffer.

2.5 | Cell proliferation assay

For CCK-8 assay, 10 μl of CCK-8 reagent (MedChemExpress) was added to each well and incubated at 37°C for 1 h before measuring absorbance at a wavelength of 450 nm. For the EdU assay, the Cell-Light™ EdU Apollo488 in vitro kit (RiboBio) was used, according to manufacturer's instructions.

2.6 | Transwell assay

For cell migration ability detection, 1×10^5 GC cells were resuspended in 1 ml of serum-free culture media and 150 μl was seeded onto the upper chamber. For cell invasion ability detection, 100 μl of 300 μg/ml ABW® Matrigengel (ABWbio) was added to the membrane of the upper chamber before cell seeding. A culture medium containing 20% FBS was added to the bottom chamber. After 24 h, cells passing through the membrane were stained with crystal violet and counted under the microscope.

2.7 | In vivo growth experiments

All experimental procedures were approved by the Animal Ethics Committee of Central Hospital Affiliated to Shandong First Medical University (#JNCH2021-20). BALB/c nude mice (male, 5 weeks old) were randomly divided into five groups ($n = 6$). HGC27 cells (2×10^6; WT, knockout, or stable cell lines) suspended in 100 μl of PBS were subcutaneously injected into right flanks of the mice. Tumor volumes were calculated every 3 days as length×width^2×0.5. After 24 days, the mice were sacrificed, and the subskin grafts were dissected and weighed.

2.8 | Immunohistochemistry staining

Immunohistochemistry was performed as described previously[27] on GC tissue microarrays purchased from Shanghai Outdo Biotech Company (#HstmA160CS01) with anti-TRIM27 and anti-TUFT1 antibodies. Two observers independently scored the tissues on a scale of 0–3 according to the percentage of immunoreactive cells and staining intensity.

2.9 | Intracellular calcium concentration detection

Cells cultured in 96-well plates were washed with PBS, and 50 μl of 2 μM Fluo-4 AM (Beyotime) was added to each well and incubated at 37°C for 30 min. Cells were washed with PBS and the fluorescence intensity of Fluo-4 was captured under the fluorescence microscope.

2.10 | Immunofluorescence

Cells were fixed in 4% paraformaldehyde for 15 min at room temperature and then permeabilized with 0.15% Triton X-100 for 15 min. Cells were blocked with 3% BSA for 30 min, incubated with primary antibodies (1:100) in 3% BSA at 4°C overnight, and stained with CoraLite488/594-conjugated secondary antibodies (Proteintech) for 1 h at room temperature. The samples were observed at room temperature using a confocal microscope (Leica).

2.11 | Quantification and statistical analysis

The intensity of the immunoblot bands and fluorescent signals was measured using ImageJ software (NIH). Statistical analyses were performed using GraphPad Prism 7 software. Statistical data were analyzed using paired or unpaired two-tailed Student's t-test and represented as mean±SEM. P value <0.05 was considered statistically significant. Asterisks represent statistical significance: ***$P<0.001$, **$P<0.01$, *$P<0.05$. The statistical details of each experiment, including statistical significance and n values, are provided in the figure legends.

3 | RESULTS

3.1 | TUFT1 is SUMOylated at K79

To identify SUMOylated proteins in GC cells, we performed liquid chromatography tandem mass spectrometry screening following SUMO-modified protein enrichment of normal gastric mucosa and GC cells, and noticed that TUFT1 was one of the candidates with high confidence. TUFT1 expression was upregulated in GC cells relative to the normal gastric mucosa cells (Figure 1A), which is consistent with the published study.[28] We confirmed SUMOylation of TUFT1 by immunoprecipitation of total SUMOylated proteins from different GC cell line lysates with an anti-SUMO1 antibody, and this modification was marginally detected in normal gastric mucosa cells (Figure 1A). To further identify the SUMOylation sites of TUFT1, lysine residues were mutated to the non-SUMOylatable arginine (R) residue, and the SUMOylation level of exogenous TUFT1 was remarkably reduced when K79 was mutated (Figure 1B), indicating that K79 is the main SUMOylation site of TUFT1. Subsequent sequence analysis showed that the amino acid sequences around the SUMOylation site were well-conserved, with high homology among species (Figure 1C).

3.2 | SUMOylation of TUFT1 is essential for promoting proliferation, migration, and invasion of GC cells

Since TUFT1 promotes the malignant behavior of various cancer cells, we investigated whether SUMOylation influences the biological functions of TUFT1. EdU and CCK-8 assays indicated that overexpression of TUFT1-WT significantly increased the proliferation of AGS and HGC27 cells (Figure 1D–G). However, overexpression of the non-SUMOylatable mutant TUFT1 (TUFT1-KR) did not promote GC cell proliferation (Figure 1D–G). Accordingly, the results of the Transwell assays showed that TUFT1-WT overexpression remarkably enhanced the migration and invasion of GC cells, which was significantly impaired in the TUFT1-KR transfection groups (Figure 1H,I). These results indicate that SUMOylation of TUFT1 at K79 is a prerequisite for its oncogenic effect in GC.
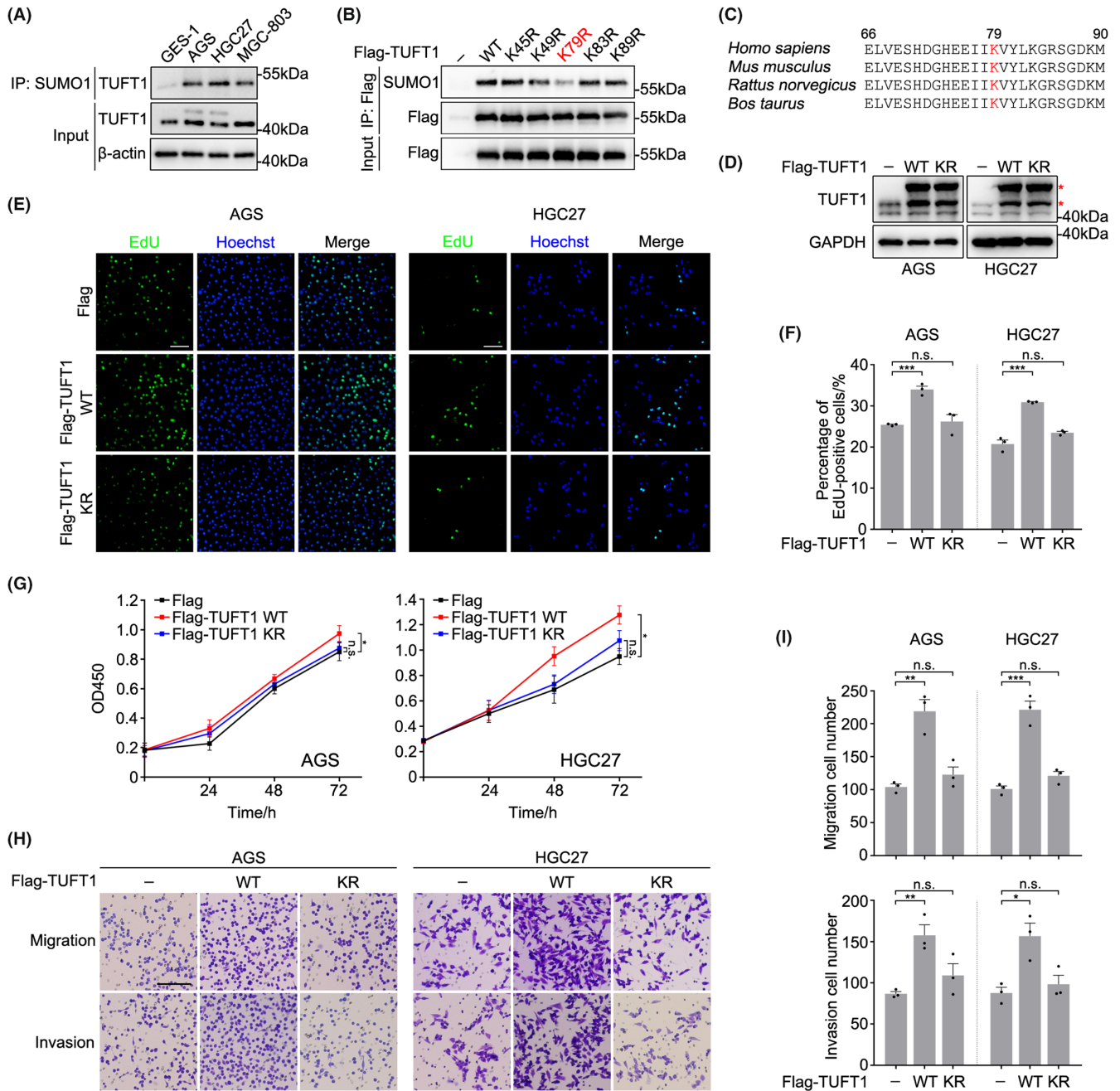
FIGURE 1 SUMOylation of TUFT1 at K79 is essential for promoting the proliferation, migration, and invasion of gastric cancer cells. (A) Lysates of gastric cancer cells (AGS, HGC27, MGC-803) and gastric mucosa cells (GES-1) were subjected to immunoprecipitation (IP) with anti-SUMO1 antibody and immunoblotted with anti-TUFT1 antibody. β-actin was used as a loading control. (B) HEK293T cells overexpressing TUFT1-WT or point mutants were subjected to immunoprecipitation with anti-Flag antibody and immunoblotted with anti-SUMO1 antibody. (C) Comparison of TUFT1 amino acid sequences around the SUMOylation site (shown in red) among species. (D) Lysates of AGS and HGC27 cells overexpressing TUFT1 were immunoblotted with anti-TUFT1 antibody. Asterisks indicate exogenous TUFT1. GAPDH was used as a loading control. (E, F) EdU assays were performed in AGS and HGC27 cells overexpressing TUFT1-WT or TUFT1-KR to evaluate cell proliferation. DNA was stained with Hoechst (blue). Scale bar, 50 μm. Quantification is shown in (F) ($n = 3$, five randomly selected fields). (G) CCK-8 assays were performed in AGS and HGC27 cells overexpressing TUFT1-WT or TUFT1-KR to evaluate cell proliferation ($n = 3$). (H, I) Transwell assays were performed in AGS and HGC27 cells overexpressing TUFT1-WT or TUFT1-KR to evaluate cell migration and invasion. Scale bar, 100 μm. Quantification is shown in (I) ($n = 3$, five randomly selected fields). Data are presented as means ± SEM. *P* values were determined by unpaired (F, I) or paired two-tailed Student's *t*-test (G). ***$P < 0.001$, **$P < 0.01$, *$p < 0.05$; n.s., not significant.

To further confirm the importance of TUFT1 SUMOylation, we generated TUFT1 knockout AGS and HGC27 cell lines by a CRISPR-Cas9 approach (Figure S1). TUFT1 knockout remarkably suppressed the proliferation, migration, and invasion of GC cells, and this phenotype could only be rescued by transient re-introduction of TUFT1-WT but not TUFT1-KR (Figure 2A–E). A similar phenotype was observed in AGS cells transfected with TUFT1 siRNA, and this phenotype could only be rescued by siRNA-resistant TUFT1-WT transfection (Figure S2A–E). Overall, these results suggest that SUMOylation is essential for TUFT1 to promote the malignant behavior of GC cells.

3.3 | TUFT1 SUMOylation is necessary for AKT/mTOR signaling pathway activation

Previous studies have indicated that the AKT/mTOR signaling pathway is one of the key pathways activated by TUFT1 to promote tumor growth and metastasis.[18,20,22] Based on these results, we detected whether AKT activation is influenced by TUFT1 SUMOylation. Consistent with our hypothesis, the ability of TUFT1-KR to activate AKT was notably suppressed compared with that of TUFT1-WT (Figure 3A,B). Meanwhile, the phosphorylation state of 70-kDa
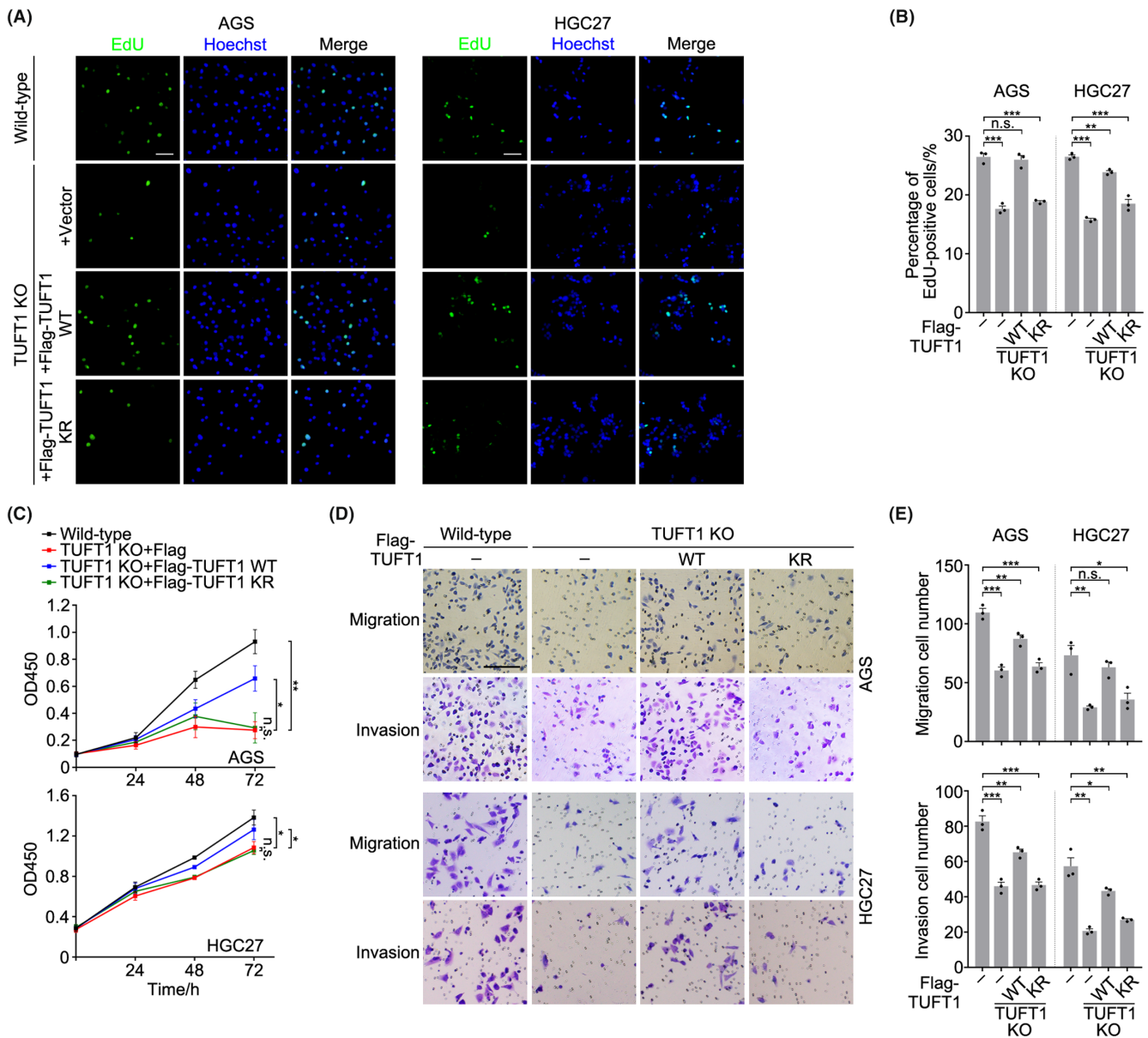
FIGURE 2 The oncogenic effect of TUFT1 is impaired in the absence of SUMOylation. (A, B) EdU assays were performed in WT or TUFT1 knockout (KO) AGS and HGC27 cells rescued by exogenous TUFT1-WT or TUFT1-KR. DNA was stained with Hoechst (blue). Scale bar, 50 μm. Quantification is shown in (B) (n = 3, five randomly selected fields). (C) CCK-8 assays were performed in WT or TUFT1 KO AGS and HGC27 cells rescued by exogenous TUFT1-WT or TUFT1-KR (n = 3). (D, E) Transwell assays were performed in WT or TUFT1 KO AGS and HGC27 cells rescued by exogenous TUFT1-WT or TUFT1-KR. Scale bar, 100 μm. Quantification is shown in (E) (n = 3, five randomly selected fields). Data are presented as means ± SEM. P values were determined by unpaired (B, E) or paired two-tailed Student's t-test (C). ***P < 0.001, **P < 0.01, *P < 0.05; n.s., not significant.
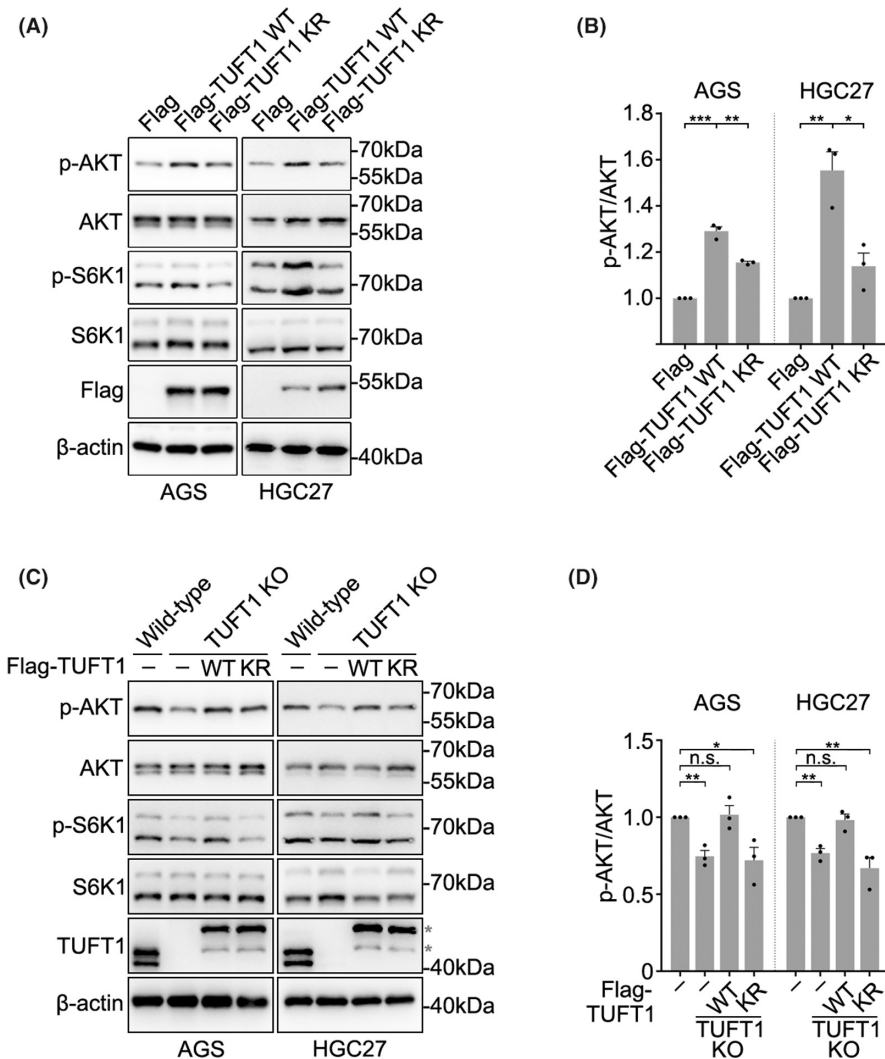
FIGURE 3 TUFT1 SUMOylation is necessary for AKT/mTOR activation. (A, B) Evaluating the effects of TUFT1 overexpression on AKT/mTOR signaling activation in AGS and HGC27 cells by immunoblotting. Phospho-AKT (p-AKT) indicates AKT activation and 70-kDa ribosomal S6 kinase (S6K1) is a downstream effector of mTOR. Quantification is shown in (B) ($n = 3$). (C, D) Evaluating the effects of TUFT1 KO on AKT/mTOR signaling activation in AGS and HGC27 cells by immunoblotting ($n = 3$). Exogenous TUFT1-WT or TUFT1-KR were transiently transfected into the KO cells. Asterisks indicate exogenous TUFT1. Quantification is shown in (D) ($n = 3$). Data are presented as means ± SEM. P values were determined by unpaired two-tailed Student's t-test. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$; n.s., not significant.

ribosomal S6 kinase (S6K1), a downstream effector of mTORC1, was not elevated as much (Figure 3A). Accordingly, TUFT1-KR could not restore the blockage of AKT/mTOR pathway activation caused by TUFT1 knockout or knockdown (Figures 3C,D and S2F,G). These results indicate that SUMOylation of TUFT1 promotes the proliferation, migration, and invasion of GC cells by activating the AKT/mTOR signaling pathway.

3.4 | TUFT1 SUMOylation is regulated by TRIM27

Since SUMOylation is of great importance in regulating the biological function of TUFT1, we aimed to determine the E3 SUMO ligase that mediates TUFT1 SUMOylation. By searching related protein interaction databases, we noticed that TRIM27, a zinc finger protein with SUMO transferase activity,[29] may interact with TUFT1. Interactions between TUFT1 and TRIM27 were detected by co-immunoprecipitation assays (Figure 4A,B). Recombinant TUFT1 and TRIM27 expressed in and purified from bacteria bound to each other in vitro (Figure S3A), further suggesting their direct interactions. To investigate whether TUFT1 is a substrate for TRIM27, we transfected

AGS cells with TRIM27 siRNA and found that SUMOylation of TUFT1 was remarkably decreased after TRIM27 knockdown (Figure 4C). We further co-overexpressed TRIM27 and TUFT1 (WT or KR), and the immunoblot results showed that overexpression of TRIM27 significantly increased SUMOylation of TUFT1-WT but not TUFT1-KR (Figure 4D), indicating that the K79 residue of TUFT1 is SUMOylated by TRIM27. Since TRIM27 possesses both ubiquitin ligase activity and SUMO transferase activity,[29,30] we examined whether TRIM27 participates in the ubiquitin-proteasome-mediated degradation of TUFT1. The mutation at K79 did not block the ubiquitination of TUFT1 (Figure S3B). TRIM27 overexpression or knockdown did not alter the ubiquitination level of TUFT1 (Figure S3C,D), indicating that TRIM27 only participates in TUFT1 SUMOylation.

Since several studies have demonstrated that TRIM27 promotes esophageal and colorectal cancer progression by activating the AKT signaling pathway,[31,32] we next investigated whether TRIM27 promoted GC progression and AKT activation by SUMOylating TUFT1. Indeed, TRIM27 overexpression remarkably enhanced the proliferation, migration, and invasion of WT GC cells, and this phenotype was almost abolished in TUFT1 knockout or knockdown groups (Figures 4E–H and S3E–I). Accordingly, overexpression of TRIM27
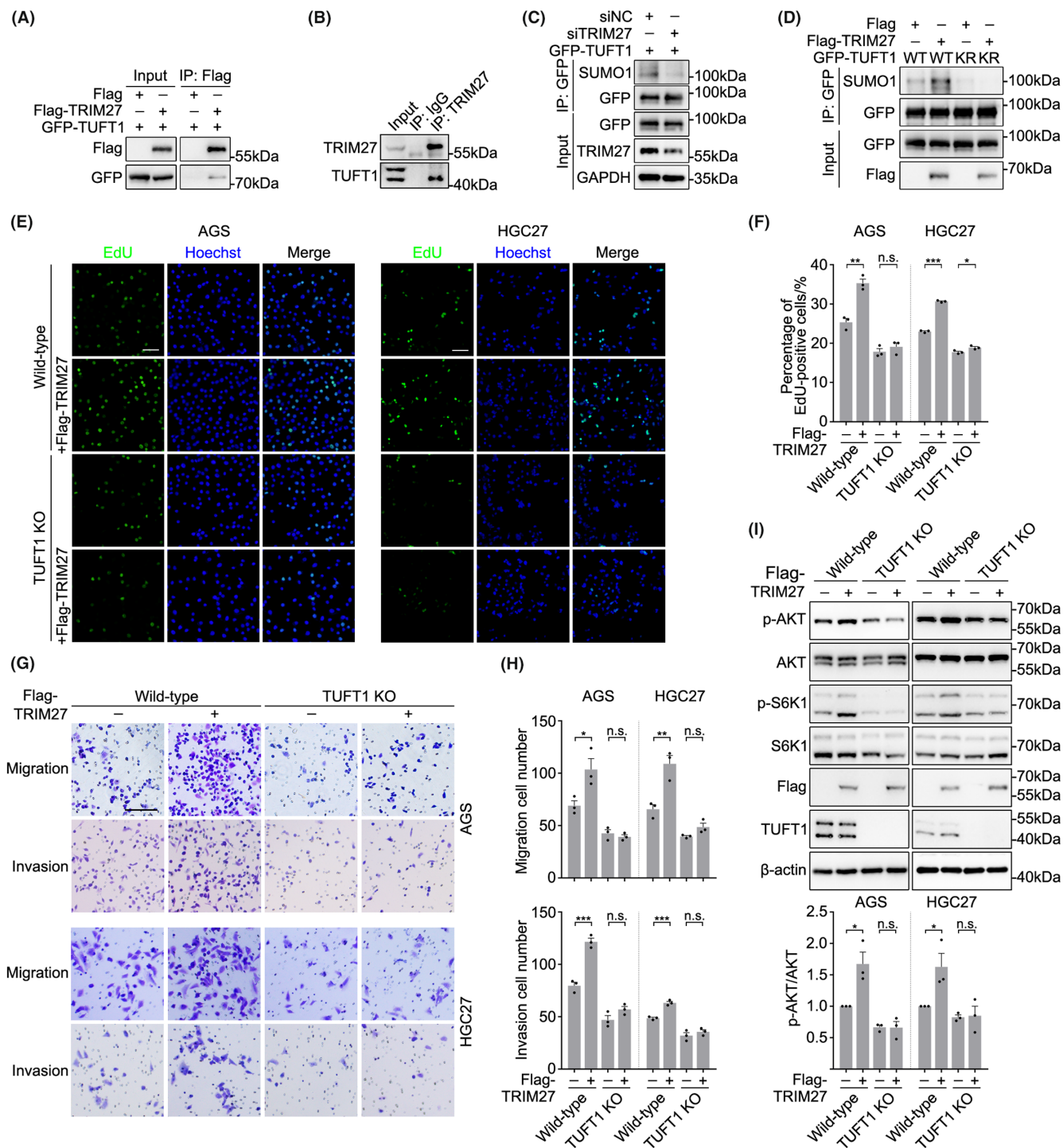
FIGURE 4 TUFT1 SUMOylation is regulated by TRIM27. (A) HEK293T cells overexpressing Flag-TRIM27 and GFP-TUFT1 were subjected to immunoprecipitation with an anti-Flag antibody and immunoblotted with anti-Flag and anti-GFP antibodies. (B) TRIM27 was co-immunoprecipitated with TUFT1 in AGS cells. The samples were immunoblotted with anti-TRIM27 and anti-TUFT1 antibodies. (C) Negative control (siNC) or TRIM27 knockdown (siTRIM27) AGS cells overexpressing GFP-TUFT1 were subjected to immunoprecipitation with anti-GFP antibody and immunoblotted with anti-SUMO1 antibody. GAPDH was used as a loading control. (D) AGS cells overexpressing GFP-TUFT1 (WT or KR) and Flag-TRIM27 were subjected to immunoprecipitation with anti-GFP antibody and immunoblotted with anti-SUMO1 antibody. (E, F) EdU assays were performed in WT or TUFT1 KO AGS and HGC27 cells overexpressing TRIM27. DNA was stained with Hoechst (blue). Scale bar, 50 μm. Quantification is shown in (F) ($n = 3$, five randomly selected fields). (G, H) Transwell assays were performed in WT or TUFT1 KO AGS and HGC27 cells overexpressing TRIM27. Scale bar, 100 μm. Quantification is shown in (H) ($n = 3$, five randomly selected fields). (I) Evaluating the effects of TRIM27 overexpression on AKT/mTOR activation in WT and TUFT1 KO AGS and HGC27 cells by immunoblotting. Quantification is shown below ($n = 3$). Data are presented as means ± SEM. P values were determined by unpaired two-tailed Student's t-test. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$; n.s., not significant.

activated the AKT signaling pathway in WT but not TUFT1-deficient GC cells (Figures 4I and S3J,K), suggesting that TRIM27 and TUFT1 promote the malignant behavior and AKT activation in GC cells via the same pathway.

3.5 | TUFT1 interact with TRIM27 through its N-terminus

Having confirmed the physical and regulatory relationships between TUFT1 and TRIM27, it was essential to determine the binding region of TUFT1 to TRIM27. We constructed a series of truncation mutants based on the functional domains of TUFT1, and co-immunoprecipitation assays revealed that the first 59 amino acids of TUFT1 were indispensable for its binding affinity to TRIM27 (Figures S4 and 5A). Consistent with this conclusion, the SUMOylation level of the TUFT1 ΔN mutant (60–390 amino acids; TUFT1-ΔN) was remarkably decreased (Figure 5B), although the SUMOylation site (K79) was incorporated. Thus, TUFT1-ΔN did not promote proliferation, migration, invasion, or AKT activation in GC cells (Figure 5C–H), similar to the phenotype observed in the TUFT1-KR mutant groups.

To confirm that TUFT1 SUMOylation deficiency impaired tumor growth in vivo, TUFT1 knockout HGC27 cells stably expressing GFP-TUFT1 (WT, KR, or ΔN) and control cells were subcutaneously injected into nude mice to induce ectopic tumor formation. Compared with WT cells, the volume and weight of tumor tissues remarkably decreased in mice injected with TUFT1 knockout cells (Figure 6A–C). In addition, TUFT1-KR and TUFT1-ΔN insignificantly restored tumor growth compared to that with TUFT1-WT (Figure 6A–C), further confirming that TRIM27-mediated SUMOylation is crucial for the oncogenic effect of TUFT1.

3.6 | Upregulation of the TRIM27/TUFT1 axis is associated with poor outcomes in GC

We further investigated the potential effects of TUFT1 SUMOylation on the prognosis of patients with GC. Since there was no available antibody specific for SUMOylated TUFT1, we assessed the correlation between TRIM27/TUFT1 expression in GC samples and patient outcomes instead. We performed immunohistochemical staining on commercial tissue microarrays of GC samples with TUFT1 and TRIM27 antibodies, and scored every sample on a scale of 0–3 based on the percentage of immunoreactive cells and the staining intensity. Scores of 0–1 were marked as low, while scores of 2–3 were marked as high (Figure 6D and Table S2). Patients with high TRIM27 and TUFT1 expression tended to develop more advanced clinical stages accompanied by higher rates of lymph node metastasis (Figure 6E,F). Meanwhile, there was no correlation between TUFT1 expression and GC malignancy in patients with low TRIM27 expression (Figure 6E,F), indicating that the expression of TRIM27

is a precondition for assessing the oncogenic function of TUFT1 in GC progression. We assessed the correlation between TRIM27/TUFT1 expression and patient survival. Cohorts of patients with GC collected from published studies[33,34] were divided into two groups according to the protein level of TRIM27. Consistent with the above results, TUFT1 expression was negatively correlated with OS only in the TRIM27 high expression group (Figure 6G). In summary, the TRIM27/TUFT1 axis well reflects outcomes and survival of patients with GC and might be a promising biomarker for GC diagnosis.

3.7 | SUMOylation of TUFT1 affects the perinuclear accumulation of lysosomes and mTORC1

Having confirmed the importance of TUFT1 SUMOylation in GC progression, we investigated the molecular mechanism of TUFT1 SUMOylation-induced AKT activation in GC. It is reported that TUFT1 activates the AKT signaling pathway by elevating the concentration of intracellular calcium in hepatocellular carcinoma cells.[20] However, the ability of the TUFT1-KR mutant to elevate the concentration of intracellular calcium in AGS cells was not impaired compared with that of TUFT1-WT (Figure 7A), indicating that TUFT1 SUMOylation deficiency blocks AKT activation downstream of the intracellular calcium signal. On the other hand, a recent study reported that TUFT1 directly activated mTOR, bypassing AKT phosphorylation.[19] Mechanically, TUFT1 forms a complex with Rab GTPase-activating protein 1 (RABGAP1) and inhibits Rab GTPases. Inhibition of Rab GTPases promotes the perinuclear accumulation of mTORC1, which is indispensable for mTORC1 activation.[19,35] Co-immunoprecipitation assays showed that the binding affinity of TUFT1-KR or TUFT1-ΔN to RABGAP1 compared to that with TUFT1-WT was significantly decreased (Figure 7B). Accordingly, we observed diffuse localization of lysosomes and mTOR in TUFT1 knockout AGS cells, and only TUFT1-WT restored perinuclear accumulation (Figure 7C). Together, TRIM27-mediated SUMOylation is of great importance for TUFT1 to promote the malignant behavior of GC cells by activating the AKT/mTOR signaling pathway. On the one hand, TUFT1 SUMOylation is a necessary upstream signal for AKT activation. On the other hand, SUMOylated TUFT1 forms a complex with RABGAP1 and directly activates mTOR by promoting its perinuclear accumulation (Figure 7D).

4 | DISCUSSION

TUFT1 has been proven to play a critical role in tumor growth, metastasis, and drug resistance in various cancers and is associated with worse outcomes and poorer prognosis.[18,21,25,36,37] However, the effects of post-translational modifications of TUFT1 on its oncogenic function remain unexplored. In this study, we demonstrated, for the first time, that TUFT1 is SUMOylated at K79. SUMOylation deficiency significantly impairs the ability of TUFT1 to promote the proliferation, migration, and invasion of GC cells by blocking
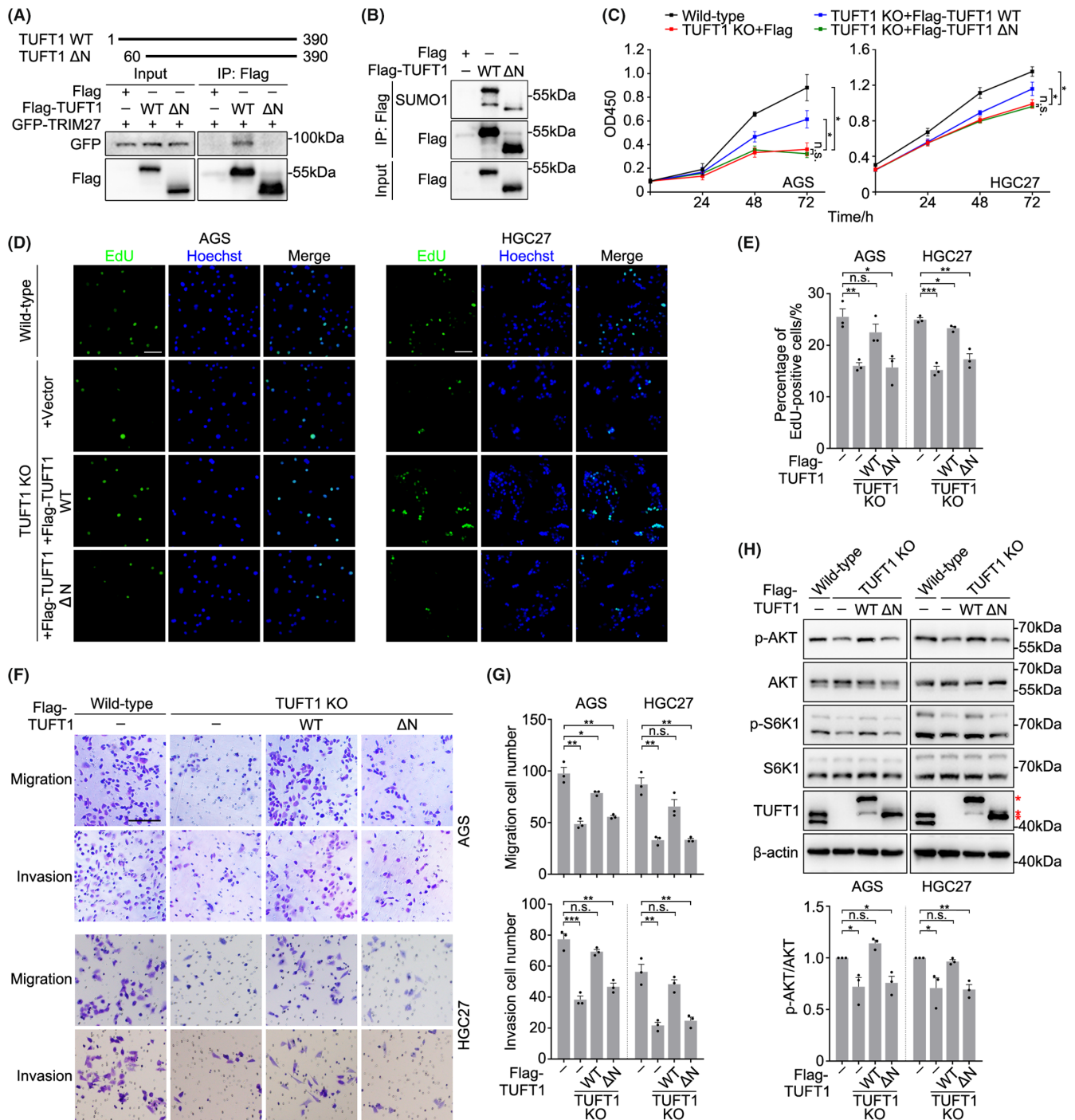
FIGURE 5 TUFT1 interacts with TRIM27 through its N-terminus. (A) HEK293T cells co-overexpressing GFP-TRIM27 and Flag-TUFT1 (WT or ΔN) were subjected to immunoprecipitation with anti-Flag antibody and immunoblotted with anti-GFP and anti-Flag antibodies. (B) AGS cells overexpressing Flag-TUFT1 (WT or ΔN) were subjected to immunoprecipitation with anti-Flag antibody and immunoblotted with anti-SUMO1 antibody. (C) CCK-8 assays were performed in WT or TUFT1 KO AGS and HGC27 cells rescued by exogenous TUFT1-WT or TUFT1-ΔN ($n = 3$). (D, E) EdU assays were performed in WT or TUFT1 KO AGS and HGC27 cells rescued by exogenous TUFT1-WT or TUFT1-ΔN. DNA was stained with Hoechst (blue). Scale bar, 50 μm. Quantification is shown in (E) ($n = 3$, five randomly selected fields). (F, G) Transwell assays were performed in WT or TUFT1 KO AGS and HGC27 cells rescued by exogenous TUFT1-WT or TUFT1-ΔN. Scale bar, 100 μm. Quantification is shown in (G) ($n = 3$, five randomly selected fields). (H) Evaluating the effects of TUFT1 KO on AKT/mTOR activation in AGS and HGC27 cells by immunoblotting. Exogenous TUFT1-WT or TUFT1-ΔN were transiently transfected into the KO cells. Asterisks indicate exogenous TUFT1. Quantification is shown below ($n = 3$). Data are presented as means ± SEM. P values were determined by unpaired (E–G, H) or paired two-tailed Student's t-test (C). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$; n.s., not significant.
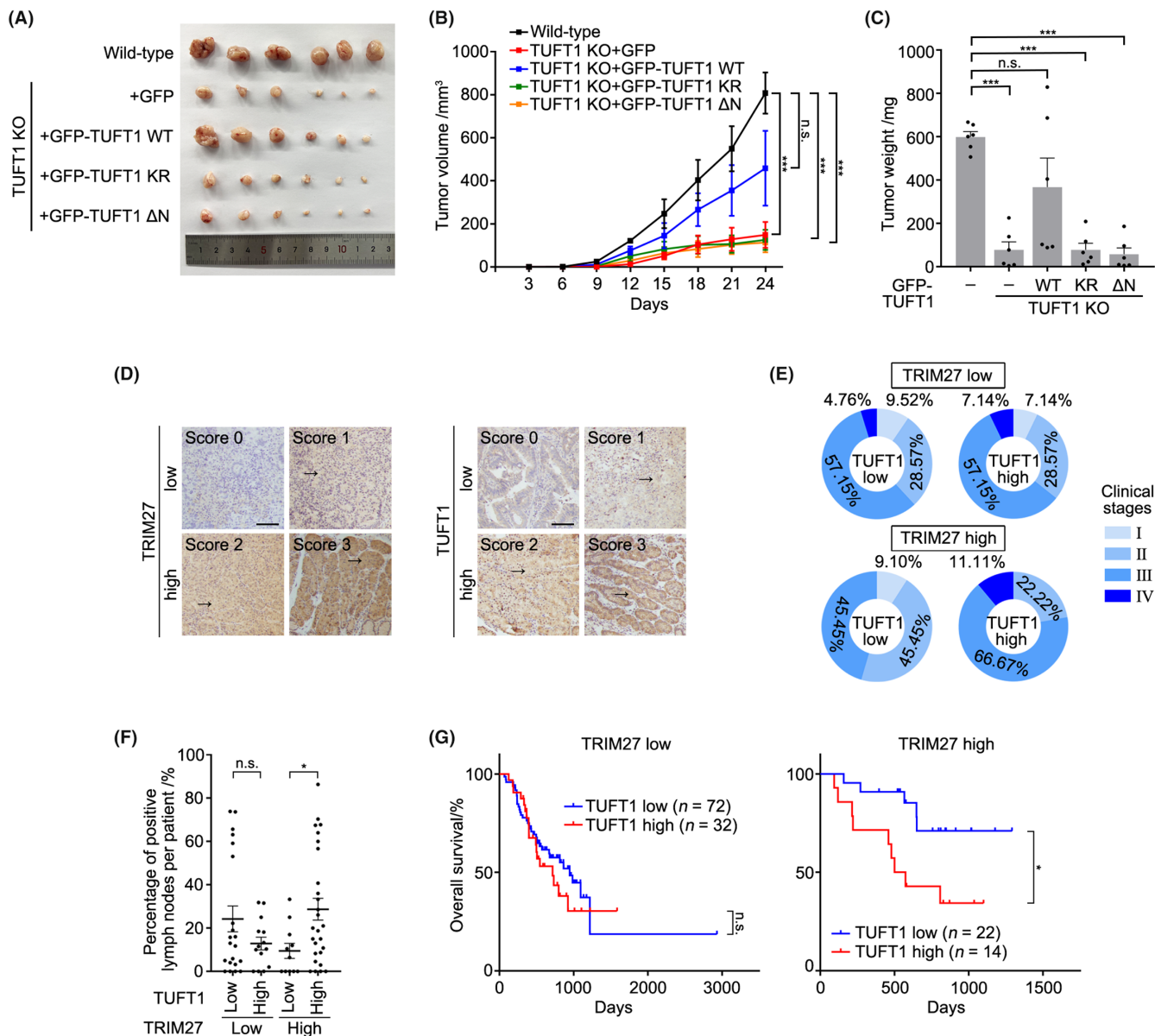
FIGURE 6 Upregulation of the TRIM27/TUFT1 axis is associated with poor outcomes in GC. (A–C) WT, TUFT1 knockout, and stable rescue HGC27 cells were injected subcutaneously into the right flanks of nude mice ($n = 6$). Representative photographs of excised tumors are shown in (A). The tumor volume was measured every 3 days (B). The tumor weight was measured at the end of experiment (C). (D) Representative images of immunohistochemistry staining of TRIM27 and TUFT1 in GC tissue microarrays. Arrows indicate the cytoplasmic staining. Scale bar, 100 μm. (E, F) Relationship between TUFT1 expression and clinical stages (E) or lymph node metastasis rate (F) in TRIM27 low and high expression subgroups, using the GC patient cohort in (D). (G) Survival analysis of TRIM27/TUFT1 expression in GC. Data are presented as means ± SEM. P values were determined by unpaired two-tailed Student's t-test (B, C–F) or two-sided log-rank test (G). ***$P < 0.001$, *$P < 0.05$; n.s., not significant.

the activation of the AKT/mTOR signaling pathway, indicating that SUMOylation determines the oncogenic effect of TUFT1.

Elevated TUFT1 or TRIM27 expression has been proven to be associated with unfavorable clinical features and poor prognosis in GC (Figure S5A,B).[19,38] Through analysis of clinical samples from tissue microarrays and public databases, we further uncovered that TUFT1 expression only correlates with tumor malignancy and OS of patients with high TRIM27 expression (Figures 6D–G and S5C), indicating that the joint detection of TRIM27 and TUFT1 expression reflects GC progression more accurately. In fact, we also found

that, in addition to GC, the TRIM27/TUFT1 axis reflects patient OS in triple-negative breast cancer, ovarian cancer, and lung adenocarcinoma (Figure S6),[39–42] further confirming that SUMOylation of TUFT1 might be a promising therapeutic target or biomarker for tumor progression.

The AKT/mTOR signaling pathway is one of the core pathways activated by TUFT1 to promote tumor progression.[18,20,43] Herein, we demonstrated that SUMOylated TUFT1 regulates this pathway through two distinct mechanisms. First, SUMOylated TUFT1 is an upstream signal for AKT activation. In future studies, we will

FIGURE 7 SUMOylation of TUFT1 affects the perinuclear accumulation of lysosomes and mTORC1. (A) The concentration of intracellular calcium was detected in AGS cells overexpressing TUFT1. Data are presented as means ± SEM. *P* values were determined by unpaired two-tailed Student's *t*-test. ***P < 0.001; n.s., not significant. (B) HEK293T cells overexpressing Flag-TUFT1 (WT, KR, or ΔN) were subjected to immunoprecipitation with anti-Flag antibody and immunoblotted with anti-RABGAP1 and anti-Flag antibodies. (C) Immunostaining of LAMP1 (red) and mTOR (green) in WT or TUFT1 KO AGS cells. DNA was stained with DAPI (blue). Scale bar, 5 μm. (D) Schematic of TRIM27/TUFT1 axis in promoting GC progression. TRIM27-mediated TUFT1 SUMOylation is a necessary upstream signal for AKT activation. In addition, SUMOylated TUFT1 forms a complex with RABGAP1 and directly activates mTOR by promoting its perinuclear accumulation.
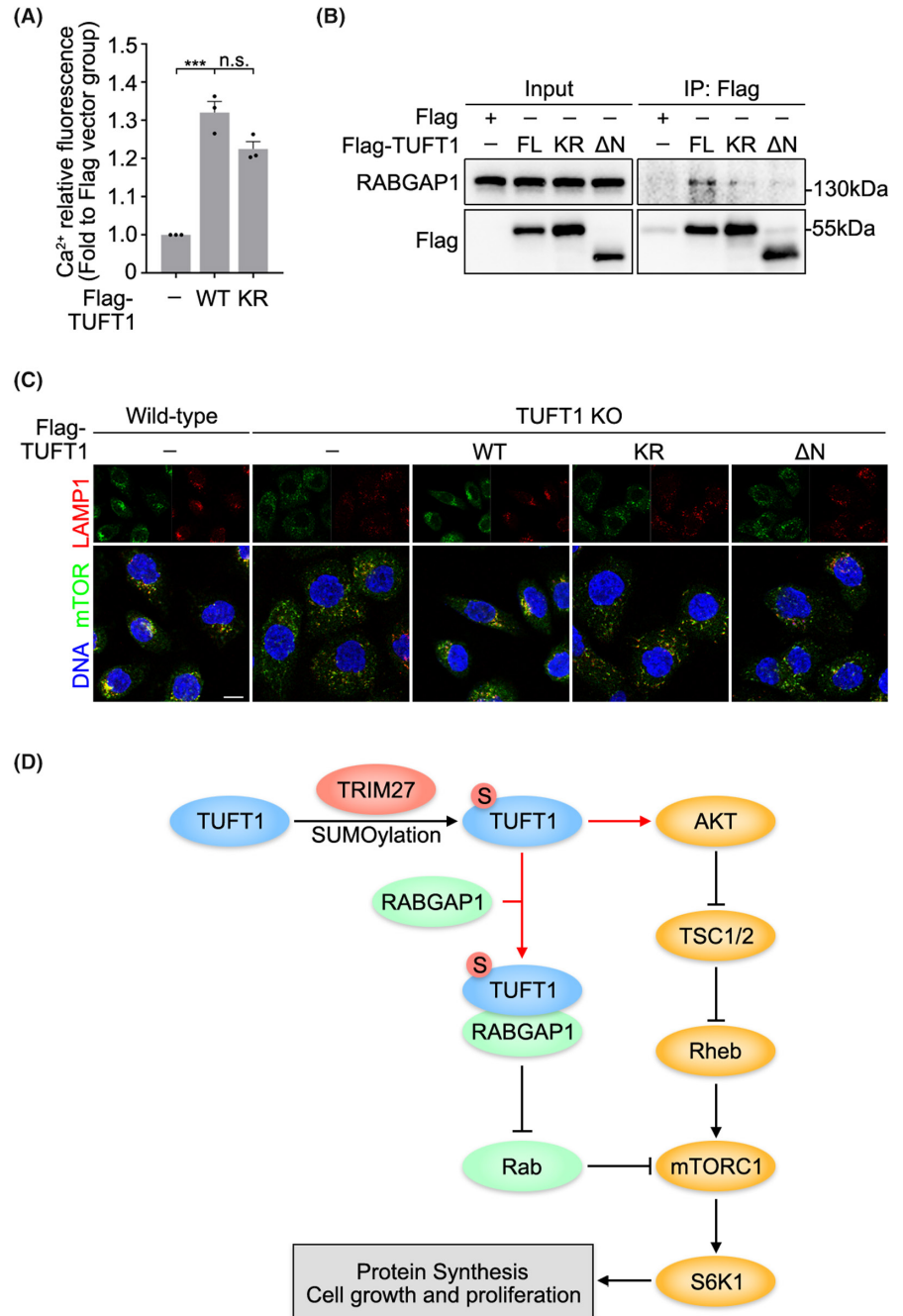
investigate how SUMOylated TUFT1 activates AKT in GC cells. Second, SUMOylated TUFT1 directly activates mTOR by forming a complex with RABGAP1, which further inhibits Rab GTPases and promotes perinuclear accumulation of mTORC1. Loss of SUMOylation remarkably decreased the binding affinity of TUFT1 to RABGAP1 (Figure 7B). In addition to the AKT/mTOR signaling pathway, TUFT1 promotes tumor growth, metastasis, and drug resistance through other pathways such as HIF-1/Snail and Rab5/Rac1/β-catenin.[21,23,25] Moreover, TUFT1 upregulates the expression of LncRNA DANCR, promoting the malignancy and invasiveness of triple-negative breast cancer.[24] Whether SUMOylation of TUFT1 influences these downstream effectors in GC needs further investigation.

TRIM27 plays an oncogenic role in various types of cancer, and its expression positively correlates with tumor malignancy and anticancer drug resistance.[32,44–46] In colorectal, esophageal, and ovarian serous carcinoma cells, TRIM27 promotes tumor growth and metastasis by activating AKT.[31,32,47] Herein, we found that TUFT1 is a new intermediate between the TRIM27 and AKT signaling pathways in GC. TRIM27 interacted with TUFT1 and promotes AKT/mTOR signaling activation by SUMOylating TUFT1 (Figure 4A–D,I). Consistent with this conclusion, the ability of TRIM27 to promote cell proliferation, migration, and invasion was almost abolished in TUFT1-deficient GC cells (Figures 4E–H and S3E–I). On the other hand, it was found that TRIM27 regulates GC cell proliferation and 5-fluorouracil resistance through the

Hippo-baculoviral IAP repeat-containing protein 5 (BIRC5) pathway[38]; whether there is any crosstalk between the two pathways requires further studies.

Based on previous studies and our results, SUMOylation is closely related to tumor growth, survival, metastasis, and drug resistance as it regulates the function of core signaling transduction proteins or transcription factors in GC. We believe that a comprehensive quantitative SUMOylome mass spectrometry analysis of GC clinical samples or cell lines is of great necessity to investigate the mechanism of tumorigenesis and progression from a new perspective, contributing to the exploration of early diagnosis and targeted therapies of GC.

AUTHOR CONTRIBUTIONS

T.W. performed most of the molecular biology experiments, cell biology experiments, clinical sample analysis, and data analysis. L.M., Y.G., and M.Z. conducted some molecular biology and cell biology experiments. S.F. and H.W. performed some clinical sample analysis. Y.Z. and Y.W. are the senior authors who conceived and designed the project. T.W., Y.Z., and Y.W. wrote the manuscript.

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DISCLOSURE

The authors declare no potential conflicts of interest.

ETHICAL APPROVAL

Approval of the research protocol by an Institutional Reviewer Board: The research protocol was approved by the Ethics Committee of Central Hospital Affiliated to Shandong First Medical University (#SZR2021-017-01) and the Ethics Committee of Shanghai Outdo Biotech Company (#YBM-05-02).

Informed consent: All samples were obtained with written informed consent from participants.

REGISTRY AND THE REGISTRATION

No of the study/trial: None.

ANIMAL STUDIES

All experimental procedures were approved by Animal Ethics Committee of Central Hospital Affiliated to Shandong First Medical University (#JNCH2021-20).

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