

RESEARCH ARTICLE



# ALKBH5-mediated m6A demethylation of TXNDC5 drives malignant progression in gastric cancer

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## ABSTRACT

**Background:** Gastric cancer (GC) remains a leading cause of cancer-related mortality worldwide. N6-methyladenosine (m6A) modification plays a critical role in post-transcriptional gene regulation. This study aimed to elucidate the molecular mechanism by which the RNA demethylase ALKBH5 regulates GC progression through m6A modification of thioredoxin domain-containing protein 5 (TXNDC5).

**Methods:** Differential expression models of ALKBH5 and TXNDC5 were established in GC cells using RNA interference and gene overexpression. Methylated RNA immunoprecipitation (MeRIP-qPCR), qPCR, and Western blot were performed to assess ALKBH5-mediated m6A modification and its effect on TXNDC5 expression. Functional assays, including proliferation, migration, and invasion, as well as a xenograft mouse model, were used to evaluate their roles in GC progression.

**Results:** ALKBH5 was significantly upregulated in GC tissues and cells. Overexpression of ALKBH5 stabilized TXNDC5 expression in an m6A-dependent manner, thereby promoting malignant phenotypes. Conversely, ALKBH5 knockdown increased m6A methylation of TXNDC5, reduced TXNDC5 protein expression, and suppressed GC cell proliferation, migration, and invasion. In vivo experiments confirmed that loss of ALKBH5 impaired tumor growth.

**Conclusions:** Our findings demonstrate that the ALKBH5–TXNDC5 axis drives GC progression through m6A-dependent regulation, highlighting ALKBH5 as a potential therapeutic target for GC.

## PLAIN LANGUAGE SUMMARY

Stomach cancer is a serious disease that is often hard to treat. In this study, we focused on a protein called ALKBH5, which helps cancer cells grow. We found that ALKBH5 increases the level of another protein, TXNDC5, making stomach cancer cells stronger and more likely to spread. When ALKBH5 was blocked, TXNDC5 decreased, and the cancer cells became weaker. These results show that ALKBH5 May drive stomach cancer growth and could be a new target for treatment in the future.

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## KEYWORDS

TXNDC5; gastric cancer;  
ALKBH5; m6A;  
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## 1. Introduction

Gastric cancer, a malignancy of significant global health concern, is characterized by an annual incidence exceeding 1 million cases and approximately 760,000 deaths worldwide. Though advancements in clinical interventions and population-level prevention strategies have driven a progressive decline in both morbidity and mortality rates, GC persists as the fifth most prevalent neoplasm globally and ranks third among cancer-related fatalities [1–4]. This persistent burden predominantly stems from three interrelated factors: diagnostic delays caused by nonspecific early symptomatology; aggressive tumor biology marked by rapid progression and high metastatic potential at initial presentation [5]. The pathogenesis of GC involves a multifactorial interplay between environmental exposures and genetic susceptibility. Notably, *Helicobacter pylori* infection synergizes with dietary risk factors (e.g., high-salt diets, carcinogen-containing preserved foods), tobacco use, and excessive alcohol consumption to drive malignant transformation. Complementing these exogenous risks, hereditary

predisposition and premalignant conditions (notably chronic atrophic gastritis and gastric intestinal metaplasia) further amplify carcinogenic progression [6–8]. Despite elucidation of these etiological drivers, the early detection rate remains below 30% in clinical practice, resulting in advanced-stage diagnoses for most patients. Even with combined surgical resection, chemotherapy, and radiotherapy, the 5-year survival rate scarcely exceeds 30% [9]. This diagnostic dilemma primarily arises from the absence of robust noninvasive screening modalities. While endoscopic biopsy with histopathological verification remains the diagnostic benchmark, its inherent invasiveness and substantial resource requirements preclude widespread implementation, particularly in low-to-middle-income regions [2,10,11]. Emerging liquid biopsy technologies (e.g., circulating tumor DNA profiling) show preclinical promise; however, technical limitations in sensitivity and specificity currently restrict their clinical utility [12]. Therapeutic management of localized GC hinges on radical surgical resection as the cornerstone of curative intent. Nevertheless, postoperative local recurrence and

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**Article highlights**

- Identifies the ALKBH5–TXNDC5 axis as a novel and critical regulator of gastric cancer progression.
- Demonstrates that ALKBH5 stabilizes TXNDC5 expression through m6A-dependent post-transcriptional regulation.
- Shows that targeting ALKBH5–TXNDC5 signaling suppresses gastric cancer cell proliferation, migration, invasion, and tumor growth in vivo.
- Provides mechanistic insights linking RNA m6A demethylation to malignant phenotypes in gastric cancer.
- Highlights ALKBH5 as a promising therapeutic target with potential translational value for gastric cancer treatment.

distant metastasis rates lead to persistently high therapeutic failure rates [5]. Conventional platinum/fluoropyrimidine-based chemotherapy extends median survival in advanced disease, yet often culminates in dose-limiting toxicities and acquired drug resistance [13]. Recent molecular insights have catalyzed a therapeutic paradigm shift: Targeted therapeutic strategies, including HER2-directed agents (e.g., trastuzumab) and angiogenesis inhibitors (VEGF/VEGFR2 blockers), have demonstrated progression-free survival improvements in biomarker-defined subgroups [14,15], whereas immuno-oncology interventions – particularly PD-1/PD-L1 checkpoint inhibitors – enhance anti-tumor immune surveillance, achieving durable clinical responses in patients with microsatellite instability-high (MSI-H) or PD-L1-positive tumors [16]. Notwithstanding these advances, interpatient heterogeneity in therapeutic response – attributable to tumor molecular diversity and dynamic immune microenvironment modulation – remains a critical challenge, necessitating precision biomarkers to guide individualized regimens [17].

As the most abundant posttranscriptional modification in eukaryotic mRNA, N6-methyladenosine (m6A) refers to adenosine methylation at the N6 position, dynamically regulated by writer complexes (e.g., METTL3/METTL14), erasers (FTO/ALKBH5), and readers (YTHDF1/YTHDF2). This methylation machinery orchestrates RNA splicing, nuclear export, stability, and translational efficiency, thereby dictating cellular fate [18,19]. As a key m6A demethylase, ALKBH5 plays a crucial role in multiple cancers by specifically removing methyl groups from RNA molecules and regulating target gene expression [20]. Mechanistically, FOXM1-AS has been shown to facilitate ALKBH5-FOXM1 nascent transcript interaction, driving glioblastoma stem-like cell maintenance [21]. Meanwhile in gastric cancer, Wang et al. identified an oncogenic ALKBH5-m6A-ZKSCAN3-VEGFA signaling axis promoting tumor progression [22]. However, this pro-tumorigenic role conflicts with findings by Hu et al., who demonstrated ALKBH5 as a metastasis suppressor via m6A-dependent stabilization of PKMYT1 mRNA and subsequent activation of the ALKBH5-PKMYT1-IGF2BP3 network [23]. Despite these advances, the mechanisms underlying ALKBH5-mediated regulation of gastric cancer proliferation and metastasis remain incompletely characterized, particularly regarding its downstream target networks. Filling this knowledge gap could enable precision therapies targeting m6A modifications.

Thioredoxin domain-containing protein 5 (TXNDC5), an endoplasmic reticulum stress sensor and member of the protein disulfide isomerase (PDI) family, regulates protein folding and redox homeostasis by catalyzing disulfide bond formation and isomerization [24–26]. Emerging evidence implicates TXNDC5 in the tumorigenesis. For instance, HERG1 was reported to promote esophageal squamous cell carcinoma progression via PI3K/AKT-mediated TXNDC5 upregulation [27]. Notably, METTL3 has been shown to modulate TXNDC5 expression in an m6A reader-dependent manner [28], providing a rationale for investigating m6A-TXNDC5 interplay in gastric cancer. Nevertheless, the expression profile, functional role, and upstream regulatory mechanisms of TXNDC5 in gastric carcinogenesis remain elusive, with no existing evidence linking ALKBH5 to TXNDC5 through m6A-dependent pathways.

Our study elucidated the pathogenic significance of the ALKBH5-TXNDC5 axis in GC demonstrating that ALKBH5 governs TXNDC5 expression via m6A-dependent mechanisms. These findings advance our understanding of epigenetic crosstalk in gastric tumorigenesis and context-dependent ALKBH5 regulatory networks, while providing potential diagnostic biomarkers and therapeutic targets.

## 2. Methods

### 2.1. Database

Transcriptome RNA-seq data of 450 STAD cases (normal samples, 36cases; tumor samples, 410 cases) and the corresponding clinical data were downloaded from TCGA and GEO database. (<https://portal.gdc.cancer.gov/>, <https://www.ncbi.nlm.nih.gov/geo/>).

### 2.2. Survival analysis

The Kaplan – Meier method was utilized to generate the survival curves, and the log-rank test was applied to assess statistical significance, with a p-value of less than 0.05 considered indicative of a significant difference. Survival data were obtained from publicly available gastric cancer cohorts, including GSE14210, GSE15259, GSE22377, GSE29372, GSE62254, and GSE51105.

### 2.3. Tissue samples collection

In this study, 8 pairs of GC and adjacent non-tumor tissue samples were collected from GC patients who underwent gastrectomy at the general surgery of Shandong Provincial Hospital Affiliated to Shandong First Medical University. Samples were quickly placed in liquid nitrogen for refrigeration. The newly diagnosed GC patients had not received radiotherapy or chemotherapy prior to surgery. Informed consent was obtained for all individuals. Ethics approval was obtained from the Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong First Medical University.

## 2.4. Cell culture

The human GC cell lines used were purchased from ATCC. GC cells were grown in RPMI 1640 medium with 10% FBS and 1% Penicillin – Streptomycin. Cells were identified with STR profiles. Mycoplasma decontamination was conducted once every two months.

## 2.5. Lentiviral infection

Following the manufacturer's instructions, cells were incubated with an appropriate concentration of viral supernatant for 24 hours. Subsequently, 2 µg/mL puromycin was added to select for stable knockdown cell lines. The knockdown efficiency was verified by Western blot (WB) analysis. Small interfering RNAs (siRNA) against TXNDC5 (si-1: 5'-GCCAAGAAGCUGUGAAGUAtt3,' si-2: 5'-CCAAAGUCUAUGUGGCUAAtt3,' si-3: 5'-CACCAUUGCAGAA GGAAUAtt3') and scrambled siRNA negative control were synthesized by the company. Stable lentiviruses for ALKBH5 knockdown (shA: 5'-ACCCAGCTATGCTTCAGAT3,' shB: 5'-CTGCCCCGA AAGGTGAAGAT3,' shC: 5'-GTGTCCGTGCTTCTTTA3') and overexpression were constructed using the pcDNA3.1 vector. The ALKBH5 overexpression plasmid and its mutant variant H204A were also generated with the same backbone. The ALKBH5-H204A plasmid and corresponding lentiviruses were procured from a commercial supplier. Transient transfection was performed with liposome 2000 according to the manufacturer's instructions. The transfected cells were incubated and harvested for subsequent detection.

## 2.6. Crystal violet assay

Log-phase cells were digested, centrifuged, and counted before seeding 3,000 cells per well in a 24-well plate, with three replicates per group. After ensuring cell attachment, the time was marked as 0 hours, and cells were collected at 0, 2, 4, and 6 days. Cells were fixed with 4% paraformaldehyde for 15 minutes, stained with crystal violet for 15 minutes, and air-dried. Then, 10% acetic acid solution was added to each well, and absorbance was measured at 450 nm using a microplate reader. A growth curve was then plotted.

## 2.7. Colony formation assay

Log-phase cells were digested, centrifuged, and counted before seeding at a density of 1000 cells per well in a 6-well plate. Cells were cultured under standard conditions for 14 days. Afterward, cells were fixed with 4% paraformaldehyde for 15 minutes, stained with crystal violet for 15 minutes, and photographed for colony counting.

## 2.8. Transwell migration and invasion assay

Log-phase cells were digested, centrifuged, resuspended in serum-free medium, and counted. A total of  $1 \times 10^4$  cells per well were seeded into the upper chamber of a Transwell insert, while 600 µL of complete medium was added to the

lower chamber. After incubation, cells were fixed with 4% paraformaldehyde for 15 minutes and stained with crystal violet for 15 minutes. The stained cells were then photographed and counted under a microscope. For the invasion assay, Matrigel was precoated in the upper chamber and solidified for 30 minutes in an incubator before proceeding with the same steps as in the migration assay.

## 2.9. Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA was extracted according to the TRIzol reagent manual. Reverse transcription into cDNA was performed following the protocol of the reverse transcription kit. qRT-PCR was conducted according to the SYBR Green PCR kit manual, using GAPDH as an internal control. Each experiment was repeated three times.

## 2.10. Western blots analysis

Soluble protein was extracted using RIPA buffer. An equivalent amount of protein (20–50 µg) was separated on a 12.5% SDS-PAGE gel and then transferred onto PVDF membranes via electrophoresis. The membranes were blocked in blocking buffer for 1 hour and subsequently incubated with specific primary antibodies at 4°C overnight, with GAPDH serving as a loading control. Following incubation with the primary antibodies, the membranes were treated with the corresponding HRP-conjugated secondary antibodies. The protein signals were detected using a chemiluminescence system. The bands resulting from gel imaging were quantified by ImageJ software. The antibodies used were as follows: Anti-ALKBH5 (Abcam Cat# ab195377, RRID:AB\_2827986), Anti-TXNDC5 (ABclonal Cat# A14152, RRID:AB\_2761010), Anti-GAPDH (Proteintech Cat# CL488-60004, RRID:AB\_2919223).

## 2.11. Immunohistochemistry (IHC)

All of slides were placed in a 60°C incubator for 20 min, deparaffinized in xylene and rehydrated in gradient ethanol. The slides were incubated with 3% hydrogen peroxide for 10 min, followed by antigen retrieving using 0.01 M citrate buffer (pH 6.0) for 30 min. After blocking with 5% BSA, slides were incubated overnight at 4°C with relevant primary antibody. After that, the secondary biotin-conjugated antibody was applied for 1 h in room temperature. The IHC staining was visualized using diaminobenzidine reaction, counterstained with hematoxylin. Protein expression was quantitatively assessed using Integrated Optical Density (IOD) measured by Image-Pro Plus software.

## 2.12. Methylated RNA immunoprecipitation (MeRIP-qPCR)

MeRIP-qPCR was performed strictly according to the kit manufacturer's instructions.

### 2.13. Xenograft model establishment

BALB/c nude mice (4–6 weeks, 18–22 g) were used following ethical guidelines. MKN45 cells with stable ALKBH5 knockdown or overexpression, confirmed  $\geq 95\%$  viable by trypan blue exclusion, were resuspended in serum-free medium/Matrigel (1:1) at  $1 \times 10^7$  cells/mL. Each mouse received 100  $\mu$ L cell suspension subcutaneously in bilateral inguinal regions (left: experimental; right: control) using a 29 G insulin syringe at a 15° angle, with injection sites pressed for 15 s to prevent leakage. Mice were euthanized by cervical dislocation when tumors reached endpoint size or showed necrosis/ulceration. Tumors were excised, weighed (accuracy 0.1 mg), and photographed for morphological analysis.

### 2.14. Statistical analysis

Results were expressed as mean  $\pm$  standard deviation (Mean  $\pm$  SD). Differences between groups were analyzed using the t-test. Bioinformatics and statistical analyses were performed using R software (RRID:SCR\_001905) and GraphPad Prism (RRID:SCR\_002798), and graphical representations were generated accordingly. ImageJ software (RRID:SCR\_003070) was used for quantitative analysis. Statistical significance was indicated as follows:  $p < 0.05$  (\*), representing significant differences;  $p < 0.01$  (\*\*),  $p < 0.01$  (\*\*),  $p < 0.001$  (\*\*\*)  $p < 0.0001$  (\*\*\*\*), representing highly significant differences.

## 3. Results

### 3.1. ALKBH5 is upregulated in GC and Correlates with gc progression

To evaluate the clinical relevance of ALKBH5 in GC we first analyzed TCGA datasets, which revealed significant upregulation of ALKBH5 in tumor tissues compared to normal controls (Figure 1(A)). Subsequent survival analysis demonstrated that elevated ALKBH5 expression strongly correlated with poor prognosis (Figure 1(B)). To validate these findings in clinical samples, we obtained paired tumor and adjacent normal tissues from our affiliated hospital which were subjected to IHC staining and western blotting. Consistent with bioinformatics data, ALKBH5 protein levels were markedly higher in tumor tissues (Figure 1(C,D)). Furthermore, we extended our analysis to in vitro models using the normal gastric epithelial cell line GES-1 as a control. Western blot analysis across five GC cell lines (HGC27, MKN45, AGS, MKN74, and SGC7901) confirmed sustained ALKBH5 overexpression in malignant cells compared to normal counterparts (Figure 1(E)). Collectively, these multi-level findings establish that ALKBH5 is aberrantly upregulated in GC and exhibits significant clinical relevance.

### 3.2. ALKBH5 facilitates the progression of GC cells both in vitro and in vivo

To systematically investigate the oncogenic function of ALKBH5 in GC progression, we developed isogenic models through stable knockdown (KD) and overexpression (OE) of

ALKBH5 in HGC27 and MKN45 cell lines (Figure 2(A,B)). Functional characterization revealed that ALKBH5-KD substantially attenuated proliferation, whereas ALKBH5-OE cells exhibited markedly enhanced self-renewal capability (Figure 2(C–E)). Extending these observations to metastatic properties, Transwell migration and Matrigel invasion assays demonstrated that the migratory and invasive capacities of ALKBH5-KD group was suppressed, while ALKBH5-OE reliably promoted both malignant behaviors (Figure 2(F)). In vivo validation using subcutaneous xenograft models further corroborated this regulatory hierarchy: ALKBH5-KD tumors displayed diminished growth kinetics with reduced terminal mass (Figure 2(G)), contrasting sharply with ALKBH5-OE xenografts that manifested accelerated tumorigenic progression and increased weight (Figure 2(H)). Taken together, these results indicate that the inactivation of ALKBH5 significantly inhibits the proliferative activity of GC cells in vitro and tumorigenic potential in vivo, and that its gene expression level correlates significantly with the degree of tumor malignancy.

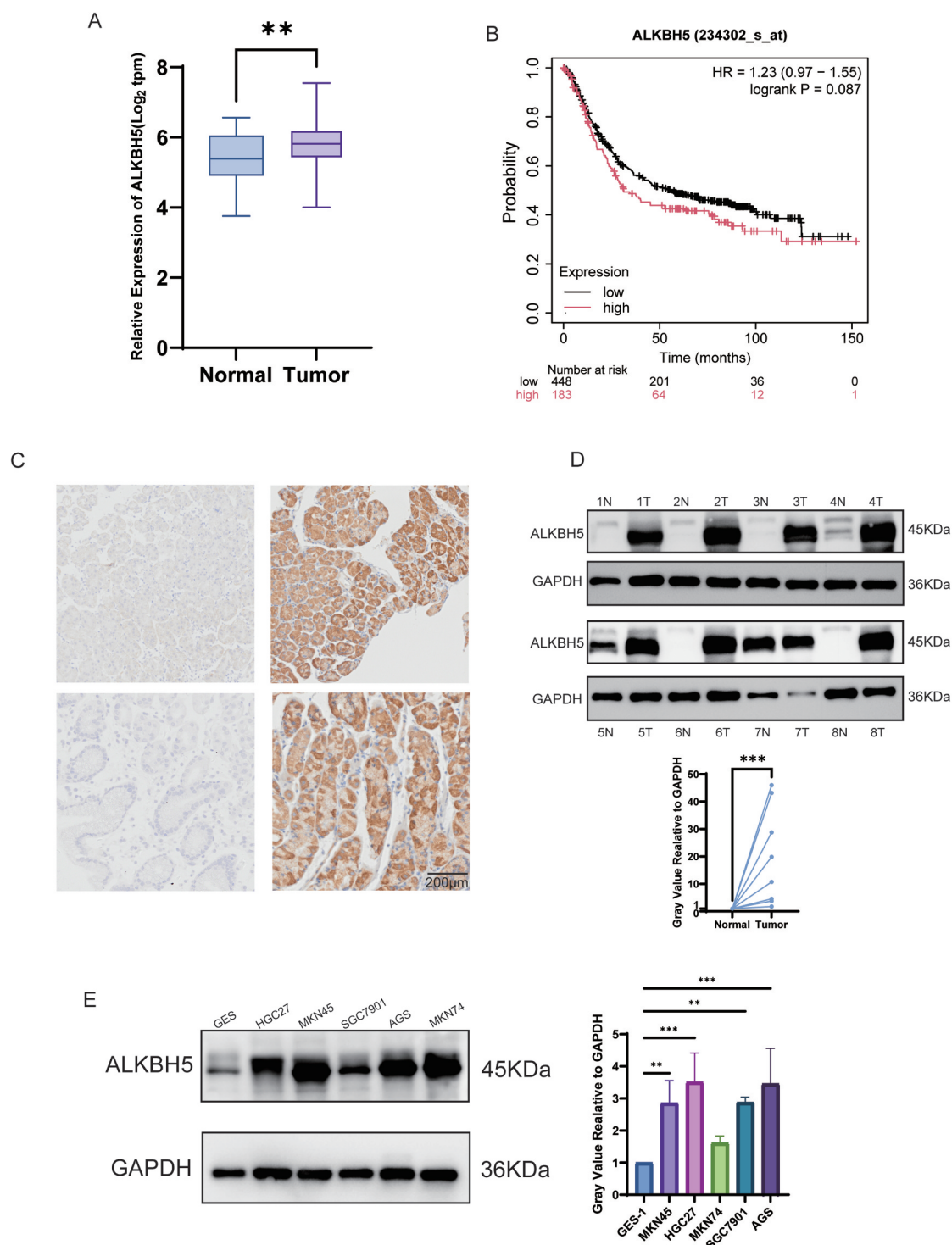
### 3.3. TXNDC5 is highly expressed and promotes the advancement of GC cells in vitro

By reviewing the literature, we identified TXNDC5 as a potential downstream target of ALKBH5 that may play an important role in GC. Consequently, we used immunohistochemistry to assess TXNDC5 expression in GC tissues. As shown in Figure 3(A), TXNDC5 expression was higher in GC tissues than in adjacent non-cancerous tissues. We also discovered in public databases that TXNDC5 expression was elevated compared with normal tissues (Figure 3(B)). Furthermore, we found that TXNDC5 was highly expressed in GC cell lines (Figure 3(C)). To clarify its functional role, we knocked down TXNDC5 in GC cells (Figure 3(D)). We then conducted colony formation assays on these knockdown cells. As shown in Figure 3(E), inhibiting TXNDC5 significantly reduced the colony-forming ability of GC cells. In addition, we explored the effect of TXNDC5 on cell migration and invasion. Similarly, our findings indicated that TXNDC5 knockdown markedly impaired both migratory and invasive capacities (Figure 3(F)). Altogether, these results suggest that TXNDC5 is overexpressed in GC and that its functional inactivation can significantly curb the progression of GC cells.

### 3.4. ALKBH5 indirectly regulates GC cell progression through TXNDC5

To confirm the regulatory relationship between ALKBH5 and TXNDC5 in GC development, we first analyzed publicly available GC datasets. We observed a positive correlation between ALKBH5 and TXNDC5 expression levels (Figure 4(A)). Subsequent experimental validation showed that knocking down ALKBH5 decreased TXNDC5 protein expression. Building on these observations, we generated TXNDC5-overexpressing cell lines with ALKBH5 knockdown to test functional interactions (Figure 4(B)). Since ALKBH5

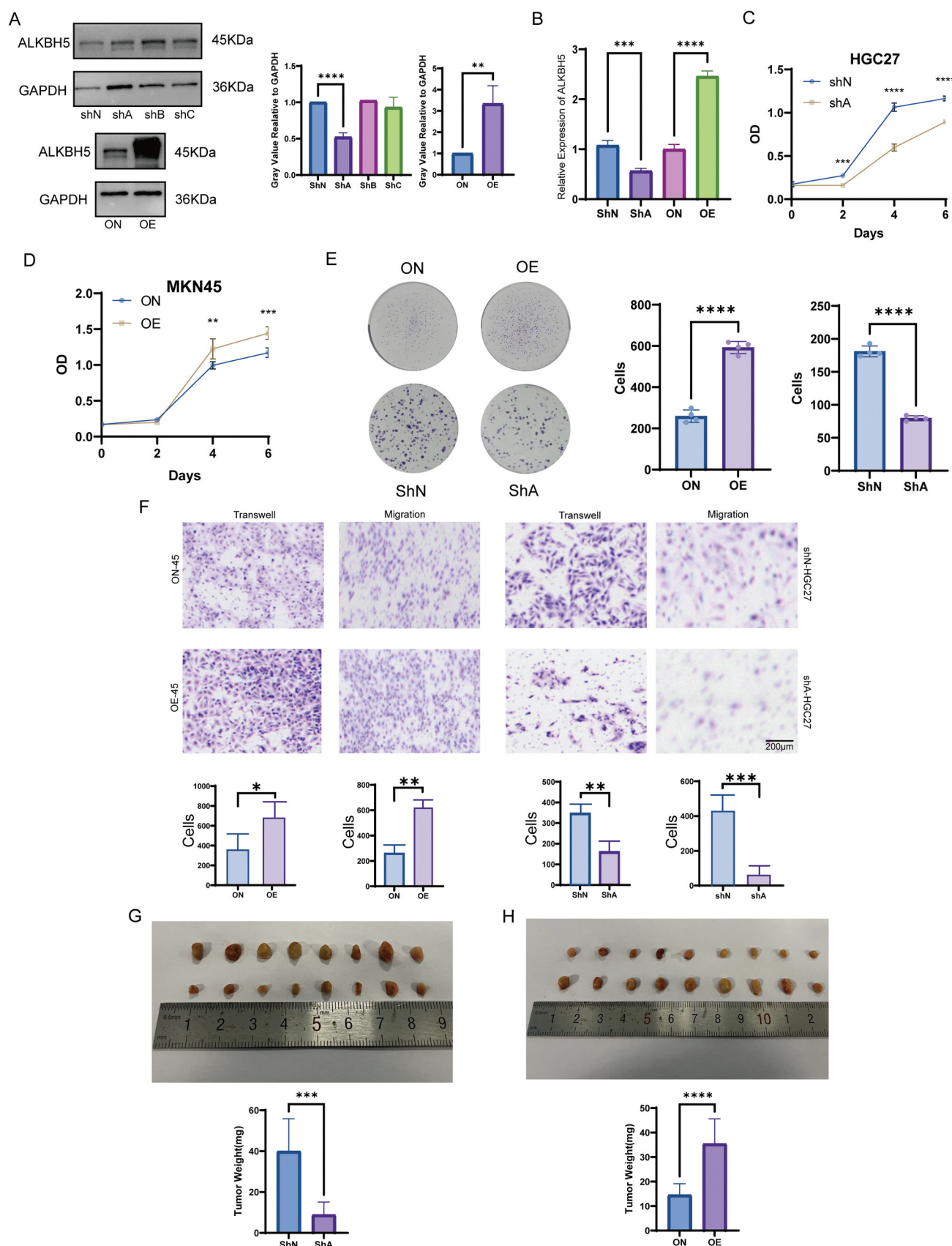




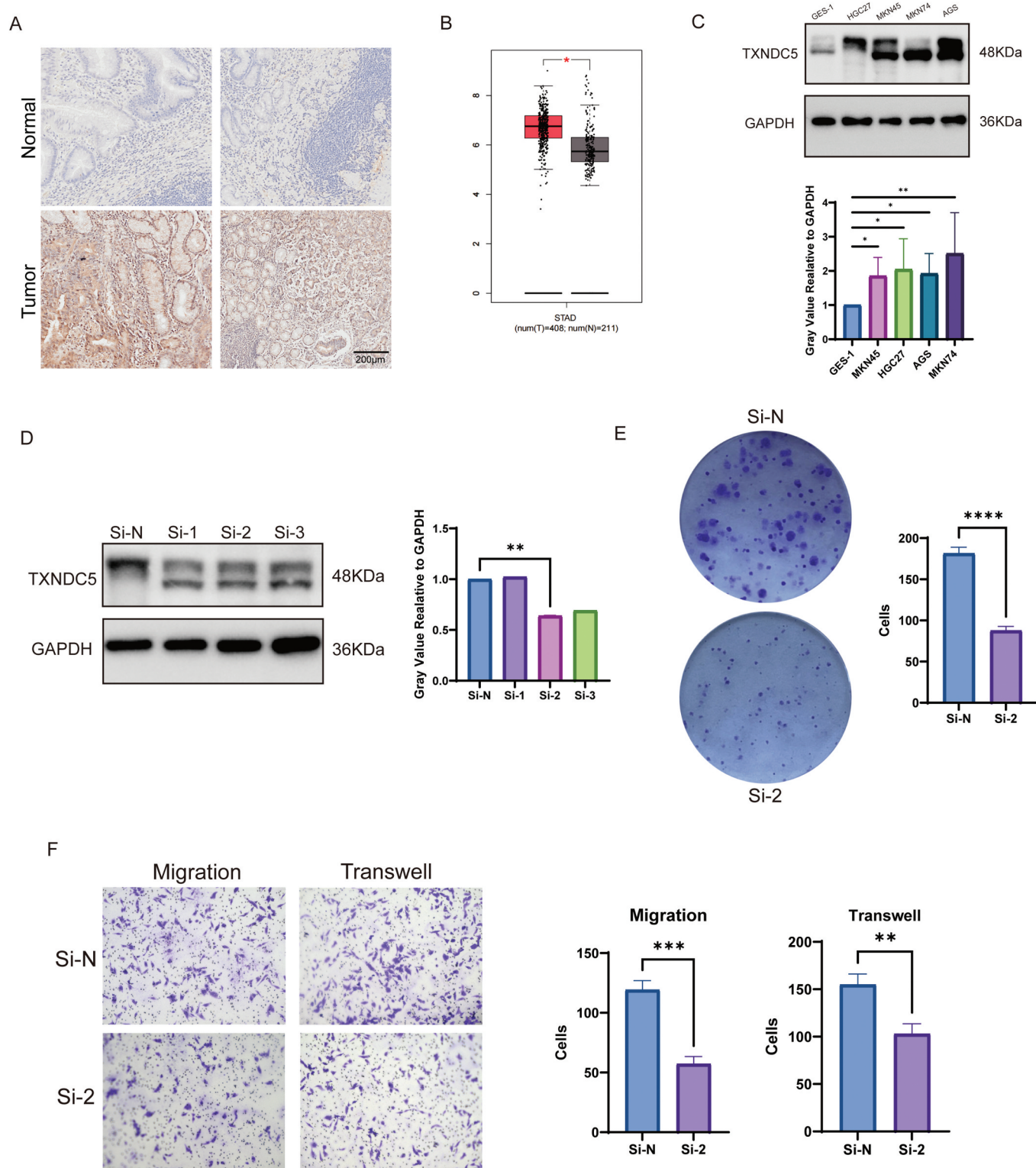
**Figure 1.** ALKBH5 expression and survival analysis in gastric cancer (A) Comparative mRNA expression analysis of ALKBH5 between gc tumors and non-cancerous gastric tissues. (B) Kaplan-meier survival analysis comparing high vs. low ALKBH5 expression in the TCGA gastric cancer cohort. (C) Representative immunohistochemical staining micrographs depicting ALKBH5 expression in human gc and paired adjacent normal tissues. (D) Quantitative analysis of ALKBH5 protein expression in matched tumor-adjacent tissue pairs. (E) Differential ALKBH5 mRNA levels between immortalized gastric mucosal epithelial cells and gc cell lines. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

is a demethylase, we employed an actinomycin D assay to evaluate the stability of TXNDC5 mRNA in both ALKBH5-knockdown and ALKBH5-overexpressing cells. The results

showed that TXNDC5 mRNA stability declined in ALKBH5-knockdown cells compared with the control group (Figure 4(C)), whereas it rose in cells overexpressing



**Figure 2.** Functional characterization of ALKBH5 modulation in vitro and in vivo (A,B). Western blot analysis (a) and quantitative RT-PCR validation (b) of ALKBH5 knockdown and overexpression efficiency in gc cell lines. (C,D). Proliferation capacity assessed by crystal violet staining assay in ALKBH5-modulated HGC27 and MKN45 cells. (E) Colony formation assay of ALKBH5-transfected gc cells. (F) Transwell migration and Matrigel invasion assays evaluating metastatic potential in modified gc cells. (G,H). Subcutaneous xenograft models showing tumor morphology (g) and comparative tumor weights (h) at endpoint. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

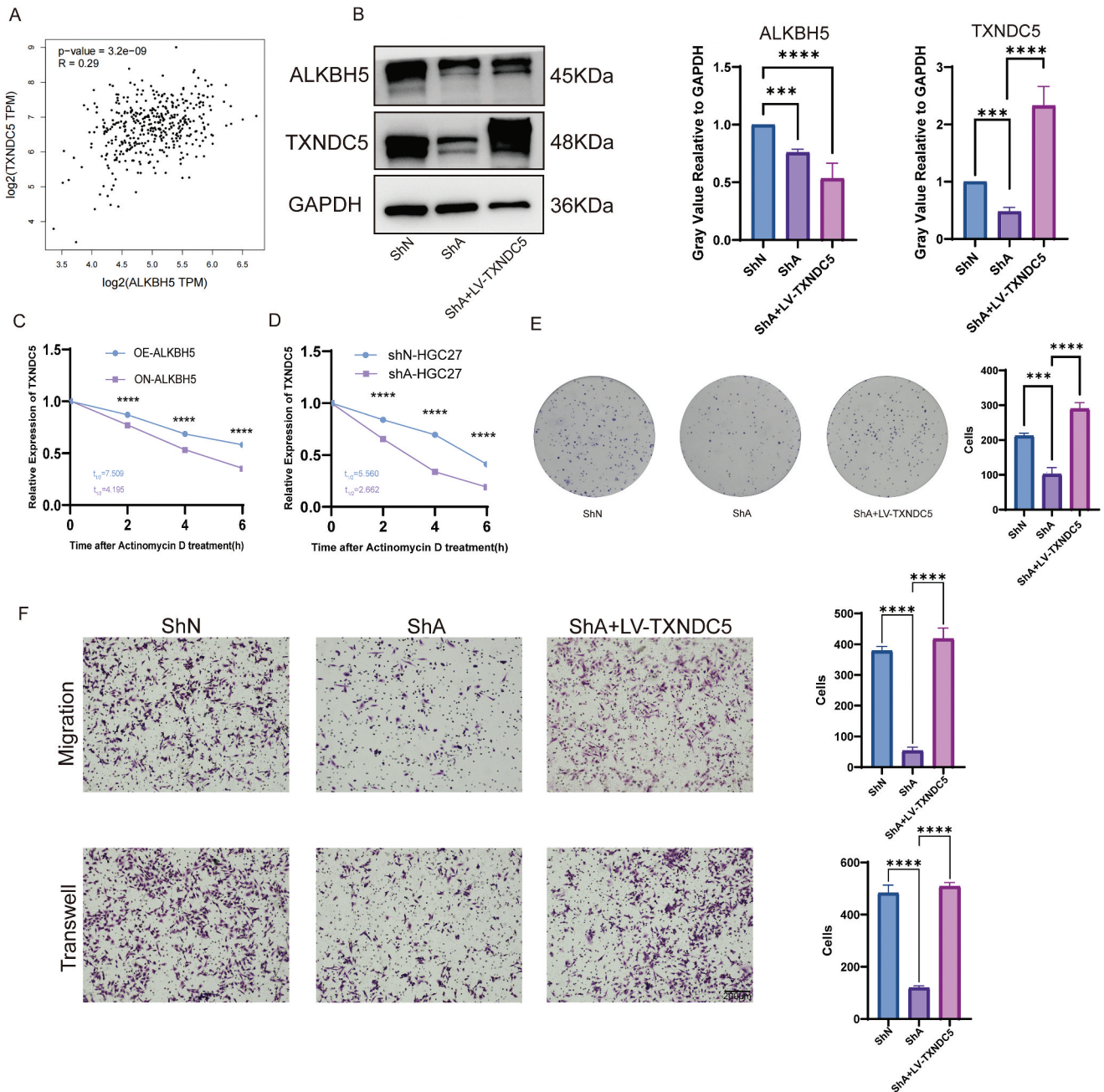


**Figure 3.** TXNDC5 expression profiling and functional validation (A) Immunohistochemical localization of TXNDC5 in gc and matched normal mucosa. (B) TCGA-STAD dataset analysis of TXNDC5 mRNA expression in gastric adenocarcinoma. (C) TXNDC5 transcript levels in gc cell lines relative to GES-1 controls. (D) Western blot confirmation of TXNDC5 silencing efficiency. (E) Colony-forming capacity of TXNDC5-depleted cells. (F) Metastatic potential analysis in TXNDC5-knockdown cells. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

ALKBH5 (Figure 4(D)). Importantly, functional recovery experiments demonstrated that restoring TXNDC5 expression rescued the defective colony-forming ability caused by ALKBH5 knockdown (Figure 4(E)). This compensatory

effect was also observed in metastatic behaviors, TXNDC5 overexpression effectively restored the impaired invasion and migration capacities induced by ALKBH5 suppression (Figure 4(F)). These experimental findings collectively





**Figure 4.** TXNDC5 as a downstream effector of ALKBH5 regulation (A) Spearman correlation analysis between ALKBH5 and TXNDC5 transcript levels in TCGA-GC cohort. (B) Reciprocal expression patterns of ALKBH5 and TXNDC5 in combinatorial manipulation models. (C,D). mRNA stability assays using actinomycin D (5  $\mu\text{g/mL}$ ) treatment in ALKBH5-depleted cells. (E) Rescue colony formation assay with concurrent ALKBH5 knockdown and TXNDC5 overexpression. (F) Metastatic potential restoration assays in ALKBH5-inhibited/TXNDC5-overexpressing cells. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

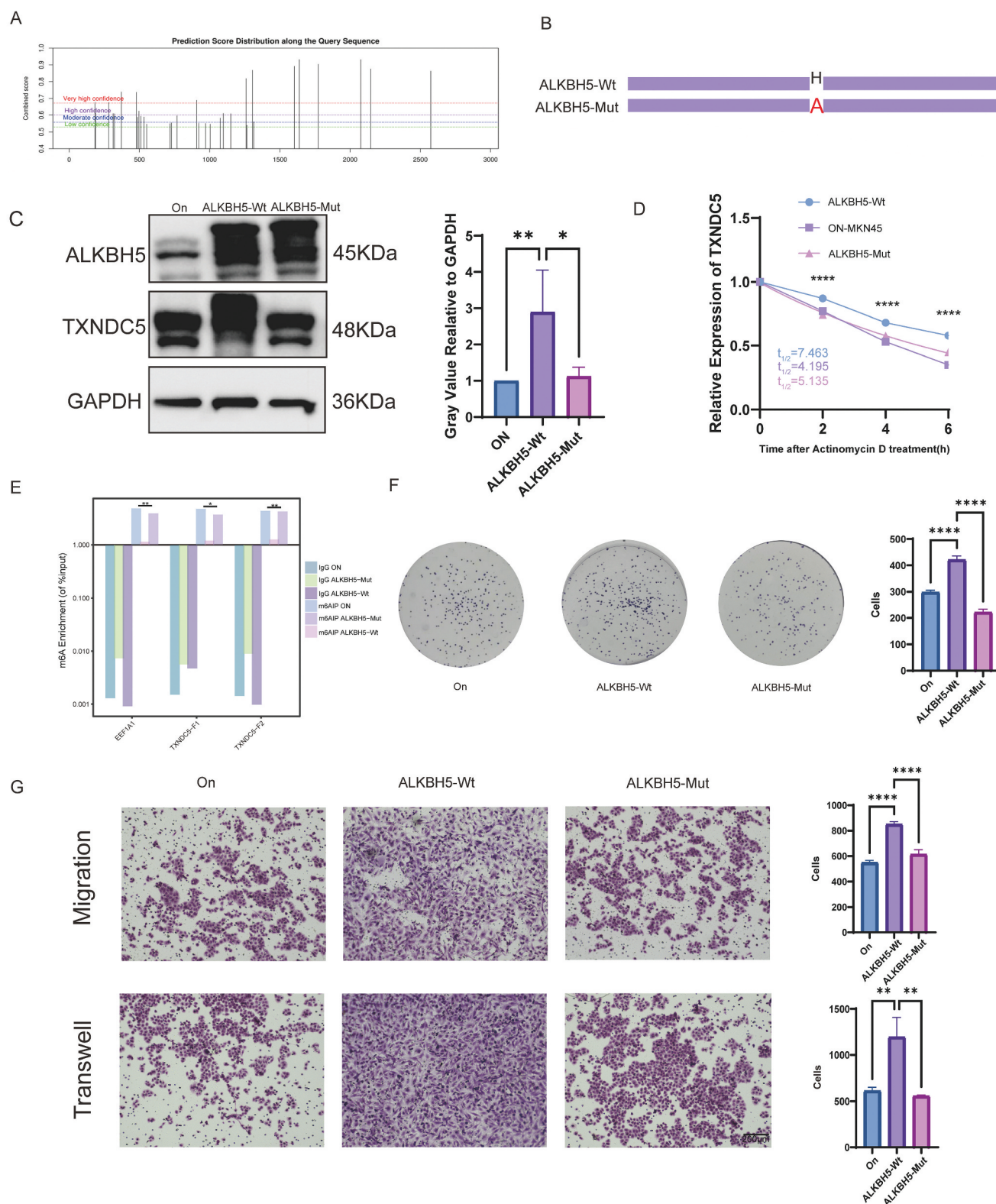
confirm TXNDC5 as a downstream effector of ALKBH5 mediated regulation.

### 3.5. m6A-Dependent regulation of TXNDC5 by ALKBH5 promotes GC malignancy

Through online analysis [29], we identified multiple high-confidence m6A methylation sites in TXNDC5 (Figure 5(A)). Therefore, we constructed an ALKBH5 H204A mutant plasmid which result in enzymatically inactive (Figure 5(B)) and transfected it into GC cell lines. Notably, TXNDC5 protein abundance remained

unaffected by the ALKBH5 mutation (Figure 5(C)). To determine mRNA stability dynamics, we conducted actinomycin D treatments and observed accelerated TXNDC5 mRNA decayed in mutant cells compared to ALKBH5-Wt controls (Figure 5(D)). MeRIP-qPCR analysis further revealed the enrichment of TXNDC5 transcripts by m6A-specific antibodies was diminished in mutant cell lines. (Figure 5(E)). Functional characterization revealed that ALKBH5-mutant cells exhibited decreased colony formation compared to wild-type counterparts (Figure 5(F)). Parallel observations were made for metastatic behaviors and mutant cells showed attenuated migration/invasion relative to wild-type controls (Figure 5(G)).





**Figure 5.** m6A-Dependent regulation of TXNDC5 by ALKBH5 (A) m6A modification sites of TXNDC5. (B) Schematic of catalytically inactive ALKBH5 mutants (H204A) generated via site-directed mutagenesis. (C) TXNDC5 expression comparison between wild-type and ALKBH5-mutant gc cells. (D) mRNA decay kinetics of TXNDC5 following actinomycin D treatment in mutant vs. wild-type cells. (E) MeRIP-qPCR demonstrating TXNDC5 mRNA enrichment in anti-m6A immunoprecipitation. (F,G). Functional recovery assays in ALKBH5-mutant models: colony formation (f) and Transwell migration/invasion (g). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

#### 4. Discussion

In this study, we investigated the mechanism of the demethylase ALKBH5 in GC. We systematically examined how ALKBH5 regulates the downstream target gene TXNDC5 through m6A modification and explored the biological significance of this process. Specifically, we established stable knockdown and overexpression models of ALKBH5 and TXNDC5 in GC cell lines, including HGC-27 and MKN45. We measured m6A modification levels on TXNDC5 mRNA employing methylated RNA immunoprecipitation (MeRIP). Additionally, we evaluated the effects of these modifications using cell proliferation, migration, and invasion assays. Furthermore, we established a mouse xenograft model to confirm the regulatory role of ALKBH5 and TXNDC5 in tumor growth *in vivo*.

At the beginning of our research, we analyzed clinical samples and public databases. We found that ALKBH5 was highly expressed in GC tissues, and high ALKBH5 expression correlated with poor survival. These database analyses provided initial evidence that ALKBH5 might promote GC progression. Previous studies have indicated conflicting roles for ALKBH5 in different cancer types. For example, ALKBH5 acts as a tumor suppressor in certain lung and liver cancers by reducing oncogene expression [30–32]. Conversely, it promotes tumor cell self-renewal and invasiveness in glioblastoma and breast cancer [21,33]. Thus, ALKBH5 does not have a fixed role as either a tumor suppressor or an oncogene. Its function likely depends on the tumor type or specific downstream targets.

To clarify the role of ALKBH5 in GC, we employed RNA interference and overexpression strategies in multiple GC cell lines. We included empty vector and negative controls to ensure experimental specificity and comparability. After altering ALKBH5 expression, we assessed various functional indicators of GC progression, including proliferation, colony formation, migration, and invasion. Our results indicated that ALKBH5 knockdown significantly reduced these malignant features in GC cells, whereas overexpression had the opposite effect. These findings demonstrate that ALKBH5 positively regulates GC cell growth and mobility, consistent with our earlier database analyses. In addition, our xenograft model experiments confirmed that ALKBH5 knockdown decreased tumor growth in mice, providing strong *in vivo* evidence supporting our *in vitro* findings.

We also focused on TXNDC5, an important downstream target of ALKBH5. Previous studies primarily linked TXNDC5 to endoplasmic reticulum stress and protein folding, indicating that it might contribute to tumor progression by affecting protein stability [24,25,28]. However, the specific biological role of TXNDC5 across different cancers was unclear. In our experiments, we first confirmed that TXNDC5 expression was significantly increased in GC tissues. Then, through functional assays, we demonstrated that knocking down TXNDC5 markedly reduced cell proliferation, colony formation, migration, and invasion. Thus, we concluded that TXNDC5 acts as an oncogene in GC. Although we did not explore downstream molecular pathways beyond TXNDC5, our findings provide a solid foundation for future investigations into the ALKBH5–TXNDC5 signaling axis.

To further clarify the relationship between ALKBH5 and TXNDC5, we examined changes in TXNDC5 protein expression following ALKBH5 knockdown or overexpression. We found that knocking down ALKBH5 significantly reduced TXNDC5 protein levels, while overexpressing ALKBH5 increased them. Based on this observation, we hypothesized that ALKBH5 might regulate GC malignancy through TXNDC5. To verify this hypothesis, we performed rescue experiments by overexpressing TXNDC5 in ALKBH5-knockdown cells. The results showed that restoring TXNDC5 partially reversed the reductions in cell proliferation and migration caused by ALKBH5 knockdown. These findings strongly support the existence of a direct upstream – downstream relationship between ALKBH5 and TXNDC5. Confirming similar results in other cell lines or tissues could further validate the broader relevance of this signaling axis.

ALKBH5 is a key demethylase involved in m6A RNA modification that selectively removes m6A methylation marks from RNA transcripts, thereby controlling their expression [20]. Based on this known function, we evaluated TXNDC5 mRNA stability after knocking down ALKBH5. Our data indicated that ALKBH5 depletion reduced TXNDC5 mRNA stability, resulting in lower protein expression and decreased the capacities of cell proliferation and invasion. Additionally, MeRIP-qPCR experiments confirmed that ALKBH5 directly regulated TXNDC5 mRNA methylation. Specifically, knocking down ALKBH5 increased m6A methylation levels on TXNDC5 transcripts, leading to accelerated transcript degradation. Conversely, overexpressing ALKBH5 improved TXNDC5 transcript stability. Previous studies reported that mutating the 204th amino acid residue (H204A) of ALKBH5 abolishes its demethylation activity [34]. In our study, we constructed an ALKBH5 H204A mutant and found that cells expressing this mutant showed significantly reduced TXNDC5 protein levels compared with wild-type ALKBH5 cells. Furthermore, these mutant cells exhibited weaker proliferation, migration, and invasion capabilities. These experiments strongly demonstrated that ALKBH5 promoted GC malignancy at least partially through demethylating and stabilizing TXNDC5 mRNA.

Our findings provided a representative example of how epigenetic modifications influence cancer development. m6A modification is a key post-transcriptional regulatory mechanism maintained by methyltransferases (“writers”), demethylases (“erasers”), and binding proteins (“readers”) [18]. ALKBH5, acting as an eraser, precisely controls mRNA stability, splicing, and translation efficiency [20]. Some previous studies had shown that ALKBH5 could suppress tumor growth in certain cancers [32], whereas others have indicated that it promotes cancer progression [21,35]. Our study clearly demonstrated that ALKBH5-mediated demethylation of TXNDC5 promotes GC progression. This finding expands our understanding of ALKBH5’s diverse roles across different cancers.

Currently, GC treatment mainly relies on surgery, chemotherapy, radiotherapy, and emerging targeted and immunotherapy strategies, but patient outcomes remain unsatisfactory. Identifying novel therapeutic approaches, such as targeting ALKBH5, could provide important breakthroughs. Based on our results, developing specific small-molecule inhibitors against ALKBH5 or using gene-editing techniques to reduce ALKBH5

activity may effectively decrease TXNDC5 and other downstream oncogenic targets. These approaches could potentially reduce tumor growth and metastasis in GC.

However, our study had several limitations. First, we mainly used in vitro cell lines and subcutaneous xenografts, which may not fully represent complex clinical conditions such as metastasis and immune interactions. Future studies should include orthotopic or patient-derived xenograft (PDX) models to better mimic clinical disease progression. Second, although we analyzed clinical samples and databases, the sample size was relatively small. Given the substantial molecular heterogeneity of GC, larger multi-center studies will be required to enhance the generalizability of our findings. Finally, although we confirmed TXNDC5 as a critical ALKBH5 target, ALKBH5 likely regulates other transcripts as well. Future studies using multi-omics techniques, such as single-cell sequencing, RIP-seq, and proteomics, could better define the full range of ALKBH5-regulated targets.

In summary, our study demonstrates that ALKBH5 promotes GC progression through demethylation and stabilization of TXNDC5 transcripts. Our experiments provide valuable insights into epigenetic regulation in cancer and lay an important foundation for future research on targeted therapies. Although further validation is required, our findings offer a significant step toward translating epigenetic mechanisms into improved clinical outcomes for patients with GC.

## 5. Conclusion

In summary, our study demonstrates that ALKBH5 promotes gastric cancer progression by regulating TXNDC5 expression in an m6A-dependent manner. Overexpression of ALKBH5 enhances TXNDC5 stability and facilitates malignant phenotypes, whereas ALKBH5 depletion increases m6A methylation, suppresses TXNDC5 expression, and inhibits tumor growth both in vitro and in vivo. These findings identify the ALKBH5–TXNDC5 axis as a critical driver of gastric cancer progression and suggest ALKBH5 as a promising therapeutic target for future clinical intervention.

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## Author contributions

Wenkun Peng: Conceptualization, Data curation, Formal analysis, Visualization, Investigation, Software, Methodology, Writing – original draft, Writing – review & editing, Project administration. Xiaoquan Wei: Investigation, Visualization, Writing – review & editing. Feifei Zhou: Writing – review & editing. Hongwei Xu: Project administration, Resources, Funding acquisition, Supervision, Writing – review & editing.

## Disclosure statement

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes

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## Ethical Declaration

Ethics approval and consent were obtaining from clinical patients. The study was conducted in accordance with the Declaration of Helsinki.

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## Data availability statement

The datasets analyzed during this study are available in the TCGA database (<https://portal.gdc.cancer.gov>).

The participants of this study did not give written consent for their data to be shared publicly, so due to the sensitive nature of the research supporting data is not available.

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