Neoplasia 36 (2023) 100863

Contents lists available at ScienceDirect

Neoplasia

journal homepage: www.elsevier.com/locate/neo

FOXM1 increases hTERT protein stability and indicates poor prognosis in gastric cancer

Qingyun Tang[1], Cheng Liu[1], Shengwei Zhang, Lijiao He, Yunyi Liu, Jing Wang, Xiaoyan Zhao*, Xinzhe Li*

Department of Gastroenterology, Xinqiao Hospital, Third Military Medical University, Chongqing, 400037, China

ARTICLE INFO

Editor name: Prof Alnawaz Rehemtulla

Keywords:
Gastric cancer
FOXM1
hTERT
ubiquitination
Wnt/β-catenin

ABSTRACT

Gastric cancer is one of most lethal diseases across the world. However, the underlying mechanism of gastric cancer carcinogenesis and development is still not fully known. Forkhead box M1 (FOXM1) belongs to the FOX family and has crucial roles in transactivation of multiple oncogenes in several cancer types, including gastric cancer. Recent studies have also shown the non-transcriptional function of FOXM1 via protein–protein interactions. Human telomerase reverse transcriptase (hTERT) is the core subunit of telomerase that facilitates cancer initiation and progression by maintaining cell immortalization, promoting cell proliferation and inhibiting cell apoptosis. However, the relationship between FOXM1 and hTERT in gastric cancer is still unclear. In our study, we found that FOXM1 and hTERT were convergent to the cell cycle-related pathways and they were positively related with advanced gastric cancer stages and poor outcomes. Simultaneous high levels of FOXM1 and hTERT predicted the worst prognosis. FOXM1 could increase hTERT protein rather than mRNA levels in a non-transcriptional manner. Mechanistically, FOXM1 interrupted the interaction between the E3 ligase MKRN1 and hTERT and decreased hTERT protein degradation. Further studies revealed that FOXM1 interacted with hTERT through its DNA-binding domain (DBD) region. Finally, we found that hTERT played important roles in FOXM1-mediated activation of the Wnt/β-catenin pathway to promote gastric cancer cell proliferation. Taken together, we found a novel non-classical function of FOXM1 to increase hTERT protein stability. Targeting the FOXM1-hTERT pathway may be a potential therapeutic strategy in treating gastric cancer.

Introduction

Gastric cancer is one of the leading causes of cancer death worldwide. Several therapeutic strategies have been used for the treatment of advanced gastric cancer or early gastroesophageal carcinoma, such as the traditional and adjuvant chemotherapy, or surgical and endoscopic resection [1,2]. Recently, the gastric microbiota and disordered immune function have been implicated in gastric cancer therapy. Besides, novel materials have also been used in endoscopic submucosal dissection [3–5]. Although *H. pylori* infection is an important risk factor for gastric cancer, the underlying mechanism of gastric carcinogenesis and tumor progression is not entirely known. A currently recognized molecular characterization for gastric adenocarcinoma dictated four molecularly distinct gastric cancer subtypes [6]. Each of the subtypes has unique characteristics, such as recurrent PIK3CA mutations, RHOA mutations or focal amplification of receptor tyrosine kinases. Therefore, the detailed molecular mechanism for gastric cancer initiation and progression still requires further investigation.

Forkhead box M1 (FOXM1) is a versatile transcription factor that belongs to the FOX family [7]. It is involved in multiple cellular processes such as cell proliferation, differentiation and apoptosis. FOXM1 is highly expressed in several cancer types and promotes cancer angiogenesis, invasion and therapy resistance, which result in a poor prognosis [8]. The FOXM1 protein consists of an N-terminal repressor domain (NRD), a forkhead DNA-binding domain (DBD), and a C-terminal transcriptional activation domain (TAD) [9]. Among the four FOXM1 splice variants, FOXM1b has a relatively higher transforming ability [10]. FOXM1 is a critical regulator of numerous downstream targets, which contribute to almost all cancer hallmarks [8]. FOXM1 has been closely linked to gastric cancer. For instance, FOXM1 is overexpressed in gastric cancer, and its inhibition induces cellular senescence [11]. Dysregulation of FOXM1 or the FOXM1-STMN1 axis contributes to gastric cancer progression [12,13]. In addition, FOXM1 mediates the biological processes of other upstream factors in gastric cancer, such as MET signaling molecules [14]. Besides, Hu et al. revealed that FOXM1 elicited oxaliplatin resistance by upregulating Mcl-1 in gastric cancer cells [15].

* Corresponding authors.
E-mail addresses: zhaoxiaoyan2018@126.com (X. Zhao), lixinzhe73@126.com (X. Li).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.neo.2022.100863
Received 31 May 2022; Received in revised form 29 November 2022; Accepted 2 December 2022

Interestingly, FOXM1 is able to influence biological process in a transcription-independent manner by interacting with different proteins [16]. For instance, the interaction between FOXM1 and SMAD3/SMAD4 favors the TGF-β-dependent signaling to promote cancer metastasis [17].

Human telomerase reverse transcriptase (hTERT) is the catalytic subunit of telomerase. hTERT plays pivotal roles in cancer initiation, progression, metastasis and therapy resistance [18,19]. It has been shown that hTERT is positively associated with VEGF in gastric cancer samples. hTERT increases VEGF gene expression by interacting with the transcription factor Sp1 [20]. hTERT inhibition activated p38 MAPK, which stimulated COX2 transcription, and the combination of hTERT depletion and celecoxib synergistically suppressed gastric cancer cells [21]. Besides, the clinical drug proton pump inhibitor (PPI) pantoprazole repressed gastric cancer metastasis by downregulating hTERT, which is worthy of further clinical investigation [22]. It has been previously demonstrated that hTERT promoted the binding of FOXM1 to its binding elements in the ITGB1 gene. In addition, hTERT enhanced the ubiquitination of another FOX family member, FOXO3a, and finally facilitated the invasion of gastric cancer cells [23]. However, whether FOXM1 also regulates hTERT in gastric cancer and the potential mechanism are currently unknown. In this study, we found that high expression of both FOXM1 and hTERT dictates a poor prognosis in gastric cancer patients. Mechanically, FOXM1 could non-canonically upregulate hTERT level in a non-transcriptional manner which is independent of the classical transactivating function of FOXM1. In addition, hTERT plays a crucial role in FOXM1-elicited Wnt/β-catenin activation. Targeting the FOXM1-hTERT axis may be a novel strategy for the prevention and treatment of gastric cancer.

Results

FOXM1 and hTERT share common downstream genes but their mRNA levels are not correlated

To explore the relationship between FOXM1 and TERT, we first analyzed their expressions in 34 types of human cancer. As shown in Fig. 1A, FOXM1 was significantly upregulated in 33 cancer types, while TERT was markedly increased in 29 cancer types compared with normal tissues. Besides, high levels of FOXM1 or TERT was positively with advanced pathologic stages and histologic grades in gastric cancers (Figure S1A-S1D). The receiver operating characteristic (ROC) curve also indicated an obvious positive relationship between high FOXM1 or TERT expressions with advanced gastric cancer pathologic stages (Figure S1E and S1F). Subsequently, by comparing the top 25% samples with higher FOXM1 (or TERT) expression with the bottom 25% samples with lower FOXM1 (or TERT) expression, we identified 344 (or 156) upregulated genes, respectively (Fig. 1B-C). The Venn diagram showed that 30 genes were collectively upregulated between the FOXM1 and TERT groups (Fig. 1D). In addition, GO term and KEGG pathway enrichment analyses of the overlapping genes revealed that the FOXM1 and TERT-upregulated genes are involved in the cell cycle and meiosis process (Fig. 1E and F). Interestingly, the mRNA level of FOXM1 and hTERT was not significantly correlated in TCGA Stomach Adenocarcinoma (STAD), suggesting the importance of post-transcriptional regulation in association between FOXM1 and hTERT (Fig. 1G). These results indicated that FOXM1 was associated with hTERT during cell cycle process and the mechanism may lie in the post-transcriptional level.

FOXM1 enhanced hTERT protein stability by inhibiting ubiquitination-mediated degradation

We next explored whether FOXM1 could regulate hTERT expression and the underlying mechanism. Knockdown of FOXM1 decreased the protein level of hTERT (Fig. 2A and S2A), whereas overexpression of FOXM1 increased the protein level of hTERT (Fig. 2C and S2B) in gastric cancer cell lines. However, either knockdown or overexpression of FOXM1 did not affect the mRNA levels of hTERT (Fig. 2B, and D and S2C-S2F) at different time points. The above evidences suggested that FOXM1 may upregulate hTERT by enhancing its protein stability post-transcriptionally. Ubiquitination is crucial in post-transcriptional regulation. Thus, we investigated whether FOXM1 influenced hTERT ubiquitination. As shown in Fig. 2E, overexpression of FOXM1 dramatically inhibited hTERT ubiquitination. Besides, MG132 reverses the effect of FOXM1 loss on hTERT levels, revealing that endogenous FOXM1 regulated endogenous hTERT exactly by the ubiquitin-proteolysis mechanism (Fig. 2F). The E3 ligases MDM2 and MKRN1 have been shown to ubiquitinate hTERT and reduce its protein stability [24,25]. To further uncover which E3 ligase was involved in FOXM1-mediated suppression of hTERT ubiquitination, Co-IP assays were performed. Results indicated that while FOXM1 did not affect the interaction between MDM2 and hTERT (Fig. 2G), FOXM1 significantly reduced the interaction between MKRN1 and hTERT (Fig. 2H). In addition, we found that FOXM1 and hTERT interacted with each other (Fig. 2I). In summary, FOXM1 enhanced hTERT protein stability and increased it protein level by inhibiting MKRN1-mediated ubiquitination.

FOXM1 could increase hTERT protein levels independent of its transcriptional activity

FOXM1 canonically regulates downstream signaling as a transcription factor. We have shown that FOXM1 could enhance hTERT protein stability by inhibiting ubiquitination-mediated degradation. To further investigate how FOXM1 performed this function, FOXM1-Mut was constructed by mutating three key amino acids within the DBD of wild-type FOXM1b (Fig. 3A) ([26,27]). Next, we constructed a luciferase reporter plasmid containing six copies of the Forkhead consensus sequence (AAACAAACAAAC), which could indicate the transcriptional activity of FOXM1. Fig. 3B showed that FOXM1-Mut had remarkably attenuated transcriptional activity compared to FOXM1-WT. In addition, the promoter activity of CCNB1, a FOXM1 target gene, was also suppressed by FOXM1-Mut, which also demonstrated that this mutant is defective for its transcriptional activating function (Fig. 3C). However, FOXM1-Mut could evidently enhance hTERT protein level in gastric cancer cells (Fig. 3D). Additionally, FOXM1-Mut, similarly as FOXM1-WT, was able to interfere with the interaction between hTERT and MKRN1 but not between hTERT and MDM2 (Fig. 3E and F). Moreover, both FOXM1-Mut and FOXM1-WT strongly inhibited the ubiquitination of hTERT (Fig. 3G). Taken together, FOXM1 could suppress MKRN1-mediated hTERT ubiquitination and increase hTERT protein levels independent of its canonical transcriptional activity.

hTERT played key roles in FOXM1-mediated activation of Wnt/β-catenin pathway

Wnt/β-catenin is one of the crucial pathways that promotes cancer cell proliferation via governing cell cycle-associated markers. FOXM1 has been shown to activate the Wnt/β-catenin pathway [16,28]. As shown in Fig. 4A, FOXM1-Mut increased the mRNA levels of c-myc, cyclin-D1 and proliferating cell nuclear antigen (PCNA), three key proliferation-related markers of the Wnt/β-catenin pathway ([29,30]). Knockdown of hTERT significantly reversed the impact of FOXM1-Mut-mediated activation of Wnt/β-catenin pathway (Fig. 4A). In addition, although FOXM1-Mut did not elevate the Wnt/β-catenin-governed proliferation markers compared with the Control group in hTERT-negative osteosarcoma U2OS cells (Fig. 4B), FOXM1-Mut significantly upregulated these markers in hTERT-overexpressing U2OS cells (Fig. 4B). Furthermore, analysis of Gastric Cancer data from the TCGA also revealed the positive relationship between FOXM1 or TERT with the Wnt/β-catenin signaling genes (Fig. S3A). Finally, we found that FOXM1-Mut remarkably promoted the colony formation of gastric cancer cells, while

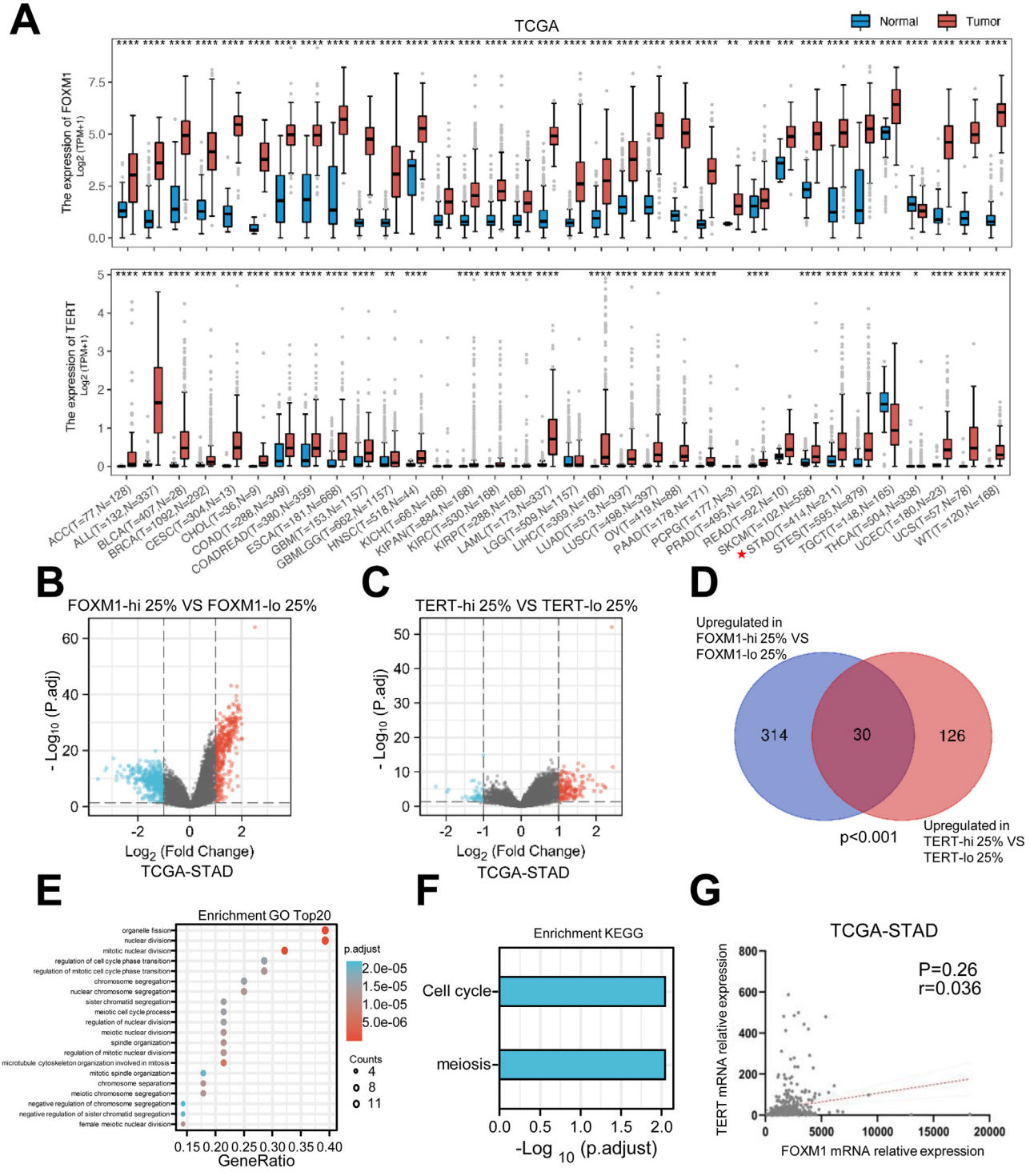

Fig. 1. FOXM1 and hTERT share common downstream genes but their mRNA levels are not correlated. (A) Expression of FOXM1 and TERT across 34 cancer types and the corresponding normal tissues using data from The Cancer Genome Atlas (TCGA) database. Data was analyzed by the Wilcoxon test. (B) Volcano plots that displays differentially expressed genes between samples with higher FOXM1 expression (top 25%) and lower FOXM1 expression (bottom 25%) from the TCGA gastric cancer database (Stomach Adenocarcinoma, STAD). Red represents the upregulated genes, and blue represents the downregulated genes. (C) Volcano plots that shows the expression of TERT as that in (B). (D) Venn diagram illustrating the overlapping upregulated genes between FOXM1 and TERT by analyzing the data of STAD from (B) and (C). The probability distribution of the overlapping genes was done based on the Poisson Distribution. The overlapping genes were displayed in Supplementary Table 3. (E and F) GO term analysis (E) and KEGG pathway enrichment analysis (F) of the overlapping upregulated genes in (D) from STAD. (G) The correlation between FOXM1 mRNA and TERT mRNA by analyzing the TCGA-STAD. Data was analyzed by Pearson's test.

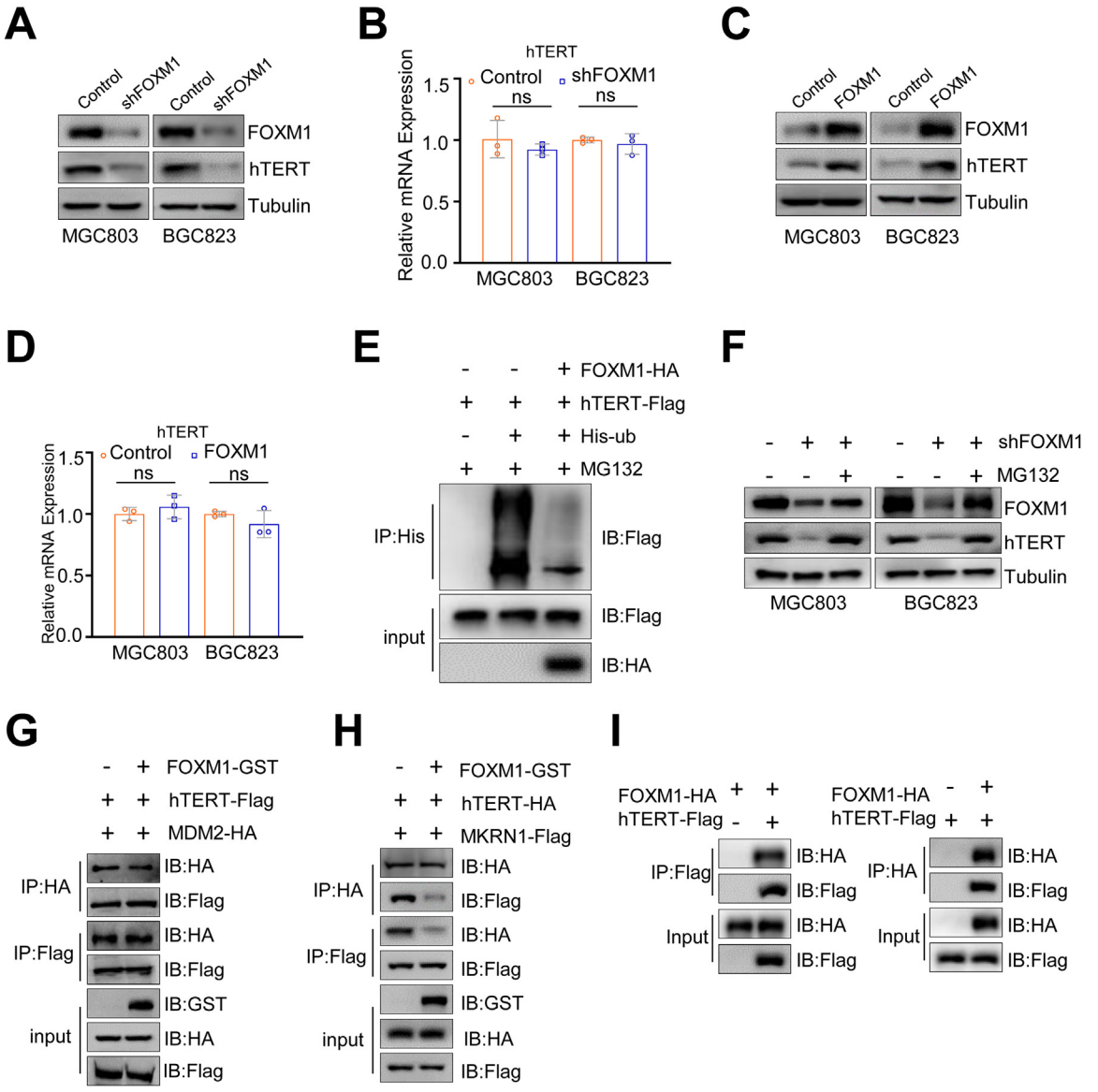

Fig. 2. FOXM1 enhanced hTERT protein stability by inhibiting ubiquitination-mediated degradation. (A or C) Different gastric cancer cells (MGC803 and BGC823) were transfected with shFOXM1 plasmids (A) or FOXM1 overexpressing plasmids (C) for 48h, then Western blot were performed with the antibody against FOXM1, hTERT or Tubulin. (B or D) Different gastric cancer cells (MGC803 and BGC823) were transfected with shFOXM1 plasmids (B) or FOXM1 overexpressing plasmids (D) for 48h, then qRT-PCR were performed to detect the mRNA level of hTERT or Tubulin. The relative levels of hTERT mRNA were shown. (E) HEK293T cells were transfected with His-ub and/or FOXM1-HA in the presence of hTERT-Flag, and then incubated with MG132 for another 8 h before cells were harvested. After that ubiquitination of hTERT was detected with anti-Flag antibody following His immunoprecipitation. The input was detected with anti-Flag or anti-HA antibody, respectively. (F) MGC803 and BGC823 cells were transfected with shNC or shFOXM1 for 48 hours, with or without MG132 added for another 8 hours before harvesting. Then Western blot were performed with the antibody against FOXM1, hTERT or Tubulin. (G) HEK293T cells were transfected with hTERT-Flag and MDM2-HA with or without FOXM1-GST, then co-immunoprecipitation (Co-IP) assays were performed using the HA and Flag antibody. Western Blot was performed using the immunoprecipitates or input with the antibody against HA, Flag or GST. (H) HEK293T cells were transfected with hTERT-HA and MKRN1-Flag with or without FOXM1-GST, then Co-IP assays were performed using the HA and Flag antibody. Western Blot was performed using the immunoprecipitates or input with the antibody against HA, Flag or GST. (I) HEK293T cells were transfected with FOXM-HA or hTERT-Flag or their combinations, then Co-IP assays were performed using the HA or Flag antibody. Western Blot was performed using the immunoprecipitates or input with the antibody against HA or Flag.

silencing hTERT attenuated the proliferative ability of FOXM1-Mut (Fig. 4C). In summary, these findings provided evidence that hTERT played key roles in FOXM1-mediated upregulation of Wnt/β-catenin-governed proliferation markers.

FOXM1 interacted with hTERT through its DBD region

To further investigate the detailed region of FOXM1 protein that mediated the FOXM1-hTERT interaction, we constructed the N-terminal deleted, DBD deleted and C-terminal deleted construct on the basis of FOXM1-Mut, respectively (Fig. 5A). The interaction between FOXM1 and hTERT was dramatically decreased when the DBD domain was truncated, while the deletion of N-terminal or C-terminal had no influence (Fig. 5B). Besides, only the DBD-truncated construct remarkably increased the ubiquitination of hTERT and promoted the its protein degradation (Fig. 5C and D). In addition, DBD deletion attenuated the Wnt/β-catenin pathway genes compared with the full-length vector (Fig. 5E). Finally, cell colony formation assays also confirmed the

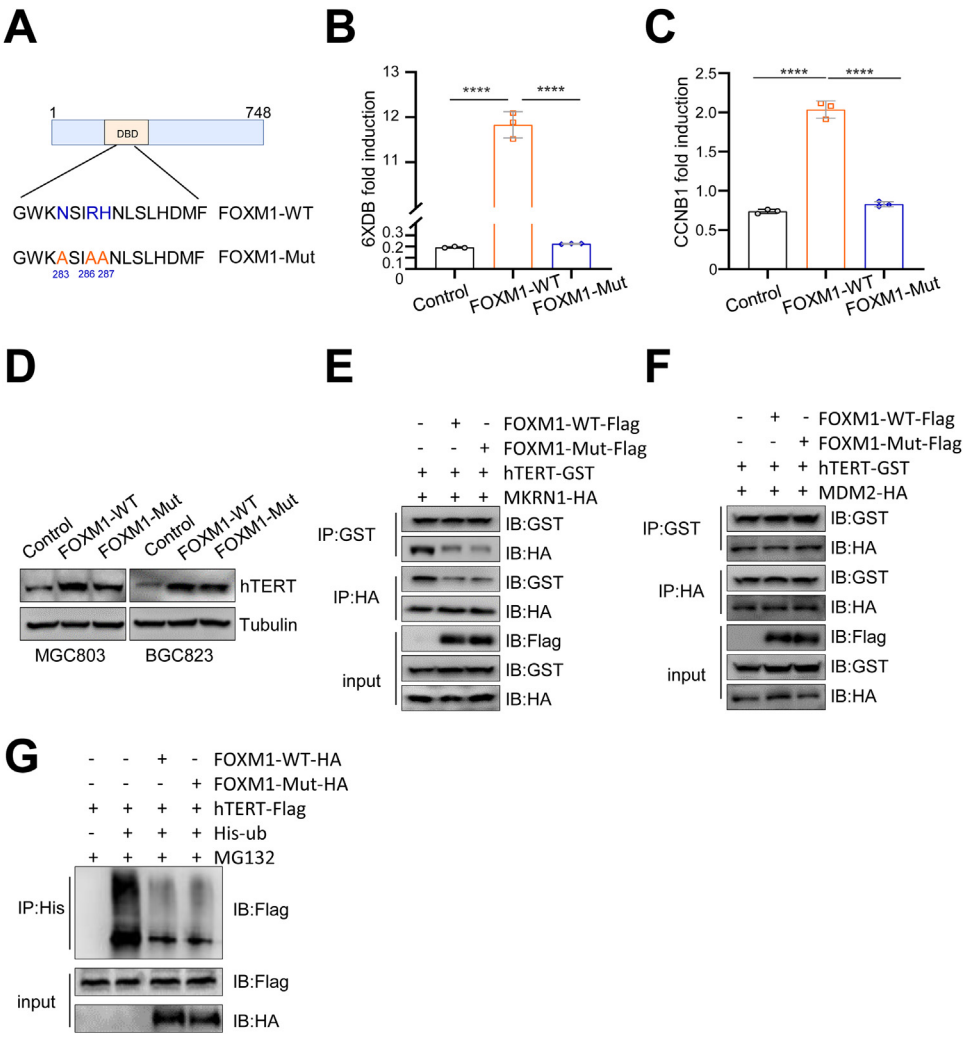

Fig. 3. FOXM1 could increase hTERT protein levels independent of its transcriptional activity. (A) A schematic depiction of the transcription active site and the mutation of FOXM1. (B) The activity of a 6xDB luciferase reporter in HEK293T cells transfected with wild-type (WT) or mutant (mut) FOXM1. (C) The luciferase reporter assays for CCNB1 promoter activity in HEK293T cells transfected with WT or mut FOXM1. (D) MGC803 and BGC823 cells were transfected with FOXM1-WT or FOXM1-Mut, then Western Blot analysis was performed with the antibody against hTERT or Tubulin. (E) HEK293T cells were transfected with hTERT-GST and MKRN1-HA, with or without the co-transfection of FOXM1-WT-Flag or FOXM1-Mut-Flag, then Co-IP was performed using the GST or HA antibody. Western Blot was performed using the immunoprecipitates or input with the antibody against GST, HA or Flag. (F) HEK293T cells were transfected with hTERT-GST and MDM2-HA, with or without the co-transfection of FOXM1-WT-Flag or FOXM1-Mut-Flag, then Co-IP was performed using the GST or HA antibody. Western Blot was performed using the immunoprecipitates or input with the antibody against GST, HA or Flag. (G) HEK293T cells were transfected with FOXM1-WT-HA or FOXM1-Mut-HA in the presence of His-ub and hTERT-Flag, and then incubated with MG132 for another 8 h before cells were harvested. After that ubiquitination of hTERT was detected with anti-Flag antibody following His immunoprecipitation. The input was detected with anti-Flag or anti-HA antibody, respectively.

inhibitory effect of DBD loss on FOXM1-mut-driven cancer cell proliferation (Fig. 5F). Taken together, FOXM1 interacted with hTERT through its DBD region to promote cancer cell proliferation.

High levels of FOXM1 and hTERT indicated poor prognosis in gastric cancer samples

We further analyzed the clinical association of FOXM1 and hTERT by analyzing the immunofluorescence staining microarray data of human gastric cancer tissue. The ROC curves showed the areas under the curve (AUC) of the FOXM1-based and hTERT-based prediction, which suggested that both FOXM1 and hTERT could effectively predict prognosis of gastric cancer patients (Fig. 6A and B). Moreover, survival analysis revealed that high expression of FOXM1 or hTERT was associated with poor prognosis of gastric cancer patients (Fig. 6C and D). Importantly, simultaneous high expression of both FOXM1 and hTERT indicated the worst prognosis, whereas simultaneous low expression of them indicated the best prognosis (Fig. 6E). Taken together, high levels of FOXM1 and hTERT indicated poor prognosis in gastric cancer samples.

Discussion

Both FOXM1 and hTERT are well recognized for their versatile functions in promoting cancer malignancies. However, the relationship between the two key factors is still not clear. In this study, we unraveled that FOXM1 was able to enhance hTERT protein in a non-transcriptional way in gastric cancer cells. FOXM1 is described as a functional transcription factor that transactivates multiple oncogenes. In addition, emerging evidence has shown that FOXM1 also influences cancer development by direct protein-protein interactions [16]. For instance, the interaction between nucleophosmin (NPM) and FOXM1 plays important roles in maintaining FOXM1 in the cytoplasm and in maintaining the levels of FOXM1 [31,32]. In addition, MELK regulates FOXM1 phosphorylation and activation, which is dependent on the direct binding of PLK1 with FOXM1 [33]. Furthermore, the interaction between FOXM1 and SMAD3 is crucial for SMAD3/SMAD4 complex function and maintains the TGFβ signaling pathway during breast cancer invasion [17]. In our study, we also found that FOXM1 could exert a transcription-independent function to upregulate hTERT protein levels rather than mRNA levels by interacting with the E3 ligase MKRN1, which has been reported to degrade hTERT by the ubiquitination-proteasome pathway [25]. Besides, hTERT was pivotal for FOXM1-enhanced expression of Wnt/β-catenin-governed proliferation markers. Recent studies have shown that hTERT directly enhances the Wnt/β-catenin pathway as a cofactor in the β-catenin transcriptional complex [34], which supported our results. Previous studies showed that FOXM1 could transactivate c-myc [35], and c-myc was also able to transcriptionally upregulate hTERT [36]. In our study, c-myc mRNA was upregulated by FOXM1-Mut. The mechanism mainly lied in that FOXM1-Mut increased hTERT protein levels and hTERT activated the Wnt/β-catenin pathway to upregulate c-myc. It was because FOXM1-Mut is transcriptionally incapable. Besides, the decrease of

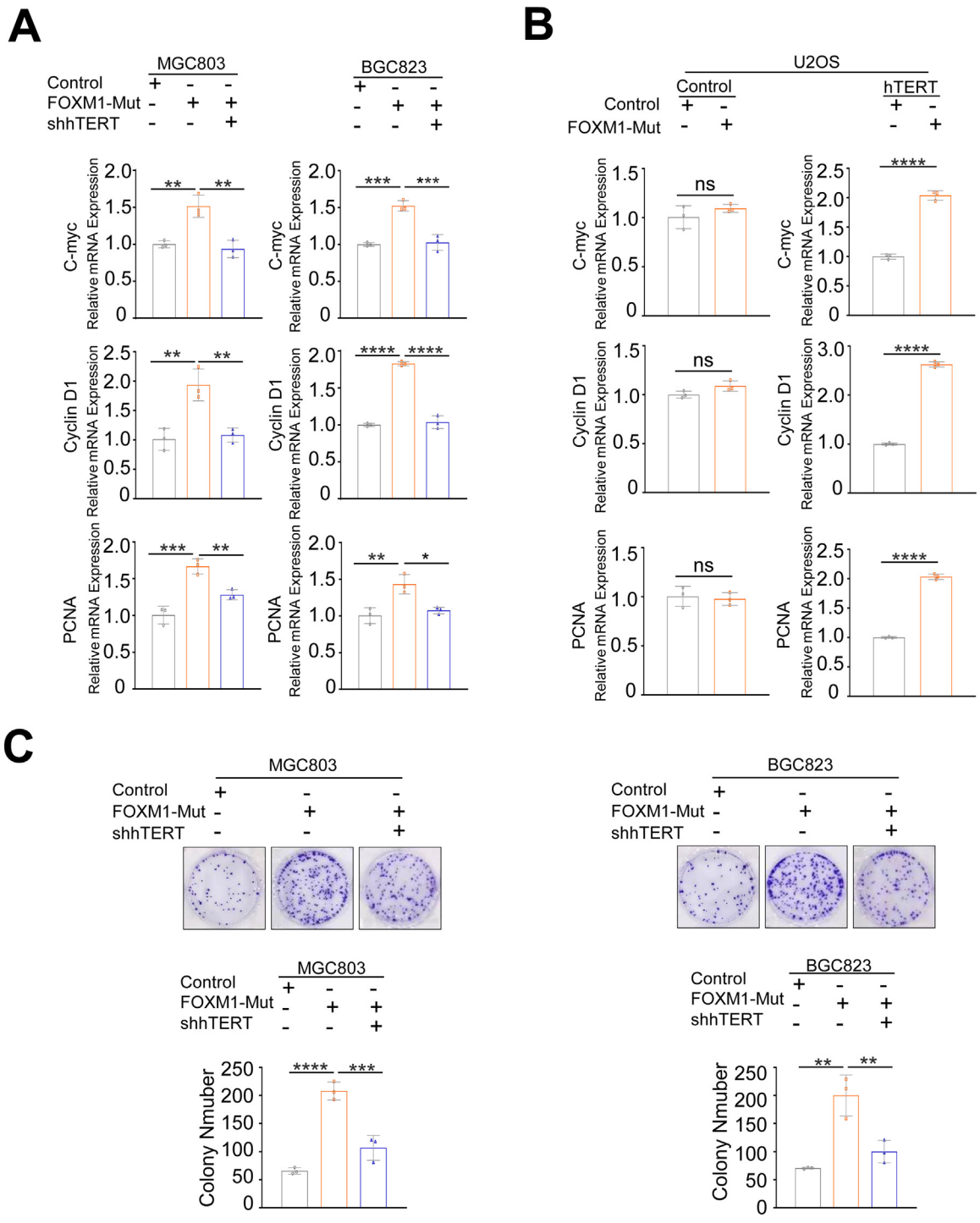

Fig. 4. hTERT played key roles in FOXM1-mediated activation of Wnt/β-catenin pathway. (A) MGC803 and BGC823 cells were transfected with FOXM1-Mut with or without the co-transfection of shhTERT, then Wnt/β-catenin pathway genes were analyzed by qRT-PCR. (B) U2OS cells with or without hTERT-overexpressing were transfected with FOXM1-Mut, then Wnt/β-catenin pathway genes were analyzed by qRT-PCR. (C) MGC803 and BGC823 cells were transfected with FOXM1-Mut with or without the co-transfection of shhTERT, then colony formation assays were performed (top). The number of cell colonies was quantified (bottom).

c-myc mRNA by hTERT knockdown (Fig. 4A) was comparable to that by the DBD region deletion (Fig. 5E). Although c-myc mRNA level was increased by FOXM1-Mut, the hTERT mRNA level was not significantly upregulated in our cellular context. It can be explained that there were a plenty of hTERT upstream regulators, and FOXM1-mut may also upregulate other hTERT suppressors or downregulate other hTERT activators. Taken together, our data revealed an important alternative regulation manner of FOXM1 to enhance hTERT protein levels and may broaden the knowledge of non-classical FOXM1 function. Importantly, the FOXM1-hTERT axis was clinically relevant, because high levels of both FOXM1 and hTERT predicted the worst outcome in gastric cancer patients. Interestingly, we found that the overlapping genes of FOXM1 and TERT-upregulated targets were also involved in the meiosis process. Currently, there were few studies about the relationship between FOXM1 or hTERT with meiosis [37–39], which is worthy of further investigating.

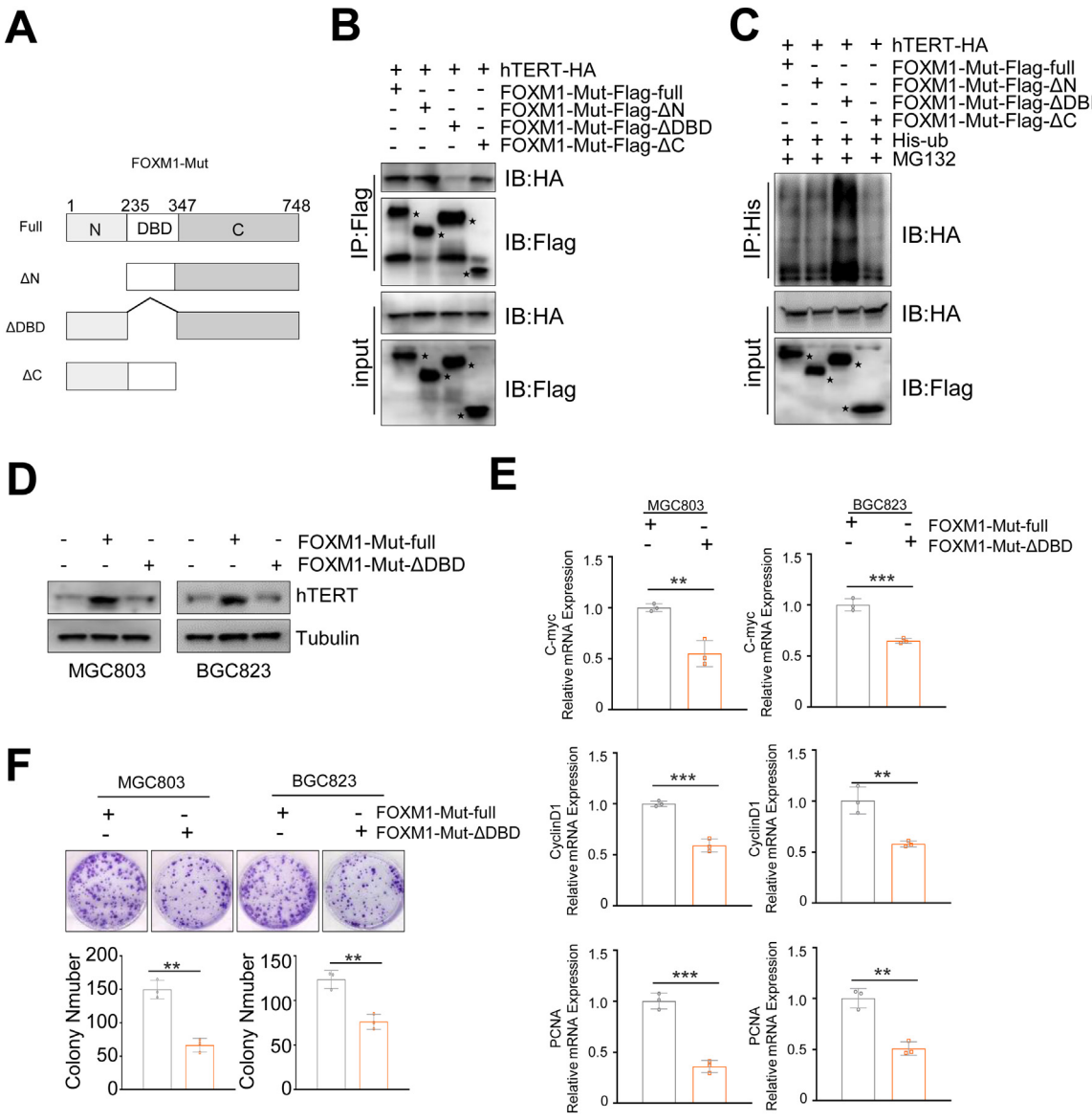

Fig. 5. FOXM1 interacted with hTERT through its DBD region. (A) A schematic depiction of the full-length and different truncated form (N-terminal deleted, DBD deleted and C-terminal deleted) of FOXM-mut protein. (B) HEK293T cells were transfected with hTERT-HA and different form of FOXM1-Mut-Flag constructs shown in (A), then Co-IP was performed using the Flag antibody. Western Blot was performed using the immunoprecipitates or input with the antibody against HA or Flag. (C) HEK293T cells were transfected with His-ub, hTERT-HA, and different form of FOXM1-Mut-Flag constructs shown in (A), then cells were incubated with MG132 for another 8 h before harvested. After that ubiquitination of hTERT was detected with anti-HA antibody following His immunoprecipitation. The input was detected with anti-HA or anti-Flag antibody, respectively. (D) MGC803 and BGC823 cells were transfected with full-length FOXM1-Mut or DBD-truncated FOXM1-Mut, then Western Blot analysis was performed with the antibody against hTERT or Tubulin. (E) MGC803 and BGC823 cells were transfected with full-length FOXM1-Mut or DBD-truncated FOXM1-Mut, then Wnt/β-catenin pathway genes were analyzed by qRT-PCR. (F) MGC803 and BGC823 cells were transfected with full-length FOXM1-Mut or DBD-truncated FOXM1-Mut, then colony formation assays were performed (top). The number of cell colonies was quantified (bottom).

hTERT expression is frequently observed in most human malignancies, and high levels of hTERT are linked to advanced tumor staging and unfavorable prognosis. Our group has summarized the regulation of hTERT from different layers, including the transcriptional and post-transcriptional levels, etc. [40]. Ubiquitination is one of the core regulatory avenues at the post- transcriptional level. For instance, MDM2 ubiquitinates hTERT at the N-terminus and promotes its degradation in a proteasome-dependent manner [24]. MKRN1 overexpression facilitates hTERT degradation and attenuates telomerase activity [25]. Interaction between hTERT and the chaperone-associated ubiquitin ligase CHIP promotes hTERT cytoplasm retention and ubiquitination [41]. TERT interacts with the EDD-DDB1-VprBP E3 ligase complex and is then degraded by the ubiquitination-proteasome pathway [42]. In our study, we found that FOXM1 could increase hTERT protein levels in a post-transcriptional manner. We further revealed that FOXM1 interfered with the binding of MKRN1 to hTERT, which interrupted ubiquitination-mediated hTERT degradation in gastric cancer cells. The phenotype was also obvious when the FOXM1 transcriptional activity was diminished. Interestingly, TERT promoter mutation, a frequent event in most cancer types, was observed at a low frequency in a large cohort of gastric cancers, which may suggest the importance of post-transcriptional regulation of hTERT in gastric cancer [43].

Taken together, our results established a novel link between the two important oncogenes in gastric cancers. FOXM1 could non-canonically increase hTERT protein levels by impeding the ubiquitination by E3 ligase MKRN1 and further repressing hTERT degradation. FOXM1 and

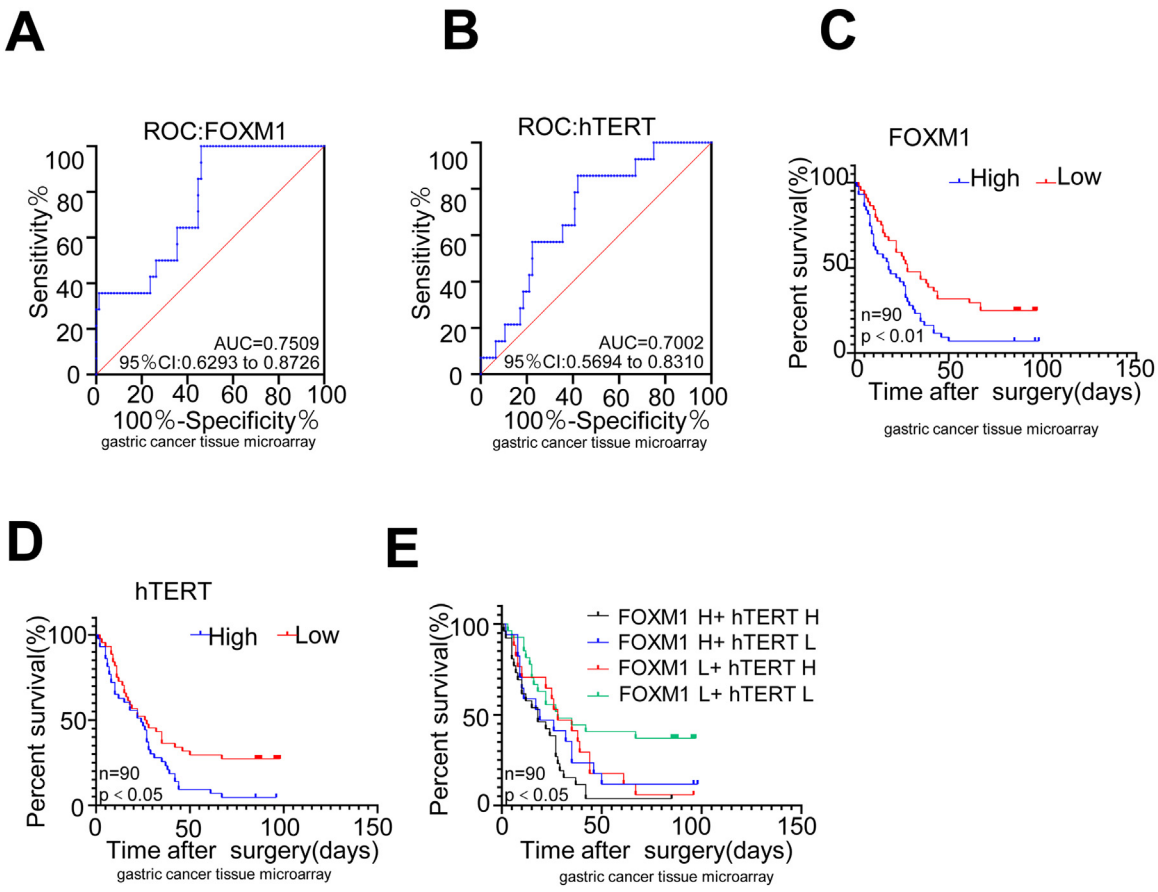

Fig. 6. High levels of FOXM1 and hTERT indicated poor prognosis in gastric cancer samples. (A, B) The ROC curves of FOXM1(A) or hTERT (B) for predicting the diagnosis of gastric cancer patients via analyzing the immunofluorescence staining microarray data of human gastric cancer tissue. (C, D) Kaplan-Meier analysis of patients survival according to different FOXM1 (C) or hTERT (D) expression. Log-rank (Mantel-Cox) test was used. (E) Kaplan-Meier analyses of patients survival according to different expression combinations of FOXM1 and hTERT. Log-rank (Mantel-Cox) test was used.

hTERT were both increased in human gastric cancer samples and their simultaneous upregulation predicted a poor prognosis. Notably, although both FOXM1 and hTERT have been shown to activate the Wnt/β-catenin signaling [28,34], we demonstrated that hTERT played an important role in FOXM1-mediated activation of Wnt/β-catenin pathway genes. In brief, our study provides novel rationale for gastric cancer treatment by targeting the FOXM1-hTERT axis.

Materials and methods

Cell culture and transfection

Cells were cultured in Gibco Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 5% CO_2 at 37 °C. The BGC823 and HEK293T cell lines were from American Type Culture Collection (Manassas, Virginia, USA). MGC803 and MKN45 cells were from Shanghai Fuheng Technology Co., Ltd. Cell lines were transfected using polyethyleneimine (PEI) and were collected at indicated time after transfection.

RNA extraction and qRT-PCR

Total RNA was extracted from cells using RNAiso Plus reagent (Takara, Otsu, Japan), and cDNA was synthesized using the PrimeScript RT reagent kit (Takara). Then quantitative real-time PCR (qRT-PCR) was carried out by using SYBR Premix Ex Taq II (Takara) with the ABI StepOnePlus 7500 Real-Time PCR system (Applied Biosystems). The primers used for qRT-PCR are listed in Supplementary Table 1.

Western blot and antibodies

Proteins were extracted with RIPA lysis buffer (P0013B, Beyotime, Shanghai, China) containing protease inhibitor (4693159001, Roche, Indianapolis, USA) and quantified by an enhanced BCA protein assay kit (P0009, Beyotime, Shanghai, China). Western blot analysis was performed according to a previously described standard protocol [23]. Anti-FOXM1 antibody (20459) was purchased from Cell Signaling Technology (Boston, USA). Anti-hTERT antibody (32020) was purchased from Abcam (Cambridge, UK). Anti-tubulin antibody (AF1216) was purchased from Beyotime. Anti-HA antibody (H6908) was purchased from Sigma-Aldrich (St. Louis, USA). Anti-Flag antibody (20543-1-AP) and anti-GST antibody (3G12B10) were purchased from Proteintech (Wuhan, China). These primary antibodies were used at a 1:1,000 dilution for immunoblotting analysis.

Luciferase reporter assays

The pGL4.26 [luc2/minP/Hygro] plasmid was used to generate the reporter containing six copies of the FKH binding consensus [44]. The pGL4.26-6xDB reporter plasmid and the pGL3-CCNB1 reporter plasmid was from Youbio Cooperation (Changsha, China). For reporter assays, pGL4.26-6xDB or pGL3-CCNB1 constructs and the pRL-TK plasmid were co-transfected into HEK293T cells with Lipofectamine 3000 (Invitrogen). Twenty four hours after transfection, the HEK293T cells were lysed and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, E1910). The luciferase activity was normalized to the pRL-TK activity.

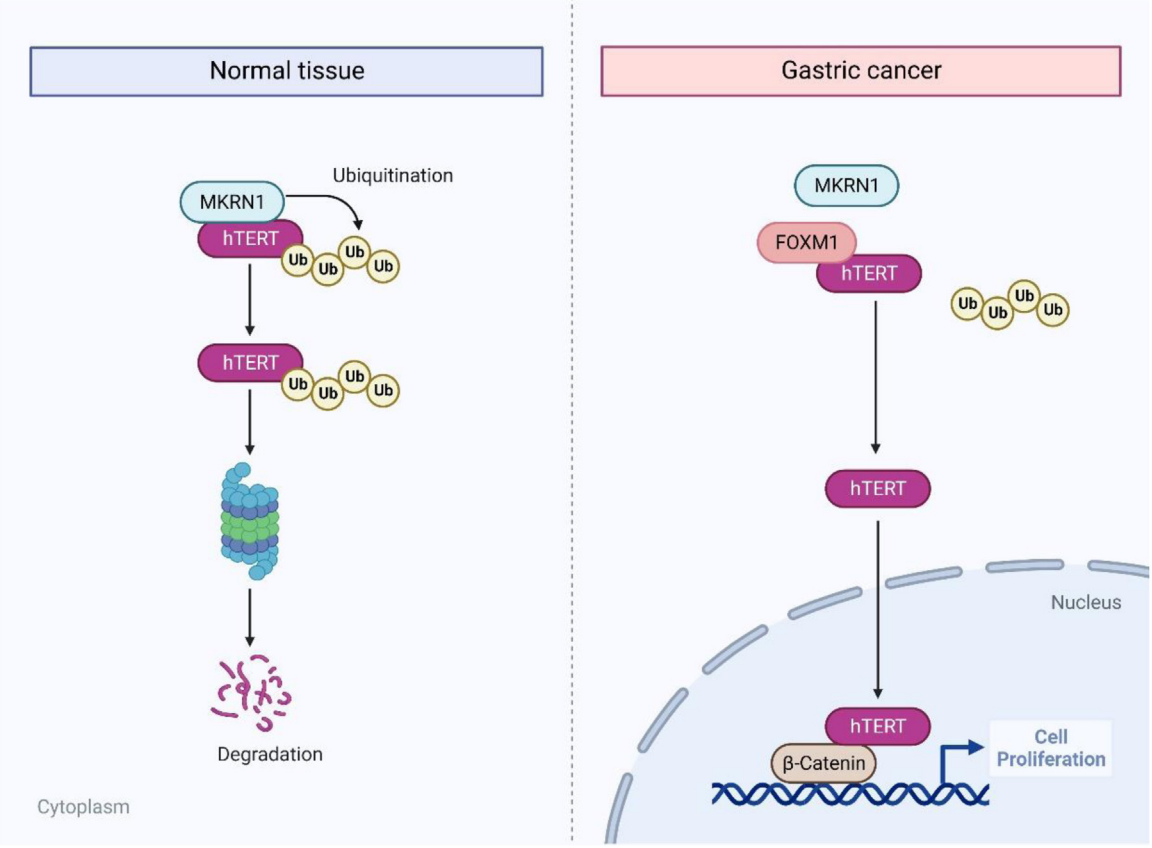

Fig. 7. A schematic model for the role of the FOXM1 to inhibit hTERT ubiquitination and degradation. (Left) In normal tissues, the E3 ubiquitin-protein ligase MKRN1 ubiquitinates hTERT and negatively regulates the hTERT protein stability. (Right) In gastric cancer tissues where FOXM1 is high, FOXM1 protein prevents the binding of hTERT with MKRN1 and stabilizes hTERT, which finally promotes expression of Wnt/β-catenin-downstream proliferation-associated genes to facilitate cancer cell proliferation.

Co-immunoprecipitation

Cells were lysed in EBC buffer (50 mM Tris pH 7.5, 120 mM NaCl, and 0.5% NP-40) supplemented with protease inhibitors. Then the lysates were incubated with anti-HA (Sigma, A2095), anti-Flag (Sigma, A2220) or anti-GST agarose beads (ThermoFisher, 16101) for 4 h at 4°C. The immunoprecipitates were washed three times with NETN buffer (20 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA and 0.5% NP-40) before being resolved by SDS-PAGE and immunoblotted with the indicated antibodies.

In vivo ubiquitination assay

His-ubiquitin and the indicated plasmids were transfected into HEK293T cells and treated with 20 μM MG132 for 8 h before harvesting. Thirty-six hours after transfection, the cells were lysed in buffer A (6 M guanidine-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, and 10 mM imidazole [pH 8.0]) and sonicated on ice. After centrifugation, the supernatants were incubated with nickel-nitrilotriacetic acid (Ni-NTA) matrices (QIAGEN, 30210) for 3 h at room temperature. Subsequently, the beads were successively washed twice with buffer A, twice with buffer A/TI (buffer A: buffer TI=1:3), and once with buffer TI (25 mM Tris-HCl and 20 mM imidazole [pH 6.8]). Then the immunoprecipitates were resolved by 8% SDS-PAGE and analyzed by immunoblotting using the indicated antibodies.

Colony formation assays

Cells were seeded at 600 cells per well in six-well plates and incubated for 10-16 days. Once visible colonies formed, the colonies were washed twice with PBS and fixed with 4% paraformaldehyde for 30 min. After staining with crystal violet solution for 30 min, the plates were washed and dried, and colony numbers were counted.

Tissue microarray and immunofluorescence

Tissue microarray of gastric cancer were purchased from Shanghai Outdo Biotech Co., Ltd., Shanghai, China. Detailed clinicopathological characterization is summarized in Supplementary Table 2. Standard Immunofluorescent (IF) staining procedures were performed using a specific antibody against FOXM1 (Abcam 207298) or hTERT (Abcam 230527) (1:50 dilution) for 1 hour incubation at room temperature [45]. All images were acquired using the TissueFAXS Spectra Systems by TissueGnostics and then analyzed by StrataQuest analysis software (TissueGnostics). The overall IF scores of FOXM1 or hTERT were calculated using the following formula: overall score = percentage of positive cells × mean fluorescence intensity of positive cells.

Gene expression and enrichment analysis

The pan-cancer TCGA dataset were used to analyze the expressions of FOXM1 and TERT across 34 cancer types as well as normal tissues. KEGG and GO enrichment analyses of differentially expressed genes were analyzed using the XIANTAO platform.

Identification of differentially expressed genes (DEGs)

Differentially expressed gene analysis was performed using the R package LIMMA (v3.40.2). To reduce false-positives, the adjusted

P value was used in the analysis. "Adjusted P < 0.05 and |log 2 (Fold Change)| ≥ 1" were defined as thresholds for the screening of differentially expressed mRNAs. The Venn diagram web tool (http://bioinformatics.psb.ugent.be/webtools/Venn/) were used to generate the Venn diagram.

Spearman's correlation analysis

Spearman's correlation analysis was performed with the Corrplot package. We downloaded the FPKM data matrix of TCGA-STAD RNA-seq data from the UCSC-Xena database. Then the HALLMARK_WNT_BETA_CATENIN_SIGNALING entry was obtained from the MSigDB database. Spearman's correlation was used to analyze the association between TERT or FOXM1 expression and WNT pathway-related genes.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software 9.0. Comparisons between groups normally distributed were determined using the unpaired Student's test or One-way ANOVA. Comparisons between groups not normally distributed were determined using Mann-Whitney test or Kruskal-Wallis test. Survival analysis was performed using Kaplan-Meier analysis and the log-rank test. The Pearson's test was used to assess the correlation between FOXM1 mRNA level and hTERT mRNA level of GC patients. * means P < 0.05, ** means P < 0.01, *** means P < 0.001, **** means P < 0.0001, ns means not significant. P < 0.05 was considered statistically significant.

Authorship contribution statement

Li X. and Zhao X. devised, coordinated and supervised the project. Tang Q. and Liu C. performed all the experiments with the help from Zhang S., He L., Liu Y. and Wang J. Tang Q. and Liu C. analyzed the data and wrote the manuscript. All authors read and participated in revising the manuscript.

Declaration of Competing Interest

All authors declare that there are no competing interests.

Acknowledgement

This work was supported by the National Natural Science Foundation of China (81902516).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neo.2022.100863.

References

[1] S.S. Joshi, B.D. Badgwell, Current treatment and recent progress in gastric cancer, CA Cancer J. Clin. 71 (2021) 264–279.
[2] E.C. Smyth, M. Nilsson, H.I. Grabsch, N.C.T. van Grieken, F. Lordick, Gastric cancer, The Lancet 396 (2020) 635–648.
[3] K. Li, A. Zhang, X. Li, H. Zhang, L. Zhao, Advances in clinical immunotherapy for gastric cancer, Biochim. Biophys. Acta Rev. Cancer 1876 (2021) 188615.
[4] O.A. Stewart, F. Wu, Y. Chen, The role of gastric microbiota in gastric cancer, Gut. Microbes 11 (2020) 1220–1230.
[5] C. Fan, K. Xu, Y. Huang, S. Liu, T. Wang, W. Wang, W. Hu, L. Liu, M. Xing, S. Yang, Viscosity and degradation controlled injectable hydrogel for esophageal endoscopic submucosal dissection, Bioact. Mater. 6 (2021) 1150–1162.
[6] N. Cancer Genome Atlas Research, Comprehensive molecular characterization of gastric adenocarcinoma, Nature 513 (2014) 202–209.
[7] P. Laissue, The forkhead-box family of transcription factors: key molecular players in colorectal cancer pathogenesis, Mol. Cancer 18 (2019) 5.
[8] D. Kalathil, S. John, A.S. Nair, FOXM1 and cancer: faulty cellular signaling derails homeostasis, Front. Oncol. 10 (2020) 626836.
[9] G.B. Liao, X.Z. Li, S. Zeng, C. Liu, S.M. Yang, L. Yang, C.J. Hu, J.Y. Bai, Regulation of the master regulator FOXM1 in cancer, Cell Commun. Signal 16 (2018) 57.
[10] A.K. Lam, A.W. Ngan, M.H. Leung, D.C. Kwok, V.W. Liu, D.W. Chan, W.Y. Leung, K.M. Yao, FOXM1b, which is present at elevated levels in cancer cells, has a greater transforming potential than FOXM1c, Front. Oncol. 3 (2013) 11.
[11] J. Zeng, L. Wang, Q. Li, W. Li, M. Bjorkholm, J. Jia, D. Xu, FoxM1 is up-regulated in gastric cancer and its inhibition leads to cellular senescence, partially dependent on p27 kip1, J. Pathol. 218 (2009) 419–427.
[12] J. Liu, J. Li, K. Wang, H. Liu, J. Sun, X. Zhao, Y. Yu, Y. Qiao, Y. Wu, X. Zhang, R. Zhang, A. Yang, Aberrantly high activation of a FoxM1-STMN1 axis contributes to progression and tumorigenesis in FoxM1-driven cancers, Signal Transduct. Target. Ther. 6 (2021) 42.
[13] Q. Li, N. Zhang, Z. Jia, X. Le, B. Dai, D. Wei, S. Huang, D. Tan, K. Xie, Critical role and regulation of transcription factor FoxM1 in human gastric cancer angiogenesis and progression, Cancer Res. 69 (2009) 3501–3509.
[14] P. Francica, L. Nisa, D.M. Aebersold, R. Langer, F. Bladt, A. Blaukat, D. Stroka, M.R. Martinez, Y. Zimmer, M. Medova, Depletion of FOXM1 via MET targeting underlies establishment of a DNA damage-induced senescence program in gastric cancer, Clin. Cancer Res. 22 (2016) 5322–5336.
[15] C.J. Hu, B. Wang, B. Tang, B.J. Chen, Y.F. Xiao, Y. Qin, X. Yong, G. Luo, J.W. Zhang, D. Zhang, S. Li, F. He, S.M. Yang, The FOXM1-induced resistance to oxaliplatin is partially mediated by its novel target gene Mcl-1 in gastric cancer cells, Biochim. Biophys. Acta 1849 (2015) 290–299.
[16] A.L. Gartel, FOXM1 in cancer: interactions and vulnerabilities, Cancer Res. 77 (2017) 3135–3139.
[17] J. Xue, X. Lin, W.T. Chiu, Y.H. Chen, G. Yu, M. Liu, X.H. Feng, R. Sawaya, R.H. Medema, M.C. Hung, S. Huang, Sustained activation of SMAD3/SMAD4 by FOXM1 promotes TGF-beta-dependent cancer metastasis, J. Clin. Invest. 124 (2014) 564–579.
[18] K.C. Low, V. Tergaonkar, Telomerase: central regulator of all of the hallmarks of cancer, Trends Biochem. Sci 38 (2013) 426–434.
[19] P. Martinez, M.A. Blasco, Telomeric and extra-telomeric roles for telomerase and the telomere-binding proteins, Nat. Rev. Cancer 11 (2011) 161–176.
[20] N. Liu, D. Ding, W. Hao, F. Yang, X. Wu, M. Wang, X. Xu, Z. Ju, J.P. Liu, Z. Song, J.W. Shay, Y. Guo, Y.S. Cong, hTERT promotes tumor angiogenesis by activating VEGF via interactions with the Sp1 transcription factor, Nucl. Acids. Res. 44 (2016) 8693–8703.
[21] T. Liu, X. Liang, B. Li, M. Bjorkholm, J. Jia, D. Xu, Telomerase reverse transcriptase inhibition stimulates cyclooxygenase 2 expression in cancer cells and synergizes with celecoxib to exert anti-cancer effects, Br. J. Cancer 108 (2013) 2272–2280.
[22] B. Zhang, T. Ling, P. Zhaxi, Y. Cao, L. Qian, D. Zhao, W. Kang, W. Zhang, L. Wang, G. Xu, X. Zou, Proton pump inhibitor pantoprazole inhibits gastric cancer metastasis via suppression of telomerase reverse transcriptase gene expression, Cancer Lett. 452 (2019) 23–30.
[23] C. Hu, Z. Ni, B.S. Li, X. Yong, X. Yang, J.W. Zhang, D. Zhang, Y. Qin, M.M. Jie, H. Dong, S. Li, F. He, S.M. Yang, hTERT promotes the invasion of gastric cancer cells by enhancing FOXO3a ubiquitination and subsequent ITGB1 upregulation, Gut 66 (2017) 31–42.
[24] W. Oh, E.W. Lee, D. Lee, M.R. Yang, A. Ko, C.H. Yoon, H.W. Lee, Y.S. Bae, C.Y. Choi, J. Song, Hdm2 negatively regulates telomerase activity by functioning as an E3 ligase of hTERT, Oncogene 29 (2010) 4101–4112.
[25] J.H. Kim, S.M. Park, M.R. Kang, S.Y. Oh, T.H. Lee, M.T. Muller, I.K. Chung, Ubiquitin ligase MKRN1 modulates telomere length homeostasis through a proteolysis of hTERT, Genes Dev. 19 (2005) 776–781.
[26] Z. Zhang, S.T. Xue, Y. Gao, Y. Li, Z. Zhou, J. Wang, Z. Li, Z. Liu, Small molecule targeting FOXM1 DNA binding domain exhibits anti-tumor activity in ovarian cancer, Cell Death Discov. 8 (2022) 280.
[27] D.R. Littler, M. Alvarez-Fernandez, A. Stein, R.G. Hibbert, T. Heidebrecht, P. Aloy, R.H. Medema, A. Perrakis, Structure of the FoxM1 DNA-recognition domain bound to a promoter sequence, Nucl. Acids. Res. 38 (2010) 4527–4538.
[28] C.C. Hsu, W.Y. Liao, K.Y. Chang, T.S. Chan, P.J. Huang, C.T. Chiang, Y.S. Shan, L.H. Cheng, T.Y. Liao, K.K. Tsai, A multi-mode Wnt- and stemness-regulatory module dictated by FOXM1 and ASPM isoform I in gastric cancer, Gastric Cancer 24 (2021) 624–639.
[29] Z. Zhang, Z. Xue, H. Yang, F. Zhao, C. Liu, J. Chen, S. Lu, Z. Zou, Y. Zhou, X. Zhang, Differential effects of EPA and DHA on DSS-induced colitis in mice and possible mechanisms involved, Food Funct. 12 (2021) 1803–1817.
[30] Y. Zhang, X. Wang, Targeting the Wnt/beta-catenin signaling pathway in cancer, J. Hematol. Oncol. 13 (2020) 165.
[31] I. Khan, M. Halasi, M.F. Zia, P. Gann, S. Gaitonde, N. Mahmud, A.L. Gartel, Nuclear FOXM1 drives chemoresistance in AML, Leukemia 31 (2017) 251–255.
[32] U.G. Bhat, R. Jagadeeswaran, M. Halasi, A.L. Gartel, Nucleophosmin interacts with FOXM1 and modulates the level and localization of FOXM1 in human cancer cells, J. Biol. Chem. 286 (2011) 41425–41433.
[33] K. Joshi, Y. Banasavadi-Siddegowda, X. Mo, S.H. Kim, P. Mao, C. Kig, D. Nardini, R.W. Sobol, L.M. Chow, H.I. Kornblum, R. Waclaw, M. Beullens, I. Nakano, MELK-dependent FOXM1 phosphorylation is essential for proliferation of glioma stem cells, Stem Cells 31 (2013) 1051–1063.
[34] J.I. Park, A.S. Venteicher, J.Y. Hong, J. Choi, S. Jun, M. Shkreli, W. Chang, Z. Meng, P. Cheung, H. Ji, M. McLaughlin, T.D. Veenstra, R. Nusse, P.D. McCrea, S.E. Artandi, Telomerase modulates Wnt signalling by association with target gene chromatin, Nature 460 (2009) 66–72.
[35] I. Wierstra, J. Alves, FOXM1c transactivates the human c-myc promoter directly via the two TATA boxes P1 and P2, FEBS J. 273 (2006) 4645–4667.

[36] K.J. Wu, C. Grandori, M. Amacker, N. Simon-Vermot, A. Polack, J. Lingner, R. Dalla-Favera, Direct activation of TERT transcription by c-myc, Nat. Genet. 21 (1999) 220–224.
[37] H. Murakami, H. Aiba, M. Nakanishi, Y. Murakami-Tonami, Regulation of yeast forkhead transcription factors and FoxM1 by cyclin-dependent and polo-like kinases, Cell Cycle 9 (2010) 3233–3242.
[38] E.G. Kosebent, S. Ozturk, Telomere associated gene expression as well as TERT protein level and telomerase activity are altered in the ovarian follicles of aged mice, Sci. Rep. 11 (2021) 15569.
[39] S. Prasad, S.K. Chaube, Increased telomerase reverse transcriptase expression associates with spontaneous exit from M-II arrest in rat eggs, Cell Reprogram 19 (2017) 27–34.
[40] M.M. Jie, X. Chang, S. Zeng, C. Liu, G.B. Liao, Y.R. Wu, C.H. Liu, C.J. Hu, S.M. Yang, X.Z. Li, Diverse regulatory manners of human telomerase reverse transcriptase, Cell Commun. Signal 17 (2019) 63.
[41] J.H. Lee, P. Khadka, S.H. Baek, I.K. Chung, CHIP promotes human telomerase reverse transcriptase degradation and negatively regulates telomerase activity, J. Biol. Chem. 285 (2010) 42033–42045.
[42] H.Y. Jung, X. Wang, S. Jun, J.I. Park, Dyrk2-associated EDD-DDB1-VprBP E3 ligase inhibits telomerase by TERT degradation, J. Biol. Chem. 288 (2013) 7252–7262.
[43] Y. Qu, L. Shi, D. Wang, B. Zhang, Q. Yang, M. Ji, B. Shi, P. Hou, Low frequency of TERT promoter mutations in a large cohort of gallbladder and gastric cancers, Int. J. Cancer 134 (2014) 2993–2994.
[44] D.A. Sanders, M.V. Gormally, G. Marsico, D. Beraldi, D. Tannahill, S. Balasubramanian, FOXM1 binds directly to non-consensus sequences in the human genome, Genome Biol. 16 (2015) 130.
[45] T. Wang, J. Zhang, N. Li, M. Li, S. Ma, S. Tan, X. Guo, Z. Wang, Z. Wu, L. Gao, C. Ma, X. Liang, Spatial distribution and functional analysis define the action pathway of Tim-3/Tim-3 ligands in tumor development, Mol. Ther. 30 (2022) 1135–1148.