


ORIGINAL ARTICLE

TBX20 inhibits colorectal cancer tumorigenesis by impairing NHEJ-mediated DNA repair

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Abstract

DNA high methylation is one of driving force for colorectal carcinoma (CRC) pathogenesis. Transcription factors (TFs) can determine cell fate and play fundamental roles in multistep process of tumorigenesis. Dysregulation of DNA methylation of TFs should be vital for the progression of CRC. Here, we demonstrated that TBX20, a T-box TF family protein, was downregulated with hypermethylation of promoter in early-stage CRC tissues and correlated with a poor prognosis for CRC patients. Moreover, we identified PDZRN3 as the E3 ubiquitin ligase of TBX20 protein, which mediated the ubiquitination and degradation of TBX20. Furthermore, we revealed that TBX20 suppressed cell proliferation and tumor growth through impairing non-homologous DNA end joining (NHEJ)-mediated double-stranded break repair by binding the middle domain of both Ku70 and Ku80 and therefore inhibiting their recruitment on chromatin in CRC cells. Altogether, our results reveal the tumor-suppressive role of TBX20 by inhibiting NHEJ-mediated DNA repair in CRC cells, and provide a potential biomarker for predicting the prognosis of patients with early-stage CRC and a therapeutic target for combination therapy.

KEYWORDS

colorectal carcinoma, DNA methylation, Ku70, non-homologous DNA end joining, TBX20

1 | INTRODUCTION

Colorectal carcinoma (CRC) ranks third in terms of incidence, and second in terms of mortality worldwide, accounting for ~10% of cancer-related deaths.¹ Successive accumulation of genetic alterations

(gene mutations, gene amplification, and so on) and epigenetic alterations (aberrant DNA methylation, chromatin modifications, and so on) contribute to the tumorigenesis of CRC.^{2,3} The mechanisms leading to CRC development, progression, and recurrence are complex and remain to be explored further. The transcriptional; silencing

Abbreviations: 5-Aza, 5-Aza-2'-deoxycytidine; Co-IP, co-immunoprecipitation; CRC, colorectal carcinoma; DOX, doxorubicin; DSBs, double-stranded breaks; HR, homologous recombination; IF, immunofluorescence; IHC, immunohistochemical staining; NHEJ, non-homologous DNA end joining; TFs, transcription factors; γ -H2AX, phospho-H2AX.

Jie Luo and Jie-Wei Chen contributed equally to this work.

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of tumor suppressor genes caused by DNA methylation at gene promoters is one of the main mechanisms in CRC development.² Classic tumor suppressor genes with aberrant DNA methylation, including CDKN2A, MLH1, CDH1, and VHL, are identified to be driver genes in the process of cancer formation.⁴⁻⁶ The aberrant methylation of CXLC12 even can promote the metastatic characteristics of CRC cells.⁷ Furthermore, DNA methylation has also been reported to be a biomarker for cancer recurrence.⁸⁻¹⁰

Transcription factors play a vital role in cell fate and disease development through strongly regulating the expression of essential genes.¹¹ Multiple well known oncogenic TFs, such as *c-myc*, β -catenin, KLF4, NF- κ B, STATs, and so on, have been widely studied as important drug targets.¹² However, some molecules are not targetable. We believe that restoring gene function is another possible approach to gene therapy. Therefore, we are interested in discovering potential tumor suppressor TFs for investigating the tumorigenesis of CRC.

The T-box family of TFs, which is characterized by a DNA-binding motif known as the T-domain that binds DNA in a sequence-specific manner,^{13,14} plays key roles throughout organogenesis and pattern formation in vertebrate and invertebrate embryos.^{15,16} Recent studies have shown its essential functions in tumor cell immortality, proliferation, and invasion.¹⁷⁻²⁰ As a member of T-box family, TBX20 attracted our attention for its important functions in development and angiogenesis, which indicate a potential role in tumorigenesis. TBX20 serves as a critical cardiogenic transcription factor in heart development.^{21,22} For instance, *Tbx20* regulates cardiomyocyte lineage maturation and cell proliferation at embryonic and fetal stages of murine heart development.²³ The loss of *Tbx20* in murine results in death of embryos at mid-gestation with markedly abnormal heart morphogenesis.²⁴ Consistently, TBX20 missense mutations have been identified in human patients with adult cardiomyopathies and congenital heart defects.^{25,26} TBX20 associates with a Gro/TLE-NuRD repressor complex to regulate target gene transcription within the forming heart.²⁷ In addition, *Tbx20* regulates angiogenesis through the prokineticin 2-prokineticin receptor 1 pathway and transduces cardiomyogenic differentiation of human adipose-derived mesenchymal stem cells.^{28,29} However, the biological functions, clinical significance, and molecular mechanism of TBX20 in tumors have not yet been reported.

Exogenous (chemical carcinogens, radiation, genotoxic anticancer drugs) or endogenous (radicals produced by activated immune cells such as monocytes and macrophages, DNA replication errors and inadvertent cleavage by nuclear enzymes) factors produce a variety of DNA lesions.³⁰⁻³⁵ Excess and uncontrolled lesions result in gene mutations and chromosomal damage, which are causal events in oncogenic transformation.³⁴ DNA DSBs are the most dangerous type of DNA lesions and the NHEJ pathway is one of the main pathways for the repair of DNA DSBs in human cells. In NHEJ, DSBs are first recognized by the Ku70-Ku80 heterodimer, and then recruit other NHEJ proteins to chromatin, initiating the subsequent DNA repair process.³⁶

In this study, we used a cohort of CRC tissues to identify TBX20 as a potential tumor suppressor gene. We found that TBX20 was

downregulated in CRC tissues and cell lines. DNA hypermethylation at TBX20 promoter contributed to the downregulation of TBX20 and TBX20 was degraded by E3 ubiquitin ligase PDZRN3 protein via the proteasome pathway. Furthermore, both gain- and loss-of-function assays showed that TBX20 suppressed CRC cell growth. Mechanistically, we identified Ku70 and Ku80 as binding partners of TBX20, which was mediated by the middle domain. TBX20 disrupted the interaction of Ku70 and Ku80, resulting in impaired NHEJ-mediated DSBs repair, to suppress cell growth in CRC cells. Therefore, our results reveal the mechanism and the potential clinical application of TBX20, a novel tumor-suppressive gene, in CRC.

2 | MATERIALS AND METHODS

2.1 | Patients and samples

Human CRC tissues (including 26 paired CRC tissues and adjacent normal tissues, and 118 stages I and II CRC tissues) from patients after operation between January 2010 and December 2015 were obtained from the archives of the Department of Pathology, Sun Yat-sen University Cancer Center (SYSUCC; Guangzhou, China). For inclusion, the patients were required to have a clear pathological diagnosis, the presence of follow-up data, and the absence of previous local or systemic treatment. The tumor grades and stages were defined according to the 2002 American Joint Committee on Cancer/International Union Against Cancer TNM classification system. All the samples used in this study were approved by the Committees for Ethical Review of Research Involving Human Subjects at the Sun Yat-sen University Cancer Center.

2.2 | Cell culture and transfection

The CRC cell lines HCT116, LoVo, DLD-1, SW620, SW480, RKO and CW2 were cultured in RPMI 1640 medium (Gibco) with 10% FBS (PAN-Biotech). 293T human embryonic kidney cells, EJ5-GFP cells and DR-GFP cells were cultured in DMEM (Gibco) with 10% FBS. CRC cell lines and 293T cells were purchased from ATCC. EJ5-GFP and DR-GFP cells were gifts from Professor Muyan Cai, Sun Yat-sen University Cancer Center.³⁷ All cells were cultured at 37°C in an incubator (Thermo Fisher Scientific) with 5% CO₂. All transient transfections were performed with Lipofectamine 2000 (52887, Invitrogen) according to the manufacturer's guidelines. Vector control and TBX20/PDZRN3/Ku70/Ku80-expressing plasmids were purchased from GeneCreate (Wuhan). Ubiquitin mutants were gifts from Professor Rong Deng, Sun Yat-sen University Cancer Center.³⁸ Negative control (NC) and specific siRNAs were synthesized by RiboBio. Negative control and specific shRNAs were synthesized by GeneCopoeia. TBX20si/shRNA-4: CTACCAGAATCAACTGATA; TBX20si/shRNA-5: CACTGACATTGAGAGGG; PDZRN3siRNA-1: AGAAGAAATTCACCGAATA; PDZRN3siRNA-2: GGAACGACTTTC TGGATGA.

2.3 | Methylation-specific PCR

Total DNA was extracted using the TIANamp Genomic DNA Kit (Tiagen Biotech). Bisulfite-treated DNA was prepared using the EZ DNA Methylation-Lightning™ Kit (Zymo Research). A TaKaRa EpiTaq™ HS Kit (for bisulfite-treated DNA) was used for PCR. The primers used in this experiment are listed as follows: MSP methylation pair (F: 5'-TTTGTTCGTTGTTTTT AGTC-3'; R:5'-CTTCTAACCGATCTCCATATACGTT-3'); MSP unmethylation pair (F: 5'-TGTTTTTTTGTTCGTTTGTAGTTGT-3', R: 5'-AAAGTGGTCGTTGAGGGCAA-3').

2.4 | Comet assay

Cells were treated with X-rays (10 Gy) or doxorubicin (DOX) (2 μM, HY-15142, MCE) and cultured for 6 h (X-rays) and 24 h (DOX). After mixing with 0.6% solution of LMP agarose (16520-050, Thermo Scientific) dissolved in PBS, the cells were spread on pre-coated slides (4250-2-03, R&D Systems). Then the slides were put gently into pre-cooled lysis buffer (2.5 M NaCl, 100 mM EDTA-Na₂, 10 mM Tris, 1% w/v Triton X-100, 10% w/v DMSO, pH 10) for 3 h at 4°C. After rinsing in cooled distilled water, the slides were thrown into alkaline running buffer (1 mM EDTA-Na₂, 300 mM NaOH, pH ≥ 13) for 30 min. Electrophoresis was performed at 30 V/300 mA for 25 min, followed by neutralization with 0.4 M Tris (pH 7.5). Then the cells were fixed in gradient alcohol (50%, 75%, 100%) and stained with SyBR Green (1725120, Bio-Rad). Finally, the samples were observed and photographed by inverted microscope (Eclipse Ti2, Nikon). Images were analyzed using CASP software (University of Wroclaw, Poland). Tail moment (tail moment = % tail DNA × tail length) was evaluated to analyze the differences.

2.5 | NHEJ and HR reporter assays

The I-SceI expression adenovirus was a gift from Professor Muyan Cai, Sun Yat-sen University Cancer Center.³⁷ After transfection with plasmids or siRNA for 24 h, the reporter cells were infected with I-SceI expression adenovirus for another 72 h. The proportion of GFP-positive cells was detected using a CytoFLEX flow cytometer (Beckman Coulter) and used as a standard for evaluating the efficiency of NHEJ and HR DNA repair.

2.6 | Statistical analysis

Statistical analysis was performed using SPSS 20.0 software (IBM). The correlation between TBX20 expression and clinicopathological parameters was analyzed by chi-squared (χ^2) test. The Kaplan-Meier method (the log-rank test) was used for survival analysis and univariate analysis. The Cox proportional hazards regression model was used for multivariate survival analyses. Measurements were

analyzed using two-tailed Student *t* test or one-way ANOVA. Data are presented as the mean ± SD, and *p*-values < 0.05 were considered statistically significant.

3 | RESULTS

3.1 | Transcriptional downregulation of TBX20 is mediated by promoter hypermethylation

To determine whether TBX20 expression is dysregulated and correlated with CRC tumorigenesis, we first analyzed the promoter methylation of TBX20 in an online database. Promoter methylation of TBX20 in primary CRC tumor tissues was higher than in adjacent normal tissues (Figure S1A), as well as in other cancers such as esophageal carcinoma, cervical squamous cell carcinoma, cholangiocarcinoma, and uterine corpus endometrial carcinoma (Figure S1B). We also found that a high promoter methylation level of TBX20 was correlated with advanced stages in CRC (Figure S1C). TCGA data also showed that colorectal (CRC) and uterine cancer (UCEC) patients with promoter hypermethylation of TBX20 at specific sequences had a poor prognosis (Figure S1D–F). In our study, full or partial methylation in promoter of TBX20 was detected in all six CRC cell lines by methylation-specific PCR assay (Figures 1A and S2), which was consistent with the protein expression by western blot (Figure 1B). To further confirm that promoter methylation was involved in the silencing of TBX20, four colorectal cancer cell lines (HCT116, LoVo, SW620, RKO) with downregulated TBX20 expression were treated with the DNA methyltransferase inhibitor 5-Aza-2'-deoxycytidine (5-Aza). A restored expression of TBX20 was observed in all detected CRC cells (Figure 1C,D). Moreover, frequent hypermethylation was detected in nearly 90% of the primary CRC tissues (9/10) and 40% (4/10) of adjacent normal colorectal tissues (Figure 1E). These results indicated that transcriptional downregulation of TBX20 is mediated by DNA hypermethylation.

3.2 | TBX20 is downregulated in CRC and correlated with a poor prognosis

We then examined the expression level of TBX20 in paired CRC tissues and adjacent tissues. TBX20 mRNA expression was downregulated in 61.5% (16/26) of the CRC tumor tissues (Figure 2A). Consistently, the TBX20 protein level was reduced in CRC tumor tissues compared with their adjacent normal tissues (Figure 2B). These results suggested an aberrant downregulation of TBX20 in CRC. To test whether dysregulation of TBX20 expression is correlated with CRC initiation or tumorigenesis, immunohistochemical staining (IHC) analysis was performed to evaluate the expression abundance of TBX20 in early stages I and II of tissues. We found that TBX20 protein level had been significantly downregulated in both stages I and II CRC tissues (Figure 2C). Further Kaplan-Meier analysis showed that stage II CRC patients with lower TBX20 protein levels in our

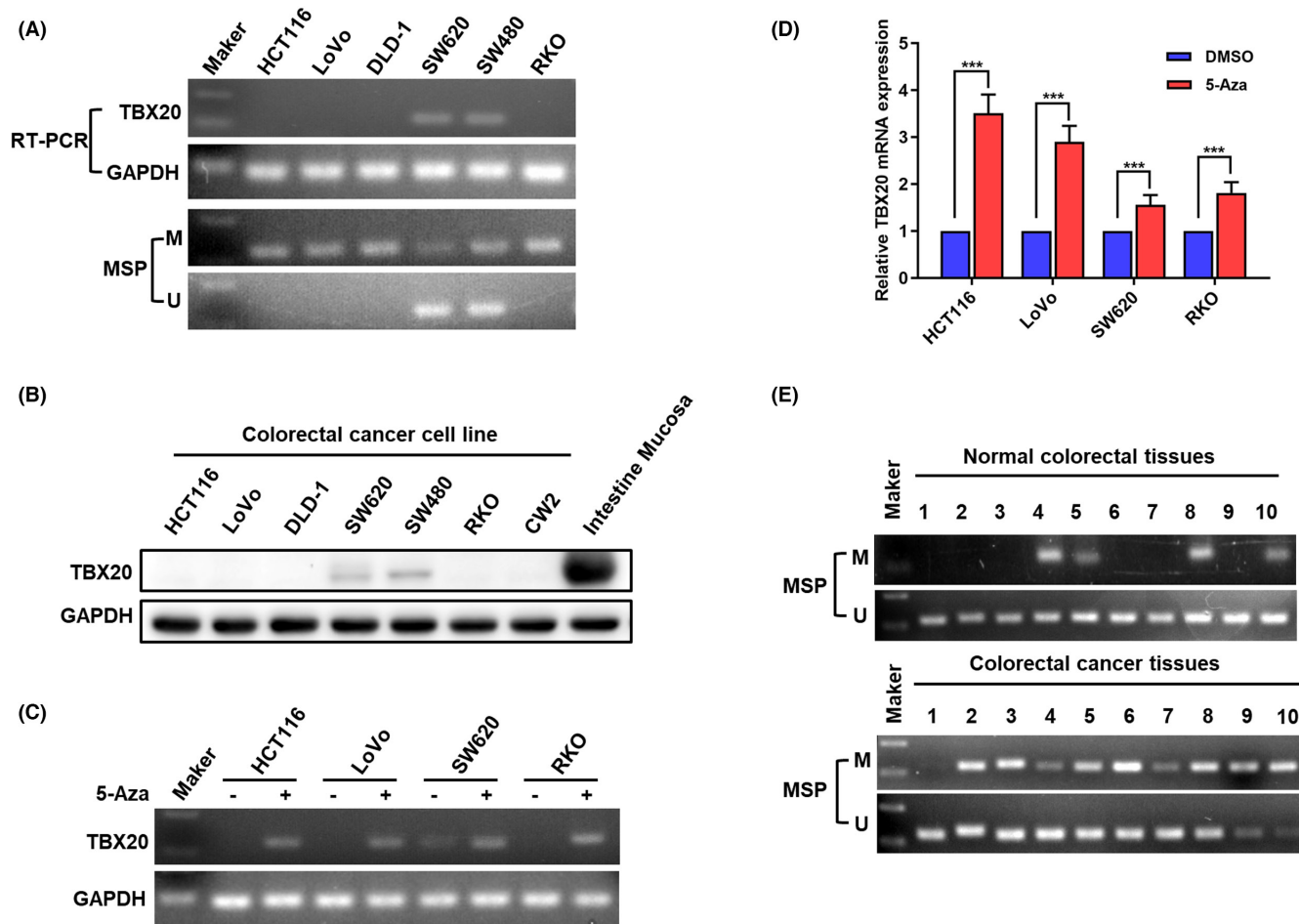


FIGURE 1 Transcriptional downregulation of TBX20 is mediated by promoter hypermethylation. (A) TBX20 expression level was detected in CRC cell lines by RT-PCR. Methylation-specific PCR (MSP) was performed to determine the status of TBX20 promoter methylation. M, indicates methylation; U, indicates unmethylation. (B) Detection of TBX20 protein level in CRC cell lines by western blot. (C, D) The mRNA expression of TBX20 was detected by RT-PCR (C) and qRT-PCR (D) after treatment with demethylation agent 5-Aza-20-deoxycytidine (5-Aza). Data are presented as the mean \pm SD of three independent experiments, *** $p < 0.001$. (E) Methylation status of TBX20 promoter in CRC tumor and adjacent non-tumor tissues from our cohort was determined by MSP assay

cohort had both shortened overall survival and disease-free survival (Figure 2E,F). Moreover, correlation analysis revealed that low expression levels of TBX20 were correlated with an advanced pT stage (Table 1). Multivariate Cox proportional hazard regression analysis also demonstrated that TBX20 represented an independent prognostic factor for the overall survival of stage II CRC patients (hazard ratio 0.036, 95% confidence interval (CI) 0.004–0.292, $p = 0.002$, Table 2), which suggest that downregulation of TBX20 is a biomarker for high risk in patients with stage II CRC. These data collectively indicated an important role of TBX20 in tumorigenesis of CRC.

3.3 | E3 ubiquitin ligase PDZRN3 degrades TBX20 via proteasome pathway

Another question we noticed is that the mRNA expression level of TBX20 was not consistent with the protein level in partial CRC tissues, which indicates that post-translational modification (PTM) might be an important factor affecting its expression. The

ubiquitin–proteasome system has a vital role in physiological and pathological processes, as well as in tumorigenesis.^{39–41} We first verified whether the TBX20 protein level was degraded via the proteasome pathway. After treatment with cycloheximide (CHX; a protein synthesis inhibitor), TBX20 was degraded over time in SW620 cells and this degradation could be inhibited by MG132 (proteasome inhibitor) (Figure 3A). Furthermore, co-immunoprecipitation (Co-IP) assay revealed that TBX20 exhibited ubiquitylation (Figure 3B), suggesting that TBX20 protein levels are regulated in a proteasomal-dependent manner.

The potential roles of E3 ligase-mediated ubiquitylation in colorectal carcinogenesis have been reported in a previous study.⁴² Targeting E3 ligase can restore gene function in cancer therapy. Therefore, we performed immunoprecipitation (IP) with Flag-tagged TBX20 in HCT116 cells followed by mass spectrometry (MS) analysis to find potential E3 ligase of TBX20. During the validation of candidate molecules in the list of TBX20 IP-MS, we identified an E3 ubiquitin ligase, PDZRN3 (Figure 3C). Then, we confirmed the interaction of TBX20 and PDZRN3 by Co-IP assay (Figure 3D,E). We

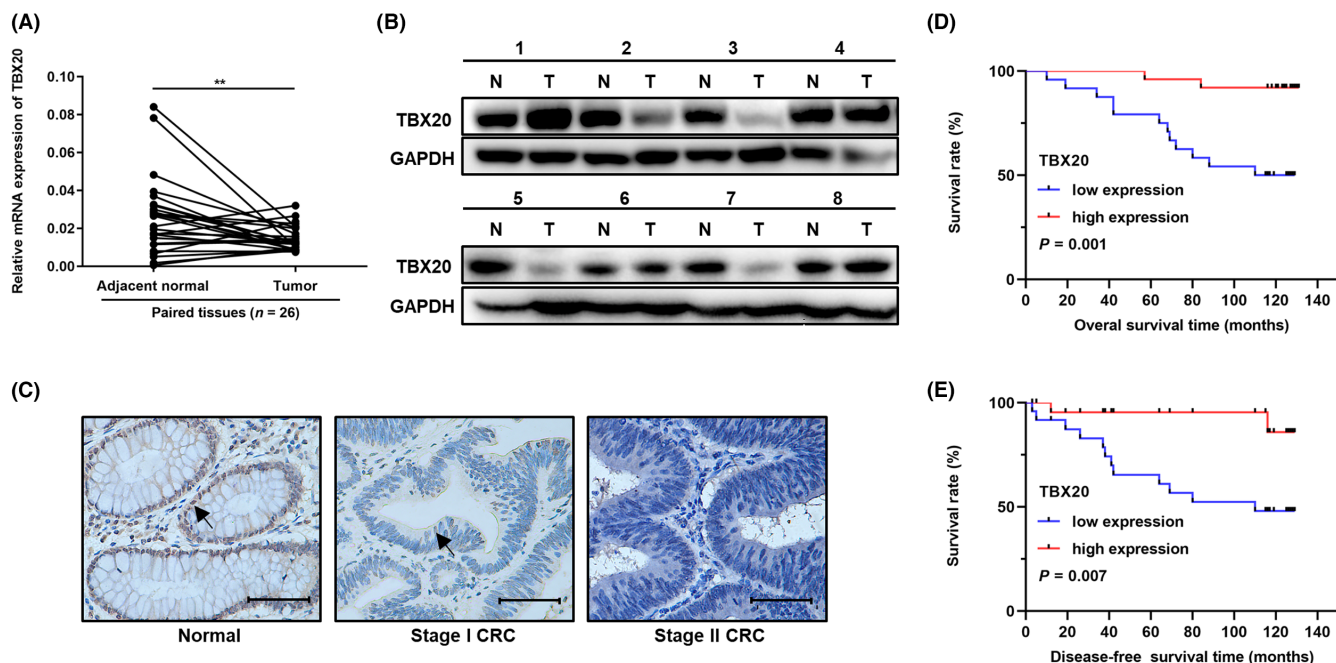


FIGURE 2 TBX20 is downregulated in CRC and correlated with a poor prognosis. (A, B) Detection of TBX20 in CRC tumor tissues (T) and paired adjacent normal tissues (N) by RT-qPCR (A, normalized to GAPDH, $n = 26$) and western blot (B, $n = 8$). (C) Representative images of immunohistochemical staining of TBX20 protein expression in CRC tumor and normal tissues. Black arrows, nuclear staining of TBX20 in colorectal epithelial cells. Scale bars, 50 μm . (D, E) Kaplan–Meier survival analysis of overall survival time (OS) (D) and disease-free survival (DFS) (E) according to TBX20 expression in 47 patients with stage II CRC (log-rank test). OS, $p = 0.001$. DFS, $p = 0.007$

also observed that the expression level of TBX20 in HCT116 and LoVo was increased after a knockdown of PDZRN3, while an inverse effect was observed in SW620 with an overexpression of PDZRN3 (Figure 3F). Moreover, we found that overexpression of PDZRN3 could enhance TBX20 ubiquitylation (Figure 3G). These results confirmed that TBX20 was ubiquitylated for degradation by PDZRN3. Ubiquitination often forms chains linking several ubiquitin units to distinct lysine (K) residues.⁴³ We then sought to determine the type of PDZRN3-mediated ubiquitination of TBX20. Using various ubiquitin mutants, we found that K6 and K63 are necessary for the formation of ubiquitin chains and that PDZRN3 ubiquitinates TBX20 mainly through chains K63 (Figures 3H and S3A,B). All the above results revealed that PDZRN3 degrades TBX20 protein via the proteasome pathway.

3.4 | TBX20 suppresses CRC cell growth

We next explored the biological function of TBX20 in CRC tumorigenesis. Here we generated two stable transfected CRC cell lines (HCT116 and LoVo cells) with TBX20 overexpression. Conversely, SW620 cells with moderate expression of TBX20 were used for loss of TBX20 function by stable transfection of shTBX20 (Figure 4A). CCK8 assay revealed that the ectopic expression of TBX20 suppressed cell proliferation in HCT116 and LoVo cells, while an inverse effect was observed in SW620 with a knockdown of TBX20 (Figure 4B). Consistently, the same result was achieved

for colony formation assay (Figure 4C). A xenograft tumor model further validated that TBX20 can suppress tumor growth of CRC cells (Figure 4D,E). The expression of TBX20 in xenograft tumors was verified by IHC staining (Figure 4F). These results revealed that TBX20 serves as a tumor suppressor in the pathogenesis of CRC.

3.5 | TBX20 interacts with Ku70 and Ku80

We further explored the underlying mechanism of TBX20 in CRC cells. Together with previous data (IP with EGFP-tagged TBX20 in 293T),²⁷ we found five coincident molecules as shown (Figure S4A). Interestingly, two of them, Ku70 and ILF2, were vital genes regulating DNA damage repair pathways. We next focused on Ku70 due to a higher abundance of protein coverage and unique spectra (Figure S4B). IP assay revealed that endogenous Ku70 and Ku80 interacted with endogenous TBX20 in SW620 cells (Figure S4C). Consistently, the interaction of endogenous Ku70 with ectopic expression of TBX20 was confirmed by Co-IP (Figure S4D) and immunofluorescence (IF) (Figure S4E) assays in HCT116 and 293T cells. As expected, we also found that exogenous TBX20 could interact with Ku80 (Figure S4C,D). Furthermore, TBX20 kept its binding capacity for Ku70 and Ku80 even after treatment with EtBr or DNase I (Figure 5A), indicating that the interaction of TBX20 with Ku70 and Ku80 does not require the presence of DNA.

As TBX20 did not affect the protein levels of Ku70 and Ku80, we guessed whether TBX20 could disrupt the interaction of Ku70

TABLE 1 Correlation between the clinicopathologic variables and TBX20 expression in colorectal carcinoma

	ALL cases	TBX20 expression		p-value*
		Low expression	High expression	
Sex				0.188
Male	24	14 (60.9%)	10 (41.7%)	
Female	23	9 (39.1%)	14 (58.3%)	
Age (years)				0.137
≤50	39	17 (43.6%)	22 (56.4%)	
>50	8	6 (75%)	2 (25%)	
Histological grade (WHO)				1.000
1	5	2 (40%)	3 (60%)	
2–3	42	20 (47.6%)	22 (52.4%)	
pT status				0.680
3	32	15 (46.9%)	17 (53.1%)	
4	15	8 (53.3%)	7 (46.7%)	
Lymphovascular space invasion				1.000
Positive	10	5 (50%)	5 (50%)	
Negative	37	18 (48.6%)	19 (51.4%)	
Perineural invasion				0.312
Positive	19	11 (57.9%)	8 (42.1%)	
Negative	28	12 (42.9%)	16 (57.1%)	

Abbreviation: WHO, World Health Organization.

*Chi-squared test.

and Ku80. Endogenous Co-IP of Ku70 in 293T cells revealed that the interaction of Ku70 and Ku80 was decreased followed by ectopic expression of TBX20, as well as endogenous Co-IP of Ku80 (Figure 5B). Exogenous Co-IP in 293T also confirmed this result (Figure 5C). Then we generated mutant constructs with a MYC tag to determine the TBX20-interaction domain of Ku70 and a HA tag for Ku80. Co-IP assay revealed that the middle domain, but not N or C termini of Ku70 and Ku80, physically interacted with TBX20 (Figure 5D,E), suggesting that the middle domain was critical for interaction with TBX20. Overall, these findings fully support the idea that TBX20 impairs the binding of Ku70 and Ku80 by interacting with the middle domain of Ku70 and Ku80.

3.6 | TBX20 impairs NHEJ-mediated DNA repair in CRC cells

The Ku70–Ku80 heterodimer protein serves as the central regulating factor during repair of DSBs. We further examined whether TBX20 could regulate the repair of DSBs in CRC cells through a series of DSB phenotype assays. It is well known that γ -H2AX levels and foci are sensitive DSB markers. IF assays (Figures 6A and S5A) and western blot (Figures 6B and S5B) showed that γ -H2AX foci and levels were higher in TBX20-overexpression HCT116 and LoVo cells at 4 h posttreatment with 4 Gy X-rays. This was confirmed by

treatment with DOX, an inducer of DSBs (Figures 6A and S5A). Next, we measured DSB levels by comet assay, in which the mean tail moment after X-ray or DOX treatment was quantified using CASP software. The results showed that TBX20-overexpression HCT116 and LoVo cells had longer comet tails at 4 h after 10 Gy X-ray radiation exposure or at 24 h after DOX treatment (Figures 6C and S5C). Additionally, we found that TBX20 overexpression significantly inhibited colony formation in HCT116 and LoVo cells after radiation and DOX treatment, suggesting that TBX20 overexpression makes colorectal cancer cells more sensitive to DSBs (Figures 6D and S5D). Taken together, these data indicated that TBX20 inhibits DSBs repair in CRC cells. In addition, an NHEJ reporter assay showed that TBX20 overexpression decreased NHEJ activity (~20%), whereas TBX20 knockdown increased NHEJ activity (~25%) (Figure 6E). But gain or loss of TBX20 did not affect HR activity (Figure 6F).

After recognizing the ends of DSBs, the Ku70–Ku80 heterodimer recruits other NHEJ proteins directly or indirectly, such as XRCC4–DNA ligase 4 (LIG4). We further detected the recruitment of NHEJ proteins on chromatin after radiation exposure by subcellular fractionation analysis. The result revealed that TBX20 overexpression reduced the recruitment of Ku70, Ku80 and LIG4 on chromatin after radiation in HCT116 cells (Figure 6G), which verified its suppressive role in DSBs repair. In general, all the above results suggested that TBX20 is likely to be involved in NHEJ, but not in HR during DSB repair, to inhibit cell growth in CRC cells.

TABLE 2 Univariate and multivariate analysis of different prognostic factors in patients with stage II CRC (Cox regression model)

Variable	Univariate analysis			Multivariate analysis	
	ALL cases	Hazard ratio (95% CI)	p-value	Hazard ratio (95% CI)	p-value
Sex			0.285		
Male	24	1.0			
Female	23	0.551 (0.184–1.645)			
Age (years)			0.752		
≤50	39	1.0			
>50	8	0.786 (0.176–3.512)			
Histological grade (WHO)			0.685		
1	5	1.0			
2–3	42	0.733 (0.164–3.280)			
pT status			0.001		<0.001
3	32	1.0		1.0	
4	15	7.457 (2.319–23.980)		11.573 (3.130–42.790)	
Lymphovascular space invasion			0.031		0.458
Positive	10	1.0		1.0	
Negative	37	3.220 (1.112–9.328)		1.666 (0.433–6.412)	
Perineural invasion			0.153		0.184
Positive	19	1.0		1.0	
Negative	28	2.169 (0.751–6.263)		0.162 (0.011–2.373)	
TBX20 expression			0.006		0.002
Low expression	25	1.0		1.0	
High expression	22	0.058 (0.008–0.447)		0.036 (0.004–0.292)	

Abbreviations: CI, confidence interval; WHO, World Health Organization.

4 | DISCUSSION

In this study, we demonstrated TBX20 as a new tumor-suppressive gene in CRC, which served as an independent prognostic factor for the survival of early-stage CRC patients. High methylation of gene promoter contributed to the downregulation of TBX20. An E3 ubiquitin ligase PDZRN3 degraded TBX20 protein via proteasome pathway. Furthermore, functional assays confirmed its tumor-suppressive role. Mechanistically, we identified Ku70 and Ku80 as interaction partners of TBX20. Importantly, TBX20 inhibited the interaction of Ku70 and Ku80 and the accumulation of NHEJ proteins on chromatin by binding the middle domain, resulting in impaired NHEJ-mediated DSBs repair, to suppress cell growth in CRC cells. It is worth mentioning that the association between TBX20 and CRC tumorigenesis needs to be further verified by knockout animal models.

Colorectal carcinoma is the second leading cause of cancer mortality worldwide.¹ The cornerstones of therapy include surgery, neoadjuvant radiotherapy, and chemotherapy.⁴⁴ Patients with stage III or IV CRC are advised to receive chemotherapy,^{44,45} but it is difficult to predict whether stage II CRC could benefit from chemotherapy.^{44,46,47} Therefore, it is important to find new strategy to identify which group of stage II CRC patients is more suitable for chemotherapy. In our study, we used a cohort of CRC tissues and found an aberrant downregulation of TBX20. Importantly, we found that stage

II CRC patients with lower TBX20 protein levels in our cohort had both shortened overall survival and disease-free survival, and further analysis suggested that TBX20 could represent an independent prognostic factor for the overall survival of CRC patients. Moreover, we demonstrated that TBX20 was frequently silenced in CRC cell lines, and function assays revealed that TBX20 suppressed CRC cells growth. These data strongly suggest that TBX20 possesses a tumor-suppressive function in CRC, and that stage II CRC patients with low TBX20 expression may benefit from chemotherapy.

We also explored the mechanism of TBX20 downregulation in CRC. There are three major distinct precursor lesion pathways leading to CRC, the traditional adenoma–carcinoma pathway (also referred to as chromosomal instability sequence), serrated neoplasia pathway and microsatellite instability.⁴⁸ Serrated neoplasia pathway is associated with epigenetic instability, characterized by the CpG island methylation phenotype, and usually resulted in transcriptional silencing of tumor suppressor genes such as CDKN2A, MLH1, and VHL.^{4,5,49} Our study confirmed that promoter hypermethylation contributed to the inactivation of TBX20. Demethylation treatment by DNA methyltransferase inhibitor 5-Aza obviously could restore the expression of TBX20. TCGA data also showed that there was a poor prognosis in CRC patients with high methylation of TBX20 promoter, suggesting that the promoter hypermethylation of TBX20 by DNA methyltransferase plays a key role in the transcriptional silence of TBX20 in CRC.

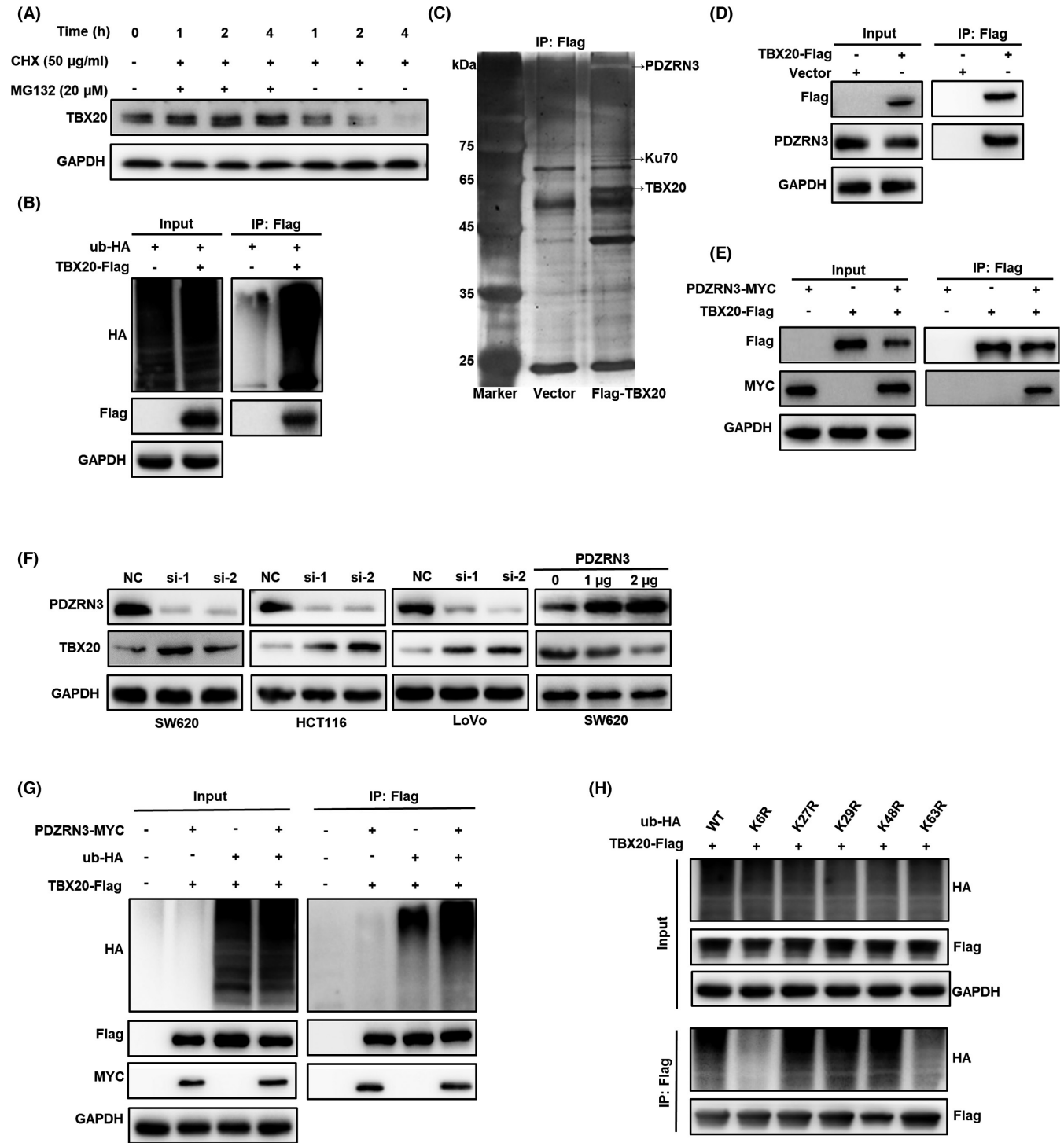


FIGURE 3 E3 ubiquitin ligase PDZRN3 degrades TBX20 protein. (A) The protein level of TBX20 in SW620 cells after treatment with CHX (50 μ g/ml) and MG132 (20 μ M). (B) The ubiquitination of TBX20 in HCT116 was detected by Co-IP. (C) A silver staining assay was used to distinguish differentiated proteins between vector and TBX20-overexpressing HCT116 cells. PDZRN3 and Ku70 are indicated by black arrows. (D, E) The interaction of endogenous (D) and exogenous (E) PDZRN3 with TBX20 overexpression in HCT116 cells was detected by Co-IP, followed by western blot analysis with the indicated antibodies. (F) TBX20 protein detection in indicated cell lines after knockdown or overexpression of PDZRN3. (G) The ubiquitination of TBX20 after PDZRN 3 overexpression by Co-IP. (H) Ubiquitin mutant plasmids were co-transfected with TBX20 in HCT116 cells, followed by Co-IP

As found for epigenetic modification (DNA methylation [and demethylation], histone modifications, and non-coding RNAs),⁵⁰ PTM is also critical for gene expression, in which ubiquitination is

one of the most studied areas.³⁹ As a key part in ubiquitination, E3 ligase has been attracting more and more attention as potential anticancer drug targets due to its pivotal role in restoring gene

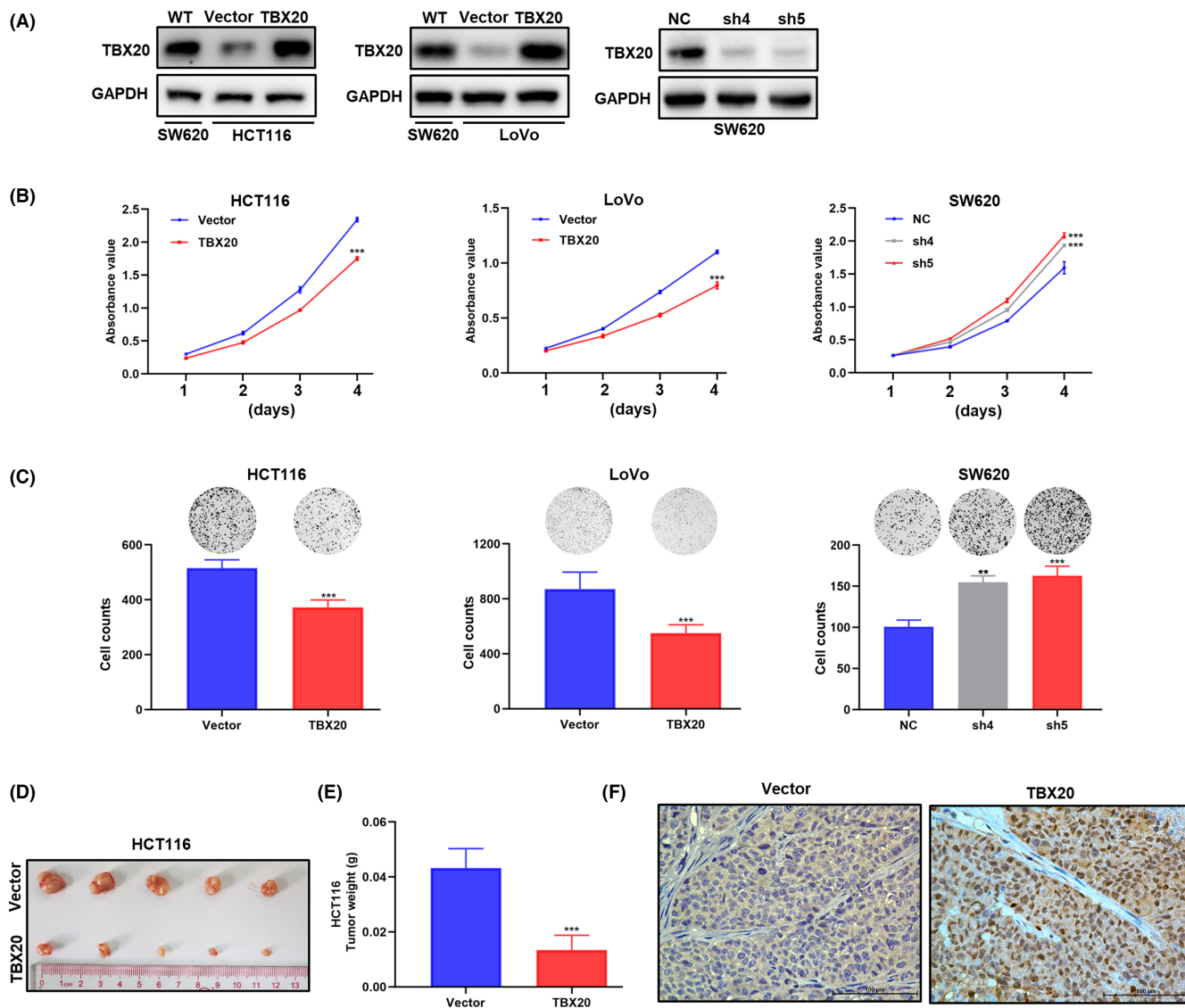


FIGURE 4 TBX20 suppresses CRC cell growth. (A) The efficiency of TBX20 overexpression (vector, control lentiviral vector; TBX20, TBX20 overexpression lentiviral vector) and knockdown (NC, negative control; sh4/5, TBX20-specific shRNA) were verified by western blot. (B) Cell proliferation ability of the indicated cells was demonstrated by CCK-8. Data are presented as the mean \pm SD of three independent experiments, *** $p < 0.001$. (C) Colony formation images of TBX20 overexpression and knockdown in indicated cells (upper panels). The colonies were counted and are presented (lower panels). Data are presented as the mean \pm SD of three independent experiments, ** $p < 0.01$, *** $p < 0.001$. (D, E) Images of the CRC xenograft tumors formed in nude mice. The tumor volume and tumor weight were measured. ** $p < 0.01$ *** $p < 0.001$. (F) Tissues from xenograft neoplasms were tested by IHC assays. Scale bars, 100 μm

function, especially of tumor-suppressive genes. A notable example includes MDM2, an E3 ubiquitin ligase of P53, which has been explored to be a therapeutic target to restoring p53-dependent tumor suppressor activity with wild-type p53 in cancers, such as acute myeloid leukemia.⁵¹ Here, we performed IP with Flag-tagged TBX20 in HCT116 cells followed by MS analysis and identified PDZRN3 as an E3 ligase of TBX20. PDZRN3 regulated the degradation of TBX20 in a proteasomal-dependent manner, and could restore the expression of downregulated TBX20, which may be a target for cancer therapy.

The Ku protein is a complex of two subunits, Ku70 and Ku80, and was described as an autoantigen recognized by the sera of patients

with autoimmune diseases.⁵²⁻⁵⁴ It plays a key role in DNA repair, as well as in other nuclear processes, such as chromosome maintenance, transcription regulation, and V(D)J recombination.⁵⁵⁻⁵⁸ The formation of the Ku70-Ku80 heterodimer is the initiating events of NHEJ. Ku70 and Ku80 have been reported to be upregulated in cervical cancer,⁵⁹ and contribute to radiation resistance and cancer growth in pancreatic cancer. Disruption of NHEJ resulted in radiation sensitivity and growth inhibition.⁶⁰ In our work, we first found the association between TBX family protein and DNA damage repair. Here, we reveal a novel regulatory mechanism for tumor suppressor genes in regulating NHEJ-mediated DSBs repair. TBX20 could interact with Ku70 and Ku80 through the middle domain. Upon DNA

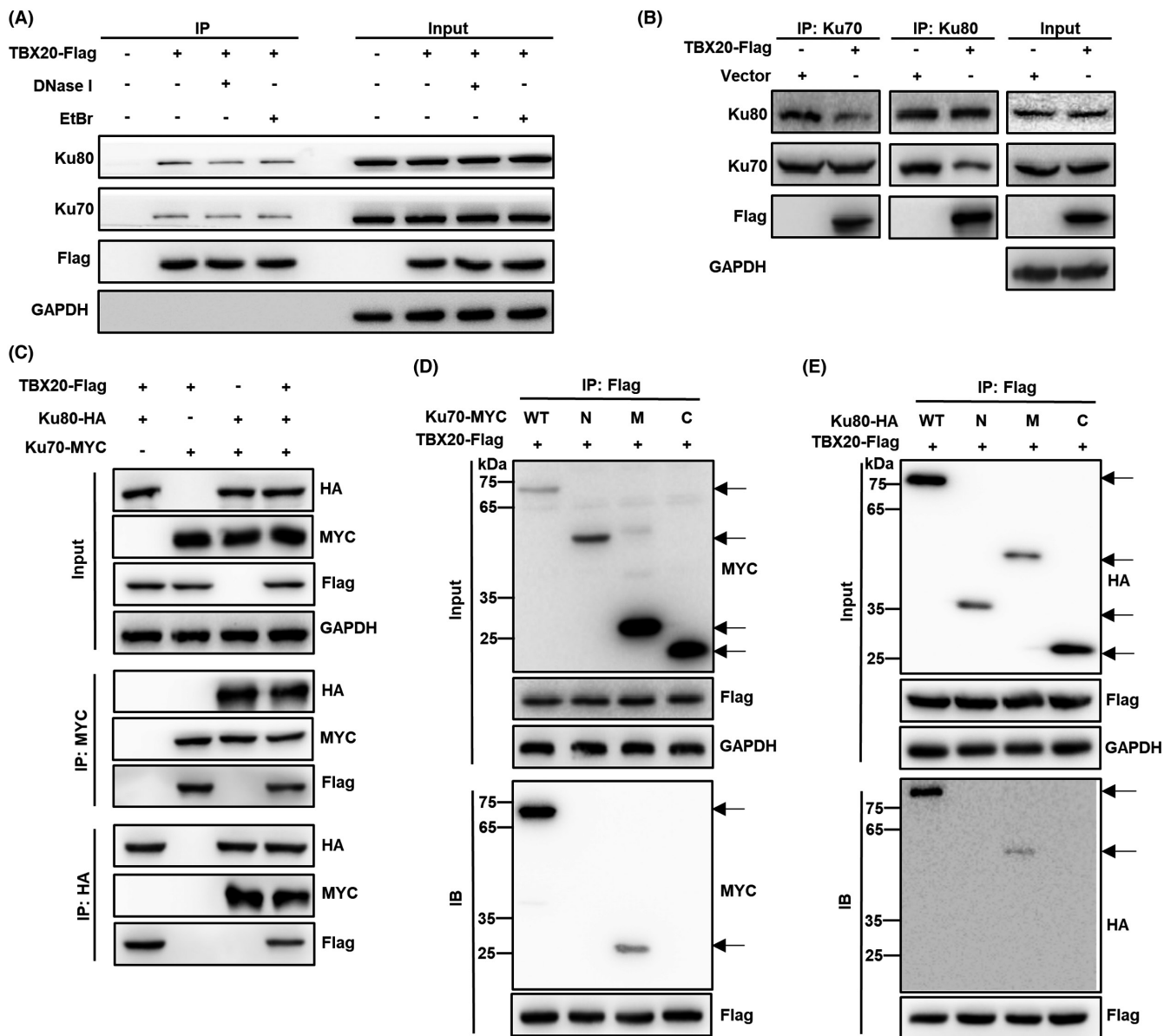


FIGURE 5 TBX20 interacts with Ku70 and Ku80. (A) DNA, ethidium bromide (EtBr, 50 μ g/ml) or DNase I (10 U) were added to 293T lysates, which were subjected to immunoprecipitation followed by western blot. (B, C) The effect of TBX20 on the interaction of Ku70 and Ku80 in 293T by Co-IP assay, followed by western blot analysis with the indicated antibodies. (D, E) Co-IP assay was performed in 293T after transfected Flag-TBX20 and Myc-Ku70 (D) (N: 1–350 aa [amino acids], M [middle domain]: 351–500 aa, C: 501–609 aa) or HA-Ku80 (E) (N: 1–240 aa, M: 241–550 aa, C: 551–732 aa) different mutant constructs. The arrows indicate expected positions of the respective proteins

FIGURE 6 TBX20 inhibits DSBs repair in CRC cells. (A, B) The effects of TBX20 on γ -H2AX foci and levels in HCT116 treated with X-rays (4 Gy, after 4 h) or DOX (2 μ M, after 24 h) by IF (A) (upper, representative IF images; down, foci number is presented as the mean \pm SD of three independent experiments) and western blot (B). Scale bar, 10 μ m. (C) Comet assay for DNA damage in HCT116 at 4 h after treatment with 10 Gy X-rays or 24 h after treatment with DOX (upper, representative pictures; lower, bar charts indicating the average tail moment per cell). Scale bars, 100 μ m. (D) Representative colony formation images of vector and TBX20 overexpression HCT116 cells treated with the indicated X-rays or dose of DOX. The colonies were counted and are presented (right panels). Data are presented as the mean \pm SD of three independent experiments, *** p < 0.001. (E, F) The effect of TBX20 on DSBs repair was assessed by reporter-based quantification of NHEJ (E) and HR (F) repair. Data are presented as the mean \pm SD of three independent experiments, ns, no significant, *** p < 0.001. (G) Western blot analysis of the indicated proteins in chromatin recruitment of vector and TBX20 overexpression HCT116 cells after irradiation at 10 Gy

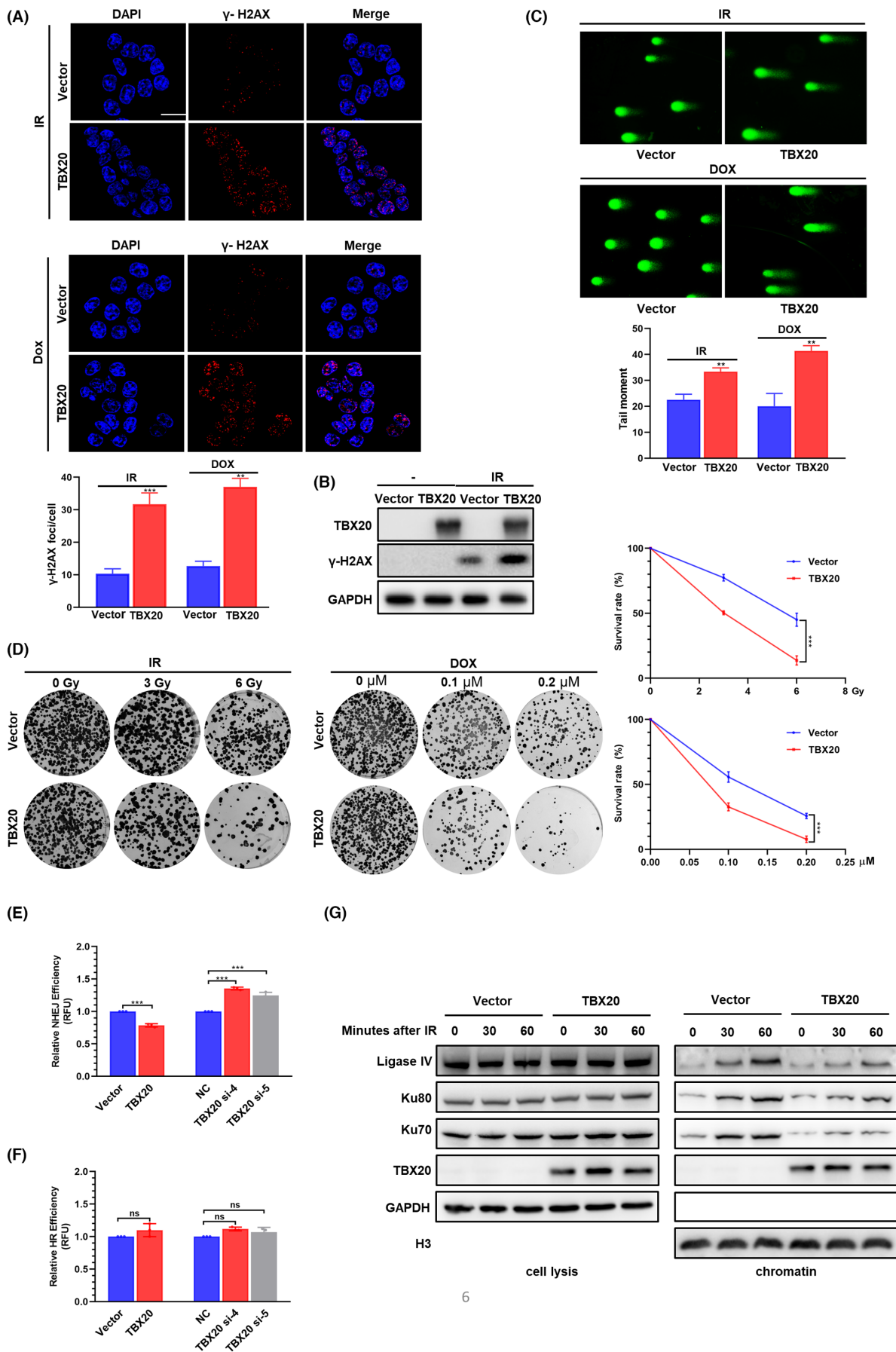
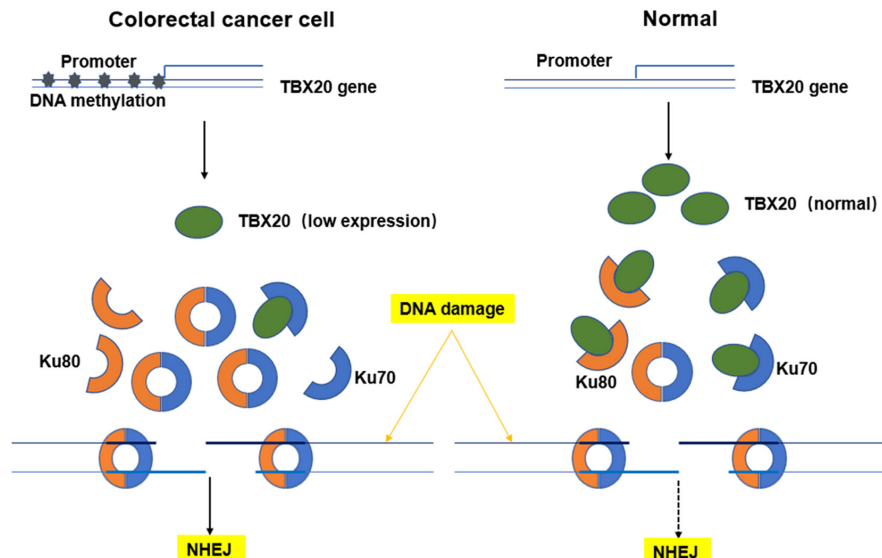


FIGURE 7 Model of TBX20 functions and mechanisms during CRC tumorigenesis



damage by radiation or DOX (a DNA damage-inducing drug), TBX20 overexpression inhibited the interaction of Ku70 and Ku80 and the accumulation of several NHEJ factors (Ku70, Ku80, Lig4) on chromatin, which resulted in impaired NHEJ, but had no effect on the HR pathway. These results revealed that TBX20 serves an important role in DSBs repair.

NHEJ is active in all stages of the cell cycle,⁶¹ and is considered to be responsible for rapid DSB repair of up to 85% of radiation-induced damage.⁶² Studies have found that NHEJ is correlated with the radioresistance of cancer cells. RFC4 mediates radioresistance in CRC by facilitating NHEJ repair.⁶³ Therefore, NHEJ pathways may be a potential target for cancer therapy. Our study confirmed that TBX20 impaired the efficiency of NHEJ-mediated DSB repair, and TBX20 overexpression could sensitize CRC cells to radiation and DNA damage-inducing drugs (DOX). Additional studies are warranted to explore whether TBX20 could improve the treatment effect of clinical chemotherapy drugs.

In summary, we uncovered for the first time the tumor-suppressive role of TBX20 involved in DNA repair in CRC. We identified that DNA hypermethylation contributed to transcriptional silencing of TBX20 in early-stage CRC, and E3 ubiquitin ligase PDZRN3 mediated the degradation of TBX20 protein via the proteasome pathway. Our study highlights the dysregulation of TBX20 in early-stage CRC tissues and the mechanism suppressing NHEJ-mediated DSBs repair by inhibiting the interaction of Ku70 and Ku80 (Figure 7), which indicates the potential of TBX20 as an effective biomarker for predicting the prognosis of patients with early-stage CRC and provides a therapeutic target for combination therapy with DNA repair inhibitors.

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DISCLOSURE

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

F.-W.W. and D.X. conceived and devised the study. F.-W.W. and J.L. designed the experiments and analysis. J.L., J.Z., K.H., S.L., J.-L.D., C.-H.C., and J.-L.L. performed the experiments. J.-W.C. and J.L. performed bioinformatics and statistical analysis. J.L., J.Z. and S.L. analyzed and interpreted the data. J.-W.C. provided CRC patients tissue samples and clinical information. D.X. and F.-W.W. supervised the research and together with J.L. wrote the manuscript. All authors approved the submitted manuscript.

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SUPPORTING INFORMATION

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