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Boris knockout eliminates AOM/DSS-induced in situ colorectal cancer by suppressing DNA damage repair and inflammation

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Abstract

The Brother of Regulator of Imprinted Sites (BORIS, gene symbol CTCFL) has previously been shown to promote colorectal cancer cell proliferation, inhibit cancer cell apoptosis, and resist chemotherapy. However, it is unknown whether Boris plays a role in the progression of in situ colorectal cancer. Here Boris knockout (KO) mice were constructed. The function loss of the cloned *Boris* mutation that was retained in KO mice was verified by testing its activities in colorectal cell lines compared with the *Boris* wild-type gene. *Boris* knockout reduced the incidence and severity of azoxymethane/dextran sulfate-sodium (AOM/DSS)-induced colon cancer. The importance of *Boris* is emphasized in the progression of in situ colorectal cancer. *Boris* knockout significantly promoted the phosphorylation of γH2AX and the DNA damage in colorectal cancer tissues and suppressed Wnt and MAPK pathways that are responsible for the callback of DNA damage repair. This indicates the strong inhibition of colorectal cancer in *Boris* KO mice. By considering that the DSS-promoted inflammation contributes to tumorigenesis, *Boris* KO mice were also studied in DSS-induced colitis. Our data showed that *Boris* knockout alleviated DSS-induced colitis and that *Boris* knockdown inhibited the NF-κB signaling pathway in RAW264.7 cells. Therefore *Boris* knockout eliminates colorectal cancer generation by inhibiting DNA damage repair in cancer cells and relieving inflammation in macrophages. Our findings demonstrate the importance of Boris in the development of in situ colorectal cancer and provide evidence for the feasibility of colorectal cancer therapy on Boris.

KEYWORDS
Boris, colitis, colorectal cancer, DNA damage repair, inflammation

1 | INTRODUCTION

Colorectal cancer[1] has overtaken lung and prostate/breast cancer as the third most common cancer worldwide.[2,3] The development of CRC is caused by complex mechanisms such as environmental risk, lifestyle, and heredity.[4] Colorectal cancer usually develops from untreated polyps from the beginning of the cecum to the end of the rectum.[5,6] Although patients could benefit from surgery, chemotherapy, and targeted therapy on epidermal growth factor receptor (EGFR) and human EGFR related 2 (HER2), relapse is a perplexing problem

Abbreviations: AOM, Azoxymethane; BORIS, Brother of Regulator of Imprinted Sites; CRC, Colorectal cancer; CTCFL, CCCTC-binding factor (zinc finger protein)-like; DSS, Dextran sulfate-sodium salt.

because of the nonspecific treatment. In total, 80% of CRC patients have no prior family history, although mutations in the *MLHs*, *APC*, and *PMS2* genes are known in the development of CRC. Targeted therapy on cancer-specific targets would be an ideal strategy.[7,8] BORIS (gene symbol CTCFL) expresses specifically in cancer cells but not in normal cells. BORIS expression specificity in cancer cells is advantageous for therapy.[9] It has been reported that BORIS is a potential immunotherapeutic target for the treatment of cervical carcinoma and lung cancer.[10] BORIS promotes colorectal cancer cell proliferation, while inhibiting apoptosis. It will be interesting to investigate if *BORIS* gene knockout completely eliminates colorectal cancer.

BORIS protein includes three parts: the N terminal section, the middle zinc finger domains, and the C terminal section.[11] The 11 middle zinc finger domains are conserved with its paralog CTCF (CCCTC-binding factor), which is essential for normal cells. The N terminal section is predicted to be the functional part for oncogenesis.[12] Truncation of *BORIS*, which lacks zinc finger domains, directed *BORIS* to the cytoplasm and still inhibited colorectal cancer apoptosis. Further deletion of the *BORIS* N section might destroy this function.

Boris knockout (KO) mice were constructed and used in this study to investigate AOM/DSS-induced colorectal cancer. The loss of amino acids (aa) after 137 aa of the Boris protein damaged the Boris function in promoting cancer cell proliferation and chemotherapy resistance. *Boris* knockout seriously eliminated in situ colorectal cancer generation under the induction of AOM and DSS. In total, 90% of the animals with *Boris* knockout had no polyp, and the other *Boris* knockout individuals had obviously smaller polyps compared with wild-type animals. The *Boris* knockout also suppressed inflammation caused by DSS treatment.[13–15] High-throughput sequencing of KO mice colon tissues compared with wild mice revealed that the *Boris* knockout inhibited the MAPK pathway. In addition, *Boris* knockout promoted apoptosis of colon tissue and relieved DSS-induced colitis. Our study demonstrated the importance of Boris in colorectal cancer development and provided evidence for the feasibility of targeted therapy on Boris.

2 | MATERIALS AND METHODS

2.1 | Cell culture and transfection

RAW264.7 and Caco-2 cell lines were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences. NIH3T3 cells were donated by LJ Zhu (Bioengineering Institute, Hangzhou Medical College, China). These cells were cultured in DMEM supplemented with 10% FBS. Cells were maintained at 37°C in a 5% CO_2 atmosphere. The drugs 5-FU (Sigma) and cisplatin (Selleck) were added to the cell culture medium to investigate the drug resistance ability of transfected cells. 5-FU (1.2 μM) and cisplatin (4 μg/mL) were used for the treatment of Caco-2 cells for 8 days. 5-FU (0.5 mM/24 h) and cisplatin (15 μg/mL/24 h) were used for the treatment of NIH3T3 cells. LPS (1 μg/mL) was applied to induce inflammation on RAW264.7 cells.

The wild-type gene of mouse *Boris* (NM_001081387.2) was synthesized by the Hangzhou Yanju bio-techne company, The *Boris* mutant that was cloned from the constructed *Boris* knockout mice was inserted into the pcDNA3.1 vector. The plasmid RC216042 with human *Boris* wild-type gene was purchased from OriGene Technologies, Inc. All constructs were confirmed by sequencing. Lipofectamine® 2000 reagent (BioSharp) was used to transfect plasmids for overexpression. Lipofectamine™ RNAiMAX (Thermo Fisher Scientific, Inc.) was used for gene knockdown according to the manual. The mouse *Boris* gene was silenced using small interfering RNA (siRNA) that targeted 5′-CAAGCAAGATGAAGCGTCA-3′, shown as si*Boris* in this study. Negative siRNA and si*Boris* were synthesized by Jima Biotechnology, China.

2.2 | Cell proliferation and apoptosis assays

The cell viability, colony formation ability, and apoptosis assays were applied to detect the difference among the transfected Caco-2 cells. In total, 1000 cells plated into each well of 96-well plates were subjected to plasmid transfection followed by the treatment of 5-FU or cisplatin for 24 h. The cell viability was measured by MTT (Solarbio, 298-93-1) according to the instructions. In total, 1000 cells plated into each well of 12-well plates were transfected and cultured for 7 days. Then cells were stained with crystal violet to examine colony formation. Caspase-Glo® 3/7 lysis substrate (Promega, G8090) was used to measure the apoptosis extent.

2.3 | RNA isolation and quantitative real-time PCR (qPCR)

Total RNAs were isolated by TRIzol reagent (Life Technologies) according to the manual. cDNAs were reverse transcribed using the Hifair® III First Strand cDNA Synthesis Kit (gDNA digester plus) (Yeasen, 11121ES60) and applied for PCR/qPCR analysis. qRT-PCR was performed using the 2×T5 Fast qPCR Mix (SYBR Green, Yeasen, 11201ES08) and a CFX connect real-time PCR detection system (Bio-Rad). *GAPDH* was used as an internal reference for normalization. The primers used in this study are listed in Table SI.

2.4 | Antibody and general reagents

Catalogs and working dilutions of the antibodies were as follows: phospho-P65 (3033S, 1:1000), P65 (8242S, 1:1000), IκBα (9242S, 1:1000), phospho-ERK (4370, 1:1000), ERK (4695, 1:1000), JNK (9252, 1:1000), phospho-JNK (9255, 1:1000), Phospho-P38 (4511, 1:1000), β-catenin (8480, 1:1000) GAPDH (5174S, 1:1000). These antibodies were purchased from Cell Signaling Technology. P38 (14064-1-AP, 1:2000) was purchased from Proteintech. Phospho-IκBα (AF2002, 1:1000) was purchased from Affinity Biosciences. Anti-phospho-γH2A.X (SAB5700329, 1:1000) was purchased from Merck Millipore. Other antibodies targeting CTCFL (sc-377085, 1:1000) were purchased from Santa Cruz Biotechnology

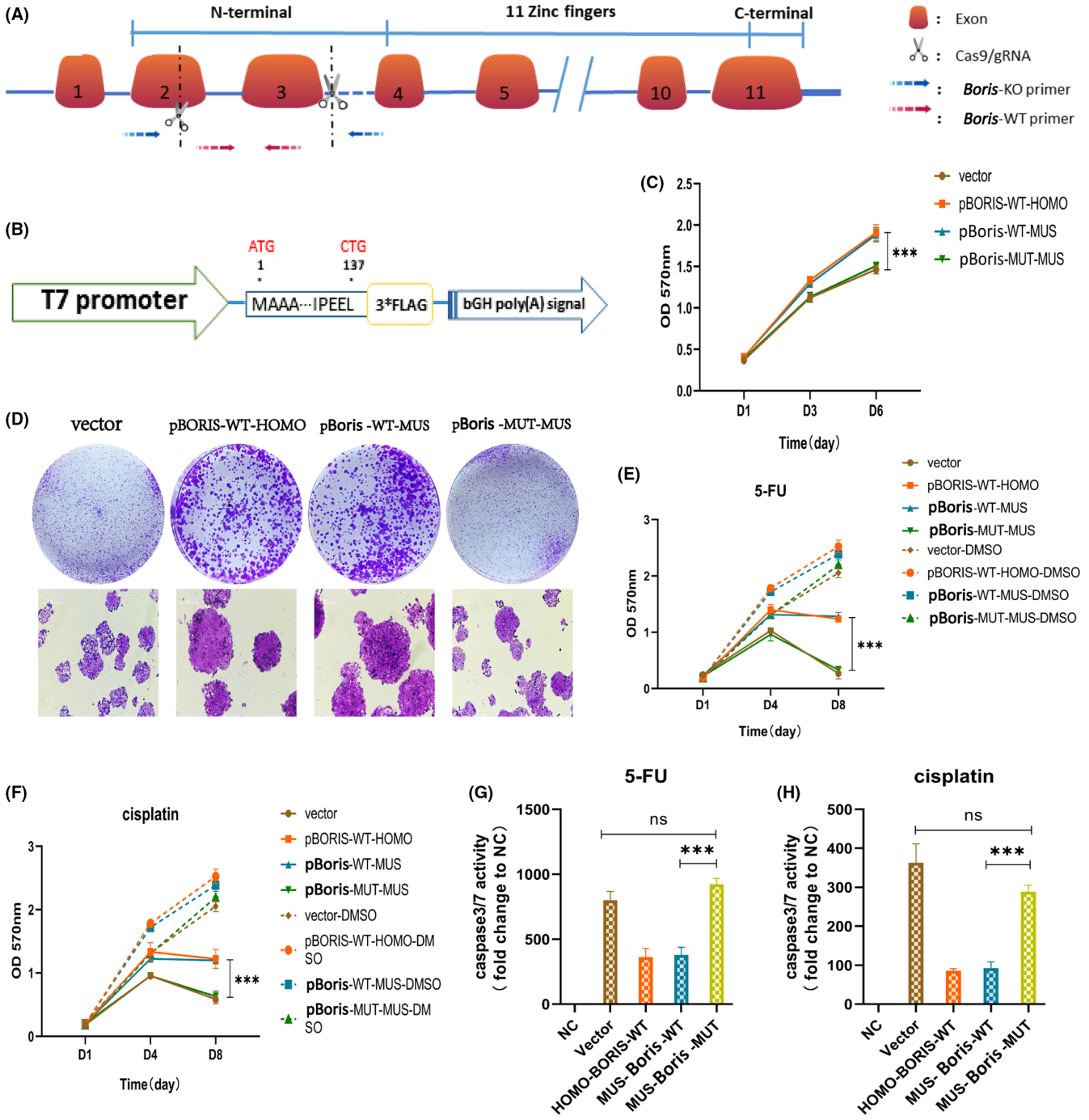


FIGURE 1 Construction of *Boris* knockout mice and verification of functional loss of Boris. (A) Schematic diagram of a deleted portion of Boris in the KO mice and the location of primers for genotype identification. (B) Schematic diagram of the *Boris* mutant, which was cloned. (C) *Boris* mutant loses the function of promoting cell proliferation. The absorbance at OD_{570} nm was detected by MTT assay after transfection of four different plasmids in Caco-2 cells (p*BORIS*-WT-HOMO: plasmid of human wild-type BORIS; p*BORIS*-MUT-HOMO: plasmid of human mutational BORIS; p*Boris*-MUT-MUS:plasmid of mouse mutational Boris). (D) *Boris* mutation loses the function of promoting colony formation. The assay was observed after transfecting four different plasmids into Caco-2 cells for 10 days. (E, F) Boris mutation loses the function of anti-apoptosis. After transfection of four different plasmids into Caco-2 cells for 24 h, the two drugs or DMSO (5-FU, 1.2 μM; cisplatin, 4 μg/mL; 2‰ DMSO) were added to a 96-well plate before detecting the absorbance at OD_{570} nm using the MTT assay every 4 days. (G, H) *Boris* mutation loses the function of anti-apoptosis. After transfection of four different plasmids into Caco-2 cells for 24 h, two drugs (5-FU, 1.2 μM; cisplatin, 4 μg/mL) were added to the 96-well plate and cultured for 24 h before detecting the luminescence. Data are shown as means ± SEM. The difference between the two groups was determined using an unpaired two-tailed Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

and LaminB was purchased from Hua An Biotechnology. Goat anti-mouse (H+L) HRP (Dawen Biotec, WD0990) and goat anti-rabbit (H+L) HRP (Dawen Biotec, WD-GAR007) are the secondary antibodies. AOM (Sigma-Aldrich, cat. no. A5486-100MG) was obtained from Sigma-Aldrich (USA). DSS (Aladdin, cat. no. D122354-100G) was purchased from Aladdin. LPS (Sigma, L2890) was purchased from Sigma.

2.5 | Animal experiment

Boris knockout (KO) mice were constructed using CRISPR/Cas gene deletion system and facilitated by Shanghai Model Organisms Center, Inc. The homozygous offspring were used in animal experiments. All mice were raised in a pathogen-free facility, and the methods of animal experiments were approved by the licensing committee of Hangzhou Medical College, China, no. 2021-076. All mice used in this study were male. The mice were divided randomly into three groups (negative control, $n = 5$; *Boris*-WT, $n = 17$; *Boris*-KO, $n = 17$). The procedure for the construction of AOM/DSS-induced in situ colorectal cancer was as follows: on the first day, AOM working solution (10 mg per kg body weight) was injected intraperitoneally into the experimental group. The sterile isotonic saline was applied to the control group comparably. In the next days, 2% (w/v) DSS in drinking water for three cycles (8 days/cycle, with a 14-day recovery after each of the DSS cycles) was administrated to induce colorectal cancer. DSS-induced colitis was constructed and compared with the process of in situ colorectal cancer progression. Two model groups (*Boris*-WT, $n = 5$; *Boris*-KO, $n = 5$) were treated with 2% (w/v) DSS in drinking water for three cycles (8 days/cycle, with a 14-day recovery after each of the DSS cycles) to induce colitis. AOM (Sigma-Aldrich, cat. no. A5486-100MG) was purchased from Sigma-Aldrich (USA). DSS (Aladdin, cat. no. D122354-100G) was purchased from Aladdin.

2.6 | Disease activity index (DAI) and histological analysis

Intestines were removed from animals and then fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, stained with H&E solution, and then examined by microscopy for histological changes. Two different investigators evaluated and determined the pathology scores of randomly numbered slides. DAI was evaluated by the scores of body weight loss (the weight before each DSS treatment compared with that after the DSS treatment: scored as 0, no change; 1, 1%–5% loss; 2, 5%–10% loss; 3, 10%–20% loss), stool consistency (0, normal; 1, soft but firm; 2, soft; 3, diarrhea), and fecal blood (0, none; 1–2, blood; and 3, gross bleeding). The lesion scoring assessment was presented as follows. Epithelium: 0, normal; 1, loss of goblet cells; 2, loss of goblet cells in large areas; 3, loss of crypts; and 4, loss of crypts in large areas. Infiltration: 0, none; 1, infiltrate around crypt basis; 2, infiltrate reaching to mucosae; 3, extensive infiltration to mucosae; and 4, infiltration of the submucosa.

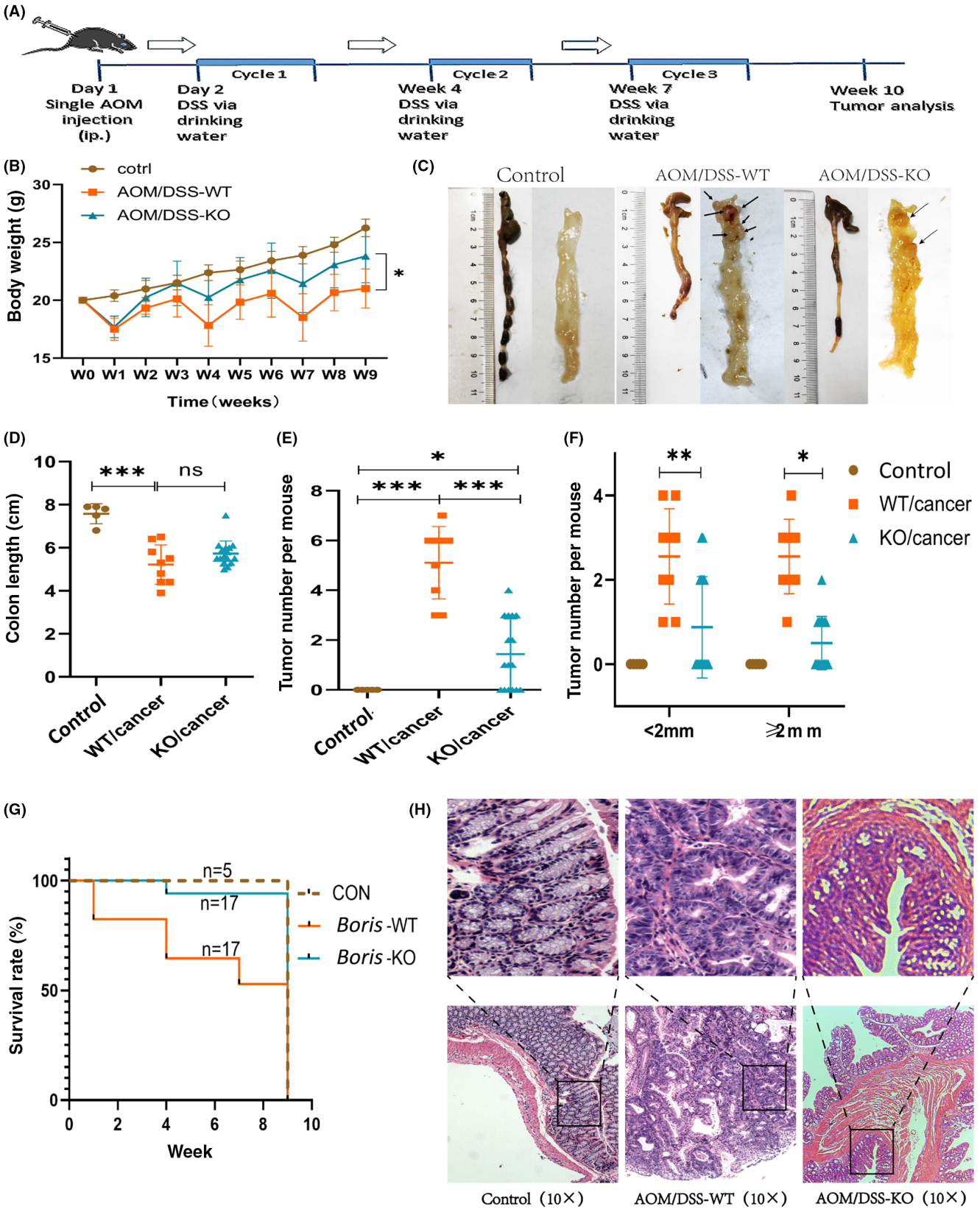
2.7 | RNA-sequencing and analysis

For sample preparation, colorectal tissues of *Boris* KO and WT CRC mice were collected, and each group of samples had three biological replicates. RNA extraction was performed using TRIzol reagent (Life Technologies) according to the protocol. A reference transcriptome analysis was performed using the MGI-SEQ platform. Briefly, 10 μg total RNA was used to purify poly(A) RNA. Samples were fragmented and reverse transcribed to create cDNA libraries. cDNAs were purified following uracil-DNA glycosylase (UDG) enzyme digestion and PCR, and the final paired-end libraries were formed (PE150). Paired-end sequencing was performed using DNBSEQ. With the final transcriptome generated, Bowtie2 and RSEM were executed to estimate the expression levels for mRNAs by calculating fragments per kilobase of exon per million mapped fragments (FPKM). Differentially expressed mRNAs, Pearson's correlation heatmap, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enriched were analyzed using the Dr Tom system (https://biosys.bgi.com/#/main). These sequence data have been submitted to the GenBank databases under accession no. PRJNA855225.

2.8 | Immunoblot assays

Lysis of intestine tissues and cells was prepared using lysis buffer containing PMSF and RIPA buffer (EMD Millipore Corp, 20-188) and then immediately subjected to western blot assay. The procedure is as follows: proteins were fractionated using SDS-PAGE, transferred to PVDF membranes, blocked in TBS containing 5% (wt/vol.) nonfat milk for 1 h rocking at room temperature, and then incubated overnight at 4°C with the indicated primary antibody. After washing with

FIGURE 2 Boris knockout eliminates AOM/DSS-induced in situ colorectal cancer. (A) The procedure of AOM/DSS-induced CRC mouse model. (B) The body weights were significantly lower in *Boris*-WT AOM/DSS animals. (C) The representative photographs of colon tissues in the three experimental groups. (D) The KO and WT groups showed a shorter length of the colon compared with the negative control group without any treatment (E, F), but *Boris*-KO has fewer tumor numbers and less size than the WT following AOM/DSS treatment. (G) The survival rate of WT is lower than for *Boris*-KO mice. (H) In addition, *Boris*-KO shows alleviated intestinal morphology disorder compared with WT in AOM/DSS-treated mice. Data are representative of three groups (negative control group: total mouse number, $n = 5$; survival mouse number, $n = 5$. Positive WT group: total mouse number, $n = 17$; survival mouse number, $n = 9$. *Boris*-KO group: total mouse number, $n = 17$; survival mouse number, $n = 16$) and are presented as means ± SEM. The significant difference between the groups was determined using an unpaired two-tailed Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.



TBST three times and 10 min each time, a secondary antibody was added and the sample incubated for 2 h at room temperature. Signals were detected after washing with TBST three times for 10 min each time; signals were presented using the Ultrasensitive ECL Kit and BIO-RAD ChemiDoc XRS+ system.

2.9 | Statistical analysis

The software GraphPad Prism 8 (GraphPad Software) was used for all statistical analyses. Two-tailed Student's *t*-test or ANOVA-Tukey test was used whenever appropriate. The variance was assessed by

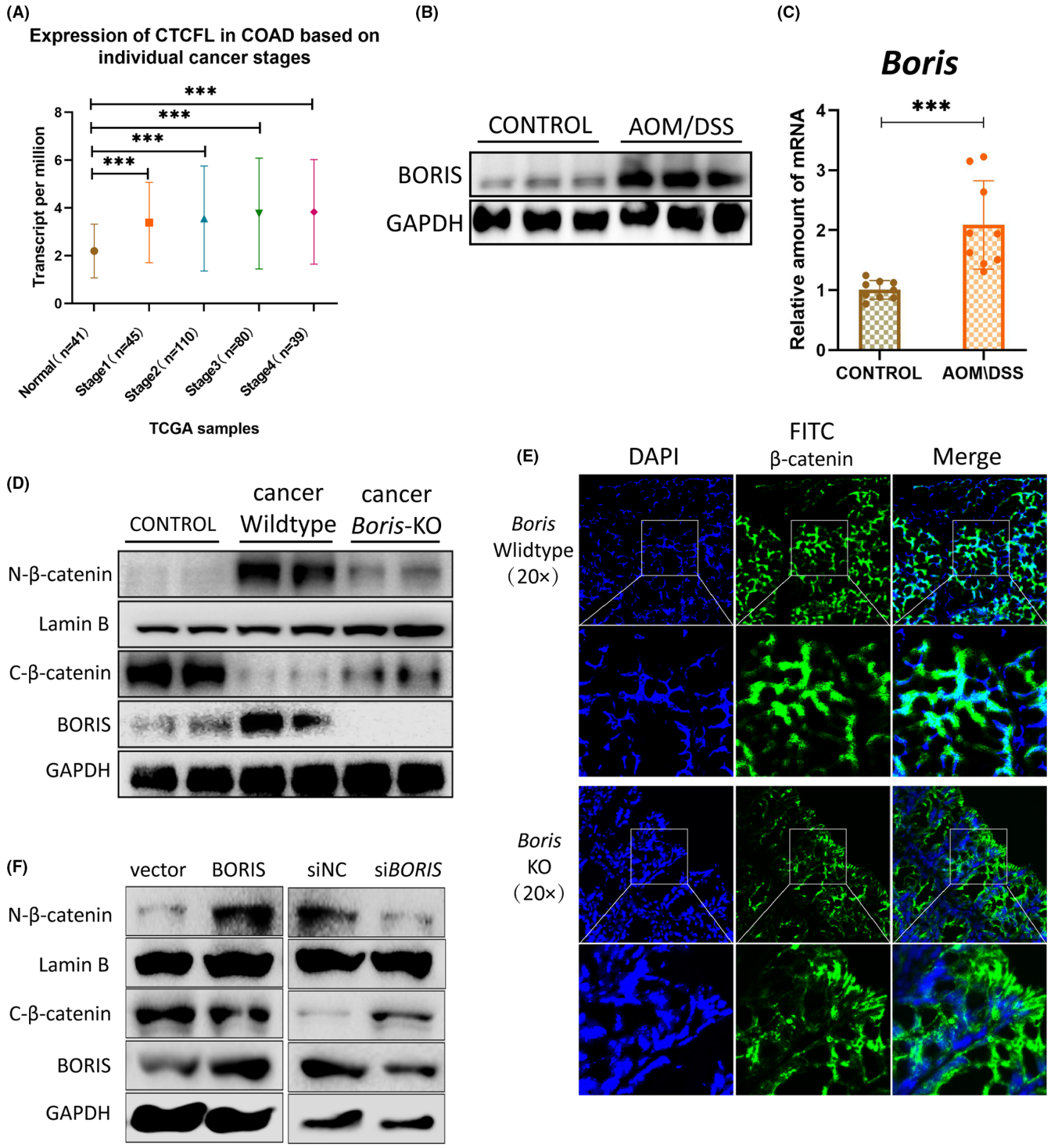


FIGURE 3 The Wnt signal pathway is inhibited by *Boris* knockout. (A) Analysis of BORIS expression in clinical colon adenocarcinoma tissues based on cancer stages. BORIS expression is higher than in normal tissue in all four cancer stages. (B, C) The expression of *Boris* in the colorectal cancer tissues, which was detected by western blot and qRT-PCR, are higher than in the negative control group. (D) Western blotting was used to determine the amount of β-catenin in the cytoplasm and nucleus of colon tissues from mice. (E) The immunofluorescence assay (×20) on mice colorectal tissues presents the subcellular localization of β-catenin. (F) After overexpressing Boris plasmids and *Boris* knockdown, protein levels of β-catenin in the cytoplasm and nucleus in Caco-2 cells were detected by western blots. Data are shown as the means ± SEM. The significant difference in this figure was determined using an unpaired two-tailed Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

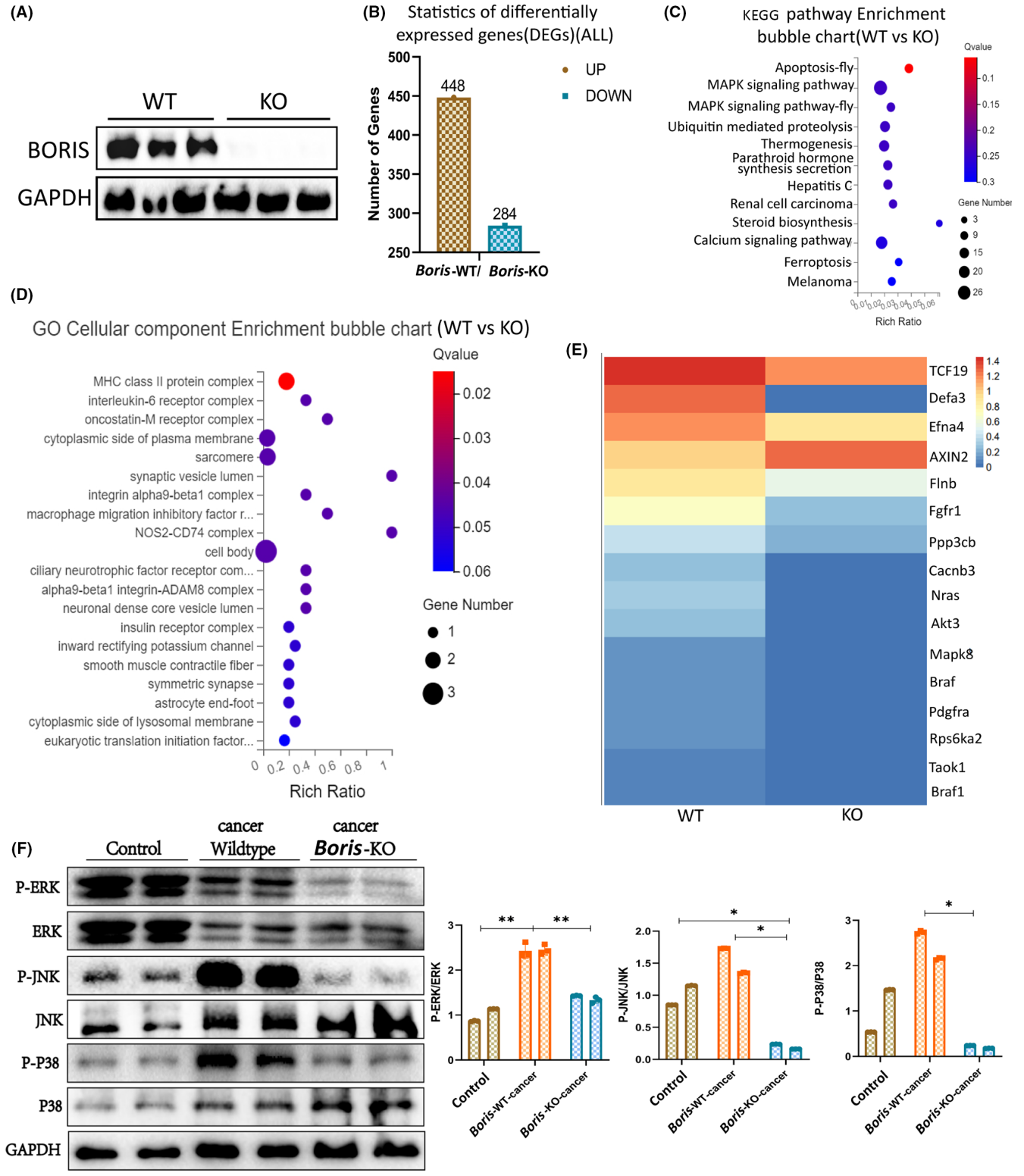


FIGURE 4 *Boris* regulates the MAPK signal pathway. (A) Western blotting was used to determine the knockout of *Boris*. (B) The number of genes regulated by *Boris* KO in colorectal tissues compared with WT is shown as upregulation and downregulation. (C) The apoptosis and MAPK signaling pathways were the top regulated pathways using KEGG pathway enrichment analysis. (D) Immune components were shown to be regulated by GO analysis. (E) Heatmap shows the changes in the genes in the MAPK pathway. (F) Western blotting was used to determine the association between Boris and the MAPK signal pathway. Data are shown as means ± SEM. The significant difference was determined using an unpaired two-tailed Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

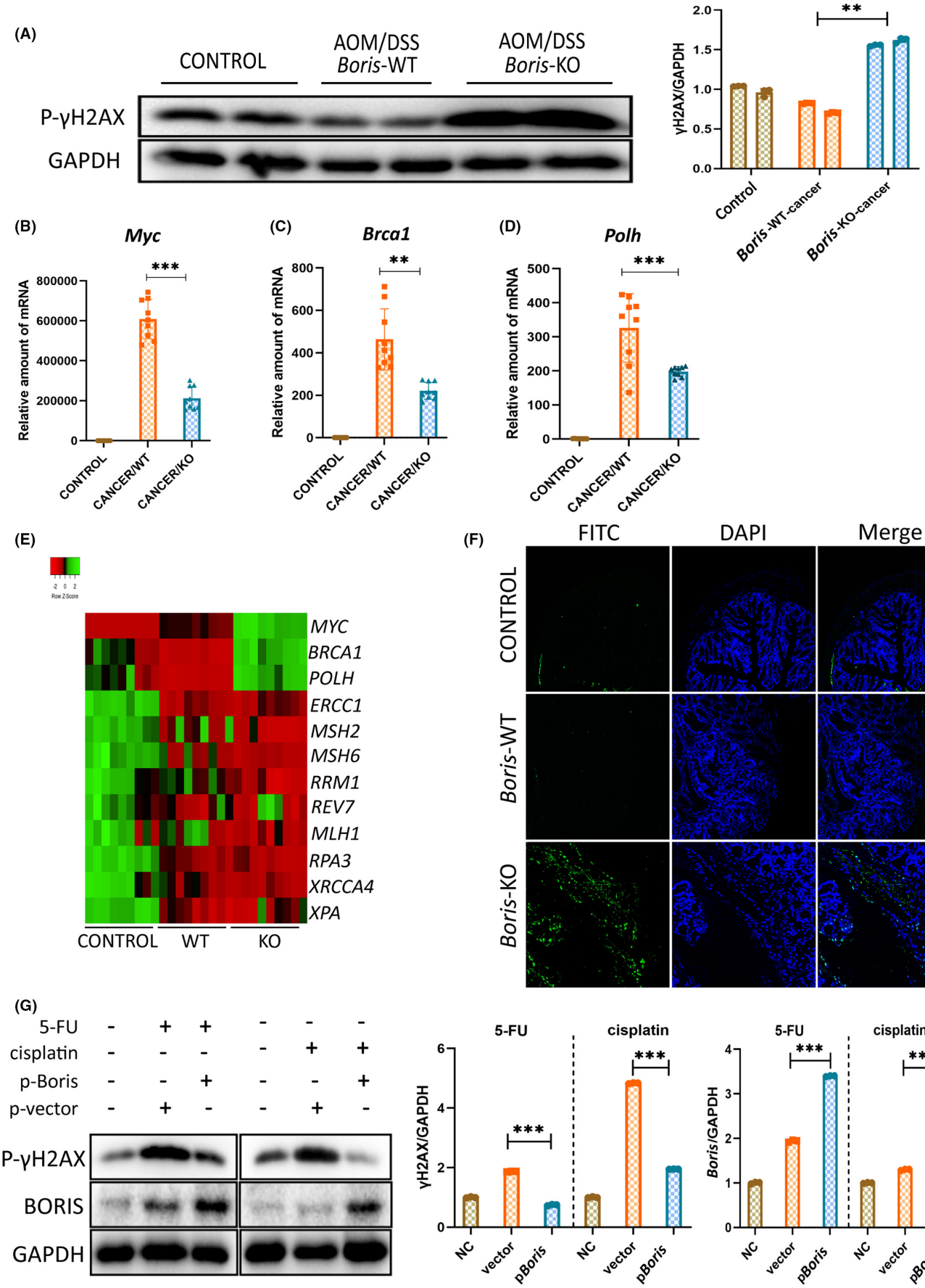


FIGURE 5 *Boris* regulates DNA damage. (A) Western blot was used to determine DNA damage in CRC tissues induced by AOM/DSS. The transcript expression levels of *MYC* (B), *BRCA1* (C), and *POLH* (D) of the colon tissues were detected using qRT-PCR. Heatmap (E) revealed a transcript expression profile of 12 genes associated with DNA damage or apoptosis. (F) TUNEL assay shows the CRC tissue apoptosis extent among the negative control without treatment, WT with AOM/DSS treatment, and *Boris* KO with AOM/DSS treatment. (G) P-γH2AX and *Boris* expression levels were detected in NIH3T3 cells, which were treated with 5-FU or cisplatin after transfection or *Boris* knockdown. Data are shown as means ± SEM. The significant difference was determined using an unpaired two-tailed Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

calculating the SEM in each group; *p*-values less than 0.05 were considered statistically significant.

3 | RESULTS

3.1 | Construction of *Boris* knockout mice and verification of functional loss of Boris

Boris KO mice were constructed using the CRISPR/Cas gene deletion systems to delete the genomic DNA between exons 2 and 3 of the *Boris* gene. *Boris* genotypes were identified using PCR and sequencing, as shown in Figure S1A. The remolding of *Boris* KO resulted in amino acid loss after 137 aa (Figure 1A). Because the deletion did not remove all of the *Boris* amino acids from the genome, it is unknown whether the remaining 137 aa of *Boris* still retained functions. The *Boris* mutant was cloned from homozygous *Boris* KO mice to test its function in colorectal cancer cells (Figures 1 and S1B).[16] Our previous studies demonstrated that *BORIS* knockdown significantly inhibited proliferation and promoted the apoptosis of colorectal cancer cells. Therefore colony formation, cell proliferation, and apoptosis assays were performed to verify the function of the *Boris* mutant in Caco-2 cells compared with the mouse *Boris* wild-type gene and human *BORIS*. The results showed that the *Boris* mutant did not enhance cell proliferation in the MTT assay (Figure 1C). Consistently, the *Boris* mutant from the KO mice did not promote colony formation (Figure 1D). Furthermore, the *Boris* mutant's effect on 5-FU or cisplatin resistance was confirmed. The *Boris* mutant lost its function to inhibit apoptosis in caspase 3/7 assays and colorectal cancer proliferation in cell viability assays. (Figure 1E–H).

3.2 | *Boris* knockout eliminates AOM/DSS-induced in situ colorectal cancer

To investigate the function of Boris on in situ colorectal cancer generation,[17,18] the AOM/DSS-induced mouse colorectal cancer model was established (Figure 2A). The positive CRC model group had significant weight loss during the first, second, and third cycles of DSS treatment compared with the negative control group. In contrast, the *Boris* KO group showed a significant remission for weight loss (Figure 2B). Then, to determine the incidence of colorectal cancer, we calculated the number and size distribution of tumors. The colon length of the *Boris* KO and *Boris* WT groups was significantly shorter than that of the control group, as shown in Figure 2 C,D, but there was no difference between the AOM/DSS-WT and AOM/DSS-*Boris* KO groups. Furthermore, the number of tumors in *Boris* WT individuals was higher than in *Boris* KO individuals (Figure 2C,E,F). The AOM/DSS-WT group had more tumors (larger than 2 mm) than the AOM/DSS-*Boris* KO group (Figure 2F). Figure 2G depicts the survival rate during administration. The AOM/DSS-WT group had ~50% survival, whereas the AOM/DSS-*Boris* KO group had only one mouse die. Histological examination also showed that *Boris* knockout significantly alleviated the typical pathological symptoms induced by AOM/DSS, presenting as an increased nucleoplasmic ratio, loss of nuclear polarity, and glandular hyperplasia (Figure 2H). These results demonstrated that the loss of *Boris* significantly relieved AOM/DSS-induced in situ tumor generation in mice.

3.3 | Wnt signal pathway is downregulated by *Boris* knockout

The Cancer Genome Atlas (TCGA) database showed that BORIS expression increased significantly in human colon adenocarcinoma (COAD) tissues in the clinic (Figure 3A).[8] This was consistent with our findings that *Boris* was significantly increased in colorectal cancer tissues in mice treated with AOM/DSS (Figure 3B,C).[19] It has been reported that most CRC occurrence is related to the activation of the Wnt signaling pathway. Therefore, we measured the level of β-catenin in the cytoplasm and nucleus of colon tissues (Figure 3D). *Boris* wild-type CRC mice had a higher accumulation of β-catenin in the nucleus and a lower amount of β-catenin in the cytoplasm compared with blank control of untreated mice. *Boris* KO mice with AOM/DSS treatment (CRC mice) showed a lower level of β-catenin in the nucleus and a higher level of β-catenin in the cytoplasm compared with *Boris* WT CRC mice. In addition, the subcellular location of β-catenin by immunofluorescence observation on colon tissue was consistent with the western blot results (Figure 3E). To confirm the relationship between BORIS and CRC, we measured the level of β-catenin in the cytoplasm and nucleus of Caco-2 cells after overexpression or knockdown of *BORIS*. The results showed that *BORIS* overexpression resulted in a higher level of β-catenin in the nucleus, and *BORIS* knockdown resulted in a decreased level of β-catenin in the nucleus. Our results demonstrated that Boris regulates the Wnt signal pathway in colon tissue and cells.

3.4 | *Boris* regulates tumorigenesis by the MAPK signal pathway

To elucidate Boris's role in colorectal cancer, the transcriptomes of AOM/DSS-treated colon tissue samples were analyzed (Figure 4A). Compared with the WT control, 732 differential genes were

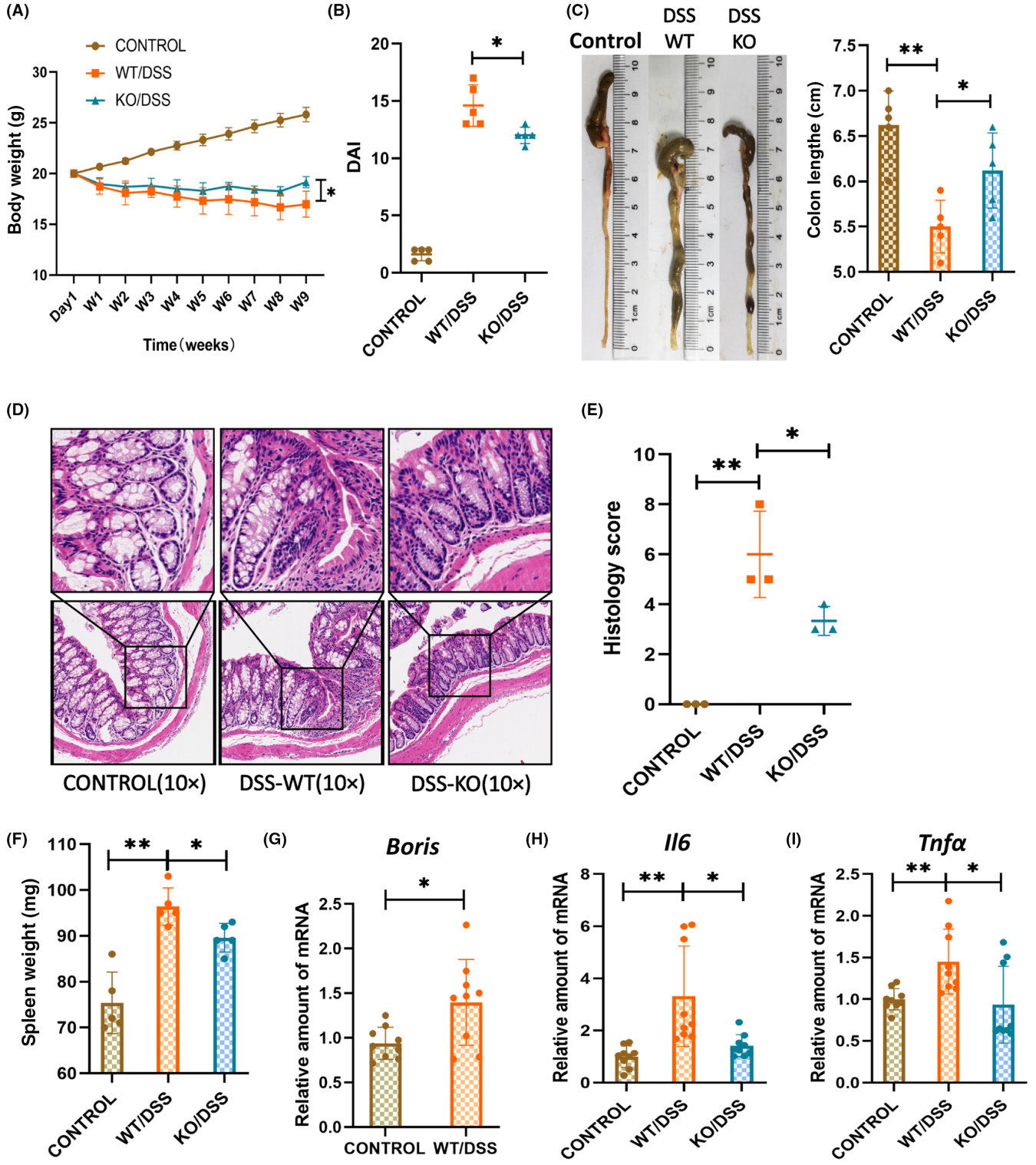


FIGURE 6 *Boris* knockout relieves colitis. The procedure of the DSS-induced mouse colitis model. There was a difference in body weight (A), DAI (B), colon length (C), histology score (D, E), and spleen weight (F) between *Boris* KO and WT mice. (G) *Boris* is highly expressed in the colon of the mouse with colitis; *IL6* (H) and *TNFα* (I) were suppressed by *Boris* knockout. Data are expressed as means ± SEM. The significant difference was determined using an unpaired two-tailed Student's *t*-test. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

detected, including 448 upregulated genes and 284 downregulated genes (fold change>2; Figure 4B). The MAPK signaling pathway and the apoptosis-related pathway were enriched in the KEGG pathway analysis of the differential genes (Figure 4C). In Gene Ontology (GO) analysis, immune-related complexes, such as the MHC class II protein complex and interleukin-6 receptor complex, were enriched in cellular component analysis (Figure 4D). As expected, the MAPK pathway genes, including *Fgfr1*, *Mapk8ip3*, *Cacnb3*, and so forth,

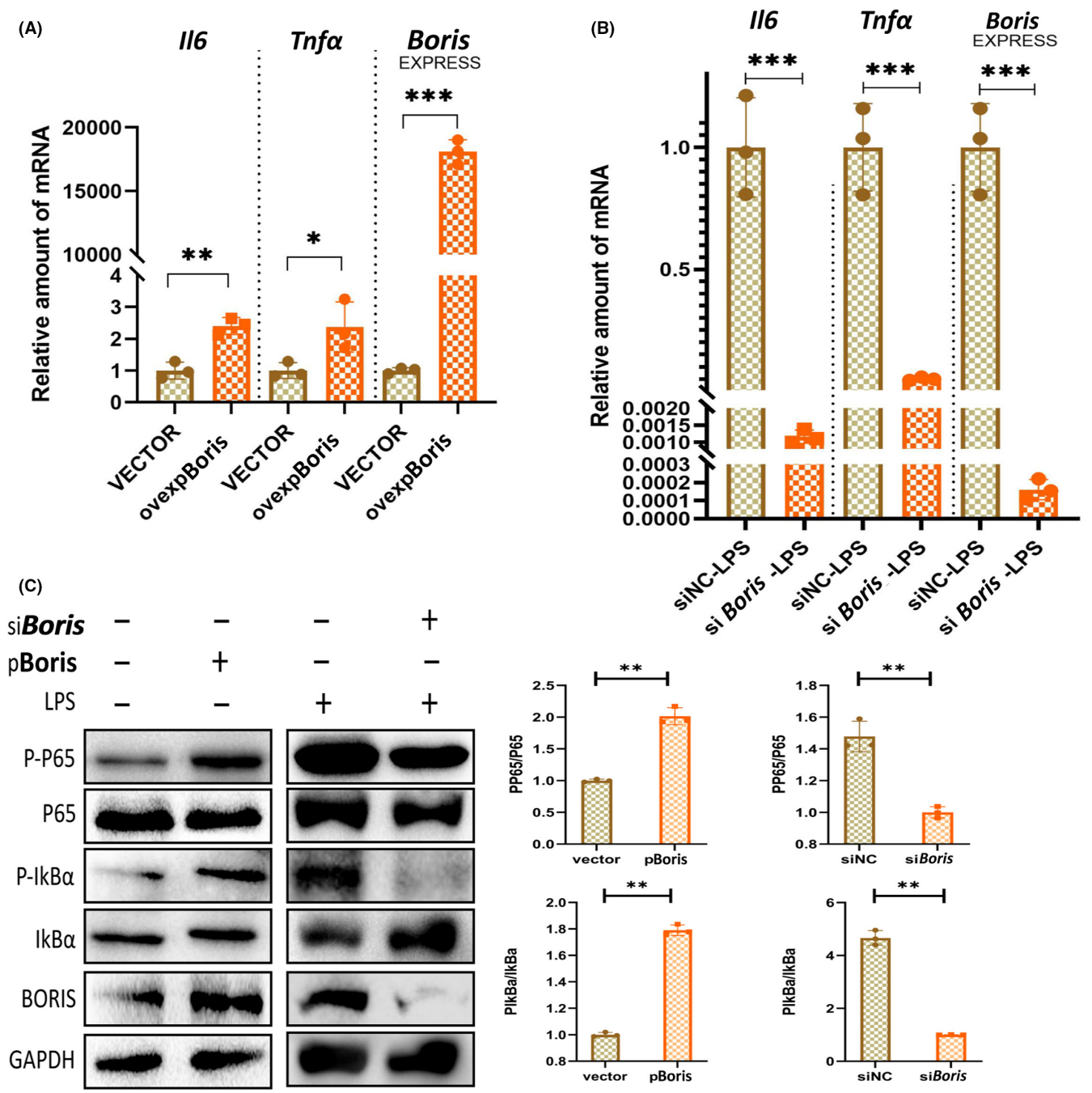


FIGURE 7 Boris promotes inflammation in macrophages. After overexpression using *Boris* plasmids (A) and *Boris* knockdown (B), the transcripts of *Il6*, *Tnfα*, and *Boris* were detected by qPCR in RAW264.7 cells. (C) The phosphorylation of P65 and IκBα and the total amounts of P65 and IκBα were detected by western blotting of RAW264.7 cells when the expression of *Boris* was modulated by transfection of overexpression plasmid or siRNA. The NF-κB pathway was induced by Boris overexpression or by LPS treatment. Data are shown as means ± SEM. The significant difference was determined using an unpaired two-tailed Student's *t*-test. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

were regulated by *Boris* (Figure 4E). Furthermore, western blot verification revealed that JNK and P38 phosphorylation in *Boris* KO colon tissues was significantly lower than in WT colon tissues (Figure 4F). In conclusion, *Boris* was found to regulate CRC through the MAPK signaling pathway.[20,21] Because the MAPK pathway reflects the occurrence of both DNA damage and inflammation,[22] we propose that Boris affects CRC by regulating both DNA damage and inflammation.

3.5 | Boris regulates DNA damage

We proposed that *Boris* knockout leads to DNA damage repair, thereby relieving colorectal cancer development. Phospho-γH2AX levels, which reflect DNA damage, were significantly higher in the *Boris* KO tissue than in the other two groups. However, there was less phospho-γH2AX in the WT tissue than in the negative control (Figure 5A). Our results demonstrated that Boris enhances DNA

repair under the treatment of AOM and DSS.[23,24] Therefore, 12 genes responsible for DNA damage repair were examined. Three genes, *MYC*, *BRCA1*, and *POLH*, were significantly downregulated after *Boris* knockout compared with the WT control (Figure 5B–E). This suggests that DNA damage repair was affected by the *Boris* knockdown. The TUNEL assay for detecting DNA damage levels revealed that the extent of DNA damage in *Boris* KO colon tissues was significantly greater than in the other two groups (Figure 5F).[16,23,25] Based on our findings from previous studies that BORIS promotes DNA damage repair in epithelial cells H1299, HCT116 and Caco-2,[26] here we used fibroblasts that usually exist in the tumor microenvironment and are responsible for tissue repair to confirm the effect of Boris on DNA damage. BORIS was transfected into NIH3T3 cells that were treated with 5-FU or cisplatin to induce DNA damage. Consistently Boris overexpression suppressed phospho-γH2AX compared with the negative control (Figure 5G). In summary, we demonstrated that Boris enhanced DNA damage repair and the loss of *Boris* led to cancer cell apoptosis.

3.6 | *Boris* knockout relieves colitis

Because *Boris* has been shown to regulate inflammation in AOM/DSS-induced CRC in pathway analysis (Figure 4), *Boris* KO mice were used to investigate if the loss of *Boris* affected DSS-induced colitis. We used the same dose of DSS and scheduled treatment as for AOM/DSS-induced CRC. Over the course of the administration, there were differences in body weight and DAI scores between the positive WT-DSS group and the *Boris* KO-DSS group (Figure 6A,B). *Boris* KO significantly impacted colon length (Figure 6C). When the colitis tissue sections were examined by H&E staining, we found that crypt structure destruction or disappearance, goblet cell loss, and inflammatory infiltration of varying degrees occurred in WT groups, but a milder pathological degree was present in KO groups. (Figure 6D,E).

In addition, the spleen weights differed significantly between the WT and *Boris* KO groups (Figure 6F). The expression of Boris in colitis was increased dramatically by the induction of DSS in WT mice, and the inflammatory factors of *Il6* and *Tnfα* decreased in colitis in the *Boris* KO group (Figure 6H,I). These findings showed that Boris expression was induced in colitis and that *Boris* knockout alleviated colitis and inflammation in animals.

3.7 | Boris promotes inflammation in macrophages

Boris locates in the nucleus and cytoplasm of cancer cells to promote tumorigenesis. As DSS-induced colitis was reported to be induced by the activation of the NF-κB signaling pathway, we used LPS to activate the cells and the NF-κB pathway in macrophages and to verify the relationship between Boris and NF-κB activation.[27] Boris promoted the expression of inflammation factors in RAW264. 7 cell (Figure 7A,B). The western blot results revealed that the P-P65/P65 and P-IκBa/IκBa ratios in the LPS-stimulated siNC group were significantly higher than in the control and *Boris* KO groups. Conversely, levels in the empty vector group without LPS treatment was lower than in the group overexpressing Boris without LPS (Figure 7C). These findings suggested that Boris provokes inflammation in macrophages.

4 | DISCUSSION

In this study, we constructed in situ colorectal cancer induced by AOM/DSS and colitis induced by DSS in *Boris* KO mice. Our findings revealed that Boris is important for the development of CRC and colitis (Figures 2 and 6). Boris regulates the Wnt signaling pathway (Figure 3). By comparing human BORIS, mouse Boris, and the *Boris* mutant cloned from *Boris* KO mice, we discovered that murine Boris had the same functions as human BORIS in promoting cancer cell proliferation and resistance to 5-FU and cisplatin treatment. Furthermore, the *Boris* mutant lost all these activities (Figure 1).

The tumor formation process induced by AOM/DSS was similar to that of humans.[28] We observed that high expression of Boris was induced in both clinical CRC samples and mouse in situ colorectal tissues (Figure 3A–C). This suggests that Boris relates to the progression of CRC. Our data confirmed that the loss of BORIS inhibited CRC in mice. Considering the mechanism that the Wnt signaling pathway was usually activated in AOM plus DSS-induced colorectal cancer, the subcellular location of β-catenin was examined by modulating Boris expression. Our results demonstrated that Boris regulated the Wnt pathway (Figure 3D–F). Our evidence determined the importance of Boris in the development of CRC.

The synergistic effects of AOM on genomic damage and DSS on inflammation led to colorectal cancer.[17] Disorders of persistent inflammatory reaction and aberrant DNA repair reaction promoted tumorigenesis. We noticed that eight mice died in the WT group within 1 week of AOM/DSS treatment, but no mice died using DSS treatment. AOM but not DSS should be the main cause of mortality in AOM/DSS-treated mice. DNA damage repair regulated by Boris should have an important effect. By investigating phospho-γH2AX and performing TUNEL assays on mice tissues, which reflect the extent of DNA damage, we demonstrated that Boris promoted DNA repair. These results were consistent with other finding that the *Boris*-WT but not *Boris*-MUT inhibits cancer cell apoptosis and resists chemotherapy (Figure 1). DSS-induced inflammation is the facilitator of tumorigenesis. Boris expression was also upregulated in inflammation. *Boris* knockout indeed suppressed colitis, which is consistent with results in RAW264.7 cells (Figures 6, 7, and S1C,D).

According to transcriptome analysis of mice colorectal tissues, the MAPK pathway is regulated by *Boris*. By further examination of colorectal cancer tissues from mice, we determined that *Boris* knockout led to the suppression of the MAPK pathway, which is presented as phosphorylation withdrawal of ERK, JNK, and p38

(Figure 4F).[20] The MAPK pathway is known to be activated by DNA damage. However, our data showed that *Boris* knockout led to DNA damage (Figure 5). It is contradictory that the loss of Boris promoted DNA damage but also inhibited the response of the MAPK pathway. Considering that MAPK responded to DNA damage and promoted subsequent cell proliferation and tissue repair, Boris deficiency might inhibit the MAPK pathway and cause a wide range of prohibition of colon tissue repair and the maximum extent of inhibition on colorectal cancer.[29] It has been reported that the Wnt pathway promotes DNA damage repair in colorectal cancer cells. Our results that *Boris* knockdown inhibits the Wnt pathway in Figure 3 also support our prediction that Boris deficiency caused a wide range of prohibition of colon tissue repair.

In conclusion, BORIS was highly expressed in CRC and promoted the development of tumors. *Boris* knockout alleviated in situ CRC generation by relieving colitis and suppressing DNA damage repair. Our findings provide new insights into the development of CRC and provide new strategies for CRC therapy.

AUTHORSHIP CONTRIBUTIONS

Bo-Wen Zuo (formal analysis: lead; investigation: lead; methodology: lead; writing – original draft: lead). Wan-Xin Yao (data curation: supporting; formal analysis: supporting; visualization: supporting). Meng-Die Fang (formal analysis: supporting; funding acquisition: lead investigation: supporting). Juan Ren (formal analysis: supporting; investigation: supporting). Ling-Lan Tu (formal analysis: supporting). Run-Jie Fan (formal analysis: supporting). Yan-Mei Zhang (conceptualization: lead; funding acquisition: lead; methodology: lead; data curation: lead; writing – original draft: lead).

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest.

ETHICS STATEMENT

- Approval of the research protocol by an Institutional Reviewer Board: N/A.
- Informed Consent: N/A.
- Registry and the Registration No. of the study/trial: N/A.
- Animal Studies: We declare that all animal experimental protocols were approved by the licensing committee of Hangzhou Medical College, China. License number: 2021-076.

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SUPPORTING INFORMATION

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

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