

## Original Research

## BORIS-mediated generation of circular RNAs induces inflammation

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## ARTICLE INFO

## Keywords:

Circular RNA  
BORIS  
RNA and DNA binding motif  
inflammation  
circRNA vaccine

## ABSTRACT

Circular RNAs (circRNAs), which are more stable than linear mRNAs and long non-coding RNAs (LncRNAs), are detected in body fluids such as plasma, serum, and exosomes. Disease-associated circRNAs have significant clinical roles due to their diagnostic and prognostic values. Brother of regulator of imprinting site (BORIS) promotes cancer progression and is specifically highly expressed in the majority of carcinoma. However, the mechanism underlying the regulation of circRNAs by the oncoprotein BORIS and their role in regulating inflammation and immunity remain to be further explored. Vaccines prepared from circRNAs extracted from cancer cells showed that circRNAs induced inflammation and prevented cancer progression. Serum from animals injected with cancer cell-derived circRNAs vigorously reacted with cells that expressed cancer-specific antigen BORIS or cancer extracted circRNAs. It has been implicated that cancer-related circRNAs could be used as antigens to activate immune responses to prevent cancers and stimulate NF- $\kappa$ B signaling pathway by up-regulating and inducing TLR3. In the study we also found that BORIS regulated the expression of circRNAs and interacted with RNA motifs and the CCCTC binding factor (CTCF) motif adjacent to circRNA splicing sites to enhance the formation of circRNAs. Thus, our study delineated the novel mechanism by which cancer-specific antigen BORIS regulated circRNAs and identified that circRNAs could serve as a vaccine for cancer prevention.

## Introduction

Circular RNAs (circRNAs) are more stable than linear RNAs, such as mRNAs and lncRNAs. Abundant circRNAs are detectable in tissues and body fluids and many cancer-associated circRNAs were reported to be potentially important for diagnosis or prognosis of cancer [1]. CircRNAs usually regulate the expression of mRNAs by sponging microRNAs (miRNAs) [2]. Previous literatures also highlight how circRNAs regulate the expression of pathogen recognition receptors, such as retinoic-acid-inducible gene-1 (RIG-I) and melanoma-differentiation-associated gene 5 (MDA5) and the phosphorylation of protein kinase R (PKR) [3–5]. It has been suggested that circRNAs might provoke inflammation. However, an interesting aspect that needs further investigation is whether cancer-specific circRNAs provoke inflammation to prevent cancer progression.

CircRNAs are derived from exons, intron, or combined exons and introns. The classic splicing sites are usually involved in the generation of circRNAs [6]. Inhibition of spliceosome assembling suppresses circRNAs expression, indicating that the generation of circRNAs depended on the classic splicing factors [7], like the RNA splicing factor RNPS1 that regulates circRNAs expression. Alu repeat elements also

enhance the expression of circRNAs [8]. It has also been reported that abnormally expressed RNA binding protein quaking (QKI) promotes the expression of unique circRNAs by binding to cis-acting elements adjacent to the splicing sites of circRNAs in epithelial-mesenchymal transition [9]. However, only a few circRNA parental genes contain Alu repeat elements or QKI binding sites. Moreover, none of the splicing factors, Alu elements, or QKI protein are particular for cancer, making it hard to explain the underlying mechanism in cancer that leads to the expression of such circRNAs.

CircRNAs generation is similar to the process of chimeric RNA fusion, which utilizes ordinary splicing sites and is regulated by spliceosomes. CircRNAs are known to be regulated by splicing factors like RNPS1, which also regulates chimeric *CTNNBIP1-CLSTN1* RNA fusion [10]. CircRNAs are generated by back-splicing, while chimeric fusion RNA is generated by trans-splicing or cis-splicing [11]. The factors which regulate chimeric RNA fusions might also regulate the generation of circRNAs. CTCF, which is conserved from *C.elegans* to mammals, is essential for the 3D structure construction of the genome by binding CTCF motifs to mediate loops generation [12,13]. CTCF deficiency promotes chimeric RNA fusions that give rise to products such as *SLC45A3-ELK4* and *CTNNBIP1-CLSTN1* [14–16]. CTCF binds insulators

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adjacent to splicing sites of *SLC45A3* and *ELK4* to inhibit *SLC45A3-ELK4* fusion [17,18]. BORIS is the paralogue of CTCF and is specifically highly expressed in the majority of carcinoma but not in the adjacent normal tissues except for testis [19,20]. BORIS was reported to enhance the expression of chimeric fusion RNAs. Considering that BORIS promotes but CTCF suppresses cancer progression, we hypothesized that BORIS might have an opposite function from CTCF, and they might, in combination, regulate the generation of circRNAs.

In this study, we used circRNAs extracted from cancer cells as a vaccine to investigate whether cancer cell-related circRNAs provoke inflammation and prevent cancer progression. We also investigated the regulations of circRNAs by factors that regulate the formation of chimeric RNA. We constructed a plasmid that contains circRNA splicing sites and BORIS binding sites to investigate the regulation mechanism of circRNAs. Thus, these results suggest circRNAs can serve as a novel vaccine, which prevents tumor progression and induces immunity, may provide valuable clinical significance and therapeutic potential.

## Materials and methods

### Cell culture, transfection, and stimulation

Human HEK293, H1299, and mouse RAW264.7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Gemini, USA). Human LNCap and K562 cells were maintained in RPMI-1640 Medium supplemented with 10% FBS. Cells were grown at 37 °C in a 5% CO<sub>2</sub> atmosphere. All cell lines used in this study were obtained from Chinese Academy of Sciences (China). Plasmid or circRNA transfection was carried out using Lipofectamine 2000 Transfection Reagent (Lip2000) (BioSharp, China), and siRNA transfection was carried out using Lipofectamine RNAiMAX Reagent (Life Technologies, USA). For treatments with different stimulators, LPS (1 µg/mL, 0.1 µg/mL), ZL0420 (10 µM), and TLR4-IN-C34 (10 µM) were directly added into the culture medium. After treatment, proteins and total RNA were collected for further analyses.

### RNA isolation and purification, RT-PCR, and qRT-PCR

Total RNA was extracted from the cultured cells using Trizol according to the manufacturer's protocol. As circRNAs are resistant to RNase R digestion [21], they were purified using RNase R (GENESEED, Guangzhou, China), which was added at a ratio of 1 U: 1 µg of total RNAs and incubated at 37 °C for 30 min. The products obtained from the treatment had a high number of circRNAs. RNase R was then inactivated at 70 °C for 10 min. The RNA was then reverse transcribed to cDNAs using the Goldenstar™ RT6 cDNA Synthesis Kit (Tsingke, China) and subjected to PCR/qPCR analysis. qRT-PCR was performed using the 2 × T5 Fast qPCR Mix (SYBR Green I, Tsingke, China) and a CFX Connect Real-Time PCR detection system (BIO-RAD, USA), according to the manufacturer's instructions. *GAPDH* and *SMARCA5* were used as internal controls for normalization. The relative quantity of circRNA and mRNA expression was calculated using the  $2^{-\Delta\Delta C_t}$  method. The expression of each examined gene was determined from three independent experiments. Primer sequences are provided in Table S1.

### Immunization of mice

Eighteen nude mice were divided equally into three groups. The three groups of mice were immunized subcutaneously with 2 µg circRNA and 5 µL Lip2000 in 200 µL Reduced Serum Media (Thermo Fisher Scientific, USA), 2 U RNase R and 5 µL Lip2000 in 200 µL Reduced Serum Media, and 5 µL Lip2000 in 200 µL Reduced Serum Media per mouse, with the preparation of mixture being the same as that for transfection. After one week,  $1 \times 10^6$  CaCO-2 cells were subcutaneously injected into the nude mice. After 2 weeks of the primary vaccination, a

booster vaccination was given. Tumors were measured twice a week. Mouse blood was collected from the eyeballs, and tumors were collected to measure their final weight after 5 weeks.

### Antibody ELISA

Mouse sera were collected through centrifugal separation. RAW264.7 cells were cultured in 96-well assay plates (Corning, USA). CircRNAs, RNase R, BORIS, and negative control plasmids were transfected, and after 2 days the plates were washed thrice with 100 µL PBS. Cells were fixed with 4% methanol for 10 min at 25 °C and blocked with 50 µL 1.5% BSA for 30 min. Serum samples from immunized mice were serially diluted in PBS and incubated on blocked plates overnight at 4 °C. Then, the wells were washed and incubated with 1: 500 rabbit anti-mouse IgG (H+L) FITC (Dawen Biotec, China) for 1 h at room temperature. The plates were washed, and fluorescence was monitored using a SpectraMax iD5 multi-mode microplate reader (485 nm/535 nm; excitation/emission wavelengths).

### ELISA measurement

RAW264.7 cells were treated for 24 h. The IL-6 and TNF-α in cell culture supernatants were measured with mouse IL-6 and TNF-α ELISA kits (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Plates were measured on a SpectraMax iD5 multi-mode microplate reader at a wavelength of 450 nm.

### RNA immunoprecipitation (RIP)

BORIS and CTCF were overexpressed in HEK293 cells for 48 h. Cells growing in 6-well plates were rinsed twice with ice-cold PBS, harvested in 1 mL ice-cold PBS, and then centrifuged at  $1000 \times g$  for 5 min at 4 °C. Cells were resuspended in 500 µL polysome lysis buffer (100 mM KCl, 5 mM MgCl<sub>2</sub>, 10 mM HEPES-NaOH pH 7.0, 0.5% Nonidet P-40 [NP-40], 1 mM dithiothreitol [DTT], 200 units/mL RNase OUT, and EDTA-free Protease Inhibitor Cocktail), and incubated on ice for 30 min, followed by 10 s (1 s on/2 s off) of sonication on ice. Cell lysates were centrifuged at  $15,000 \times g$  for 15 min at 4 °C, and the supernatants were precleared with 25 µL mouse IgG beads (PureProteome Protein A and Protein G Magnetic Beads; Merk Millipore, Germany) to eliminate any non-specific binding. Then, the precleared lysates were used for immunoprecipitation (IP) with 75 µL anti-Myc-Tag antibody beads (Cell Signaling Technology, 2276). IP was carried out overnight at 4 °C. The beads were washed six times with 1 mL of ice-cold NT-2 buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM MgCl<sub>2</sub>, 0.05% NP-40, 20 mM EDTA pH 8.0, 1 mM DTT, and 200 units/mL RNase OUT). One-tenth of the bead sample was used for western blotting. The remaining sample was used for RNA extraction and then subjected to sequencing. The RIP-sequencing output was analyzed by Shanghai Outdo Biotech Co., Ltd. (Shanghai, China). BORIS and CTCF RNA binding motifs were analyzed using HOMER (v4.11) [22].

### Co-immunoprecipitation (co-IP)

BORIS and QKI, or CTCF and QKI were overexpressed in HEK293 cells for 48 h. Cells growing in 6-well plates were rinsed twice with ice-cold PBS, harvested in 1 mL ice-cold PBS, and then centrifuged at  $1000 \times g$  for 5 min at 4 °C. Cells were resuspended in 300 µL cell lysis buffer (50 mM Tris-HCl pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 1 mM PMSF, and EDTA-free Protease Inhibitor Cocktail) and incubated on ice for 30 min, followed by 10 s (1 s on/2 s off) of sonication on ice. The cell lysates were then centrifuged at  $15,000 \times g$  for 15 min at 4 °C, and the supernatants were precleared with 5 µL mouse IgG beads (PureProteome Protein A and Protein G Magnetic Beads; Merk Millipore) to remove any non-specific binding. Then, the precleared lysates were used for co-immunoprecipitation with 25 µL antibody-beads. Co-

immunoprecipitation was carried out overnight at 4 °C. The beads were washed thrice with 500 µL of ice-cold PBS containing 0.1% Tween 20 surfactant. After washing the beads, the sample was used for western blotting. BORIS and CTCF were expressed with myc-tag, and QKI was expressed with HA-tag. Anti-Myc tag mouse monoclonal antibody (1: 1000; Cell Signaling Technology) was used to detect BORIS and CTCF, and anti-HA tag rabbit monoclonal antibody (1: 1000; Cell Signaling Technology) was used to detect QKI. After three washes with TBST buffer, membranes were incubated for 60 min at 25 °C with horseradish peroxidase (HRP)-conjugated secondary antibodies (1: 5000) (Dawen Biotec). Protein bands were visualized with a chemiluminescence imaging system (Bio-Rad).

#### Western blotting

Human HEK293, H1299, and K562 cells, and mouse RAW264.7 cells were collected and lysed 48 h after transfection to extract total proteins. The cells were lysed in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) using a Bioruptor sonicator. Proteins were separated via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes, blocked for 1 h in TBS containing 0.1% Tween 20 surfactant and 5% (wt/vol) nonfat milk with rocking at room temperature, and then incubated overnight at 4 °C with the following primary antibodies: anti-Phospho-NF-κB p65 rabbit monoclonal antibody (1: 1000), anti-NF-κB p65 rabbit monoclonal antibody (1: 1000), anti-Phospho-IκBα mouse monoclonal antibody (1: 1000), anti-IκBα rabbit polyclonal antibody (1: 1000), anti-CTCF rabbit monoclonal antibody (1: 1000), anti-Myc tag mouse monoclonal antibody (1: 1000), anti-HA tag rabbit monoclonal antibody (1: 1000) (all from Cell Signaling Technology), Anti-BORIS/CTCF antibody, clone 4A7 (1: 500) (Millipore, USA), and anti-GAPDH polyclonal antibody produced in rabbit (1: 5000) (Sigma-Aldrich). The secondary antibodies used were goat anti-mouse (H+L) HRP and goat anti-rabbit (H+L) HRP (both from Dawen Biotec).

#### Plasmid construction

Human BORIS, CTCF, and RNPS1 were PCR amplified from the cDNA of LNCap cells and cloned into the pCMV6-Entry vector (Origene, USA). Human QKI was PCR amplified from the cDNA of HEK293 cells and cloned into the pENTER vector (WZ Biosciences Inc., China). Human circSMARCA5 DNA containing QKI binding sites was PCR amplified from the genomic DNA of HEK293 cells and cloned into the EcoRI and BamHI sites of the pCMV6-Entry vector. Human BORIS binding site DNA was PCR amplified from the genomic DNA of LNCap cells and cloned into pCMV-circSMARCA5 via T-PCR. The resulting expression plasmids, circFront (in which the front QKI binding sites were replaced by the BORIS binding site), circBack (in which the back QKI binding sites were replaced by the BORIS binding site), and circAll (in which the front and back QKI binding sites were replaced by the BORIS binding site) were based on pCMV-circSMARCA5. These plasmids were used for all experiments reported in this article.

#### Bioinformatic analysis

The Hi-C data from human K562 cells showed the occurrence of BORIS on the genome. The junction sites of the fusion RNAs or circRNAs with CTCF peaks were analyzed. The genomic contacts detected by our analyses were thought to result in the formation of fusion RNAs and circRNAs. The scores represent the degree of overlap between fusion RNAs and circRNAs and the peaks mediated by CTCF. Every 0.5 score indicates one active site, representing the splicing site of fusion RNAs or circRNAs in contact with a peak. High scores indicate a high possibility that the formation of fusion RNAs and circRNAs is mediated by CTCF. The information regarding circRNAs was taken from the circBase [23]

and TRCirc [24] databases, and the scores of fusion RNAs and circRNAs related to diseases from databases CosmicFusion [25] and MNDR (v3.1) [26] were also checked and compared. Then, the gene symbols from fusion RNAs and circRNAs scored were analyzed, revealing that 13 active genes participated in the formation of both fusion RNAs and circRNAs. The distances from the CTCF or BORIS binding sites found in K562 cells to the active sites in the genome were also measured. All bioinformatics analyses were carried out using R. The results were represented using R package ggplot2.

#### Statistical analysis

GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA) was used for all statistical analyses. The student's *t*-test was used whenever appropriate. Variance was assessed by calculating the standard error of the mean in each group. *P*-values less than 0.05 were considered statistically significant.

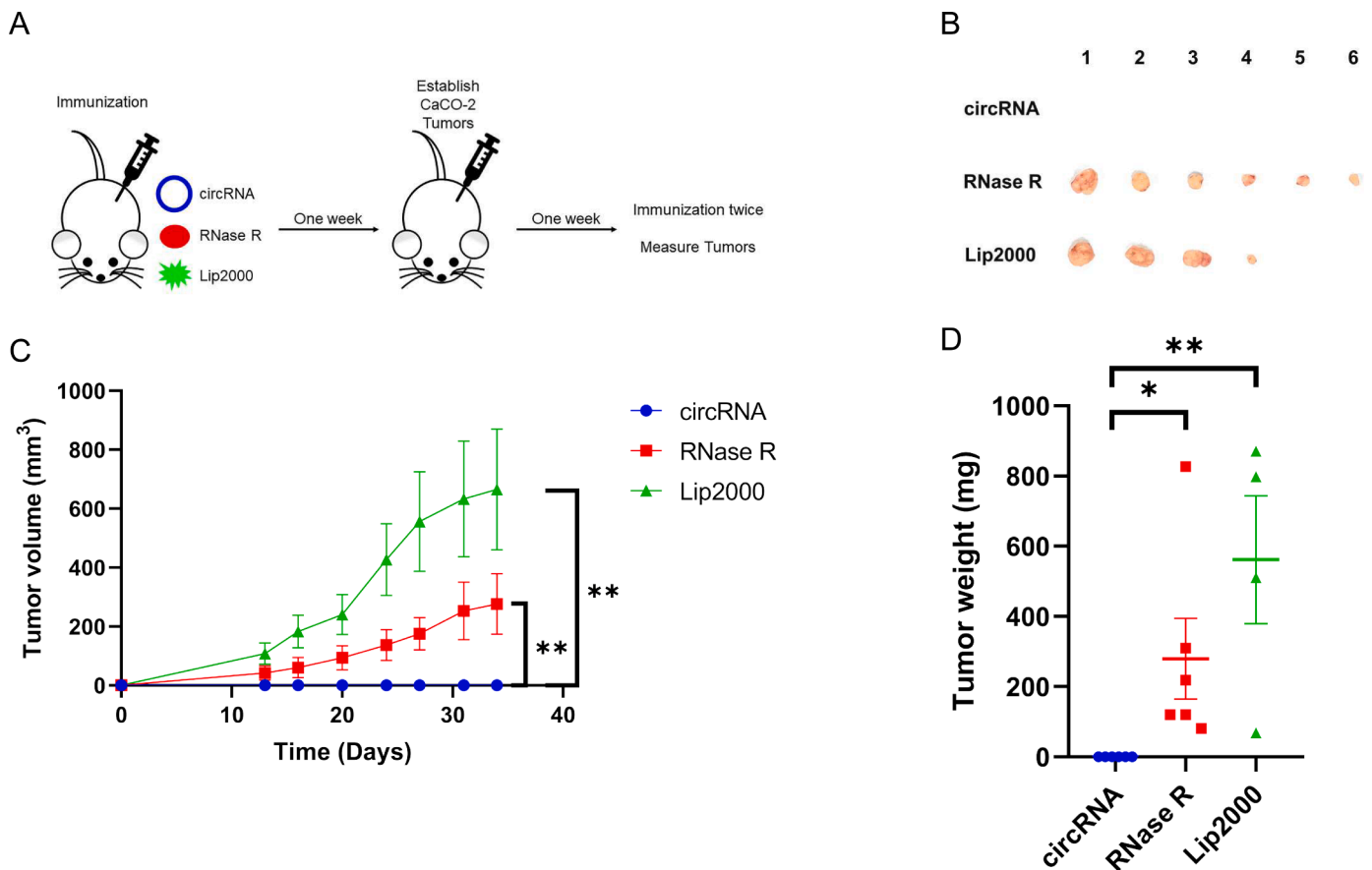
## Results

### Cancer circRNAs prevent cancer progression

According to the literature, circRNAs can activate innate immunity [3–5]. The circRNAs extracted from CaCO-2 cancer cells in this study were first used to provoke innate immunity in vivo. The total RNA extracted from CaCO-2 cells was digested with RNase R for 30 min to purify the circRNAs from CaCO-2 cells. In our study, the purified CaCO-2 circRNAs (referred to as cancer circRNAs in the following text), which served as vaccines, were injected twice subcutaneously at a concentration of 2 µg per injection 7 days before and 7 days after xenograft tumor transplantation. Subcutaneous xenograft tumors were constructed via injection of  $1 \times 10^6$  cells per transplantation in BALB/c nude mice. Three groups of treatments were investigated: 1. treatment with 100 µg cancer circRNAs/kg body weight; 2. negative control groups treated with RNase R; and 3. negative control groups treated with Lip2000. The experimental procedure is shown in Fig. 1A. Tumor volumes were measured every two days for five weeks, and tumor weights were measured after surgery at the end of the experiments. The xenograft tumors and sera from animals were collected for further analyses. The tumors collected from animals immunized with cancer circRNAs were significantly smaller than the tumors of the control groups without any cancer circRNA treatment (Figs. 1B–D).

### CircRNAs extracted from cancer cells lead to the induction of adaptive and innate immunity

Disease-specific circRNAs are usually considered as diagnostic or prognostic targets because studies have revealed that they can be detected from tissue biopsies and liquid biopsies [1]. It has been suggested that circRNAs might serve as antigens for activating a host immune response [3,4]. To determine whether inhibition of tumor growth is indeed mediated by the immunization of cancer circRNAs, serum from each animal was collected to determine the presence of antibodies against circRNAs from the cancer cells. Mouse monocyte macrophage RAW264.7 cells were transfected with cancer circRNAs or RNase R for presenting antigens; the cells transfected with RNase R served as the negative control. The transfected RAW264.7 cells were first incubated with serum from animals and then with an anti-mouse secondary antibody conjugated with Fluorescein isothiocyanate isomer (FITC). The affinities between the serum and transfected RAW264.7 cells were assessed from FITC fluorescence intensities. The experimental procedure is shown in Fig. 2A. Sera from the animals immunized with cancer circRNAs showed the highest affinity for RAW264.7 cells, which contained cancer circRNAs-related antigens (Fig. 2B). Comparatively, sera from the negative groups of animals did not bind to the cancer circRNAs-transfected RAW264.7 cells. Additionally, sera from the



**Fig. 1.** CircRNAs act as a vaccine to prevent tumor progression. (A) CircRNA vaccination was delivered via subcutaneous injection. One week later, CaCO-2 cells were established in right flanks. One week later, secondary immunization was carried out. Tumors were measured and imaged. (B) Representative images of tumors for mice vaccinated with circRNAs, RNase R, or Lip2000 prior to tumor establishment. (C) Tumor volume was monitored every week for 5 weeks. (D) Tumor weights were measured and presented at the endpoint of ELISA experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; ns, not significant;  $n = 6$ .

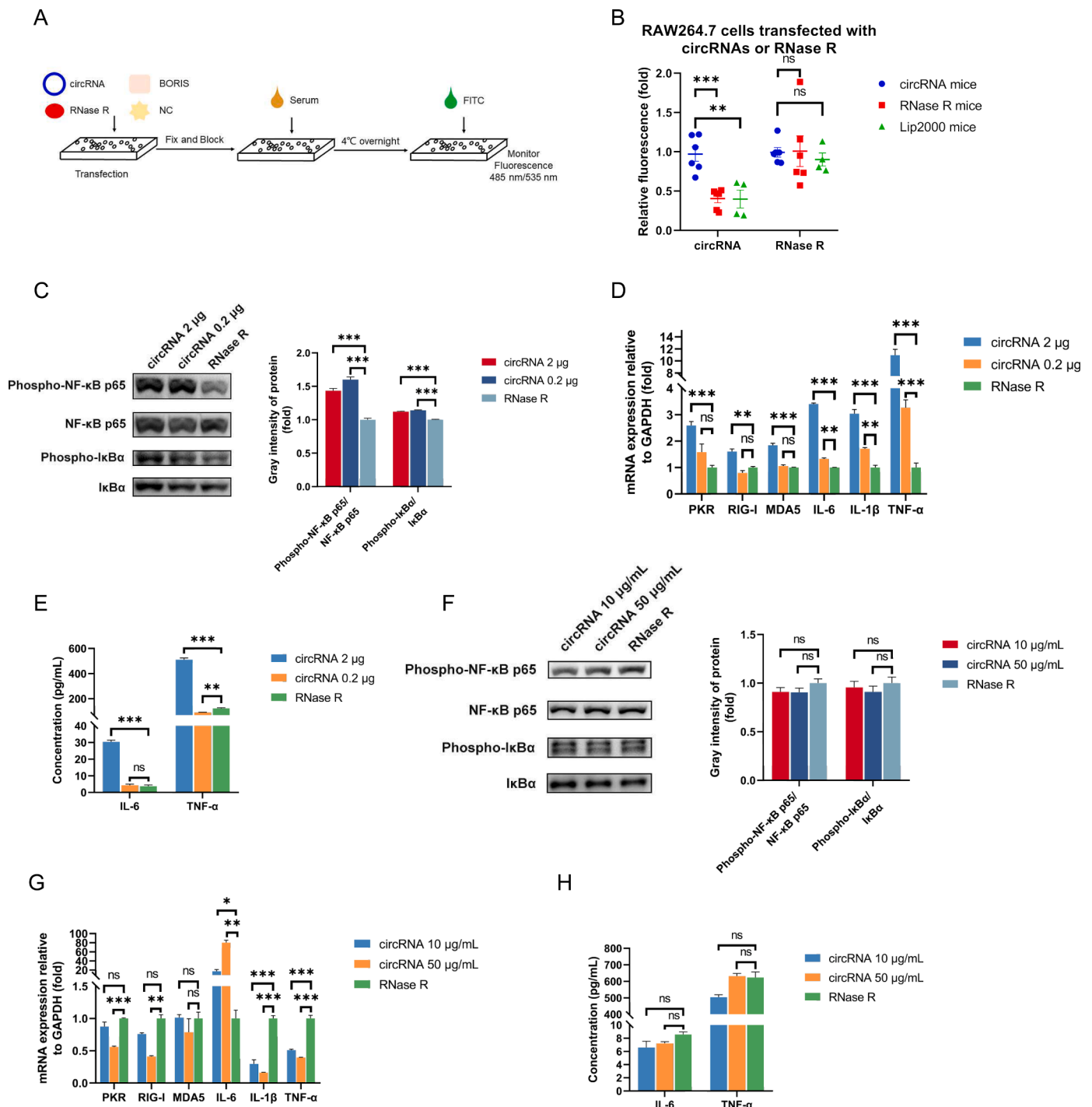
animals immunized with cancer circRNAs also did not bind to RNase R-transfected RAW264.7 cells (Fig. 2B). These results indicated that cancer circRNAs provoked adaptive immunity in mice, which activated them to produce antibodies against the antigens presented by circRNAs-transfected RAW264.7 cells.

Cancer circRNAs were added in cell culture medium or used to transfect RAW264.7 cells to determine whether the purified circRNAs can induce inflammation in the mouse model. NF- $\kappa$ B (p65) and  $\kappa$ B $\alpha$  phosphorylation levels and the gene expression of inflammatory factors such as interleukin-6 (IL-6), interleukin-1 $\beta$  (IL-1 $\beta$ ), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and innate immunity regulators such as RIG-I, MDA5, and PKR were evaluated to examine the activation of innate immunity. Lipopolysaccharide (LPS)-treated RAW264.7 cells were used as the positive control for evaluating the extension of the induction of immunity. We found that a high-dose of transfection with circRNAs activated the phosphorylation of NF- $\kappa$ B and  $\kappa$ B $\alpha$  (Fig. 2C and S1A) and enhanced the expression of IL-6, IL-1 $\beta$ , TNF- $\alpha$ , PKR, RIG-I, and MDA5 (Fig. 2D), which produced similar effects to those of LPS treatment. This in turn induced the phosphorylation of NF- $\kappa$ B and  $\kappa$ B $\alpha$  and expression of IL-6, IL-1 $\beta$ , TNF- $\alpha$ , PKR, RIG-I, and MDA5 (Fig. S2A, B and S1B). ELISA analysis verified IL-6 and TNF- $\alpha$  up-regulation by a high-dose of transfection with circRNAs in RAW264.7 cells (Fig. 2E). Comparatively, the simple addition of circRNAs to the cell culture medium failed to activate the phosphorylation of NF- $\kappa$ B and  $\kappa$ B $\alpha$  (Fig. 2F and S1C) or induce the expression of any inflammatory factor except IL-6 (Fig. 2G). ELISA analysis verified that the expression levels of IL-6 and TNF- $\alpha$  had no significant changes by the simple addition of circRNAs to the cell culture medium (Fig. 2H). These results collectively demonstrate that high-dose

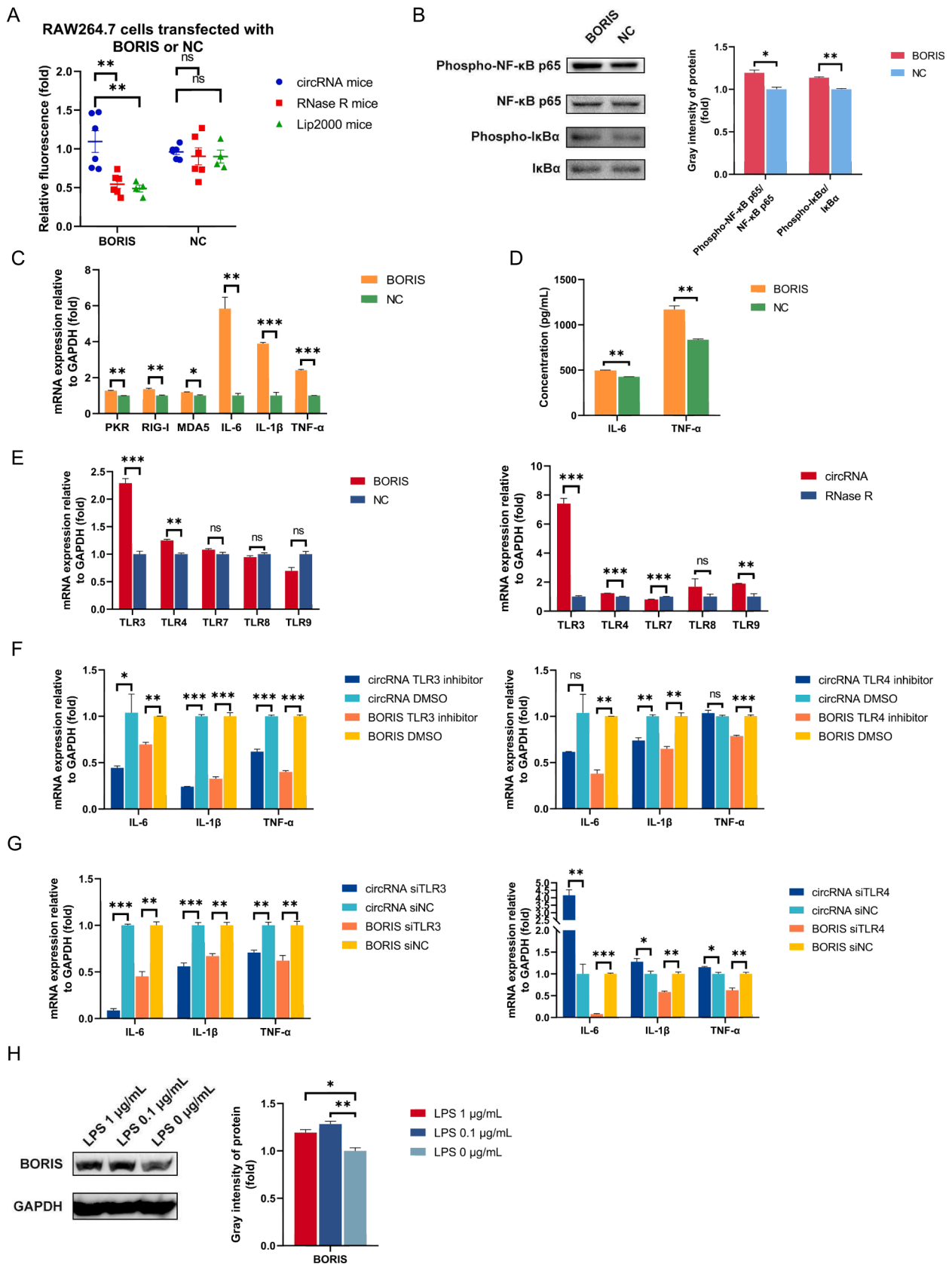
circRNA transfection with Lip2000 induces innate immunity, while the simple addition of circRNAs in cell culture medium does not. A low-dose circRNA transfection was found to induce immunity to a lower extent. These results indicate that circRNA transfection induces inflammation and immunity.

#### Sera from cancer circular RNA-immunized animals respond to BORIS overexpressed cells

BORIS is a specific antigen for cancer cells and a potential diagnostic and therapeutic target. Thus, we hypothesized that the expression of BORIS in cancer cells might modify the expression profile of circRNAs. RAW264.7 cells were transfected with BORIS and then incubated with the serum immunized by circRNAs, RNase R, or Lip2000. BORIS-transfected cells elicited a significant response to the cancer circRNAs-immunized serum (Fig. 3A). This finding suggested that BORIS induced the expression of specific circRNAs, which activated immunization in the process of cancer development. We next investigated whether the overexpression of BORIS led to inflammation. BORIS was overexpressed in RAW264.7 cells, which led to the activation of NF- $\kappa$ B and  $\kappa$ B $\alpha$  phosphorylation (Fig. 3B, S1D and S3A). This observation indicated that BORIS could play an active role in inducing inflammation. Moreover, we also investigated the pattern of expression of classic pathogen recognition receptors in BORIS-overexpressed RAW264.7 cells. Our findings revealed that IL-6, IL-1 $\beta$ , TNF- $\alpha$ , PKR, RIG-I, and MDA5 were activated (Fig. 3C), and up-regulation of IL-6 and TNF- $\alpha$  were also confirmed by ELISA analysis (Fig. 3D). We also found expression of TLR3 and TLR4 was induced, but TLR7, TLR8, and TLR9



**Fig. 2.** Transfection of exogenous circRNAs in cancer cells induces immunity in cells. (A) RAW264.7 cells were transfected with circRNAs, RNase R, BORIS, and the negative control vector (NC). The sera of the three groups of nude mice served as the primary antibody and FITC served as the secondary antibody in ELISA (Excitation wavelength—485 nm, Emission wavelength—535 nm). (B) Quantification of the FITC fluorescence signals from RAW264.7 cells transfected with circRNAs or RNase R (negative control) (n = 6). (C) Representative western blots of Phospho-NF-κB p65, NF-κB p65, Phospho-IκBα, and IκBα expression in RAW264.7 cells transfected with circRNAs from CaCO-2 cells. Gray intensity analysis of Phospho-NF-κB p65/NF-κB p65 and Phospho-IκBα/IκBα. (D) Relative mRNA levels of innate immunity and inflammatory factors from RAW264.7 cells transfected with circRNAs, as determined with qRT-PCR; The expression levels were normalized to those of *GAPDH*. (E) ELISA analysis for IL-6 and TNF-α from cell culture supernatants of RAW264.7 cells transfected with circRNAs. (F) Representative western blots of Phospho-NF-κB p65, NF-κB p65, Phospho-IκBα, and IκBα expression in RAW264.7 cells treated with circRNAs in culture medium. Gray intensity analysis of Phospho-NF-κB p65/NF-κB p65 and Phospho-IκBα/IκBα. (G) Relative mRNA levels of innate immunity and inflammatory factors from RAW264.7 cells treated with circRNAs in culture medium, as determined with qRT-PCR; The expression levels were normalized to those of *GAPDH*. (H) ELISA analysis for IL-6 and TNF-α from cell culture supernatants of RAW264.7 cells treated with circRNAs in culture medium. \*P ≤ 0.05; \*\*P ≤ 0.01; \*\*\*P ≤ 0.001; ns, not significant; n = 3.



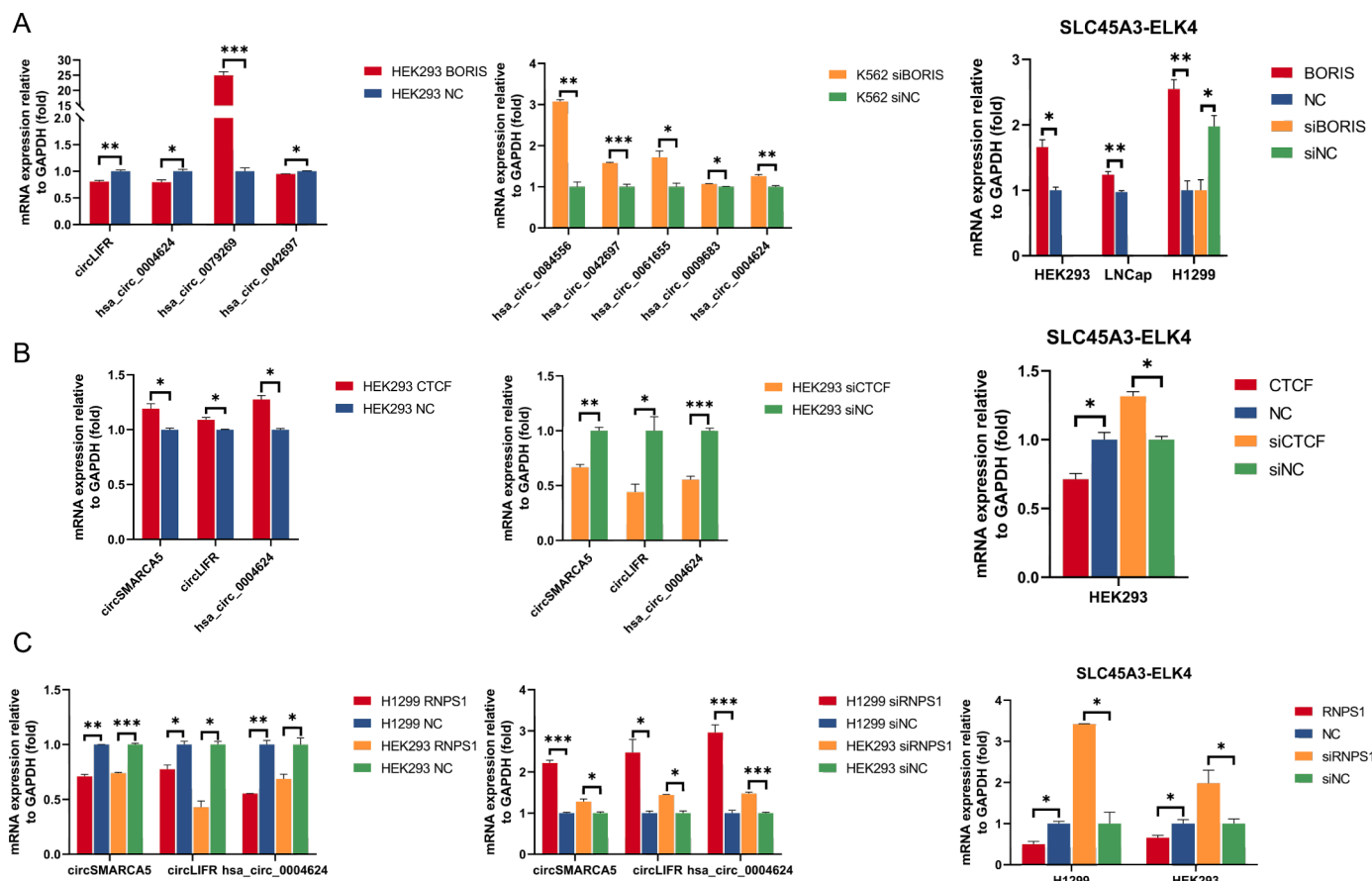
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**Fig. 3.** BORIS and circRNAs play roles in immunity through TLR3. (A) Quantification of the FITC fluorescence signals from RAW264.7 cells transfected with BORIS or the negative control vector (n=6). (B) Representative western blots of Phospho-NF-κB p65, NF-κB p65, Phospho-IκBα, and IκBα expression in RAW264.7 cells transfected with BORIS. Gray intensity analysis of Phospho-NF-κB p65/NF-κB p65, and Phospho-IκBα/IκBα. (C) Relative mRNA levels of innate immunity and inflammatory factors from RAW264.7 cells transfected with BORIS, as determined with qRT-PCR; The expression levels were normalized to those of *GAPDH*. (D) ELISA analysis for IL-6 and TNF-α from cell culture supernatants of RAW264.7 cells transfected with BORIS. (E) Relative mRNA levels of TLRs from RAW264.7 cells transfected with BORIS or circRNAs, as determined with qRT-PCR; mRNA expression was normalized to that of *GAPDH*. (F) Relative mRNA levels of inflammatory factors from RAW264.7 cells treated with a TLR3 or TLR4 inhibitor prior to the transfection of circRNAs or BORIS, as determined with qRT-PCR; expression levels were normalized to those of *GAPDH*. (G) Relative mRNA levels of inflammatory factors from RAW264.7 cells treated with TLR3 or TLR4 siRNA prior to the transfection of circRNAs or BORIS, as determined with qRT-PCR; expression levels were normalized to those of *GAPDH*. (H) Representative western blots of BORIS and GAPDH expression in RAW264.7 cells treated with LPS. Gray intensity analysis of BORIS/GAPDH. \*P ≤ 0.05; \*\*P ≤ 0.01; \*\*\*P ≤ 0.001; ns, not significant; n = 3.

were not (Fig. 3E). This result was also found by Wesselhoeft et al. [27]. Among the activated pathogen recognition receptors, TLR3 appeared to be the most intense effector (Fig. 3E). As circRNAs induced the activation of innate immunity and the expression of PKR, RIG-I, and MDA5 (Fig. 2D), the expression levels of TLR3, TLR4, TLR7, TLR8, and TLR9 were also examined after circRNA treatment. TLR3, TLR4, and TLR9 were upregulated upon circRNA treatment, with TLR3 being the most intense effector (Fig. 3E). RNase R treatment was used as the negative control treatment in these experiments. The results indicated the significant role played by TLR3 in recognizing BORIS and circRNAs.

Next, we inhibited or silenced TLR3 and TLR4 using a TLR3/4 inhibitor or TLR3/4 siRNA, respectively, to determine whether TLR3 or TLR4 play a significant role in regulating the immune response induced by cancer circRNAs and BORIS transfection. The expression levels of inflammatory factors were downregulated after treatment with the TLR3 inhibitor, while the TLR4 inhibitor showed a relatively poor effect

(Fig. 3F). Next, TLR3 and TLR4 were knocked down by siRNA, which led to the decreased expression of inflammatory factors after treatment with siTLR3 (sense: CGUUAUCACACACCAUUUAT, antisense: UAAAUUGUGUGUAUACGTT), whereas siTLR4 (sense: CGACUUACAGUUUCUACGUTT, antisense: ACGUAGAAACUGUAAGUCGTT) had a little effect on the inflammation induced by circRNA transfection (Fig. 3G and S3B). Our results collectively imply that BORIS regulated cancer circRNAs to induce an immune response mediated by TLR3. TLR4 was upregulated by both BORIS and cancer circRNA treatments. TLR4 is usually activated by bacterial infection [28]. Hence, BORIS expression was tested after LPS treatment. We found that BORIS expression was upregulated by 0 to 1 μg/mL LPS treatment (Fig. 3H). These findings collectively show potential coordination between BORIS, circRNAs, and the immunity.



**Fig. 4.** BORIS regulates the expression of fusion RNA and circRNAs. (A) Relative levels of circRNAs from BORIS-overexpressed HEK293 cells or silenced K562 cells, and fusion RNA *SLC45A3-ELK4* from BORIS-overexpressed HEK293, LNCap, and H1299 cells or BORIS-silenced H1299 cells, as determined with qRT-PCR; expression levels were normalized to those of *GAPDH*. (B) Relative levels of circRNAs and fusion RNA *SLC45A3-ELK4* from CTCF-overexpressed or silenced HEK293 cells, as determined with qRT-PCR; expression levels were normalized to those of *GAPDH*. (C) Relative abundance of circRNAs and fusion RNA *SLC45A3-ELK4* from RNPS1-overexpressed H1299 and HEK293 cells or RNPS1-silenced H1299 and HEK293 cells, as determined with qRT-PCR; expression levels were normalized to those of *GAPDH*. \*P ≤ 0.05; \*\*P ≤ 0.01; \*\*\*P ≤ 0.001; ns, not significant; n = 3.

Factors regulating chimeric RNA fusion are also responsible for regulating the generation of circRNAs

As circRNAs and chimeric fusion RNA share some common regulators, we attempted to investigate whether regulators of chimeric RNA affect the expression of circRNAs. CTCF binds to insulators on the genome and suppresses chimeric RNA fusions. BORIS is the paralogue of

CTCF and has been reported to promote cancer progression, which is the opposite of the function of CTCF [29]. The opposing behaviors of BORIS and CTCF led us to investigate the regulation of circRNAs and chimeric fusion RNAs by BORIS. To determine whether BORIS regulates circRNA generation, BORIS was silenced in K562 and H1299 cells and overexpressed in HEK293, H1299, and LNCap cells. HEK293 cells were confirmed to not express BORIS. LNCap cells were reported to express

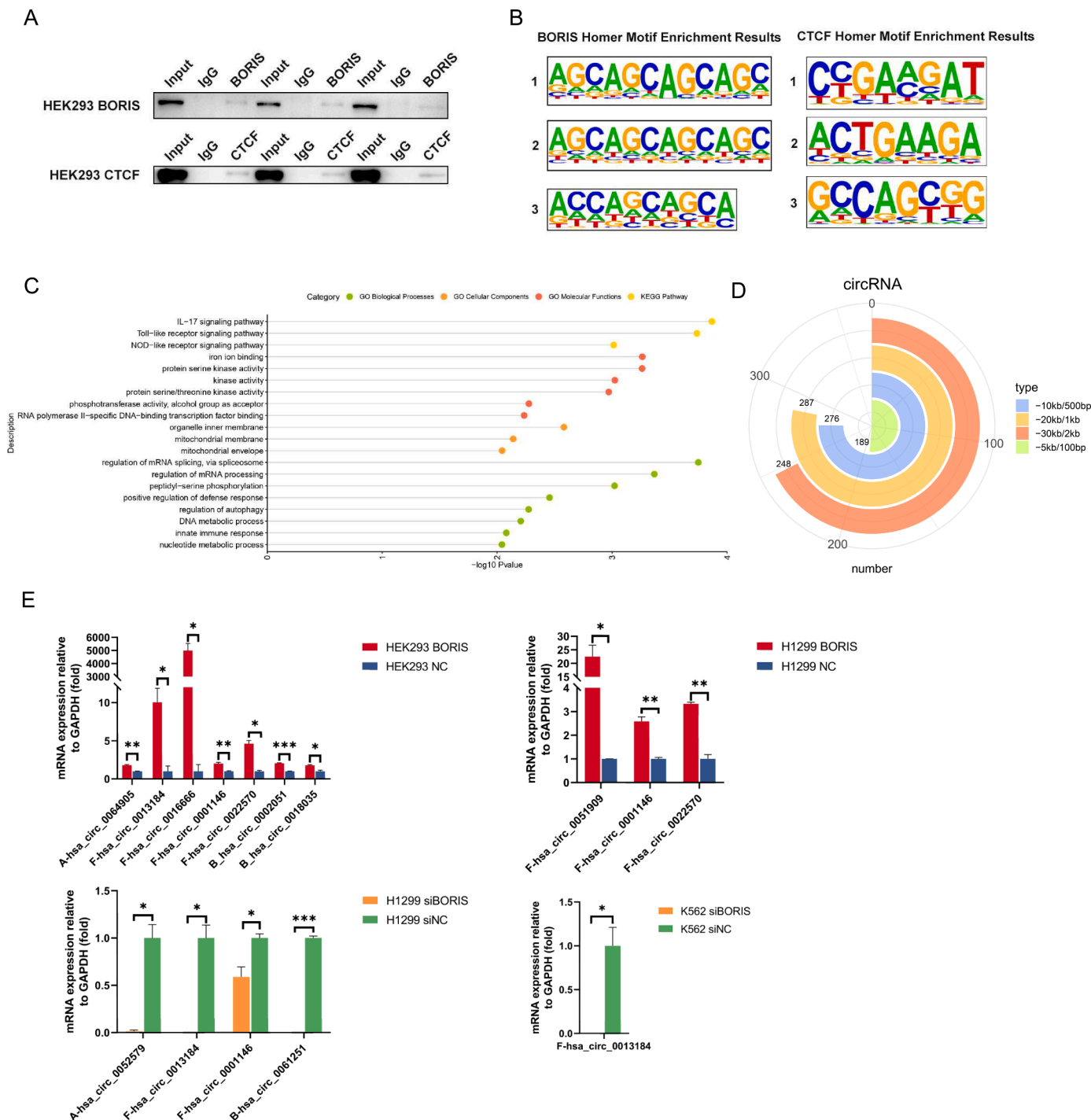


Fig. 5. BORIS binds with RNA motif to promote the expression of circRNAs. (A) BORIS- and CTCF-overexpressing HEK293 cells lysates were subjected to RIP with anti-Myc-Tag antibodies, followed by western blot analysis with anti-BORIS and anti-CTCF antibodies. IgG, immunoglobulin G. (B) BORIS and CTCF binding motif analyzed from RIP experiments using HOMER (v.4.11). (C) KEGG and GO enrichment of genes bound by BORIS. KEGG and GO enrichment *p*-value is shown. (D) Number of circRNAs at different distances from the BORIS RNA binding motif to their splicing sites. (E) Relative expression of circRNAs from BORIS-overexpressed HEK293 and H1299 cells or BORIS-silenced H1299 and K562 cells, as determined with qRT-PCR, and BORIS RNA binding regions are in front of or behind splicing sites of these circRNAs; expression levels were normalized to those of *GAPDH*. \**P* ≤ 0.05; \*\**P* ≤ 0.01; \*\*\**P* ≤ 0.001; ns, not significant; *n* = 3.

chimeric fusion RNA-like *SLC45A3-ELK4*, which was suppressed by CTCF. Accordingly, we used BORIS-overexpressed and BORIS-silenced cells to detect fusion RNA and circRNAs because of the differential expression levels and functions of BORIS in different cells. We found that BORIS promoted the expression of *SLC45A3-ELK4* and circRNAs of hsa\_circ\_0079269. Furthermore, we found that BORIS also suppressed circRNAs. These circRNAs, containing BORIS binding sites, were observed in K562 cells (Fig. 4A and S3C, Table S2). CTCF overexpression suppressed the fusion of *SLC45A3-ELK4*, but induced the generation of circSMARCA5, circLIFR, and hsa\_circ\_0004624. Conversely, CTCF silencing promoted the expression of *SLC45A3-ELK4*, but suppressed the generation of circSMARCA5, circLIFR, and hsa\_circ\_0004624 (Fig. 4B and S3D). RNPS1, which can regulate the fusion of chimeric RNA *CTNBP1-CLSTN1*, was overexpressed or silenced in H1299 and HEK293 cells. RNPS1 suppressed the expression of circSMARCA5, circLIFR, and hsa\_circ\_0004624 and the chimeric fusion RNA *SLC45A3-ELK4* (Fig. 4C and S3E). However, the mechanism of how BORIS regulates circRNAs needs further investigation.

#### *BORIS acts as an RNA binding protein to regulate the generation of circRNAs*

As circRNAs were generated by splicing from pre-messenger RNA, and the RNA binding protein of QKI can regulate circRNAs formation, BORIS might bind RNA to affect the formation of circRNAs. BORIS was indeed found to bind ribosomal RNA in a previous study [30]. HEK293 cells were overexpressed by myc-tagged BORIS plasmid to investigate whether BORIS binds RNAs. The cell lysate was incubated with a myc-tag antibody to perform RNA immunoprecipitation (RIP) for the detection of RNA-BORIS interaction. IgG pull-down was performed as the negative control treatment. As BORIS is the paralogue of CTCF and was reported to bind a similar DNA motif [18], RIP was performed in similar experiments to investigate whether BORIS and CTCF bind the same RNA (Fig. 5A). After the pull-down and extraction of RNAs in RIP, the precipitated RNAs were extracted and purified with Trizol. Then, the RNAs were analyzed using secondary sequencing. Both BORIS and CTCF were found to bind RNA. However, BORIS was found to bind the RNA motif that differed from the CTCF motif, which had nothing in common in three dependent experiments (Fig. 5B). A CCCTC motif was reported to be a CTCF binding motif on the genome [31], which suggested that CTCF might bind CCCTC RNA by DNA.

We next analyzed RNA pulled down by BORIS through GO and enrichment in KEGG pathways. Interestingly, we found that BORIS binds with genes related to Toll-like receptor signaling pathway, kinase activity, regulation of mRNA splicing via spliceosome, regulation of mRNA processing, and innate immune response (Fig. 5C). Furthermore, the distance from BORIS RNA binding sites to circRNA splicing sites was measured (Fig. 5D). We found that BORIS RNA binding sites were related to circRNAs, which BORIS potentially regulates. These results indicate that BORIS plays a vital role in regulating circRNA generation and participates in immunity-related pathways. Considering that BORIS regulated the expression of circRNAs, we proposed that BORIS-bound RNA sites regulate the generation of circRNAs. Therefore, we detected circRNAs with RNA binding sites of BORIS adjacent to their splicing sites. CircRNAs were divided into three groups according to the position of the BORIS binding sites: 1. BORIS RNA binding sites were not only present in front of but also behind the splicing sites (A); 2. BORIS RNA binding sites were present only in front of the splicing sites (F); and 3. BORIS RNA binding sites were present only behind the splicing sites (B) (Table S3). The generation of these three kinds of circRNAs can be promoted by BORIS. BORIS RNA binding sites in front of the circRNA splicing sites exerted an immense influence on the generation of circRNAs (Fig. 5E and S3C). Thus, we concluded that BORIS RNA binding sites promoted the generation of circRNAs. BORIS may recognize and bind with specific RNA binding sites to mediate their splicing site attachment and regulate the expression of circRNAs.

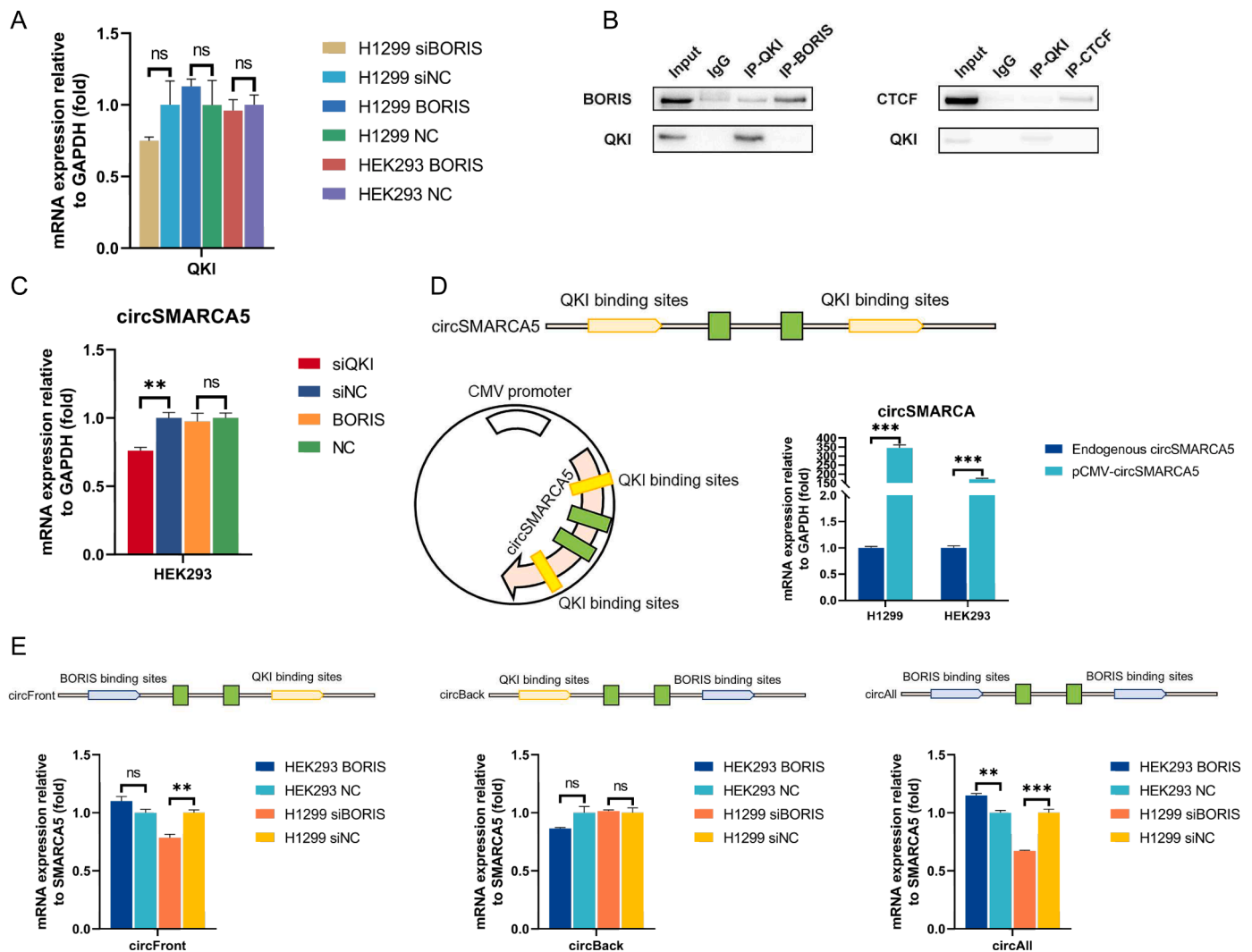
#### *BORIS regulates circRNA generation by binding to the motif-adjacent circRNA splicing sites*

As QKI was reported to bind RNA and regulate the formation of circRNAs that are not regulated by Alu elements, BORIS might bind RNA via QKI [9]. Therefore, we examined the interaction between BORIS and QKI. Our results showed that BORIS did not regulate the expression of QKI, nor did it have any protein interaction with QKI, which excludes the possibility that BORIS regulates circRNA formation by interacting with QKI (Fig. 6A, B, and S3C). CircSMARCA5 generation was reportedly promoted by QKI because of QKI binding sites adjacent to the circRNA splicing sites (Fig. S3F) [9]. We found that BORIS did not regulate endogenous circSMARCA5 expression (Fig. 6C). Furthermore, we constructed the plasmid of the *circSMARCA5* parental gene with its flanking introns, which contain QKI binding sites, and determined that QKI regulates circSMARCA5. The transfection efficiency and expression of this plasmid in HEK293 and H1299 cells were measured and compared with endogenous circSMARCA5 (Fig. 6D). Both or one of the binding sites of QKI on the plasmid were substituted with the binding site of BORIS to study the regulation of circSMARCA5 by BORIS (Fig. 6E and S3C). The BORIS binding site used here was found to bind BORIS, but not CTCF in K562 cells (Table S4). The insertion of the BORIS binding site in front of or in front of and behind the circSMARCA5 splicing sites promoted the expression of circSMARCA5 in H1299 and HEK293 cells (Fig. 6E). These results indicated that BORIS regulated circRNA expression by recognizing and binding special motifs.

#### *BORIS promotes the fusion of active genes on the human genome*

The promotion of both chimeric fusion RNA and circRNA generation by BORIS suggests that fusion active splicing sites might exist on the genome. In situ Hi-C analysis indicated that there are abundant CTCF interactions on the genome, which lead to formation of the 3D architecture of the genome [12]. BORIS was also reported to promote regulatory chromatin interactions that support specific cancer phenotypes [32]. Considering that BORIS partly binds CTCF sites on the genome, regulates chimeric RNA fusion and circRNA formation, and promotes cancer progression, we summarized the active splicing sites that were close to CTCF/BORIS binding sites. The CTCF sites close to the splicing sites according to the Hi-C results were assigned. Every 0.5 score represents splicing sites have one contact with CTCF/BORIS peak. Splicing sites of circRNAs and fusion genes were acquired from the circBase, CosmicFusion, and MNDR (v3.1) databases. The CTCF/BORIS-mediated activation of splicing sites associated with circRNAs was found to be higher than that of chimeric fusions, because the highest score is 3 for circRNA while the highest score for fusion RNA is 2 (Fig. 7A). The higher score indicated that BORIS/CTCF tends to regulate the formation of circRNAs. The splicing sites associated with diseases were also analyzed. Though fewer splicing sites were reported to be associated with diseases in the CosmicFusion and MNDR (v3.1) databases, circRNAs were found to be more regulated than the fusion genes (Fig. 7A). Upon analyzing the common active splicing sites of 5' fusion genes, 3' fusion genes, and circRNAs parental genes, 13 active splicing sites were found. The parental genes of the 13 splicing sites are presented in Fig. 7B. The distances between the CTCF/BORIS binding sites and the splicing sites of fusion genes or circRNAs are summarized in Fig. 7C. The distances between CTCF/BORIS binding sites and splicing sites of -10 kb to +500 bp and -20 kb to +1 kb from the transcription starting sites were found to be significantly different between BORIS and CTCF (Fig. 7D). This suggests that chimeric fusions or circRNA regulation differ between BORIS and CTCF in these regions.

The 13 active genes, which were identified via genome analysis of the splicing active sites, were verified for BORIS regulation. The regulation of circRNAs generated from active genes and their parental genes was examined by modifying BORIS expression via siRNA silencing or overexpression in different cells (Figs. 7E, F, S3C and D, Table S5). Then,



**Fig. 6.** Insulator bound by BORIS in front of and behind the splicing sites of circRNA induces its generation. (A) Relative mRNA levels of QKI from BORIS-overexpressed HEK293 and H1299 cells or BORIS-silenced H1299 cells, as determined with qRT-PCR; expression levels were normalized to those of *GAPDH*. (B) BORIS or CTCF and QKI co-overexpressing HEK293 cells lysates were subjected to co-immunoprecipitation with anti-Myc-Tag antibody to bind BORIS-myc or CTCF-myc and anti-HA-Tag antibody to bind HA-QKI, followed by western blot analysis with anti-BORIS, anti-CTCF and anti-HA-Tag antibodies. (C) Relative mRNA levels of circSMARCA5 from BORIS-overexpressed or QKI-silenced HEK293 cells, as determined with qRT-PCR; expression levels were normalized to those of *GAPDH*. (D) Construction of pCMV-circSMARCA5. Relative mRNA levels of endogenous circSMARCA5 from HEK293 and H1299 cells and pCMV-circSMARCA5 transfected cells, as determined with qRT-PCR; expression levels were normalized to those of *GAPDH*. (E) Construction of circ-reporter based on circSMARCA5. Relative mRNA levels of circSMARCA5 from HEK293 cells transfected with BORIS and circ-reporter and H1299 cells transfected with siBORIS and circ-reporter, as determined with qRT-PCR; expression levels were normalized to those of *SMARCA5*. \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ ; ns, not significant;  $n = 3$ .

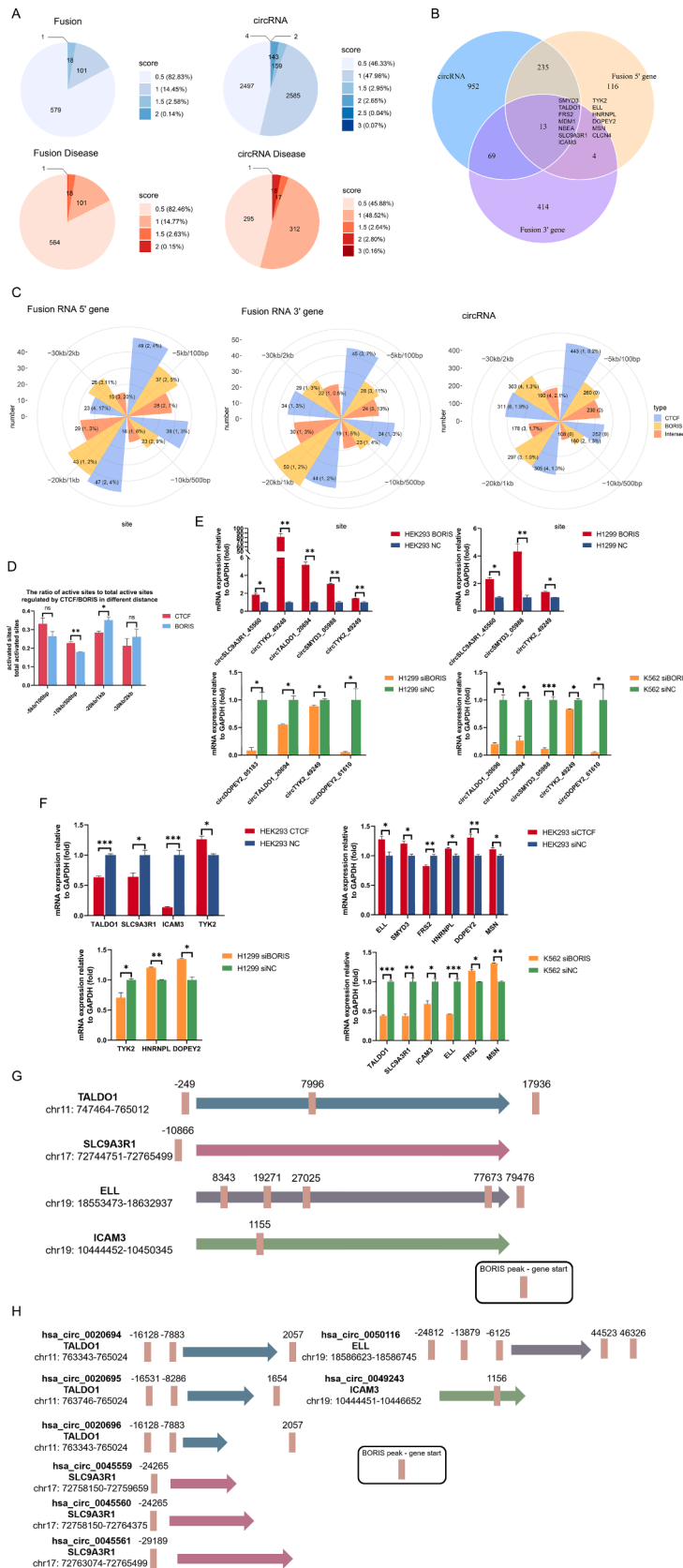
the BORIS binding motifs were screened on the genomes of active genes. BORIS binding sites found adjacent or on the genes *TALDO1*, *SLC9A3R1*, *ELL*, and *ICAM3* were induced by BORIS and suppressed by CTCF (Fig. 7G). The distance and relationship between BORIS peak and splicing sites of circRNAs are presented in Fig. 7H and Table S6. These results indicate that BORIS promotes circRNA formation through the regulation of chromosome contacts. Eleven circRNAs were detected in different cells, six of which had the binding sites of BORIS. Our results indicate that BORIS might promote the activation of circRNAs by regulating chromosome contact and binding specific motifs on the genome. Furthermore, these findings suggest that both RNA binding motifs and motifs on the genome can be recognized and bound by BORIS, through which the regulation of circRNA expression and chromosome contact are mediated.

## Discussion

circRNAs are a large class of endogenous non-coding RNA and are

abundant in mammalian cells. Most circRNAs were found to be miRNA sponges that regulate the expressions of functional genes. Additionally, some circRNAs were reported to be translated [33], with the ability to interact with RNA-binding proteins to perform biological functions [9]. The expression of circRNAs is prevalent in various tissues and body fluids, and their abnormal expression is related to tumor progression [2, 34]. Recent discoveries have revealed that circRNAs regulate NF90/NF110 to resistant viruses and stimulates RIG-I, PKR phosphorylation, and innate immune signaling pathways [3-5,35]. However, the knowledge about circRNAs remains poor. The functions and generation of circRNAs are not uncovered completely. Moreover, the annotations of circRNAs need to be expanded. The question remains if circRNAs can induce inflammation and the underlying mechanism by which circRNAs form by RNA splicing factors. Since circRNAs have some similar characteristics with chimeric fusions, such circRNAs are worth investigation for this purpose.

Our study finds that circRNAs extracted from cancer cells prevent cancer progression and induce innate immunity and inflammation.



**Fig. 7.** BORIS promotes the generation of circRNAs from active genes. (A) Scores of fusion RNAs and circRNAs and disease-related fusion RNAs and circRNAs. In the pie charts depicting the scores of fusion RNAs and circRNAs, every 0.5 score indicates one alternative splicing site of fusion RNA or circRNA contacts, with one peak of loop mediated by CTCF and BORIS. The scores represent the frequency of contact. (B) Genes that participated in the formation of fusion RNAs and circRNAs. Thirteen active genes are shown to join in the formation of both fusion RNA and circRNAs. (C) Number of gene symbols of fusion RNAs and circRNAs at different distances from the BORIS or CTCF binding motif on the genome to their splicing sites. The number and percentage of active genes are shown in parentheses. (D) Ratio of active sites to total active sites regulated by BORIS and CTCF at different distances. (E) Relative mRNA levels of circRNAs generated from active genes from BORIS-overexpressed HEK293 and H1299 cells or BORIS-silenced H1299 and K562 cells, as determined with qRT-PCR; expression levels were normalized to those of *GAPDH* (n=3). (F) Relative mRNA levels of active genes from CTCF-overexpressed or CTCF-silenced HEK293 cells and BORIS-silenced H1299 and K562 cells, as determined with qRT-PCR; expression levels were normalized to those of *GAPDH* (n=3). (G) BORIS binding sites on active genes. The number represents the distance between the BORIS binding sites and the start of the gene. (H) BORIS binding sites on circRNAs generated from active genes. The number represents the distance between the BORIS binding sites and the active sites of the circRNA. \*P ≤ 0.05; \*\*P ≤ 0.01; \*\*\*P ≤ 0.001, ns, not significant.

Additionally, innate immunity signaling induced by circRNAs in previous researches, adaptive immunity, and cancer progression prevention are part of our essential and novel findings. CircRNAs-TLR3-NF- $\kappa$ B pathway is essential for adaptive immunity that is induced by circRNAs. Also, the circular structure of circRNAs makes them more stable than mRNA and hard to degrade. These results about the immune functions regulatory role of circRNAs may lead to new vaccine technology to prevent cancer progression because circRNAs can act as immunogens. Furthermore, RNA splicing factors have been proved to regulate the formation of circRNAs like RNPS1, CTCF, and BORIS, which were just defined as chimeric fusion RNA regulators, indicating that the mechanism for circRNAs formation is similar to fusion RNAs. BORIS regulates the contact of chromosomes and binds with unique motifs adjacent to splicing sites of circRNAs to promote their generation. In conclusion, we found that BORIS bound to a particular binding motif and promoted the formation of circRNAs, and the cancer circRNAs activate innate immunity regulators and TLR3 to stimulate NF- $\kappa$ B p65 and provoke inflammation to induce immunity.

However, our research has faced some challenges. Firstly, the expression landscape of circRNAs is different in different cell lines, while some circRNAs are just expressed in cancer cells or normal cells. Hence, some circRNAs cannot be detected in 2 cell lines for comparison. Secondly, BORIS is expressed in cancer cells and exhibits various biological functions. For these reasons, circRNAs regulated by BORIS may show different functions, making it difficult to choose the correct cell lines. Further, our research also has some limitations. In this study, we have not found any specific circRNA that plays a role in preventing cancer progression, because RIP-seq results show genes bound but not circRNAs induced by BORIS. Moreover, not all circRNAs are promoted through BORIS. Some circRNAs are downregulated by BORIS, indicating that BORIS also plays other roles in circRNA regulation, such as transcriptional regulation. In addition, BORIS DNA binding sites and RNA binding sites can also participate in the generation of circRNAs. As a result, differences arise between DNA and RNA binding sites, which remain to be further studied. Finally, we could not reveal the mechanism of how circRNAs interact with TLR3 to induce NF- $\kappa$ B p65 pathway. Previous researches considered that RIG-I distinguishes circRNAs by introns and PKR recognizes circRNAs by specific duplexes [3–5], suggesting that TLR3, RIG-I, and PKR interact with different kinds of circRNAs to activate the downstream immunity pathway.

Finally, although the relationship between BORIS, circRNA, and immunity demands further investigation, our discoveries reveal the mechanism of regulation of circRNAs by BORIS and their essential combined function in adaptive immunity, which may raise critical questions about the function of circRNAs in some diseases. Systematic analyses of more such proteins acting as splicing factors or cancer biomarkers are required to understand their relation with circRNAs, thereby providing a valuable basis for identifying potential therapeutic targets.

#### CRedit authorship contribution statement

**Hao Xu:** Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. **Mengdie Fang:** Data curation, Formal analysis, Investigation, Methodology, Project administration. **Chao Li:** Data curation, Formal analysis, Investigation, Methodology, Project administration. **Bowen Zuo:** Methodology. **Juan Ren:** Resources. **Yanmei Zhang:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

#### Declaration of Competing Interest

This manuscript has not been published or presented elsewhere in part or in entirety and is not under consideration by another journal. The study design was approved by the appropriate ethics review board. We have read and understood your journal's policies, and we believe that

neither the manuscript nor the study violates any of these. There are no conflicts of interest to declare.

#### Acknowledgments

The present study is supported by grants from the Zhejiang Provincial Natural Science Foundation of China (NO. HDMY22H318024), Medical and Health Science and Technology Project of Zhejiang Province (NO. 2022RC128), Foundation of Zhejiang Academy of Medical Sciences to Yanmei Zhang and Chao Li, and Scientific Research Fund of Zhejiang Provincial Education Department (NO. Y202146054). We are very grateful to ELSEVIER language editing service for language editing.

#### Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.tranon.2022.101363](https://doi.org/10.1016/j.tranon.2022.101363).

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