# *Original Article*
# circBRAF promotes the progression of triple-negative breast cancer through modulating methylation by recruiting KDM4B to histone H3K9me3 and IGF2BP3 to mRNA

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**Abstract:** Understanding the molecular characteristics of triple-negative breast cancer (TNBC) and developing more tailored treatment approaches is crucial. Circular RNAs (circRNAs), as potential therapeutic targets, remain largely unexplored in TNBC. This study utilized circRNA microarray analysis to determine the expression of circRNAs in TNBC, analyzing nine patient specimens. The characteristics of circBRAF were examined using divergent PCR primers, Sanger sequencing, fluorescence in situ hybridization (FISH) analysis, and the application of RNase and actinomycin D. The biological function of circBRAF in TNBC was further investigated through colony formation, tube formation, and transwell assays. Crucially, the mechanisms underlying the effects of circBRAF on TNBC progression were explored via RNA immunoprecipitation sequencing (RIP-seq) data, MS2 pulldown, RNA sequencing (RNA-seq) analysis, circBRAF knockdown, histone H3K9me3 modification, and Chromatin Isolation by RNA Purification (ChIRP) tests followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). We focused particularly on hsa_circ_0007178, produced from exons 4-13 of the oncogene BRAF. Functional experiments revealed that circBRAF is crucial for the development of TNBC, with its knockdown preventing angiogenesis, metastasis, and cell division *in vitro*. Mechanistically, circBRAF interacts with KDM4B and IGF2BP3, promoting TNBC growth. Interaction of circBRAF with IGF2BP3 increased the expression of VCAN, FN1, CDCA3, or B4GALT3 by controlling mRNA stability through RNA N6-methyladenosine (m6A) modification. Furthermore, circBRAF upregulated the expression of ADAMTS14 and MMP9 through recruitment of KDM4B to enhance respective H3K9me3 modification. Furthermore, overexpression of circBRAF was able to overcome the inhibitory effects of siKDM4B and siIGF2BP3 on cell migration and invasion. Our findings suggest that circBRAF may act as an oncogene in TNBC through its specific interactions with KDM4B and IGF2BP3, implying that circBRAF could serve as a potentially effective novel therapeutic target for TNBC.

**Keywords:** circBRAF, KDM4B, IGF2BP3, RNA N6-methyladenosine, triple-negative breast cancer

## Introduction

Breast cancer is the most prevalent malignancy among women globally, and its incidence in China has risen in recent decades [1]. A subtype known as triple-negative breast cancer (TNBC) is characterized by the absence of three specific receptors: human epidermal growth factor receptor 2 (HER2), progesterone receptors (PR), and estrogen receptors (ER) [2, 3]. This subtype is associated with a poor prognosis, limited treatment options, a higher recurrence rate, and lower overall survival (OS) [4]. Therefore, a deeper understanding of the molecular and genetic characteristics of TNBC

is essential to identify potential therapeutic targets.

Circular RNAs (circRNAs), a class of noncoding RNA molecules, are distinguished from the more common linear RNA by their unique circular structure, which results from precursor mRNA back-splicing or exon-skipping processes and lacks 5' caps or 3' poly(A) tails [5]. circRNAs have attracted significant interest in molecular biology and genomics due to their exceptional stability, abundance, and evolutionary conservation and have proven to be valuable as biomarkers in many biological processes. Increasingly recognized for their roles in

modulating cellular processes and gene expression, circRNAs function as protein scaffolds or microRNA "sponges" in numerous malignancies [6, 7]. For instance, hsa_circ_001783 promotes breast cancer proliferation by sponging miR-200c-3p [8]. CircURI1 directly interacts with heterogeneous nuclear ribonucleoprotein M (hnRNPM) to regulate alternative splicing of genes involved in cell migration, thus inhibiting GC metastasis [9]. circPDIA4 has emerged as a prognostic biomarker and therapeutic target in gastric cancer [10]. circCCDC134 enhances cervical cancer tumor growth and metastasis by acting as a miR-503-5p sponge to regulate MYB expression in the cytoplasm and recruiting p65 in the nucleus [11]. The diverse roles of circRNAs in cellular processes and gene regulation highlight their importance in both health and disease, underlining the need for further research into their potential roles and mechanisms in TNBC, which could lead to the identification of new therapeutic targets.

In this study, we utilized RNA-seq to analyze the circRNA expression profile in TNBC tissues. Our findings led to the discovery of a novel circRNA, circBRAF (circBase ID: hsa_circ00071-78), which functions as an oncogene in the progression of TNBC. Mechanistically, circBRAF interacts with KDM4B and IGF2BP3, promoting TNBC growth. These findings suggest that circBRAF could serve as a novel and promising target for TNBC therapy.

**Material and methods**

*Clinical tissues*

Tissues from human breast cancer and a pair of nearby non-cancerous tissues were taken from Soochow University's First Affiliated Hospital. The primary tumor area and morphologically normal surgical margin tissue were immediately isolated from each patient by an experienced pathologist and stored in liquid nitrogen until use. The clinical research ethics committees at the First Affiliated Hospital of Soochow University approved the study (23-01/119), and all patients provided written informed permission.

*Cell cultures*

MCF-10A, MCF-7, MDA-MB-361, MDA-MB-231 and MDA-MB-436 cells were obtained from ATCC and cultured following their instructions. The culture media used for all the cells was RPMI-1640 with 10% fetal bovine serum (Invitrogen, Carlsbad, USA). Under 37°C and 5% carbon dioxide, all cells were cultivated in a humidified incubator.

*Total RNA extraction and qRT-PCR*

In brief, total RNA was isolated from tissues and cells in accordance with the manufacturer's instructions using a total RNA extraction kit (Tiangen, China). Following this, the RNA was reverse transcribed into cDNA using a Fastking cDNA Synthesis Kit (Tiangen, China). circBRAF and gene expression was found by qPCR using Ultra SYBR Mixture (Vazyme, China). The 2-ΔΔCt technique was utilised to calculate the expression of genes, with β-actin serving as an internal control. The primer sequences are shown in Table S1.

*Vector constructs and siRNA*

GenePharma (Shanghai, China) synthesised siRNAs that target circBRAF. Following two days of seeding into 6-well plates, cells were transfected with si circBRAF using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's guidelines. Following this, the cells were incubated for six hours. In order to amplify the expression of circBRAF, the full-length circBRAF sequence was cloned into the pLCDHciR vector (Geenseed Biotech, Guangzhou, China) and packaged using pMD2.G and psPAX2. Cells were transfected with overexpression plasmid using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocols. To generate the lentivirus, 293FT cells (Invitrogen) were co-transfected with psPAX2, pMD2.G, pLVX-ISG20-IRES-Neo or pLVX-SEH1L-IRES-Neo. Forty-eight hours after transfection, the lentiviral supernatants were collected and filtered through a 0.45-μm filter. The lentiviruses were added to media containing 8 μg/ml polybrene (Sigma, St. Louis, MO, USA) and transduced into cells according to the manufacturer's instructions. Stable cell strains expressing circBRAF were selected for at least 1 week using G418 (0.5 mg/ml, Invitrogen). Utilising qRT-PCR, the overexpression and depletion efficiencies were quantified. Information on the sequences is shown in Table S1.

*RNA sequence analysis*

Briefly, MDA-MB-231 cells were transfected with si-circBRAF using Lipofectamine 2000 (Invitrogen, USA). Twenty-four hours post-transfection, cells were harvested and submitted to Aksomics Company (Shanghai, China) for RNA sequencing.

*Cell colony assay*

In brief, cells were seeded into 6-well plates at a density of $1 \times 10^3$ cells per well. Cells were transfected with si circBRAF using Lipofectamine 3000 every seven days in 6-well plates. Fourteen days after seeding, the plates were fixed with 4% paraformaldehyde for 20 minutes and stained with 1% paraformaldehyde for 30 minutes. Following three washes with PBS, colonies were imaged using a digital camera (Sony, Japan).

*Cell migration and invasion assay*

Migration and invasion assays were performed in Transwell chambers (Corning, USA) according to the manufacturer's instructions. For the migration tests, the cells were extracted, resuspended in serum-free medium, and then added to the upper chamber of a Transwell membrane filter (Corning, NY, USA) or to a Transwell membrane filter covered with matrigel (Corning) for the invasion tests. As a chemoattractant, culture media containing 10% FBS was supplied to the chamber's lower compartment. Following a 24-hour incubation period, the cells were counted, photographed, and stained using methanol and 0.1% crystal violet using an Olympus microscope (Tokyo, Japan).

*Actinomycin D assay*

Briefly, cells were seeded into 24-well plates and treated with actinomycin D (5 μg/mL) (Genview, Beijing, China) for 0, 4, 8, and 12 hours. After incubation, total RNA was extracted, and qRT-PCR was performed to determine circBRAF and BRAF expression levels.

*RNase R treatment*

Total RNA from MDA-MB-231 and MDA-MB-436 cells was treated with RNase R (6 U) (Lucigen, Middleton, WI, USA) at 37°C for 10 minutes, followed by inactivation at 85°C for 5 seconds.

Subsequently, RNA was reverse-transcribed to cDNA, and qPCR was performed to assess the expression of linear BRAF and circBRAF.

*Western blot*

Cells were lysed using a protease inhibitor cocktail (Keygen, China), and proteins were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, USA). Membranes were blocked with 5% milk and incubated with primary antibodies overnight at 4°C, followed by HRP-conjugated secondary antibodies (1:5000, Abcam, UK) for one hour at room temperature. Bands were visualized using an ECL chemiluminescent reagent (Beyotime, China) after washing three times with PBS.

*Fluorescence in situ hybridization (FISH)*

Cy5-labelled probes targeting circBRAF were synthesized by GenePharma (Shanghai, China). MDA-MB-231 cells, fixed with 4% paraformaldehyde, were permeabilized with 0.5% Triton X-100 for 15 minutes at room temperature. After three PBS washes, cells were hybridized with circBRAF probes overnight at 37°C. Post-hybridization, cells were counterstained with DAPI for five minutes and imaged using a fluorescent microscope (Nikon, Japan).

*RNA immunoprecipitation (RIP) assay*

The RNA immunoprecipitation (RIP) assay was performed with a RIP kit (BersinBio, Guangzhou, China) according to the manufacturer's instructions. Briefly, MDA-MB-231 cells and MDA-MB-436 cells were lysed and incubated with beads conjugated with IGF2BP3 or KDM-4B antibodies at 4°C overnight. Coprecipitated RNAs were washed and purified for qRT-PCR. Rabbit IgG was used as a negative control.

*RNA immunoprecipitation (RIP) assay*

The RNA immunoprecipitation (RIP) assay was performed with a RIP kit (BersinBio, Guangzhou, China) according to the manufacturer's instructions. Briefly, MDA-MB-231 cells and MDA-MB-436 cells were lysed with lysis buffer and incubated with beads conjugated with IGF2BP3 or KDM4B antibodies (Abcam, UK, 1:200 dilution) at 4°C overnight. The next day, coprecipitated RNAs were washed and

purified for qRT-PCR with specific primers. Rabbit IgG was used as a negative control.

*MS2 pulldown and mass spectrometry analysis*

Briefly, plasmids pBRAF-MS2 and pMS2-GST were obtained from BersinBi (Guangzhou, China). MBA-MB-231 cells were seeded into 6-well plates for 48 hours and then co-transfected with pBRAF-MS2 and pMS2-GST. Following harvesting and lysis, 500 µl (2 µg/µl) of lysate was incubated with agarose beads (Thermo Fisher Scientific) for 4 hours at 4°C. Proteins were subsequently identified through mass spectrometry and matched with the Human Protein Reference Database (http://www.hprd.org/).

*Chromatin immunoprecipitation (ChIP) assay*

In brief, MDA-MB-231 cells were incubated with glycine for 10 min and then lysed with ChIP lysis buffer. After that, the lysates were sonicated to 800 bp fragments and then incubated with magnetic protein A/G beads conjugated with KDM4B primary antibody (Abcam, UK) at 4°C overnight. The next day, the precipitated DNA was analysed by qPCR.

*Xenograft tumor model*

Ten male BALB/c nude mice were purchased from Shanghai Animal Center and randomly separated into 2 groups (Sh circBRAF group and Sh control group). Nude mice from each group were injected with 0.1 ml of a cell suspension containing $2 \times 10^6$ MDA-MB-231 cells. One month later, the mice were sacrificed, and the tumor weight was measured. The volume of the tumor size was calculated by the formula: volume ($mm^3$) = length × width$^2$/2. Every seven days, the size of the tumor was measured. After four weeks of the experiment, the xenograft tumors were removed and weighed, and the mice were put to sleep with an intraperitoneal injection of 100 mg/kg pentobarbital sodium (Sigma, St. Louis, MO, USA).

*Statistical analysis*

Differences between two groups were evaluated using Student's t-test. *P*-values less than 0.05 were deemed significant (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Survival analysis was performed using Kaplan-Meier analysis. All statistical analyses were carried out using GraphPad Prism 8 (GraphPad Software, USA).

## Results

*circBRAF expression was upregulated in TNBC*

circRNA microarray analysis of nine patient specimens (three TNBC, three non-TNBC, and three normal controls) was performed to identify potential dysregulated circRNAs in TNBC (**Figure 1A**). The analysis revealed that, compared to non-TNBC and normal tissues, 32 circRNAs were upregulated, and 13 were downregulated in TNBC. A volcano plot depicting the variation in circRNA expression is shown in **Figure 1B**, highlighting the top 5 upregulated circRNAs. Of particular interest was hsa_circ_0007178, derived from exons 4-13 of the oncogene BRAF. The back-spliced junction of circBRAF was confirmed by Sanger sequencing, amplified using divergent PCR primers, as shown in **Figure 1C**. Primers for circBRAF were designed for the qRT-PCR experiments. The circular structure of circBRAF was confrmed by amplifcation with divergent primers, followed by Sanger sequencing, which showed that circBRAF was generated from exon 4 to exon 13 of the BRAF gene through back splicing (**Figure 1D**). Subsequent qRT-PCR validated the circRNA microarray analysis, indicating a significant upregulation of circBRAF in TNBC, as depicted in **Figures 1E** and **S1**. Similarly, circBRAF expression was higher in TNBC cell lines MDA-MB-231 and MDA-MB-436 compared to non-TNBC and normal cells, aligning with findings from human tissue samples (**Figure 1F**). Further analysis of circBRAF in TNBC included mRNA fractionation and FISH analysis to determine its localization, which was found to be distributed nearly equally between the cytoplasm and nucleus (**Figure 1G** and **1H**). The circularity of circBRAF was confirmed by qRT-PCR using oligo dT and random 6-mers; while linear BRAF could be amplified with both, circBRAF amplification required cDNA reverse transcribed using random 6-mers (**Figure 1I**). Stability comparisons between circBRAF and linear BRAF, conducted via actinomycin D and RNase R treatments, demonstrated the increased stability of circBRAF (**Figure 1J**, **1K**). For circBRAF knockdown, siRNAs targeting circBRAF were developed. siRNA significantly decreased circBRAF levels without affecting BRAF expression, with si-circBRAF-1 and si-cir-
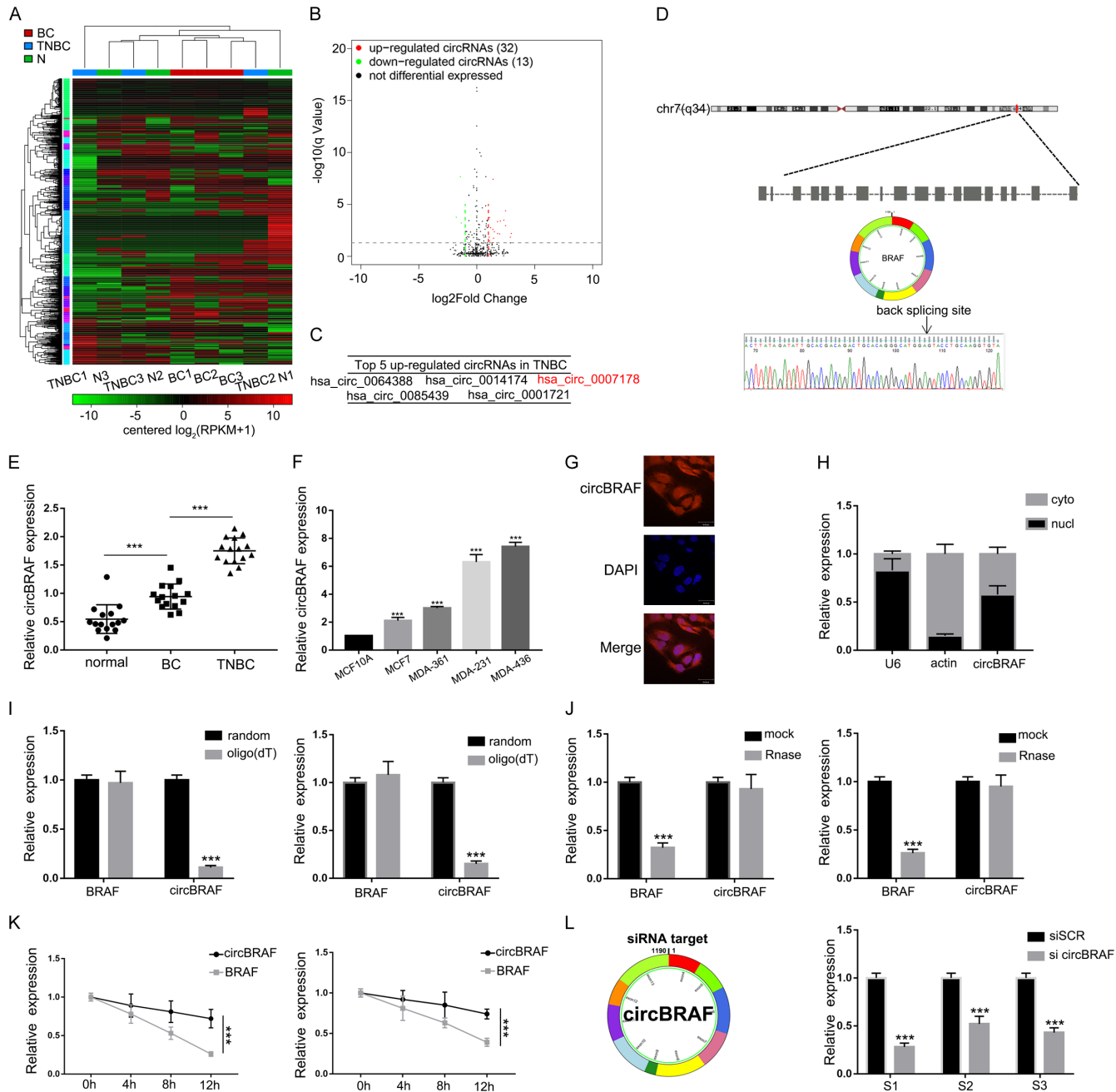
# circBRAF promotes the progression of triple-negative breast cancer

**Figure 1.** The expression of circBRAF was upregulated in TNBC. (A) A cluster heatmap and (B) a volcano plot of circRNA microarray analysis reveal the differential expression of circRNAs in human breast cancer (BC) tissues, triple-negative breast cancer (TNBC) tissues, and adjacent normal tissues (n=3). (C) The top 5 upregulated circRNAs in the microarray analysis are shown. (D) The back splicing site of circBRAF was validated by Sanger sequencing. (E) The expression of circBRAF was measured by qRT-PCR in human BC tissues (n=15), TNBC tissues (n=15) and adjacent normal tissues (n=15), showing significant upregulation of circBRAF in TNBC tissues. (F) qRT-PCR measurement of circBRAF expression in BC and TNBC cell lines demonstrates significant upregulation in TNBC cell lines. (G, H) The localization of circBRAF in MDA-MB-231 cells was assessed by FISH and mRNA fractionation assays, indicating that circBRAF is distributed nearly equally between the cytoplasm and nucleus. (I) The expression of BRAF and circBRAF was measured by qRT-PCR with random or oligo dT in MDA-MB-231 and MDA-MB-436 cells to verify the circularity of circBRAF. (J) The expression levels of circBRAF and BRAF in total RNA treated with RNase R are shown for MDA-MB-231 and MDA-MB-436 cells. (K) Stability assays of circBRAF and BRAF in MDA-MB-231 and MDA-MB-436 cells treated with actinomycin D indicate that circBRAF is more stable than linear BRAF. (L) qRT-PCR was used to measure circBRAF expression following transfection with three different siRNAs.

cBRAF-3 selected for subsequent cellular function experiments (**Figure 1L**).

*Knockdown of circBRAF significantly reduced cell proliferation, angiogenesis, and metastasis in vitro*

The biological function of circBRAF in TNBC was then investigated. The biological function of circBRAF in TNBC was explored using colony formation assays to assess the impact of circBRAF on TNBC cell growth, a tube formation assay to assess angiogenic potential, and a transwell assays to evaluate cell migration and invasion. Knockdown of circBRAF by si-circBRAF-1 and si-circBRAF-3 led to reduced proliferation of MDA-MB-231 and MDA-MB-436 cells, as shown in **Figures 2A** and S2A. The effect of circBRAF on cell angiogenesis was investigated using a tube formation assay; media conditioned by MDA-MB-231 and MDA-MB-436 cells with circBRAF knockdown resulted in decreased tube formation by human umbilical vein endothelial cells (HUVECs), as illustrated in **Figures 2B** and S2B. Transwell migration and invasion assays further demonstrated that downregulation of circBRAF significantly impaired the migratory ability of MDA-MB-231 and MDA-MB-436 cells (**Figures 2C** and S2C) and markedly suppressed TNBC cell invasion capabilities (**Figures 2D** and S2D). These results establish circBRAF as crucial for TNBC development, with its knockdown inhibiting angiogenesis, metastasis, and cell growth in vitro.

*circBRAF regulated the expression of multiple oncogenes in TNBC*

RNA-seq analysis was conducted on MDA-MB-231 cells following circBRAF knockdown to explore the mechanisms by which circBRAF influences TNBC progression (**Figure 3A**). The RNA-seq data, visualized in a volcano plot (**Figure 3B**), identified 411 downregulated and 332 upregulated genes (*P* value < 0.05, log2 fold change > 1) compared to control cells. To verify the RNA-seq results, qRT-PCR was performed on the top 5 downregulated genes (TCF12, WWP2, GDI2, FLNA, and KIF21A); qRT-PCR confirmed a decrease in the expression of these genes concurrent with the reduction of circBRAF levels (**Figure 3C**). Additionally, the TCGA database was utilized to examine the RNA expression profile, aiding in the identification of potential molecular targets of circBRAF in TNBC (**Figure 3D**). Eighteen potential targets were identified through the integration of TCGA and RNA-seq data (**Figure 3E**). Expression and survival analysis of these genes in breast cancer (BC) using TCGA datasets revealed that PTPRN expression strongly correlated with overall survival, and the expression of VCAN, FN1, CDCA3, MMP9, and ADAMTS14 was increased in BC (Figures S3 and S4). qRT-PCR further verified that the expression of VCAN, FN1, CDCA3, MMP9, ADAMTS14, and PTPRN decreased in MDA-MB-231 and MDA-MB-436 cells following circBRAF knockdown (**Figure 3F**). Western blot analysis confirmed the reduction in protein levels of the six circBRAF downstream targets in MDA-MB-231 cells transfected with si-circBRAF (**Figure 3G**). These results suggest that circBRAF may act as an oncogene by upregulating several downstream molecular targets as TNBC progresses.

*circBRAF interacted with KDM4B and IGF2BP3 in TNBC cells*

circRNAs are known to regulate cancer cell processes by binding to proteins. To identify the
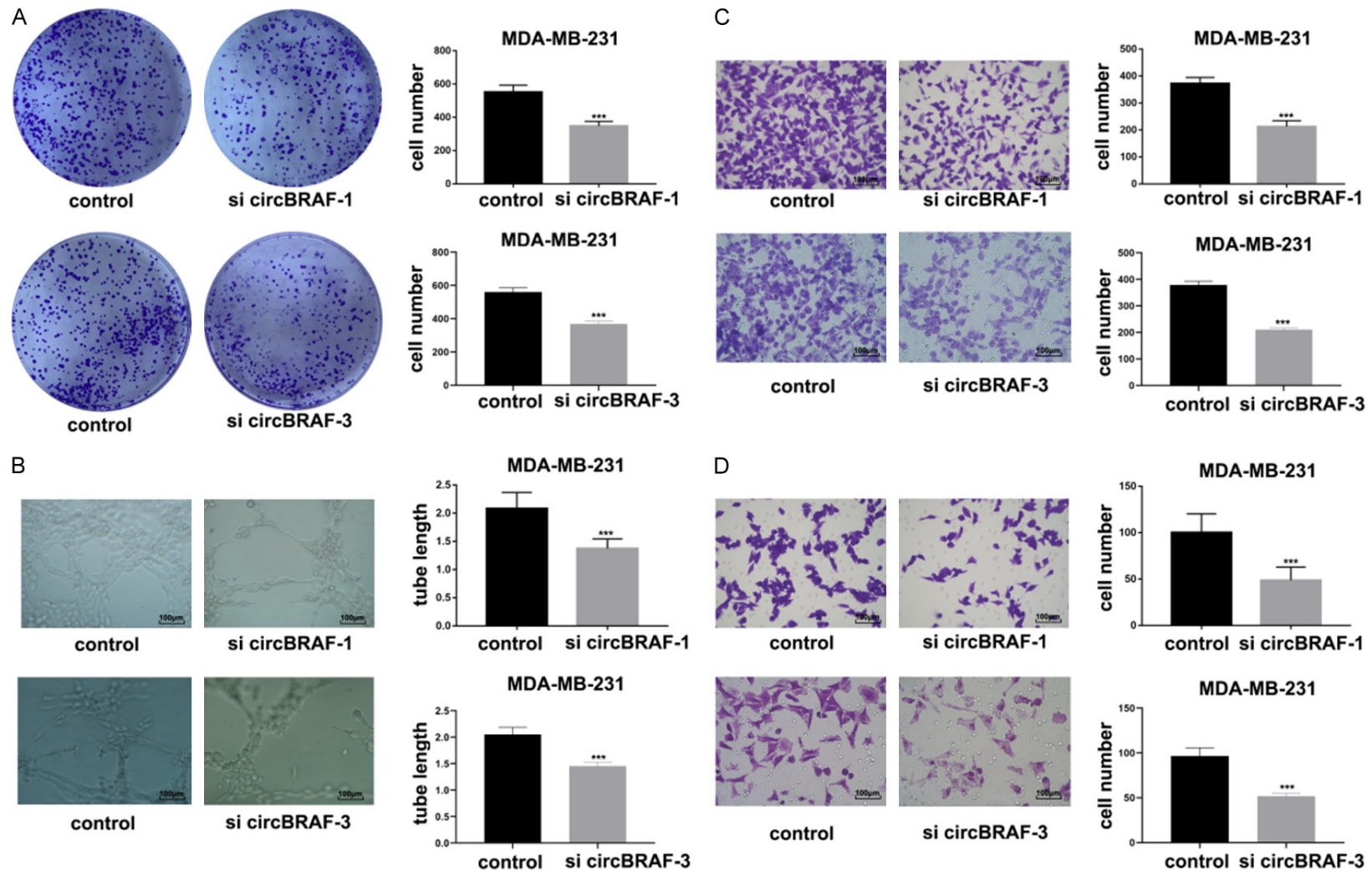
**Figure 2.** Knockdown of circBRAF decreased cell proliferation and invasion in vitro. A. Colony formation assays demonstrated that knockdown of circBRAF inhibited the proliferation of MDA-MB-231 cells. B. Tube formation assays indicated a decrease in tube formation following circBRAF knockdown. C, D. Transwell migration and invasion assays demonstrated that TNBC cell migration and invasion capacities were significantly suppressed by circBRAF knockdown.
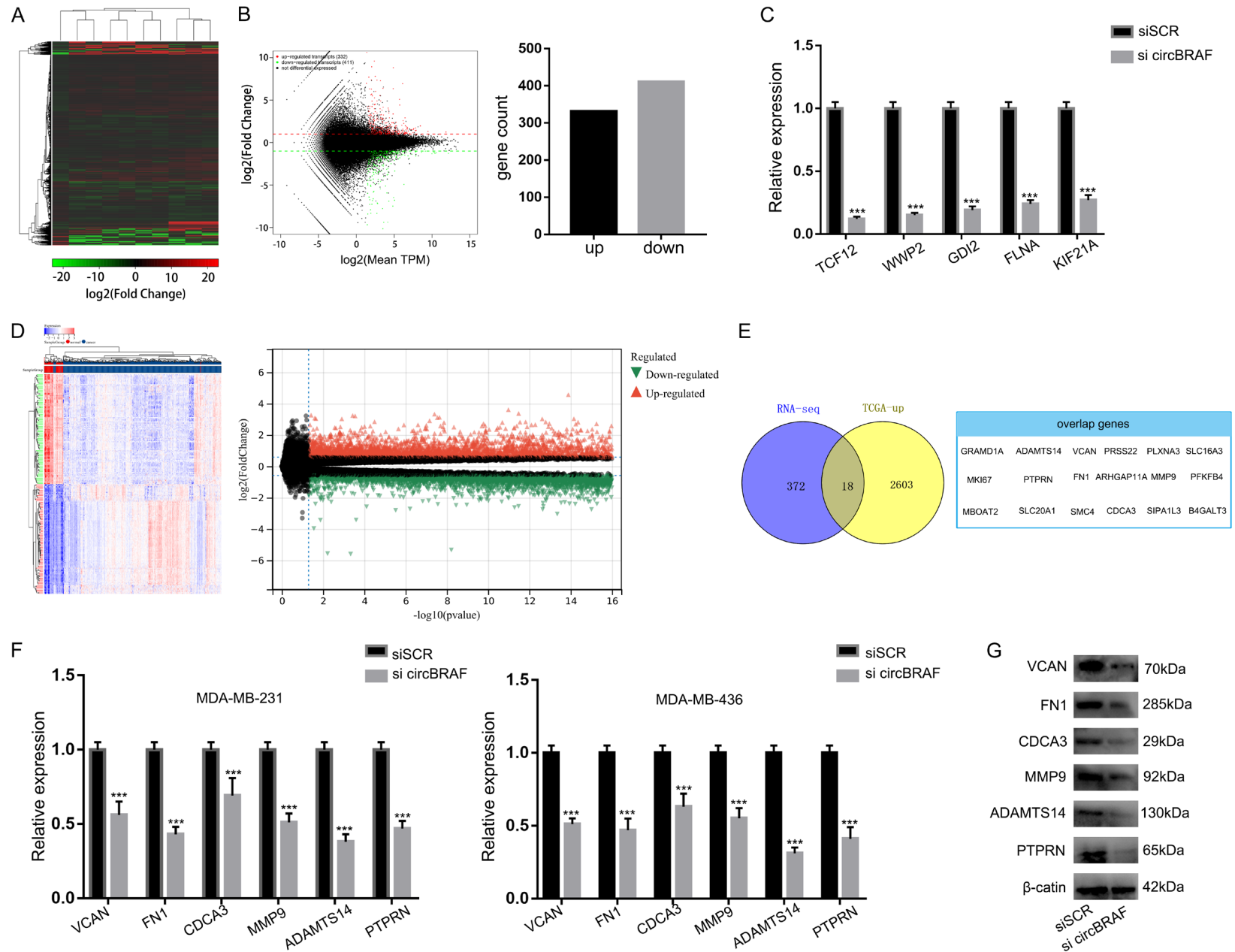
# circBRAF promotes the progression of triple-negative breast cancer

**Figure 3.** circBRAF increased the expression of multiple oncogenes. (A) A heatmap and (B) a volcano plot from mRNA-seq analysis illustrated the gene expression profile in MDA-MB-231 cells following circBRAF knockdown. (C) qRT-PCR validation confirmed the downregulation of the top 5 genes following circBRAF knockdown. (D) Heatmap and volcano plot of TCGA analysis showing the expression profile of genes in BC. (E) Venn diagram showing the potential eighteen target genes of circBRAF. (F) The qRT-PCR results confirmed that following circBRAF knockdown, the expression of VCAN, FN1, CDCA3, MMP9, ADAMTS14, and PTPRN was downregulated in both MDA-MB-231 and MDA-MB-436 cells. (G) Transfection of MDA-MB-231 cells with si-circBRAF resulted in decreased protein levels of the six circBRAF downstream targets.
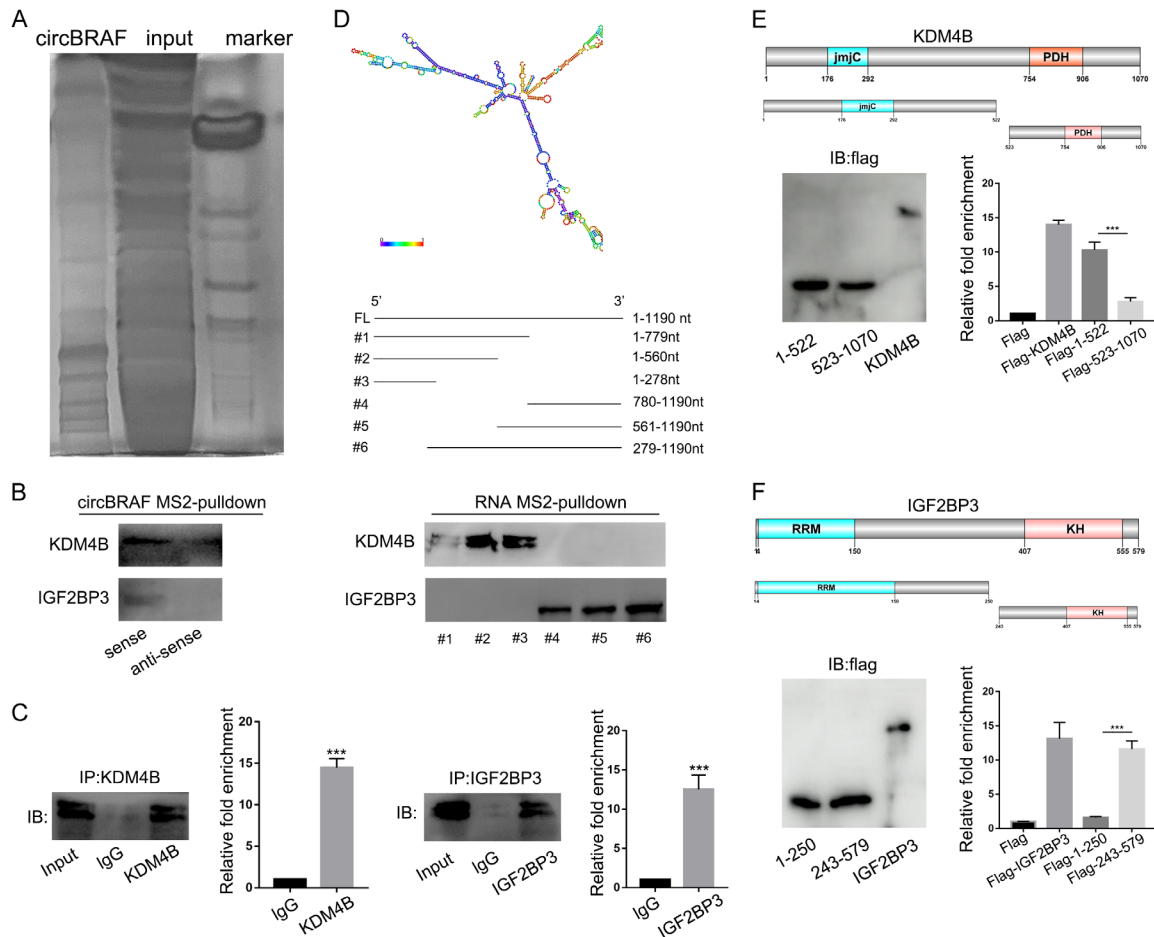


**Figure 4.** circBRAF interacted with KDM4B and IGF2BP3. A. Proteins isolated from circBRAF MS2-pulldown assays were analyzed using SDS-PAGE and visualized with silver staining. B. circRNA MS2 pulldown assays revealed that KDM4B and IGF2BP3 interact with circBRAF. C. RIP assays demonstrated interactions between circBRAF and KDM4B (right), as well as IGF2BP3 (left). D. A series of circBRAF mutants based on their secondary structure were constructed and transfected into MDA-MB-231 cells (upper). MS2 pulldown results indicated that the 1-278 nt and 780-1190 nt fragments of circBRAF facilitated its interaction with KDM4B and IGF2BP3 (lower). E, F. RIP analysis confirmed significant interactions between circBRAF and the 1-522 amino acids (aa) of KDM4B and the 243-579 aa of IGF2BP3.

binding proteins of circBRAF, a ChIRP assay followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) was conducted (**Figures 4A** and S5A, S5B). Among the identified proteins, circBRAF was found to interact with KDM4B and IGF2BP3, significant regulators in various cancers, including BC (Figure S5C,

S5D). This interaction was further confirmed by circRNA MS2 pulldown and RIP assays. The MS2 pulldown assays demonstrated that KDM4B and IGF2BP3 could bind to circBRAF (**Figure 4B**), and RIP assays confirmed the interactions of KDM4B (**Figure 4C**, right) and IGF2BP3 (**Figure 4C**, left) with circBRAF.

Furthermore, the expression of KDM4B and IGF2BP3 remained unchanged after circBRAF overexpression and knockdown (Figure S5E), indicating that circBRAF may modulate downstream target expression through interactions with KDM4B and IGF2BP3. To pinpoint the RNA regions responsible for driving circBRAF binding to KDM4B and IGF2BP3, a series of circBRAF mutants, based on secondary structure, were generated and transfected into MDA-MB-231 cells (**Figure 4D**, upper). Results from the MS2 pulldown assay indicated that the 1-278 nt and 780-1190 nt fragments of circBRAF facilitated its interaction with KDM4B and IGF2BP3 (**Figure 4D**, lower). Subsequent RIP experiments with several deletion mutants of KDM4B and IGF2BP3 demonstrated significant interactions between circBRAF and the 1-522 aa region of KDM4B (**Figure 4E**) and the 243-579 aa region of IGF2BP3 (**Figure 4F**). Collectively, these findings indicate specific interactions between circBRAF and both KDM4B and IGF2BP3.

### circBRAF regulated the expression of VCAN, FN1, CDCA3 and B4GALT3 via IGF2BP3

To elucidate the roles of IGF2BP3 and circBRAF in BC, we analyzed overlapping genes from three datasets: upregulated genes in BC from the TCGA database, mRNAs that bind to IGF2BP3 in cells according to RIP-seq data, and downregulated mRNAs in BC cells with circBRAF knockdown (**Figure 5A**). Eleven potential genes were identified, and their association with IGF2BP3 was confirmed through RIP experiments using an IGF2BP3-specific antibody, as illustrated in **Figure 5B**. Potential targets of IGF2BP3 and circBRAF were found to include GRAMD1A, VCAN, FN1, CDCA3, ARHGAP11A, MBOAT2, and B4GALT3. Subsequent analysis of these targets through western blot and qRT-PCR, following IGF2BP3 knockdown in MDA-MB-231 cells, revealed that IGF2BP3 regulated only VCAN, FN1, CDCA3, and B4GALT3 (**Figure 5C**). Additionally, IGF2BP3 knockdown led to a reduction in the mRNA stability of VCAN, FN1, CDCA3, and B4GALT3, as shown in Figure S6A. RNA pulldown assays confirmed the interaction sites between these mRNA targets and circBRAF, predicted by bioinformatics analysis (**Figures 5D** and S6B). Following circBRAF knockdown in MDA-MB-231 cells, a decrease in IGF2BP3 expression and its inter-

action with VCAN, FN1, CDCA3, and B4GALT3 was observed (**Figure 5E**). Finally, qRT-PCR and actinomycin D assays demonstrated that the stability of VCAN, FN1, CDCA3, and B4GALT3 mRNAs increased in cells overexpressing circBRAF (**Figure 5F**). These results indicate that circBRAF, by modulating mRNA stability through IGF2BP3, enhances the expression of VCAN, FN1, CDCA3, and B4GALT3.

### circBRAF increased the transcription of MMP9 and ADAMTS14 via histone H3K9me3 modification and KDM4B

According to our previous findings, circBRAF enhanced the expression of ADAMTS14 and MMP9, encouraged cell metastasis, and interacted with KDM4B and IGF2BP3. We therefore examined the histone H3K9me3 modification of MMP9 and ADAMTS14 to verify the association between circBRAF and genes related to cell metastasis (Figure S7A). The findings demonstrated that the transcription start sites of MMP9 and ADAMTS14 were highly occupied by the activated histone-protein modification H3K9me3. Next, we examined KDM4B enrichment at the MMP9 and ADAMTS14 promoters through a RIP assay (**Figure 6A** and **6E**). Knockdown of KDM4B in MDA-MB-231 led to a significant decrease in its enrichment at these promoters (Figure S7B, S7C). In both MDA-MB-231 and MDA-MB-436 cells, KDM4B knockdown resulted in inhibited expression of MMP9 and ADAMTS14 (**Figure 6B** and **6F**). Subsequent RIP assays, following KDM4B knockdown, assessed histone H3K9me3 modification levels among MMP9 and ADAMTS14 promoters (**Figure 6C** and **6G**). The results showed a significant increase in H3K9me3 modification upon KDM4B knockdown. Likewise, RIP experiments using a specific H3K9me3 antibody revealed an increase in H3K9me3 at the MMP9 and ADAMTS14 promoters following circBRAF knockdown (**Figure 6D** and **6H**). Together, these findings demonstrate that circBRAF recruits KDM4B to the promoters of MMP9 and ADAMTS14, thereby increasing the expression of these genes (**Figure 6I**).
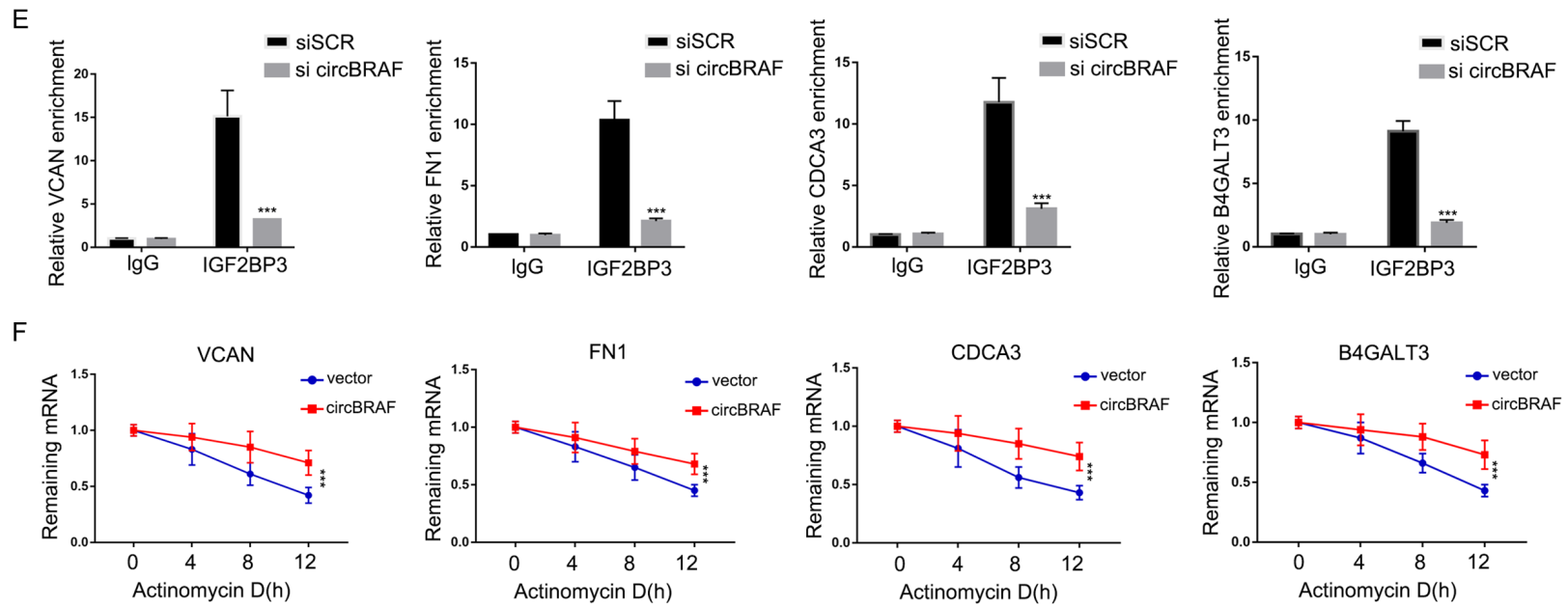
### circBRAF promoted cell proliferation, angiogenesis, and metastasis via KDM4B and IGF2BP3

We further explored whether circBRAF could influence cell functions through the modulation
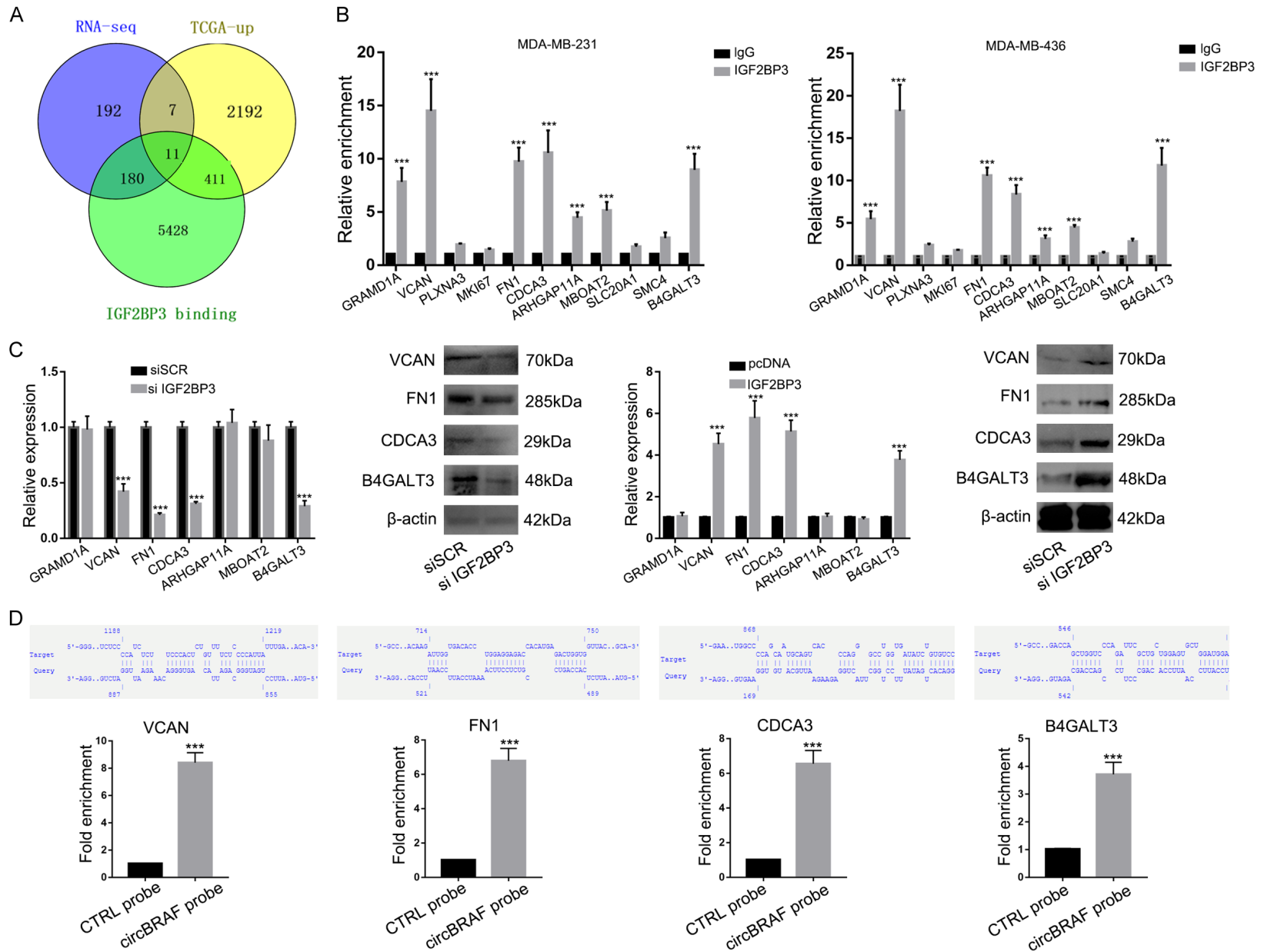
Figure 5. circBRAF regulated the expression of VCAN, FN1, CDCA3, and B4GALT3 via IGF2BP3. A. A Venn diagram identifies potential target genes of circBRAF and IGF2BP3. B. Eleven potential genes were identified and validated through RIP experiments using an IGF2BP3-specific antibody. C. qRT-PCR and western blot assays assessed the expression of potential targets, demonstrating that IGF2BP3 regulates VCAN, FN1, CDCA3, and B4GALT3. D. RNA pulldown assays validated the interactions between potential targets and circBRAF. E. RIP detected interactions between VCAN, FN1, CDCA3, B4GALT3, and IGF2BP3 following circBRAF knockdown. F. mRNA stability analysis revealed an increase for VCAN, FN1, CDCA3, and B4GALT3 in cells overexpressing circBRAF.
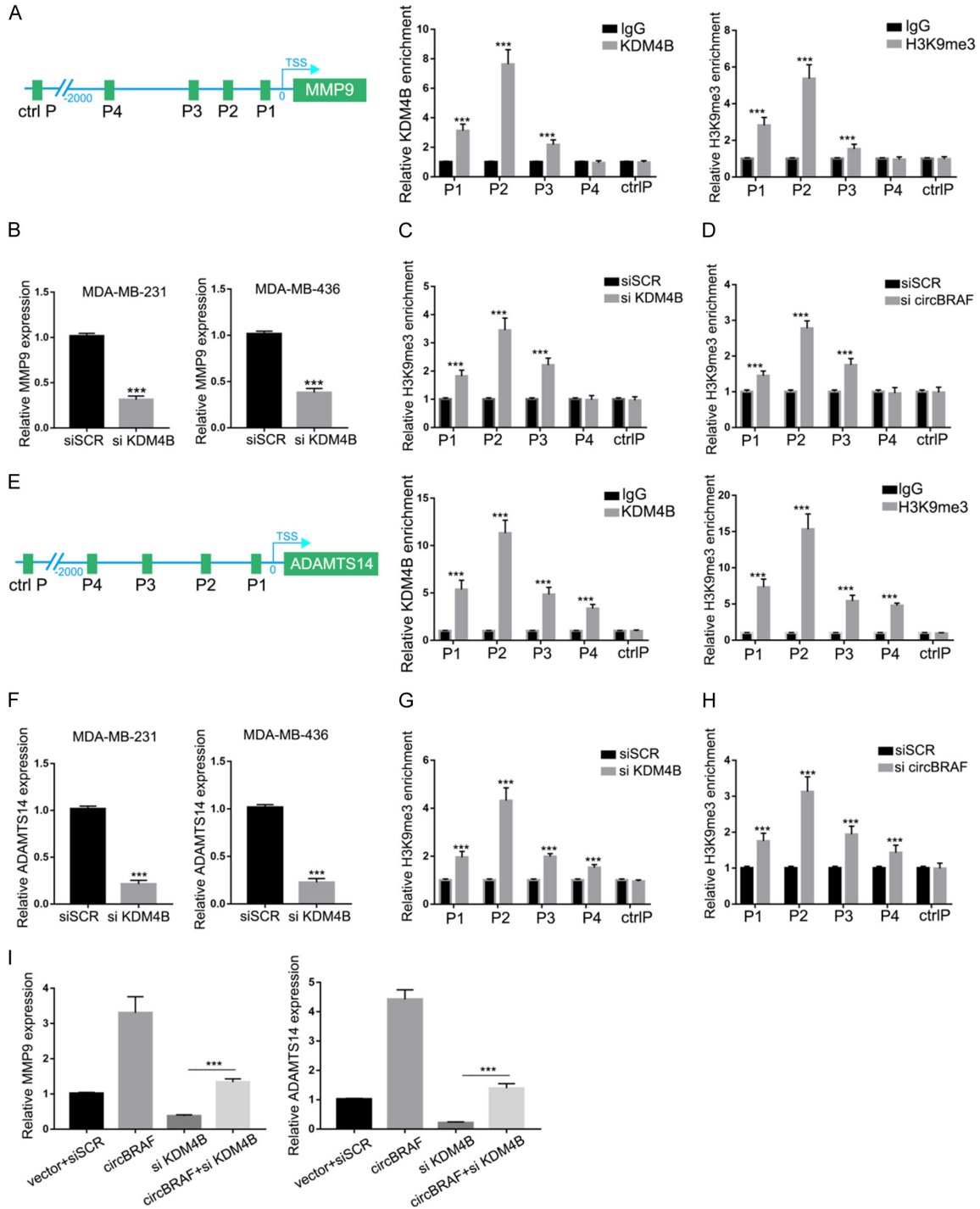
Figure 6. circBRAF increased the transcription of MMP9 and ADAMTS14 via histone H3K9me3 modification and KDM4B. A. A RIP assay assessed the enrichment of KDM4B at the promoters of MMP9 and ADAMTS14. B. In MDA-MB-231 and MDA-MB-436 cells, KDM4B knockdown led to reduced expression of MMP9 and ADAMTS14. C, D. ChIP assays detected H3K9me3 enrichment at the MMP9 promoter following KDM4B and IGF2BP3 knockdown. E. ChIP assays measured the enrichment of KDM4B and H3K9me3 on the ADAMTS14 promoter. F. The expression of AD-AMTS14 was measured by qRT-PCR after KDM4B knockdown. G, H. The enrichment of H3K9me3 on the promoter of ADAMTS14 was detected by ChIP assay after KDM4B and IGF2BP3 knockdown. I. The expression of MMP9 and ADAMTS14 was measured by qRT-PCR after circBRAF and siKDM4B co-transfection.
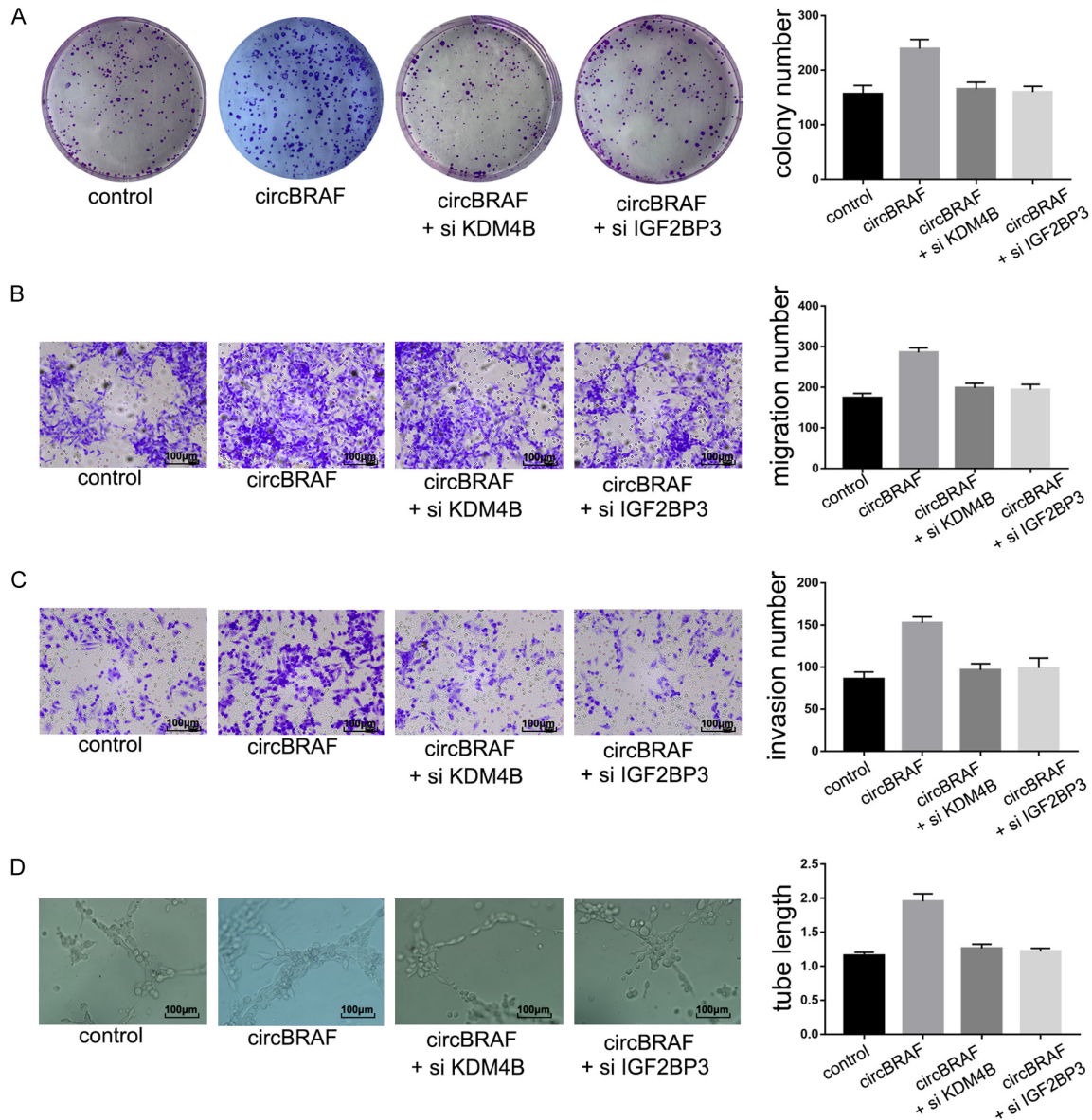
**Figure 7.** circBRAF promoted cell proliferation, angiogenesis, and metastasis via KDM4B and IGF2BP3. A. KDM4B and IGF2BP3 knockdown significantly reduced the enhanced proliferation induced by circBRAF overexpression. B, C. Knockdown of KDM4B or IGF2BP3 attenuated migratory and invasive phenotypes promoted by circBRAF in MDA-MB-231 cells. D. Overexpression of circBRAF counteracted the inhibitory effects of siKDM4B and siIGF2BP3.

of KDM4B and IGF2BP3, previously identified as regulators of various oncogenes. Initially, a colony formation assay was employed to evaluate cell growth. Results showed that the increased proliferation induced by circBRAF overexpression was significantly attenuated by the knockdown of KDM4B and IGF2BP3 (**Figure 7A**). Additionally, the knockdown of either KDM4B or IGF2BP3 diminished the enhanced migratory and invasive effects induced by circBRAF in MDA-MB-231 cells

(**Figure 7B** and **7C**). A tube formation assay was used to assess the role of circBRAF, along with KDM4B/IGF2BP3, in cell angiogenesis. The results indicated that overexpression of circBRAF counteracted the inhibitory effects of siKDM4B and siIGF2BP3 (**Figure 7D**). Moreover, the impact of circBRAF on in vivo cell proliferation was examined. Mice injected with MDA-MB-231 cells expressing circBRAF shRNA exhibited lower tumor weights and volumes compared to the control groups, as illustrated
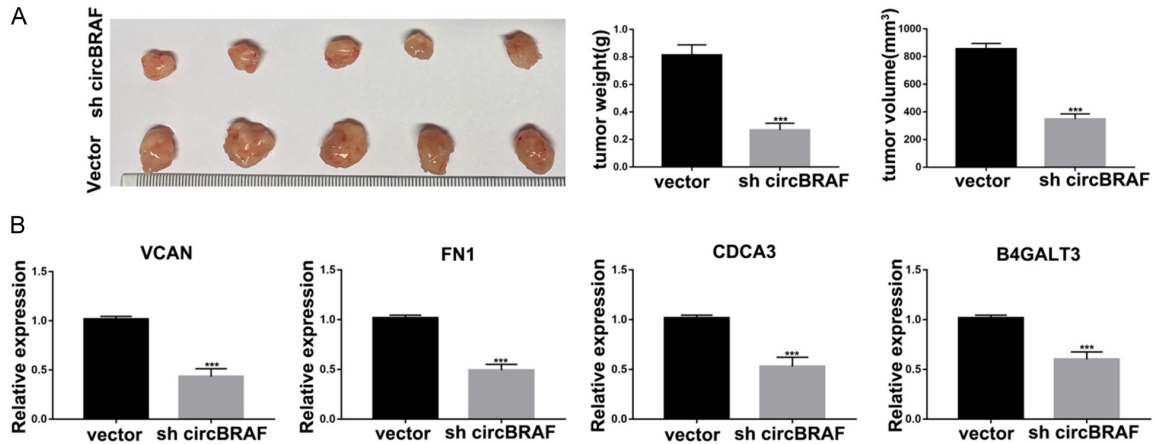
**Figure 8.** circBRAF promoted cell proliferation in vivo. A. The mice injected with MDA-MB-231 cells expressing circBRAF shRNA displayed lower tumour weights and volumes compared to control groups. B. The sh-circBRAF groups showed reduced expression of VCAN, FN1, CDCA3, and B4GALT3 relative to control groups.
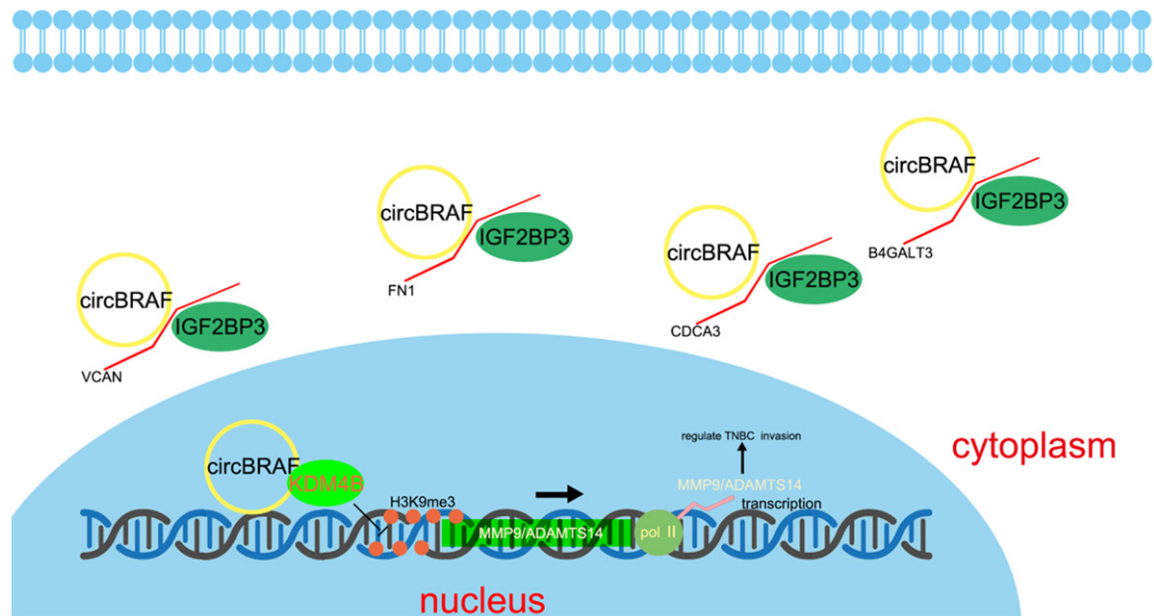


**Figure 9.** A schematic illustration of the molecular mechanism of circBRAF in promoting the development of TNBC.

in **Figure 8A**. Additionally, a reduction in the expression of VCAN, FN1, CDCA3, and B4GALT3 was observed in the sh-circBRAF groups relative to the control groups (**Figure 8B**). Collectively, our findings demonstrate that circBRAF acts as a potent positive regulator of TNBC through the mediation of IGF2BP3 and KDM4B (**Figure 9**).

## Discussion

Compared to other breast cancer subtypes, triple-negative breast cancer (TNBC) is recognized for its aggressiveness and limited therapeutic options [12]. Surgery often serves as the initial treatment step for TNBC, followed by chemotherapy. Neoadjuvant chemotherapy, administered prior to surgery, is frequently recommended to reduce tumor size, facilitate its removal, and assess the tumor's response to therapy. However, TNBC typically has a poor prognosis and shows variable responses to treatment due to its heterogeneity [13]. Understanding the molecular and transcriptomic characteristics of TNBC is crucial for developing more targeted treatment strategies. Recent

research has focused on the role of circRNAs in oncogenesis and cancer progression. Due to their tissue-specific expression and covalently closed loop structures, circRNAs are promising candidates for therapeutic targets or diagnostic markers [14]. We believe circRNAs could serve as highly effective treatments for TNBC.

In this study, the expression of circRNAs in TNBC was analyzed using circRNA microarray analysis on nine patient specimens. We focused particularly on hsa_circ_0007178, a circRNA derived from exons 4-13 of the oncogene BRAF. Most studies on BRAF have shown that the BRAF/MEK pathway is associated with the risk of breast cancer recurrence in an estrogen receptor (ER) status-dependent manner, and that a BRAF mutation could serve as a viable predictive biomarker and therapeutic target for breast cancer [15-17]. However, the roles and functions of circBRAF in TNBC remain poorly understood. Our data demonstrated elevated circBRAF expression in TNBC tissues and cells. Functional experiments demonstrated that circBRAF is essential for TNBC development. circBRAF knockdown inhibited angiogenesis, metastasis, and cell division in vitro, confirming its oncogenic role in TNBC. The mechanisms behind the effects of circBRAF on TNBC progression were investigated through RIP-seq data, histone H3K9me3 modifications, MS2 pulldown, ChIRP tests followed by LC-MS/MS, and RNA-seq analysis following circBRAF knockdown.

According to this study, circBRAF is crucial for circRNA-protein interactions, particularly with KDM4B and IGF2BP3, influencing transcriptional regulation and contributing to the functional profile of circBRAF. Our findings revealed two new functional aspects of circBRAF. Firstly, circBRAF interacts with IGF2BP3, which regulates mRNA stability through RNA N6-methyladenosine (m6A) modification, enhancing the expression of VCAN, FN1, CDCA3, and B4GALT3. Secondly, circBRAF recruits KDM4B to enhance MMP9 and ADAMTS14 expression through H3K9me3 modification.

Previous studies have indicated that IGF2BP3 enhances TNBC cell migration and invasion potential through cellular RNA m6A modifications and accelerates TNBC proliferation by destabilizing NF1 mRNA [18, 19]. These findings provide support that circBRAF, through interaction with IGF2BP3, can accelerate the development of TNBC. KDM4B, often referred to as lysine demethylase 4B, plays a role in epigenetic regulation by removing methyl groups from lysine residues on histone proteins, which are crucial for dynamic gene expression regulation [20]. In breast cancer, KDM4B increases the stemness of cancer cells and promotes epithelial-mesenchymal transition (EMT). KDM4B also inhibits PHGDH through a mechanism that reduces the enrichment of H3-K36me3 at the PHGDH promoter region [21]. Moreover, the inhibitory effects of siKDM4B and siIGF2BP3 on cell migration and invasion in TNBC were countered by overexpressing circBRAF. These results suggest that circBRAF acts through IGF2BP3 and KDM4B as a unique positive regulator of TNBC. Our research was not without its limits. Our research, however, has its limitations. Further *in vivo* studies are needed to explore the role of circBRAF in the metastasis of TNBC tumors. Furthermore, additional research is required to determine the potential therapeutic applications of circBRAF in TNBC.

In summary, our study demonstrated that circBRAF can play an oncogenic role by specifically interacting with KDM4B and IGF2BP3 in TNBC and presents a promising new potential target for the treatment of TNBC.

## Acknowledgements

## Disclosure of conflict of interest

None.

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**Table S1.** siRNA sequences in this study

| Name | Sequence (5'→3') |
| --- | --- |
| si-CircBRAF #1 | GGCATGGAGTACCTGCAAGGT |
| si-CircBRAF #2 | TGCACAGGGCATGGAGTACCT |
| si-CircBRAF #3 | CATGGAGTACCTGCAAGGTGT |
| Sh CircBRAF | GGCATGGAGTACCTGCAAGGTTTCAAGAGATCCUUGCTGGUTCUCCTUGCC |
| Sh NC | GAGUUAAAGUCAAAGUGACTTTTCAAGAGATTGUCACUUUGACUUUAACUC |
| si-KDM4B | GCGCAGAAUCUACCAACUU |
| si-IGF2BP3 | CTGGCAGAGTTATTGGAAA |
| si-NC | GAGUUAAAGUCAAAGUGACTT |
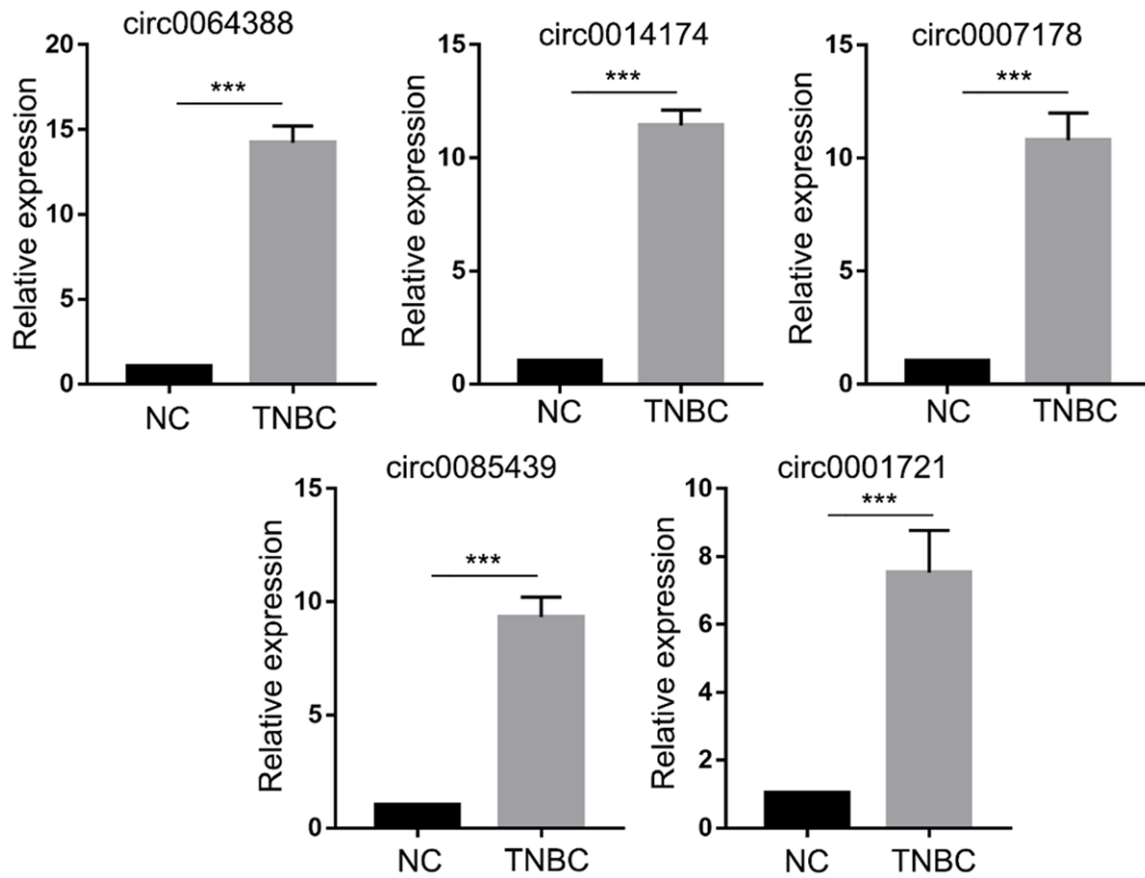


**Figure S1.** The expression of 5 candidate circRNAs was measured by qRT-PCR in TNBC tissues.
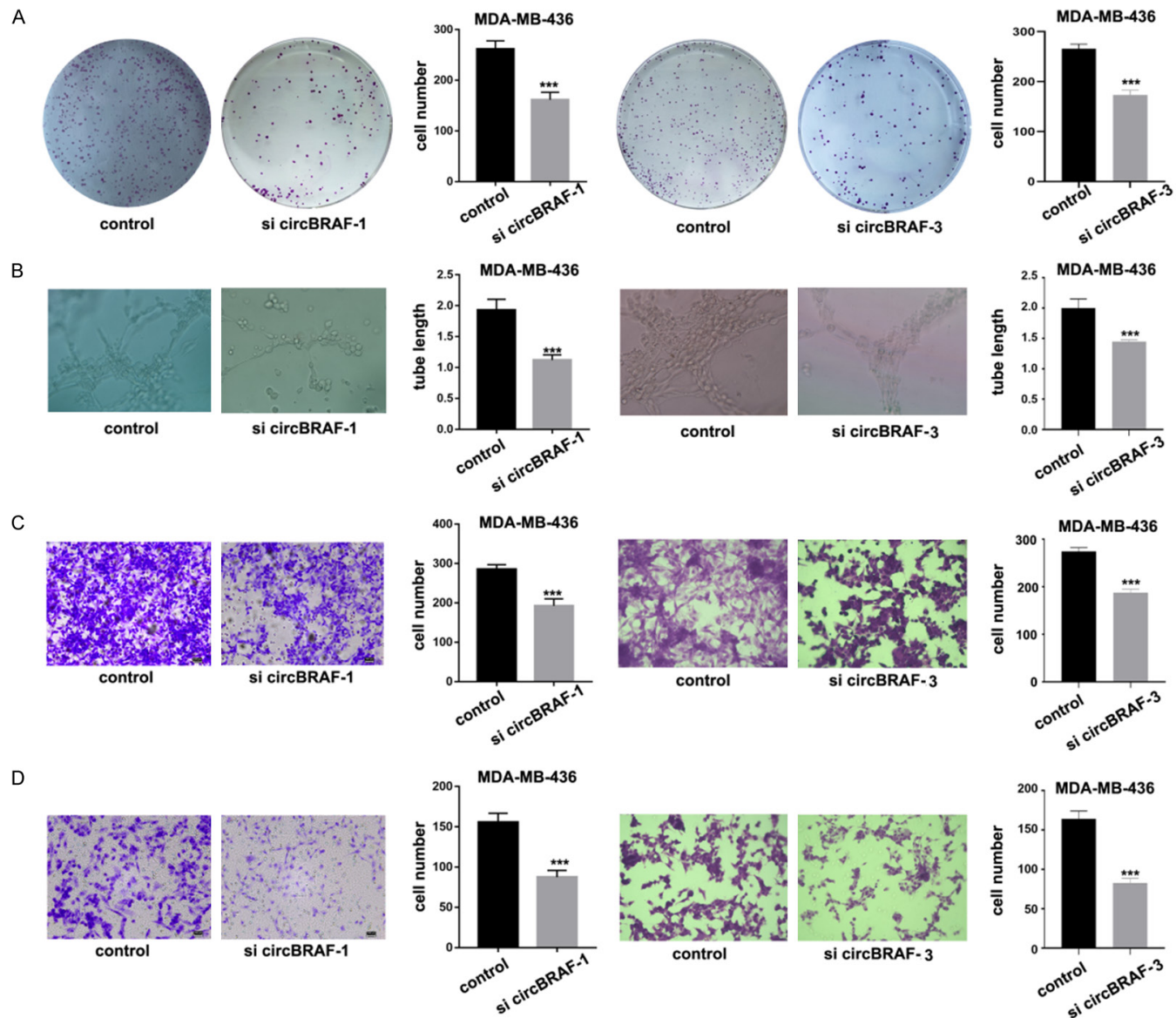
**Figure S2.** A. The results of colony formation demonstrated that circBRAF knockdown inhibited the proliferation of MDA-MB-436 cells. B. The results of the tube formation assay demonstrated that tube formation was decreased with circBRAF knockdown. C, D. The transwell migration and invasion experiment results demonstrated that TNBC cell migration and invasion capacities were markedly suppressed by circBRAF knockdown.
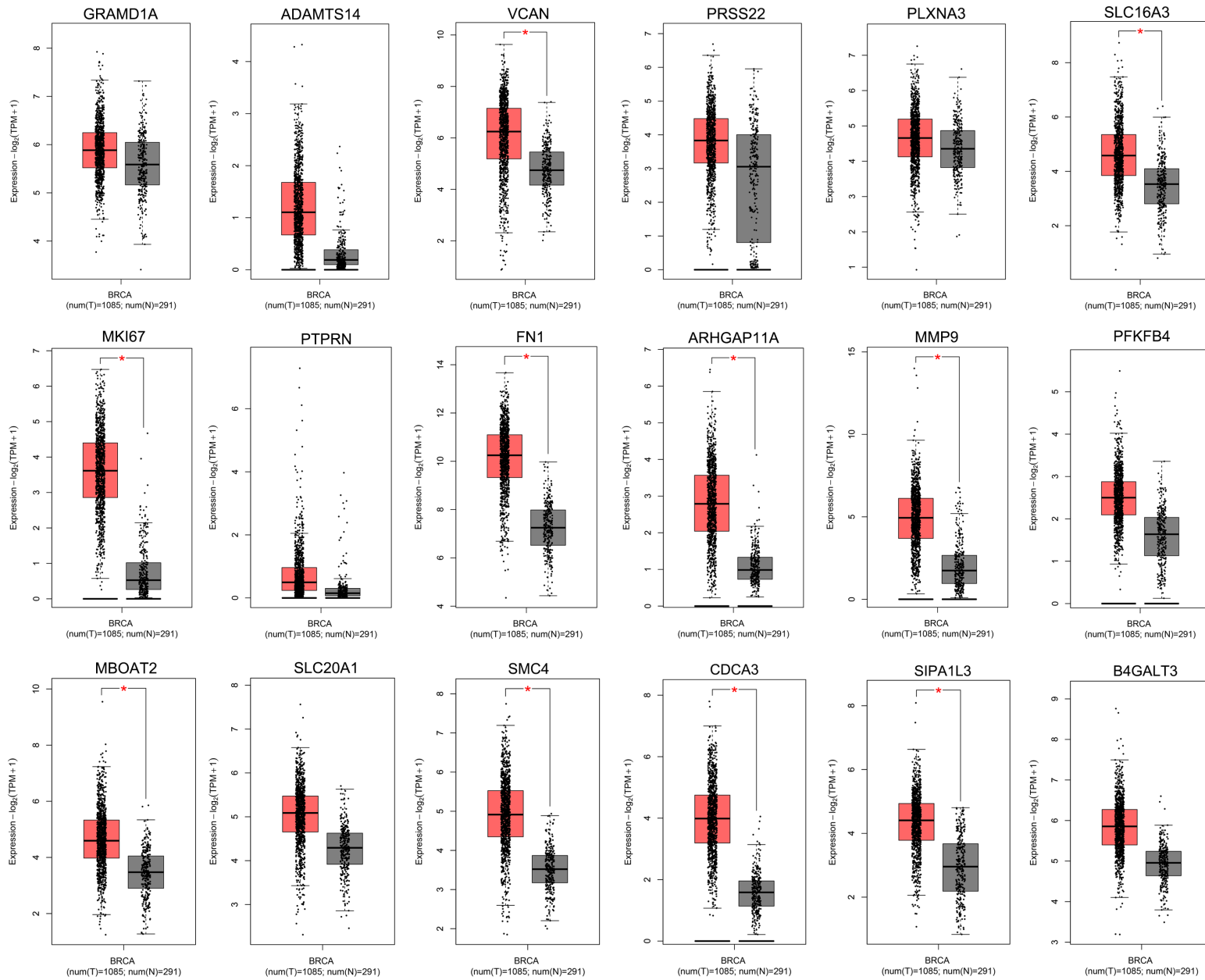
# circBRAF promotes the progression of triple-negative breast cancer

**Figure S3.** The expression of candidate targets of CircBRAF was analyzed by TCGA database.
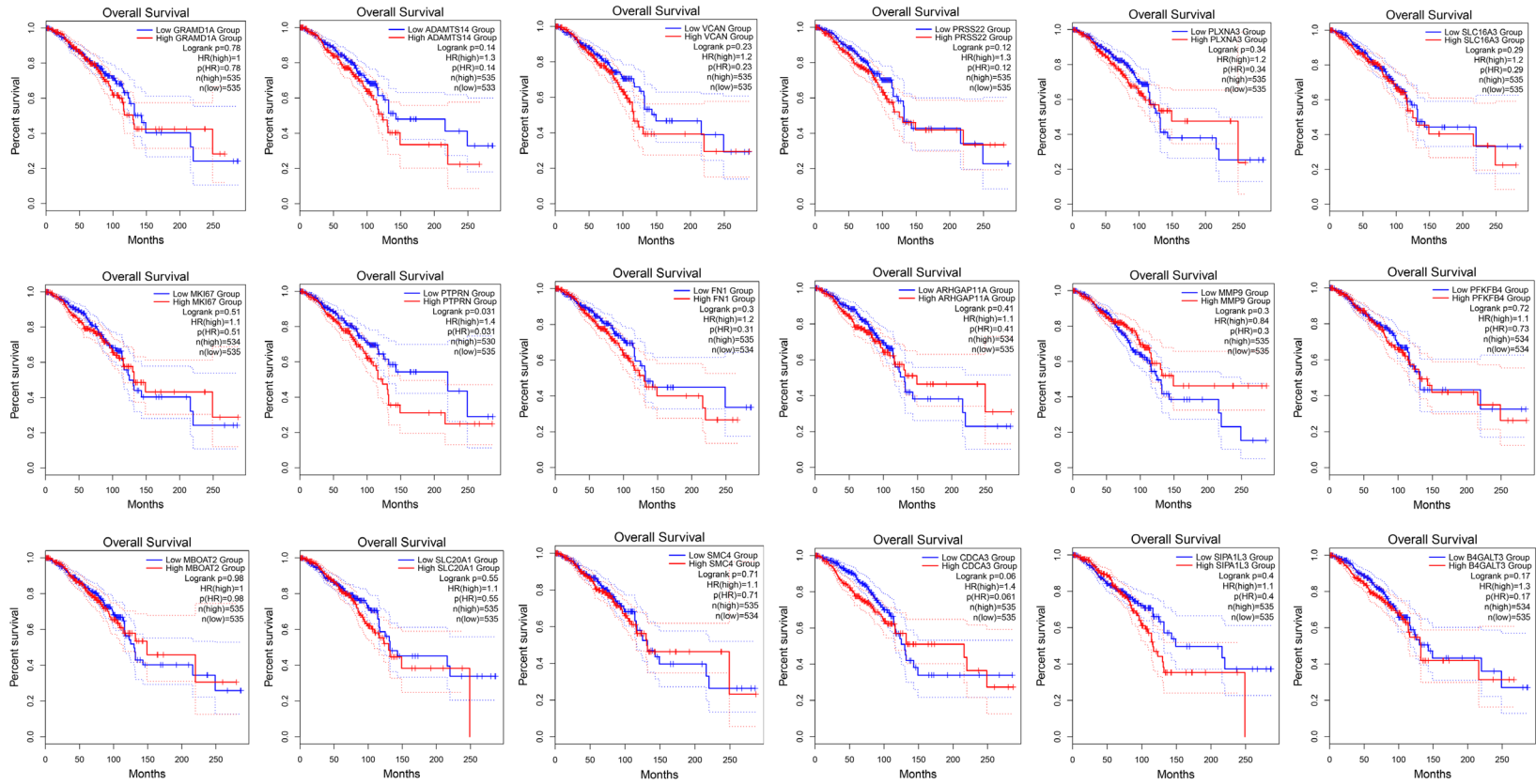


**Figure S4.** The overall survival analysis of candidate targets of CircBRAF based on TCGA database was shown.

# circBRAF promotes the progression of triple-negative breast cancer
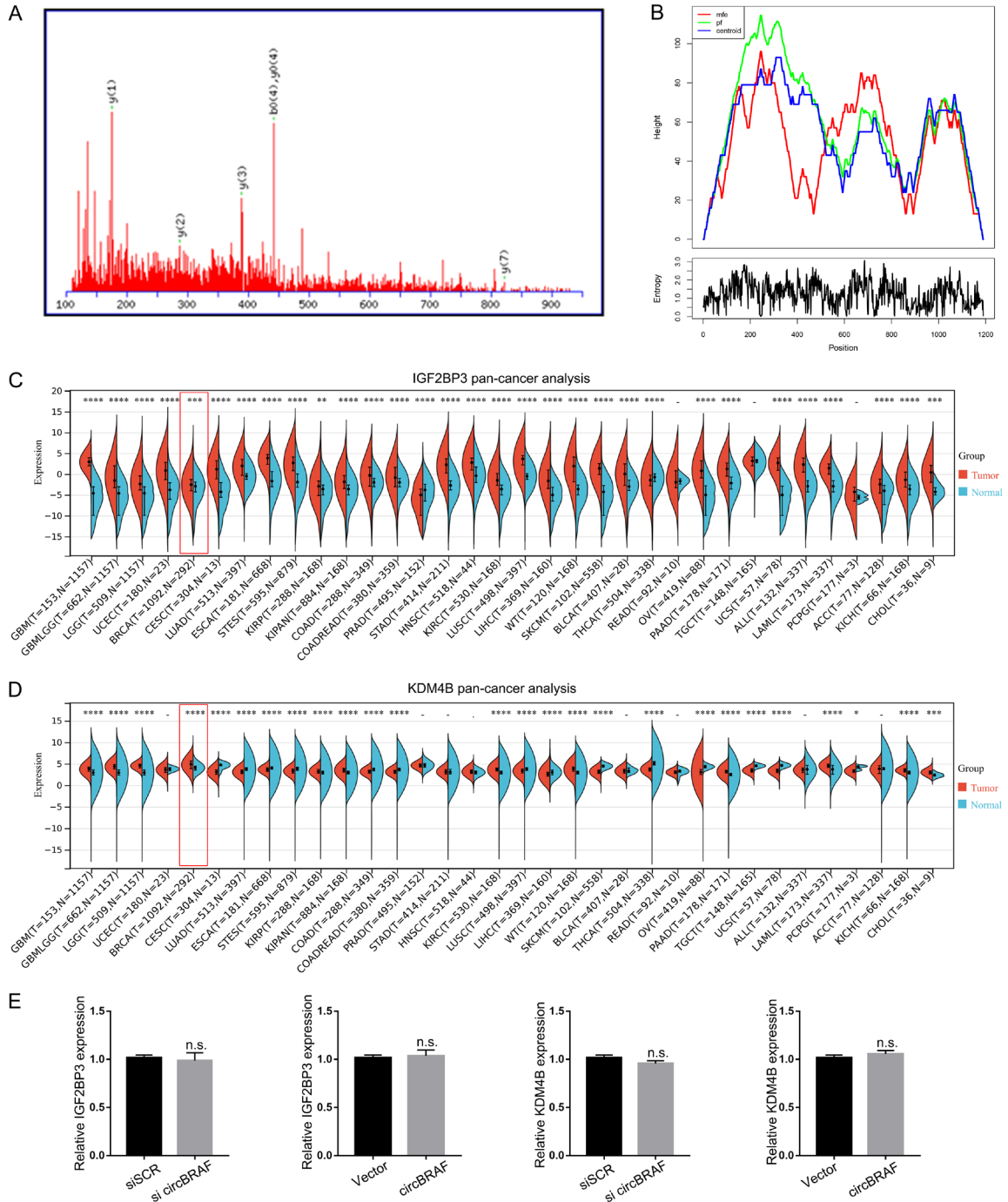


**Figure S5**. A. The potential binding proteins of CircBRAF was detected by LC-MS/MS. B. The secondary structure of CircBRAF was predicted by bioinformatic analysis. C, D. The pan-cancer analysis of KDM4B and IGF2BP3 based on TCGA database was shown. E. The effect of CircBRAF on the expression of IGF2BP3 and KDM4B was detected by qRT-PCR.

A



B
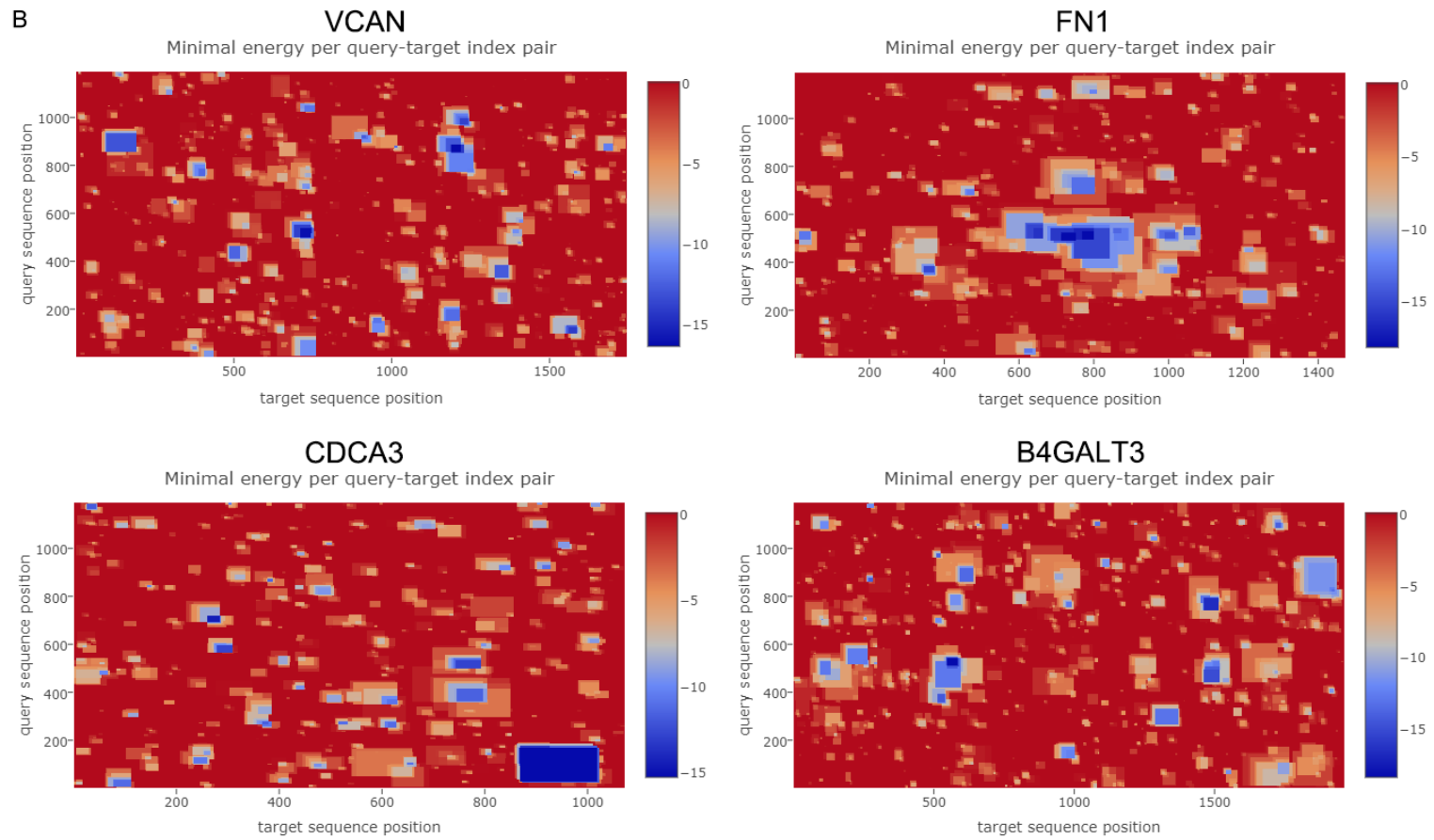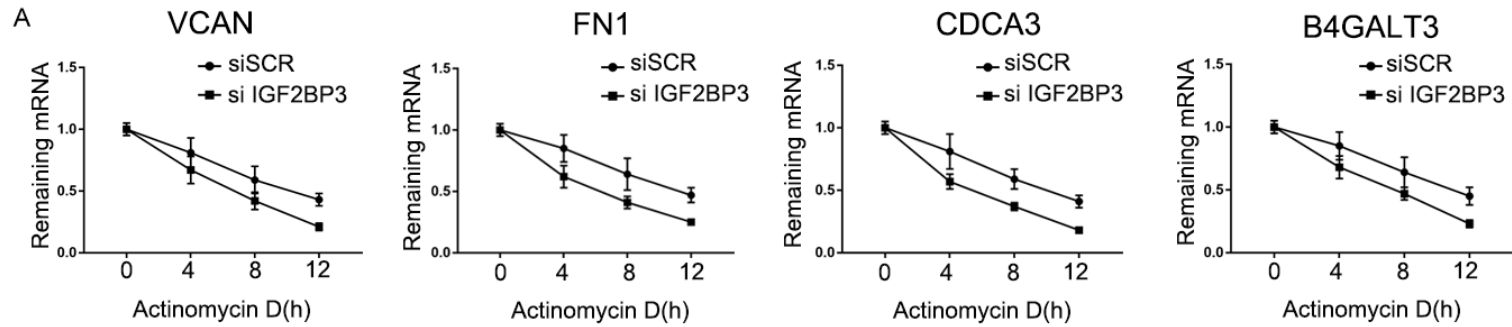
**Figure S6.** A. The expression of VCAN, FN1, CDCA3 and B4GALT3 was measured by qRT-PCR after actinomycin D treatment. B. The binding sites of circBRAF and potential targets was predicted by bioinformatic analysis.
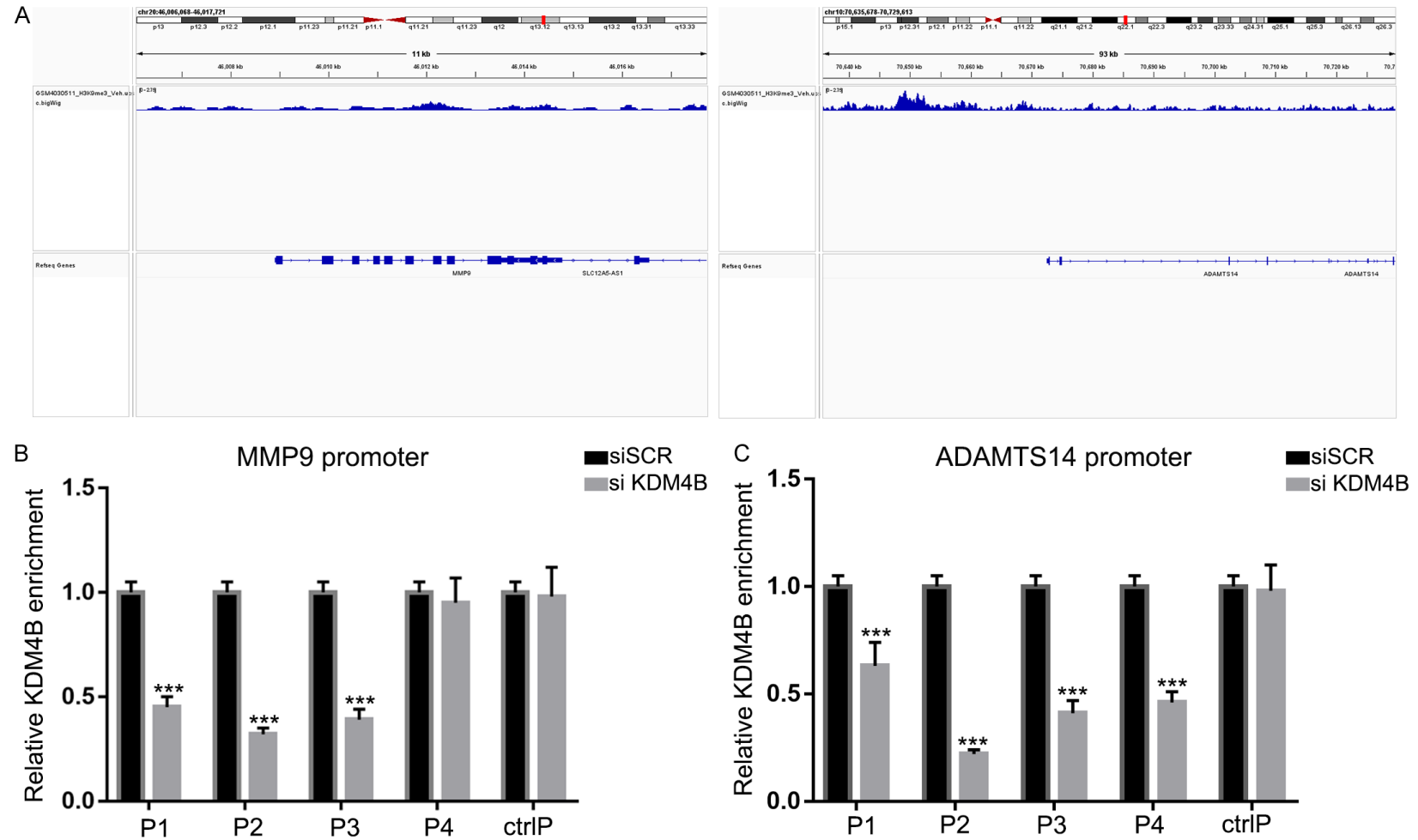


**Figure S7.** A. The H3K9me3 levels of MMP9 and ADAMTS14 was analyzed by GEO database (GSM946852). B, C. The enrichment of KDM4B on the promoters of MMP9 and ADAMTS14 was detected by ChIP assay after KDM4B knockdown.