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RBM8A, a new target of TEAD4, promotes breast cancer progression by regulating IGF1R and IRS-2

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Abstract

Background Breast cancer (BC) is the most common malignant tumor in women worldwide, and further elucidation of the molecular mechanisms involved in BC pathogenesis is essential to improve the prognosis of BC patients. RNA Binding Motif Protein 8 A (RBM8A), with high affinity to a myriad of RNA transcripts, has been shown to play a crucial role in genesis and progression of multiple cancers. We attempted to explore its functional significance and molecular mechanisms in BC.

Methods Bioinformatics analysis was performed on publicly available BC datasets. qRT-PCR was used to determine the expression of RBM8A in BC tissues. MTT assay, clone formation assay and flow cytometry were employed to examine BC cell proliferation and apoptosis in vitro. RNA immunoprecipitation (RIP) and RIP-seq were used to investigate the binding of RBM8A/EIF4A3 to the mRNA of IGF1R/IRS-2. RBM8A and EIF4A3 interactions were determined by co-immunoprecipitation (Co-IP) and immunofluorescence. Chromatin immunoprecipitation (Ch-IP) and dual-luciferase reporter assay were carried out to investigate the transcriptional regulation of RBM8A by TEAD4. Xenograft model was used to explore the effects of RBM8A and TEAD4 on BC cell growth in vivo.

Results In this study, we showed that RBM8A is abnormally highly expressed in BC and knockdown of RBM8A inhibits BC cell proliferation and induces apoptosis in vitro. EIF4A3, which phenocopy RBM8A in BC, forms a complex with RBM8A in BC. Moreover, EIF4A3 and RBM8A complex regulate the expression of IGF1R and IRS-2 to activate the PI3K/AKT signaling pathway, thereby promoting BC progression. In addition, we identified TEAD4 as a transcriptional activator of RBM8A by Ch-IP, dual luciferase reporter gene and a series of functional rescue assays. Furthermore, we demonstrated the in vivo pro-carcinogenic effects of TEAD4 and RBM8A by xenograft tumor experiments in nude mice.

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Conclusion Collectively, these findings suggest that TEAD4 novel transcriptional target RBM8A interacts with EIF4A3 to increase IGF1R and IRS-2 expression and activate PI3K/AKT signaling pathway, thereby further promoting the malignant phenotype of BC cells.

Keywords RBM8A, PI3K/AKT, Breast cancer, EIF4A3, TEAD4

Background

Breast cancer (BC) is the most commonly diagnosed malignancy in women worldwide and the second leading cause of cancer-related death in women [1]. More than 268,000 new diagnoses and more than 41,000 deaths from breast cancer are expected in the USA each year [1]. Lifestyle issues such as diet (saturated fat), alcohol consumption (excessive intake), and abnormal weight (obesity) have also been reported as risk factors for BC [2]. Many serum tumor markers for BC have been reported, but most BC patients have poor prognosis due to the high frequency of distant metastasis or tumor recurrence [3]. Therefore, it is necessary to further elucidate the molecular mechanisms involved in BC development.

RNA-binding proteins (RBP) bind to double- or single-stranded RNA to control RNA splicing, degradation, stabilization, modification, and translation [4, 5]. Existing studies have shown that RBP plays an important role in human cancer progression [6–8]. Notably, other RNPs, including the exon junction complex (EJC), have also been found to play prominent roles in cancer development [9–11].

EJC consists of three core proteins, RNA-binding motif 8 A (RBM8A), Mago homologue (MAGOH), and eukaryotic initiation factor 4A3 (EIF4A3), which regulate gene expression at multiple levels [12]. RBM8A and EIF4A3 have been reported to promote cancer development in a variety of cancers [13–19]. Studies have shown that RBM8A is upregulated in glioblastoma (GBM) tissues, and its higher expression is associated with poorer prognosis. Knockdown of RBM8A inhibited the growth and invasion ability of GBM in vitro and in vivo through the Notch1/STAT3 pathway [13]. It has also been reported that compared with normal liver tissue, RBM8A is highly expressed in hepatocellular carcinoma (HCC) tumor tissue, and it promotes tumor cell migration and invasion in HCC by activating the epithelial-mesenchymal transition signaling pathway [14]. Additionally, EIF4A3 plays an oncogenic role in colorectal cancer development through the induced expression of circ_0084615 [17]. EIF4A3-mediated biogenesis of circSTX6 promotes bladder cancer metastasis and cisplatin resistance [20]. Consistent with RBM8A, EIF4A3 was also highly expressed in HCC, promoting HCC cell proliferation and EMT function [21]. However, no studies have demonstrated the carcinogenesis and molecular mechanisms of RBM8A and EIF4A3 in BC.

In our current study, we first examined the expression of RBM8A in BC tissues. Next, we used small interfering RNA to knock down the expression of RBM8A in BC cell lines to explore the biological function of RBM8A in BC cells. To explore the molecular mechanism by which RBM8A promotes BC progression, we performed RIP-seq and searched for RBM8A-related signaling pathways based on the sequencing results. Since studies have reported that RBM8A and EIF4A3 play important roles in regulating gene expression as important components of the EJC complex, we subsequently investigated whether RBM8A and EIF4A3 have similar biological functions in BC and whether they act as a complex to jointly regulate PI3K/AKT signaling pathway was explored. To further explore the reason for the abnormal upregulation of RBM8A in BC, we identified TEAD4 as a transcription factor to activate the transcription of RBM8A in BC. Finally, we demonstrated the oncogenic roles of RBM8A and TEAD4 in vivo.

Materials and methods

Bioinformatics analysis

Gene expression data were obtained from The Cancer Genome Atlas (TCGA, https://xena.ucsc.edu) and Genotype-Tissue Expression (GTEx) public databases. The expression of RBM8A and EIF4A3 in different types of BC were analyzed using the UALCN website (https://ualcan.path.uab.edu). UCSC (https://genome.ucsc.edu/) and JASPAR (https://jaspar.elixir.no/)databases were used to analyze the transcription factors of RBM8A.

Cell culture and BC tissues

MDA-MB-231, MCF-7 and HEK-293 cells were obtained from the Biomedical Experimental Center of Xi'an Jiaotong University and were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS) at 37 °C and in 5% CO_2. BC tissues and normal tissues were collected from the First Affiliated Hospital of Xi'an Jiaotong University. All patients did not receive local or systemic treatment before surgery and gave informed consent. The study was approved by the Ethics Committee of Xi'an Jiaotong University.

Small interfering RNA (siRNA), plasmid and transfection

siRNAs (RBM8A, EIF4A3 and TEAD4) were purchased from GenePharma (Shanghai, China). The plasmid of RBM8A was purchased from Genechem (Shanghai, China) and the plasmid of TEAD4 was purchased from

miaolingbio (Wuhan, China). TEAD4 truncated mutant was constructed using the Hieff Clone® Plus Multi One Step Cloning Kit according to the manufacturer's protocol (Yeasen, Shanghai, China). All the sequences are listed in Table S1. Transfection of siRNA and plasmids were performed using jetPRIME reagent (Polyplus-Transfection, France) according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol® reagent (Genestar, Beijing, China), followed by reversing transcription using a reverse transcription kit (Yeasen, Shanghai, China). qRT-PCR was performed using SYBR Green Master Mix (Yeasen, Shanghai, China) according to the manufacturer's instructions. Relative expression levels of genes were quantified using the $2^{-\Delta\Delta Ct}$ method and normalized to the internal reference gene GAPDH. All primers used in the present study are listed in Table S1.

MTT assay

3000 cells were seeded into 96-well plates, followed by transfection, and then the plates were placed in a 37 °C, 5% CO2 incubator for continued culture. At 24, 48, and 72 h, take out a plate respectively, add 10 μL MTT (5 mg/ml) and incubate for 4 h, discard all liquid, add 150 μL DMSO, and measure the absorbance at 490 nm with a microplate reader.

Colony forming assay

1000 transfected cells were seeded into 6-well plates and cultured in a 37 °C, 5% CO_2 incubator for 10–14 days. All liquids were discarded, fixed in 4% paraformaldehyde for 30 min at room temperature and then stained with crystal violet. Photographs and counts were taken with Quantity One® software (Bio-Rad).

Cell apoptosis analysis

2×10^5 cells were seeded into 6-well plates, followed by transfection. 48 h after transfection, cells were stained with Annexin V-FITC/PI Apoptosis Detection Kit (Yeasen, Shanghai, China) according to the manufacturer's instructions, and the apoptosis rate was detected by flow cytometry (Becton-Dickinson).

Western blot analysis

RIPA buffer (Invitrogen, USA) supplemented with protease and phosphatase inhibitors was used to extract total protein from BC cells and tissues. BCA protein assay kit (GenStar, Beijing, China) was used to quantify protein concentration. Protein samples were separated by SDS-PAGE and transferred to polyvinylidene fluoride ( ) membranes (Millipore). After blocking with 5% skim milk for 60 min, the PVDF membrane was incubated overnight at 4 °C with an appropriate diluted primary antibody. The membrane was observed with a chemiluminescence detection instrument (Bio-Rad) after incubation with the specific antibody for 1 h on the second day. All antibodies used in the present study are listed in Table S1.

RNA immunoprecipitation (RIP) assay and RIP-seq

For RIP assays, the Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore) was used according to the manufacturer's instructions. In brief, MDA-MB-231 and MCF-7 cells were lysed using RIP lysis buffer supplemented with protease and RNase inhibitors. After the Protein G beads were incubated with the antibody for 4 h, they were incubated with the cell lysate overnight at 4 °C. The next day, the beads were washed extensively with wash buffer, and bound RNA was purified, followed by reverse transcription and qRT-PCR assays.

Co-immunoprecipitation (Co-IP) assay

The interaction of RBM8A and EIF4A3 was detected by Co-IP. MDA-MB-231 and MCF-7 cells were lysed using lysis buffer supplemented with protease inhibitors and PMSF, and cell lysates were incubated with antibodies overnight at 4 °C with rotation. Add pre-washed Dynabeads™ Protein G (Invitrogen, USA) and incubate at 4 °C for 4 h on the second day. After thoroughly washing the beads with PBS, add 100 μL 2× loading buffer and cook in boiling water for 10 min to separate the proteins from the beads.

Cell immunofluorescence assay

Cell immunofluorescence assay was performed with a multiplex immunohistochemistry kit (Alpha X Biotech, Beijing, China) according to the manufacturer's instructions. In brief, cells were fixed with 4% paraformaldehyde for 30 min, wished with PBST for 3 times, followed by blocking using solution of blocking for 15 min at room temperature. Cells were then incubated with primary antibodies at 37 °C for 1 h, and incubated with secondary antibody for 10 min at 37 °C. After that, cells were stained with the corresponding fluorescent dye for 5 min at room temperature in the dark. Incubation and staining of the second antibody were performed after 15 min of elution using antigen eluent following the above procedure. After staining the nucleus with 4'6-diamidino-2-phenylindole (DAPI), immunofluorescence signals were detected by ultra-high resolution two-photon laser scanning microscopy imaging system (Leica, TCS SP8 DIVE, Germany).

Chromatin immunoprecipitation (ChIP) assay

Binding of TEAD4 to the RBM8A promoter was detected by Ch-IP. MDA-MB-231 and MCF-7 cells were first cross-linked with formaldehyde and then quenched with

glycine to unreacted formaldehyde. Chromatin was cut into fragments of approximately 200 bp by sonication in lysis buffer containing protease inhibitors. Cells were collected in lysis buffer containing protease inhibitors and chromatin was sonicated into fragments of approximately 200 bp. Subsequently, cell lysates were incubated with antibodies overnight at 4 °C with rotation. Add pre-washed Dynabeads™ Protein G (Invitrogen, USA) and incubate at 4 °C for 4 h on the second day. After thoroughly washing the beads with ChIP wash buffer, add a solution of 0.1 mol/L sodium bicarbonate containing 1% SDS to separate the proteins from the beads. Samples were reverse cross-linked at 65 °C for 8 h, followed by DNA isolation using phenol/chloroform (Invitrogen, USA) and detection of promoter binding by qRT-PCR. Primer sequences used for ChIP-qRT-PCR are listed in Table S1.

Agarose gel electrophoresis

DNA samples after ChIP-qRT-PCR and RIP-qRT-PCR were separated by agarose gel. After electrophoresis the gel was exposed to UV light and photographed.

Luciferase reporter assay

6000 HEK-293 cells were seeded into 96-well plates and the siNC or siTEAD4 was co-transfected with wild-type RBM8A promoter regions reporter constructs. Firefly luciferase activity was detected using the dual luciferase assay system (Promega) 48 h after transfection. Renilla luciferase activity was used as an internal standard.

Xenograft assay

We generated MDA-MB-231 cells that stably silenced TEAD4/RBM8A and injected them and control cells subcutaneously into the bilateral groin of 5-week-old male BALB/C nude mice (Vitalriver) ($n=5$/group). Tumor size was measured every 4 days using the formula $V = (LxW^2)/2$ after tumor xenografts were observed. 27 days later, the tumors were imaged using a small animal intravital imaging system (Xenogen) and surgically resected in the xenograft model for further analysis. The study was approved by the Ethics Committee of Xi'an Jiaotong University (No. 2022−1149).

Statistical analysis

For all quantitative analyses, data are shown as mean±standard deviation (SD) from at least three separate experiments. Student's t-test was used for comparisons between two independent groups and one-way ANOVA followed by Dunnett's test was performed for multiple comparisons to analyze differences among more than two groups. Pearson's correlation analysis was performed for the correlation between TEAD4 and RBM8A. $P<0.05$ was considered statistically significant.

Results

The expression of RBM8A is upregulated in breast cancer

To evaluate the expression of RBM8A in cancer, we investigated the public datasets of TCGA and GTEx. We found that RBM8A expression is upregulated in most cancer types compared to corresponding normal tissues (Fig. 1A-B). Also, we analyzed the expression of RBM8A in BC tissues based on the TCGA and GTEx database, and the results showed that RBM8A was upregulated in BC tissues compared with normal tissues (Fig. 1C). Analysis based on the UALCN website showed that RBM8A was expressed upregulated in different types of BC (Fig. S1A). Further, qRT-PCR results were consistent with the TCGA database results (Fig. 1D). Kaplan-Meier curves showing that high expression of RBM8A is associated with poor free interval (PFI) prognosis in BC patients (Fig. 1E). The above results indicated that RBM8A was abnormally upregulated in BC and is associated with poor prognosis in BC patients.

Expression levels of RBM8A affect breast cancer cell proliferation and apoptosis

To explore the functional impact of RBM8A on BC, we manipulated its levels by small interfering RNAs targeting RBM8A in BC cell lines MDA-MB-231 and MCF-7. As shown in Fig. 2A, the silencing efficiency of the two small interfering RNAs is very high and can be used for subsequent exploration of biological functions. We observed that downregulation of RBM8A levels inhibited the proliferation and clone forming ability of BC cells (Fig. 2B-D). To investigate whether knockdown of RBM8A inhibited BC cell proliferation by regulating apoptosis, we performed apoptosis analysis using flow cytometry. The proportion of apoptotic cells was increased in MDA-MB-231 and MCF-7 cells transfected with siRBM8A compared to siNC, suggesting that knockdown of RBM8A significantly induced apoptosis in BC cells (Fig. 2E-F). Subsequently, western blotting results showed that apoptosis-associated proteins were also altered accordingly after knockdown of RBM8A in BC cells (Fig. 2G). In addition, the level of AKT phosphorylation at the S473 site was reduced after silencing RNM8A, while total AKT level did not change significantly (Fig. 2G).

RBM8A/EIF4A3 complex promotes IGF1R and IRS-2 expression

We next explored the molecular mechanism by which RBM8A promotes BC. Since RBM8A is an RNA-binding protein, we searched for downstream target genes of RBM8A by RIP-seq. Through the coding pathway enrichment analysis, 17 pathways were screened for significant differences (Fig. 3A), including the AMPK pathway, cell cycle, PI3K-Akt pathway, Hippo signaling pathway,

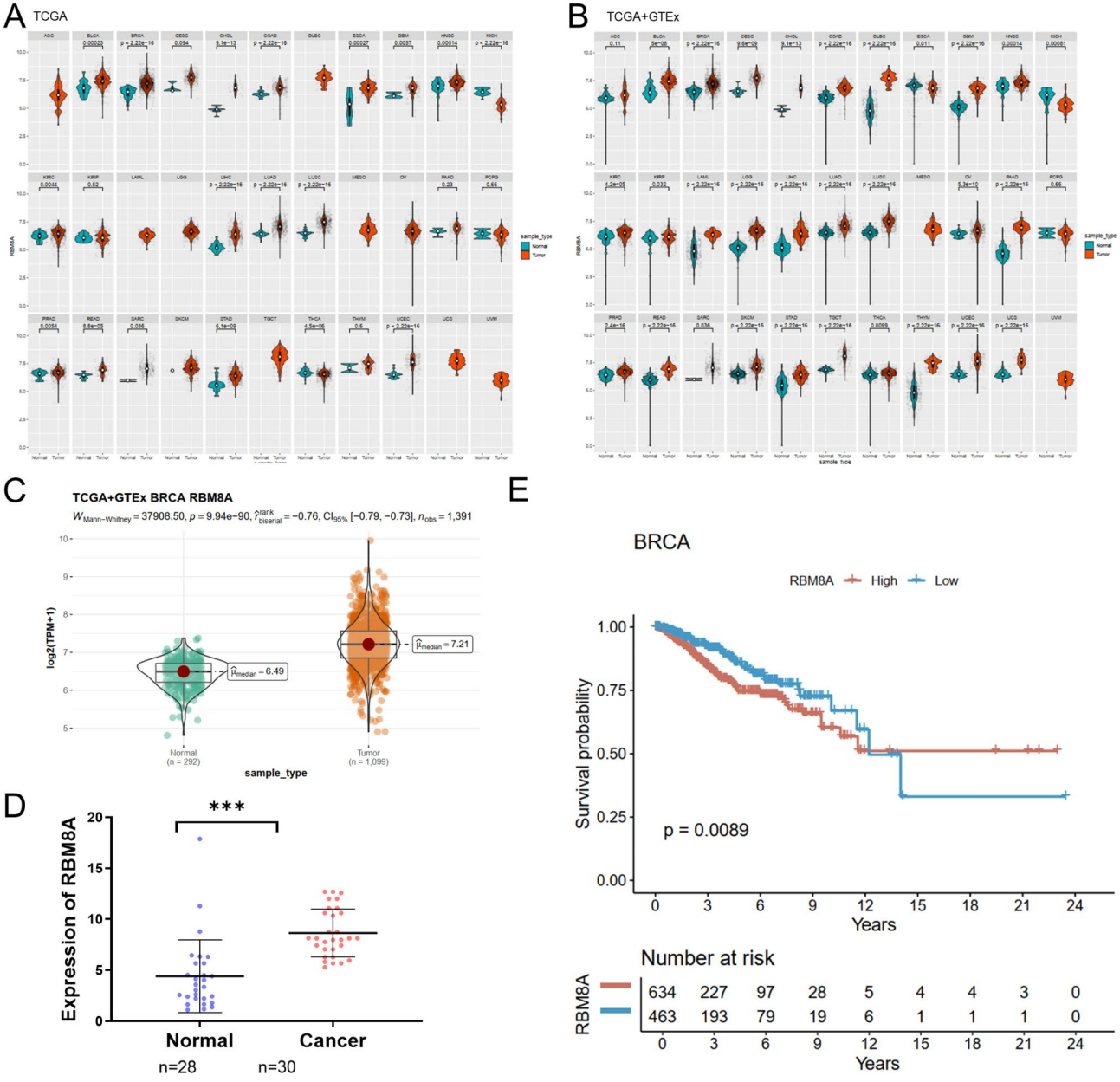

Fig. 1 The expression of RBM8A is upregulated in breast cancer. (**A**) Comparison of RBM8A expression between tumor and normal tissues across different cancer types from TCGA database. (**B**) Comparison of RBM8A expression between tumor and normal tissues across different cancer types from TCGA and GTEx databases. (**C**) The expression of RBM8A in breast cancer tissues based on the TCGA and GTEx database. (**D**) qRT-PCR was used to detect RBM8A expression in breast cancer tissues and normal tissues. (**E**) Association between the RBM8A expression and the PFI of BC patients. ACC: Adrenocortical carcinoma; BLCA: Bladder Urothelial Carcinoma; BRCA: Breast invasive carcinoma; CESC: Cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL: Cholangiocarcinoma; COAD: Colon adenocarcinoma; DLBC: Lymphoid Neoplasm Diffuse Large B-cell Lymphoma; ESCA: Esophageal carcinoma; GBM: Glioblastoma multiforme; HNSC: Head and Neck squamous cell carcinoma; KICH: Kidney Chromophobe; KIRC: Kidney renal clear cell carcinoma; KIRP: Kidney renal papillary cell carcinoma; LAML: Acute Myeloid Leukemia; LGG: Brain Lower Grade Glioma; LIHC: Liver hepatocellular carcinoma; LUAD: Lung adenocarcinoma; LUSC: Lung squamous cell carcinoma; MESO: Mesothelioma; OV: Ovarian serous cystadenocarcinoma; PAAD: Pancreatic adenocarcinoma; PCPG: Pheochromocytoma and Paraganglioma; PRAD: Prostate adenocarcinoma; READ: Rectum adenocarcinoma; SARC: Sarcoma; SKCM: Skin Cutaneous Melanoma; STAD: Stomach adenocarcinoma; TGCT: Testicular Germ Cell Tumors; THCA: Thyroid carcinoma; THYM: Thymoma; UCEC: Uterine Corpus Endometrial Carcinoma; UCS: Uterine Carcinosarcoma; UVM: Uveal Melanoma.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

etc., involving important oncogenes such as EGFR, MDM2, MET, TRK, BRAF, RAS, SHH, SMO, CDK4, FGFR3, MYC, IGF1R, IRS2, as well as oncogenes such as SMAD2/3, RB1, SMAD4, P53, etc., and the number of oncogenes' mRNAs bound by RBM8A is much higher than that of the oncogenes. Literature shows that IGF1R and IRS2 are highly expressed in breast cancer tissues, suggesting that the IGF1R-mediated PI3K/Akt pathway

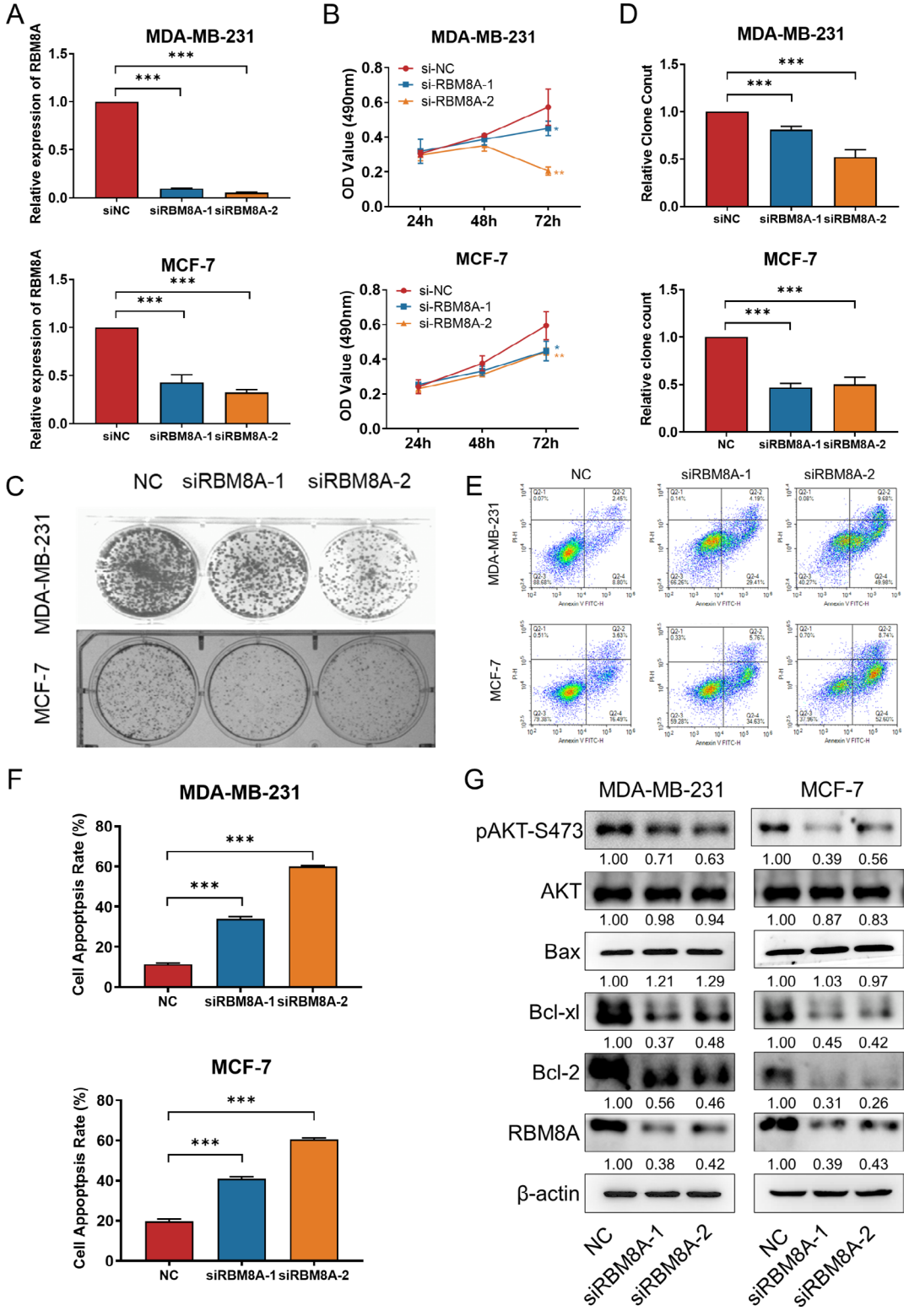

Fig. 2 Knockdown of RBM8A inhibits BC cell proliferation. (**A**) The mRNA expression of RBM8A was detected by qRT-PCR to confirm the knockdown efficiency of two siRNAs in MDA-MB-231 and MCF-7 cells. (**B**) MTT assay was used to detect the effect of RBM8A knockdown on the proliferation of BC cells. (**C**) Colony formation assay was used to detect the effect of RBM8A knockdown on the colony formation ability of BC cells. (**D**) Quantification of colony formation assay in BC cells transfected with RBM8A siRNAs. (**E**) Flow cytometry assay for apoptosis was used to detect the effect of knockdown of RBM8A on BC cell apoptosis. (**F**) Quantification of cell apoptosis assay in BC cells transfected with RBM8A siRNAs. (**G**) Western blotting was used to detect the effect of RBM8A knockdown on the expression of cell apoptosis related molecules at protein level in BC cells. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

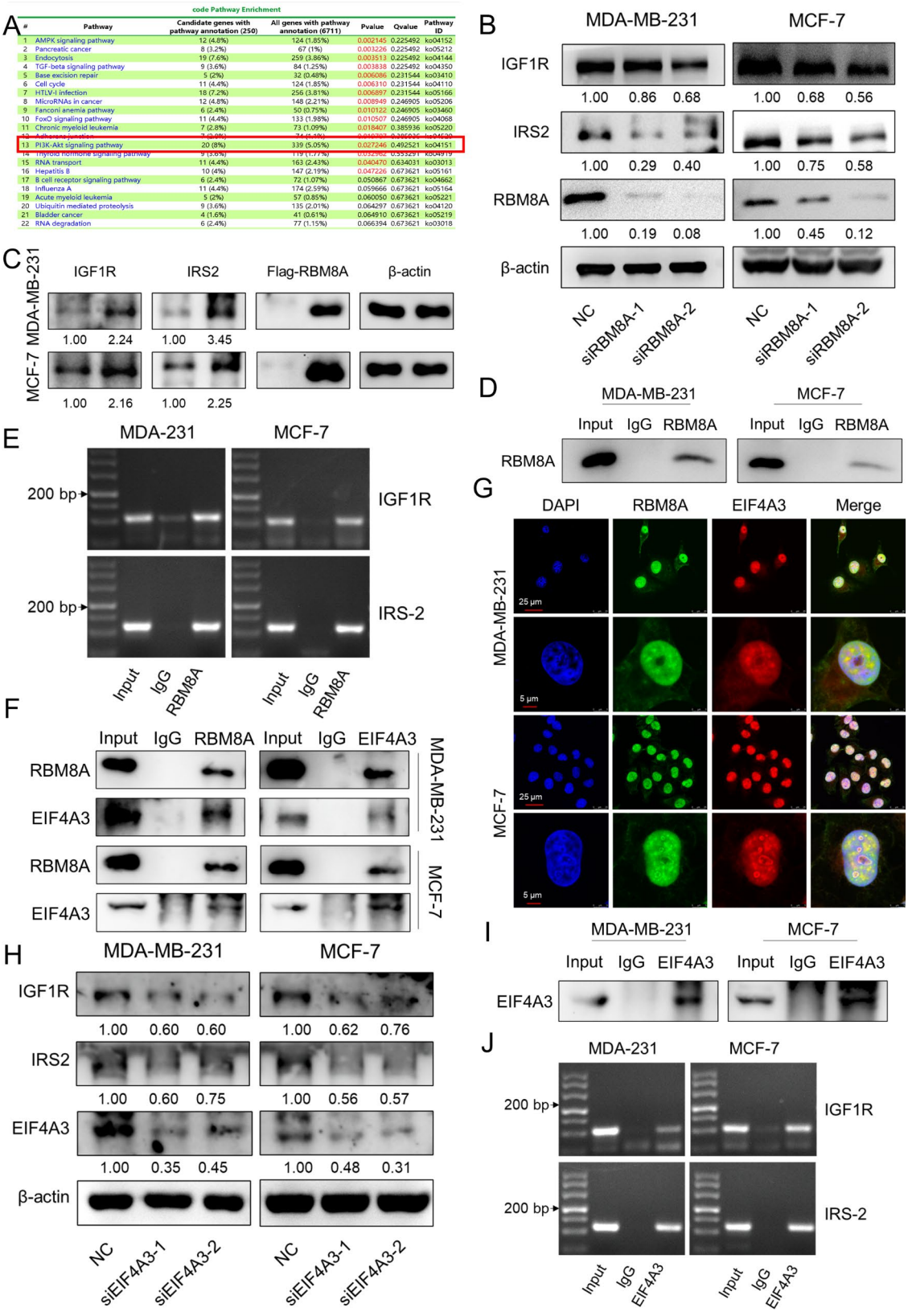

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Fig. 3 RBM8A/EIF4A3 complex promotes translation of IGF1R and IRS-2 (**A**) Pathway analysis of genes enriched by RIP-seq. (**B**) Western blotting was used to detect protein levels of IGF1R and IRS-2 in BC cells after knockdown of RBM8A. (**C**) Western blotting was used to detect protein levels of IGF1R and IRS-2 in BC cells after overexpression of RBM8A. (**D**) Western blotting was used to detect whether the target antibody pulls down the corresponding protein in RIP experiments. (**E**) After RIP experiments using RBM8A antibody, PCR and agarose gel electrophoresis were performed with primers for IGF1R and IRS-2. (**F**) MDA-MB-231 and MCF-7 cell lysates were subjected to Co-IP to detect RBM8A and EIF4A3 interaction. (**G**) Immunofluorescence assay was used to detect the co-localization of RBM8A and EIF4A3 in BC cells. (**H**) Western blotting was used to detect protein levels of IGF1R and IRS-2 in BC cells after knockdown of EIF4A3. (**I**) Western blotting was used to detect whether the target antibody pulls down the corresponding protein in RIP experiments. (**J**) After RIP experiments using EIF4A3 antibody, PCR and agarose gel electrophoresis were performed with primers for IGF1R and IRS-2

may be an important downstream target of RBM8A. Subsequently, decreased protein levels but not mRNA levels of IGF1R and IRS-2 were observed in BC cells with knockdown of RBM8A (Fig. 3B), while overexpression of RBM8A induced more IGF1R and IRS-2 proteins (Fig. 3C). In addition, we validated the two target genes IGF1R and IRS-2 screened by RIP-seq using RIP experiments in MDA-MB-231 and MCF-7 cells. As shown in Fig. 3C-D, the RNAs of IGF1R and IRS-2 could be pulled down with antibodies against RBM8A in BC cells, indicating that RBM8A could bind to the RNAs of IGF1R and IRS-2.

We further wanted to investigate whether RBM8A mediating the expression of IGF1R and IRS-2 is related to another member of the EJC complex, EIF4A3. Co-IP assay analysis revealed a physical interaction between RBM8A and EIF4A3 in BC cells (Fig. 3E). Subsequent immunofluorescence assay demonstrated significant co-localization of RBM8A and EIF4A3 in BC cells (Fig. 3F). Consistent with the RBM8A results, a decrease in IGF1R and IRS-2 protein was observed in BC cells knocked down for EIF4A3 and RNA for IGF1R and IRS-2 was also pulled down in MDA-MB-231 and MCF-7 cells with an antibody to EIF4A3 (Fig. 3G-I). The above results suggest that RBM8A/EIF4A3 complex promotes IGF1R and IRS-2 expression in BC cells.

Expression levels of EIF4A3 affect breast cancer cell proliferation and apoptosis

Based on the TCGA and GTEx dataset, we found that EIF4A3 expression was upregulated in BC tissues compared to normal tissues (Fig. 4A) and high expression of EIF4A3 is associated with poor overall survival (OS) prognosis in BC patients (Fig. 4B). Analysis based on the UALCN website showed that RBM8A was expressed upregulated in different types of BC (Fig. S1B). To investigate the functional impact of EIF4A3 on BC, we knocked down EIF4A3 levels in BC cell lines by small interfering RNAs targeting EIF4A3. As shown in Fig. 4C, the silencing efficiency of the two small interfering RNAs is very high and can be used for subsequent exploration of biological functions. MTT assay and flow cytometry detection of apoptosis showed that, consistent with knockdown of RBM8A, knockdown of EIF4A3 inhibited BC cell proliferation and induced BC cell apoptosis (Fig. 4D-E). Subsequently, Western blotting results showed that apoptosis-associated proteins were also altered accordingly after knockdown of EIF4A3 in BC cells (Fig. 4F). In addition, the level of AKT phosphorylation at the S473 site was reduced after silencing EIF4A3, while total AKT level did not change significantly (Fig. 4F). The results suggest that RBM8A/EIF4A3 complex activates the AKT signaling pathway by increasing AKT phosphorylation, which in turn induces BC cell proliferation.

TEAD4 promotes RBM8A transcription

Abnormal expression of oncogenes in tumors involves the following possible mechanisms: regulation by transcription factors, regulation by non-coding RNA targeting, and aberrant epigenetic modifications. Further, we searched for the upstream transcription factors of RBM8A through the UCSC website to explore the molecular mechanism of high expression of RBM8A in breast cancer, and found that TEAD4 may bind in the promoter region of RBM8A (Fig. 5A). Meanwhile, TCGA database analysis showed that TEAD4 was also highly expressed in BC and its expression was significantly positively correlated with RBM8A (Fig. 5B-C). Subsequently, we designed and synthesized small interfering RNAs targeting TEAD4, and observed the down-regulation of RBM8A in BC cells knocking down TEAD4 (Fig. 5D-E). Based on the UCSC database, we searched the binding sequences of TEAD4 and RBM8A promoter region, and compared the binding sequences with the binding motifs of TEAD4 on the JASPAR website to design ChIP-PCR primers, and verified whether TEAD4 binds to the RBM8A promoter region (Fig. 5F). The results of ChIP-PCR combined with agarose gel electrophoresis showed that TEAD4 could bind to the RBM8A promoter region (Fig. 5G-H). In addition, the binding sequences analyzed based on the JASPAR website were cloned into the pGL3 promoter luciferase vector to perform a dual luciferase reporter gene assay to investigate whether TEAD4 was involved in the transcriptional activation of RBM8A, and the results showed that knockdown of TEAD4 could reduce the luciferase activity of the vector in HEK-293 cells (Fig. 5I-K). We further constructed TEAD4-truncated mutants without TEA domain to explore whether TEAD4-mediated transcriptional activation of RBM8A is dependent on its TEA domain (Fig. 5L). The dual luciferase reporter assay showed that TEAD4 $^{TEA-}$ decreased

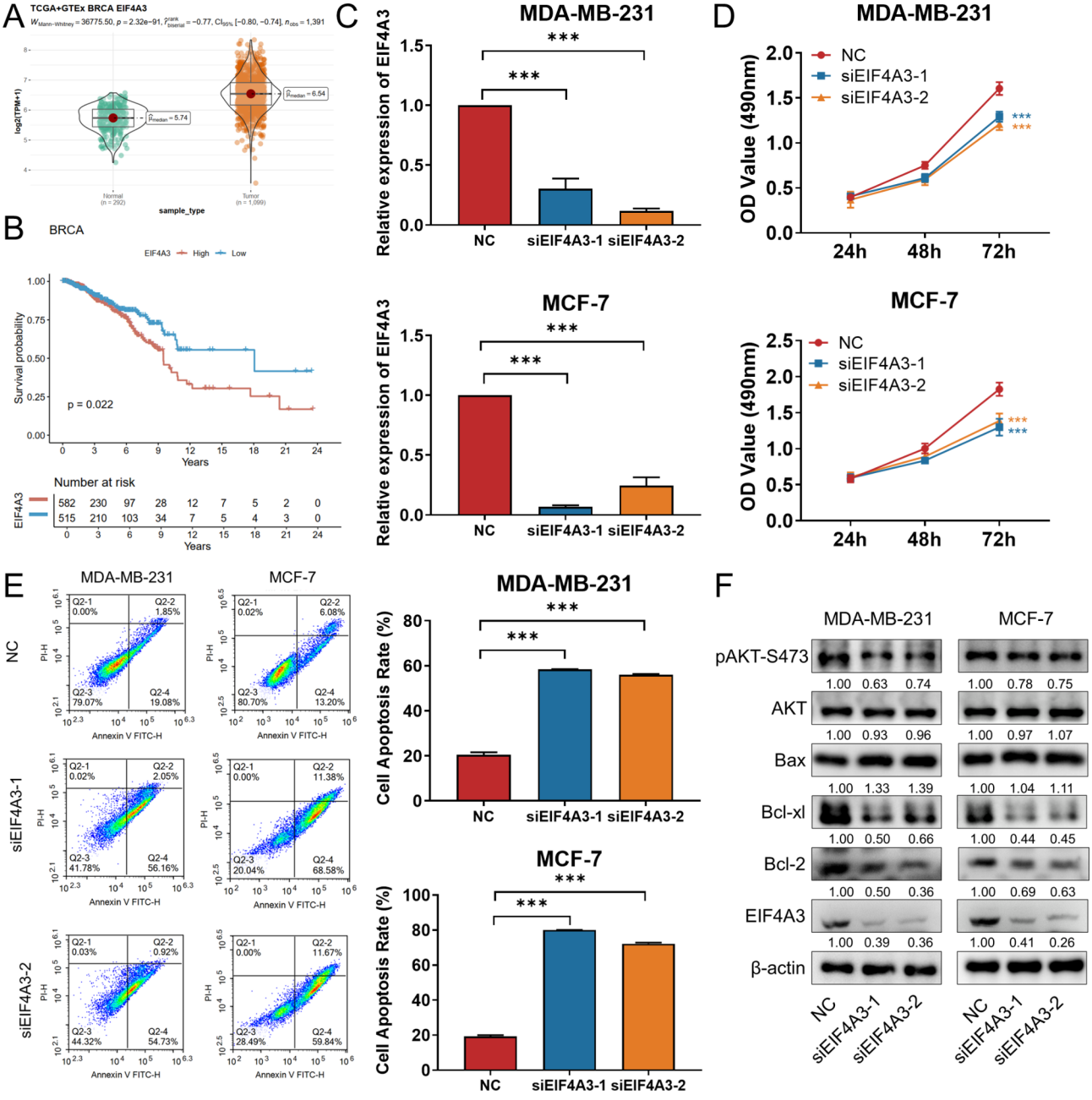

Fig. 4 EIF4A3 is upregulated in BC and Knockdown of EIF4A3 inhibits BC cell proliferation. (**A**) The expression of RBM8A in breast cancer tissues based on the TCGA and GTEx database. (**B**) Association between the RBM8A expression and the OS of BC patients. (**C**) The mRNA expression of EIF4A3 was detected by qRT-PCR to confirm the knockdown efficiency of two siRNAs in MDA-MB-231 and MCF-7 cells. (**D**) MTT assay was used to detect the effect of EIF4A3 knockdown on the proliferation of BC cells. (**E**) Flow cytometry assay for apoptosis was used to detect the effect of knockdown of EIF4A3 on BC cell apoptosis. (**F**) Western blotting was used to detect the effect of EIF4A3 knockdown on the expression of cell apoptosis related molecules at protein level in BC cells. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

the luciferase activity of the reporter vector in HEK-293 cells compared with TEAD4 ^{FL}, indicating that TEAD4 without TEA domain could not promote RBM8A transcription (Fig. 5M). Taken together, our results suggest that TEAD4 binds to the RBM8A promoter region and activates RBM8A transcription.

Knockdown of TEAD4 inhibited BC cell proliferation, and overexpression of RBM8A rescued the inhibition produced by deletion of TEAD4

To investigate the biological function of TEAD4, we performed MTT assay and flow cytometry for apoptosis detection assay, and the results showed that knockdown of TEAD4 significantly inhibited BC cell proliferation

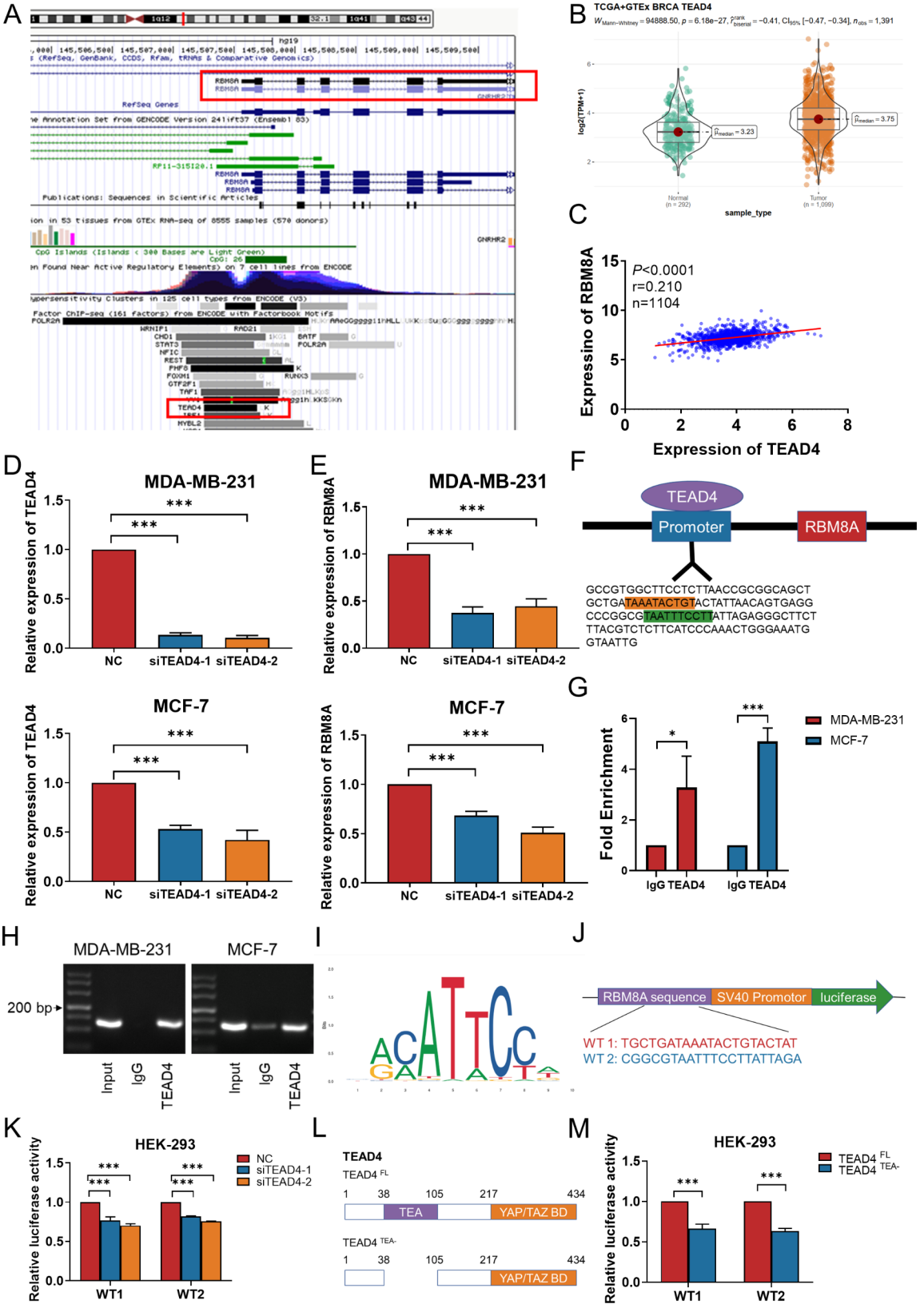

Fig. 5 (See legend on next page.)

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Fig. 5 TEAD4 binds to the promoter region of RBM8A to activate its transcription. (**A**) UCSC database analysis showed that TEAD4 could bind to the promoter region of RBM8A to regulate its transcription. (**B**) The expression of TEAD4 in breast cancer tissues based on the TCGA and GTEx database. (**C**) Correlation analysis of TEAD4 and RBM8A expression in BC based on TCGA database. (**D**) The mRNA expression of TEAD4 was detected by qRT-PCR to confirm the knockdown efficiency of two siRNAs in MDA-MB-231 and MCF-7 cells. (**E**) qRT-PCR was used to analyze the mRNA expression level of RBM8A in TEAD4 knockdown BC cells. (**F**) The UCSC database in conjunction with the JASPAR website predicts the binding sequence of TEAD4 to the promoter region of RBM8A. (**G-H**) The binding relationship between TEAD4 and the promoter region of RBM8A was verified by ChIP-qRT-PCR (**G**) and agarose gel electrophoresis (**H**). (**I**) DNA-binding motif of TEAD4 (JASPAR). (**J**) The binding sequence of TEAD4 on RBM8A predicted by JASPAR was subcloned into the pGL3 promoter luciferase vector. (**K**) Luciferase assays were performed in HEK-293 cells co-transfected with siNC/siTEAD4 and RBM8A promoter. Renilla luciferase served as the internal control. (**L**) Schematic representation of full-length (FL) TEAD4 and its deletion mutant TEA-. TEA- denotes TEA domain deletion. (**M**) Luciferase assays were performed in HEK-293 cells co-transfected with TEAD4 ^{FL}/TEAD4 $^{TEA-}$ and RBM8A promoter. Renilla luciferase served as the internal control. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

and promoted apoptosis (Fig. 6A-B). Meanwhile, Western blotting results showed that apoptosis-associated proteins were also altered accordingly after knockdown of TEAD4 in BC cells (Fig. 6C). Subsequently, TEAD4 siRNA and RBM8A overexpression plasmid were co-transfected into MDA-MB-231 and MCF7 cells to test whether the enhanced expression of RBM8A could rescue the inhibitory effect of TEAD4 knockdown on BC cell proliferation. MTT assay showed that TEAD4 knockdown inhibited BC cell proliferation, while RBM8A overexpression partially reversed the inhibitory effect of TEAD4 knockdown on BC cell proliferation (Fig. 6D). Additionally, knockdown of TEAD4 induced cell apoptosis, while overexpression of RBM8A rescued the effect of TEAD4 knockdown on BC cells (Fig. 6E). This suggests that TEAD4 promotes BC progression by activating RBM8A transcription.

TEAD4 and RBM8A promote BC tumor growth in vivo

To determine the cancer-promoting effects of TEAD4 and RBM8A in vivo, we successfully constructed the MDA-MB-231 cell line that stably knocked down TEAD4 or RBM8A, and performed xenografting assays in nude mice. As shown in Fig. 7A, the results of qRT-PCR and western blotting showed that the mRNA and protein of TEAD4 were significantly down-regulated in LV-shTEAD4 cells compared with LV-Ctrl cells, and similarly the mRNA and protein of RBM8A were significantly down-regulated in LV-shRBM8A cells compared with LV-Ctrl cells. LV-shTEAD4/shRBM8A and LV-Ctrl cells were injected subcutaneously in the groin of BALB/C nude mice bilaterally to observe the tumor growth. On the 27th day after injection, the tumors formed on the side of the LV-shTEAD4 group in the same nude mice were significantly smaller than those in the LV-Ctrl group, and the tumors formed on the side of the LV-shRBM8A group in the same nude mice were also significantly smaller than those in the LV-Ctrl group (Fig. 7B). Tumor growth curve was plotted by measuring the tumor volume during tumor formation, and the results showed that the tumor growth rate in the LV-shTEAD4/LV-shRBM8A group was slower than that in the LV-Ctrl group (Fig. 7C). Knockdown of TEAD4/RBM8A significantly reduced tumor weight compared with LV-Ctrl (Fig. 7D). Expression of TEAD4/RBM8A at the RNA and protein levels from LV-shTEAD4, LV-shRBM8A and LV-Ctrl solid tumors were detected by qRT-PCR and western blotting. The results demonstrated that TEAD4 expression was significantly lower in LV-shTEAD4 infected tumors than LV-Ctrl, and RBM8A expression was significantly lower in LV-shRBM8A infected tumors than LV-Ctrl (Fig. 7E-F). Overall, these results suggested that TEAD4 and RBM8A directly promote BC tumor growth in vivo.

In summary, these results suggested that TEAD4 novel transcriptional target RBM8A interacts with EIF4A3 to increase IGF1R and IRS-2 and activate PI3K/AKT signaling pathway, thereby further promoting the malignant phenotype of BC cells (Fig. 8).

Discussion

BC is the second leading cause of cancer-related death in women [1]. Existing cancer therapy drugs have various limitations, such as side effects, drug resistance, and lack of selectivity [22]. Therefore, it is necessary to further study BC targeted therapy molecules, which will help improve the survival rate and quality of life of BC patients.

RBPs usually participate in the occurrence and development of tumors by binding to the RNA of target genes and regulating the expression of target genes [23, 24]. Elucidating the interaction network between RBPs and cancer-associated RNA targets has provided new avenues for the pathogenesis of BC and has attracted considerable attention. Many RBPs have a major impact on BC tumorigenesis by regulating many mRNAs at the translational or post-translational level. IGF2BP3 functions as a novel oncogenic RBP in BC by stabilizing oncogenic TRIM25 mRNA [7]. POP7 regulates the stability and expression of ILF3 mRNA and promotes BC progression [25]. IGF2BP3 binds to EGFR mRNA and protects EGFR mRNA from degradation to promote BC MDA-MB-231 cell growth [26]. RBM8A, an RBP, has been reported promote cancer development in a variety of cancers [13–15]. Herein, we report that upregulation of RBM8A expression in BC tissues and silencing of RBM8A in BC cell lines can

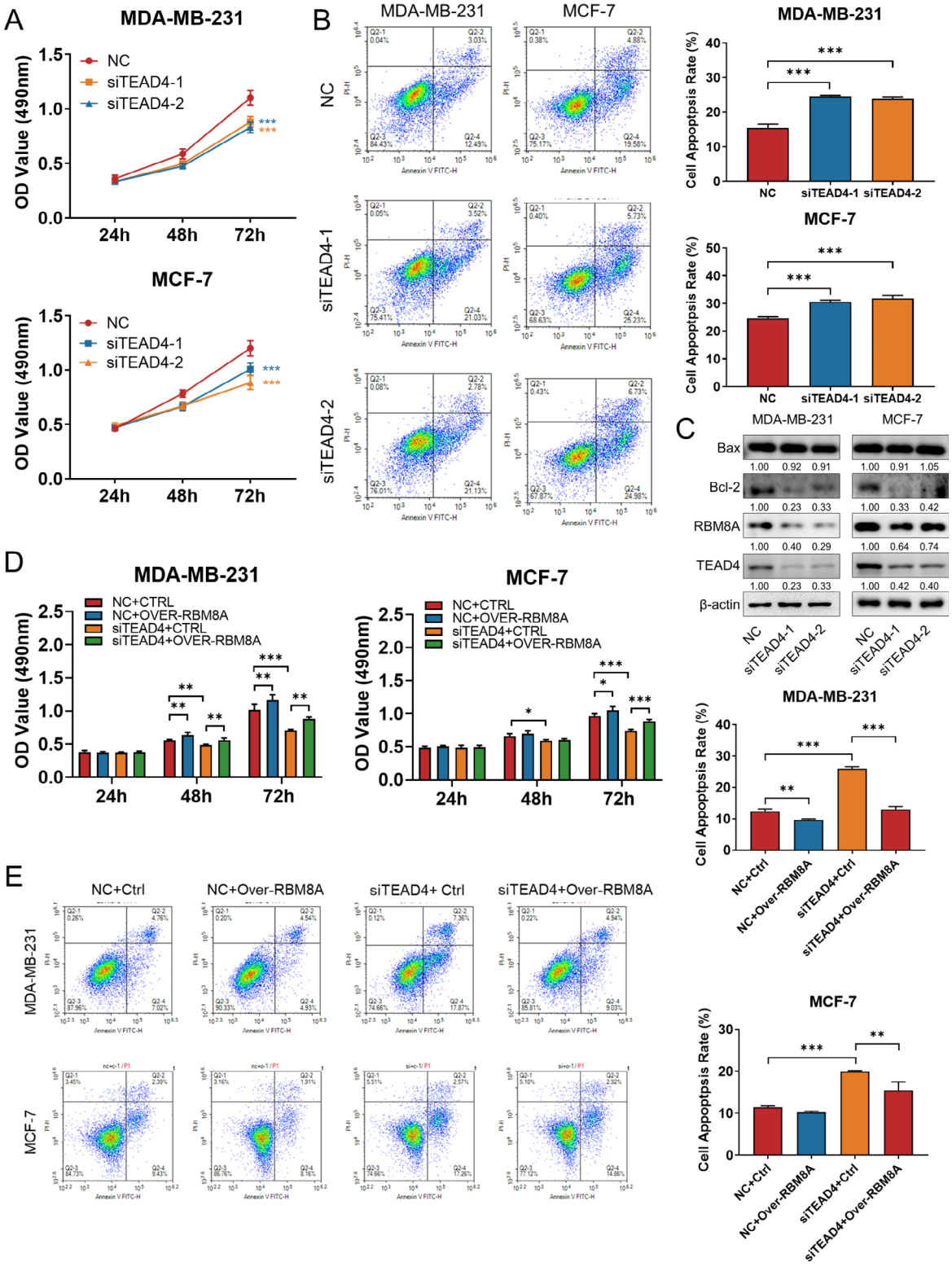

Fig. 6 Knockdown of TEAD4 inhibited BC cell proliferation, which was rescued by overexpression of RBM8A. (**A**) MTT assay was used to detect the effect of TEAD4 knockdown on the proliferation of BC cells. (**B**) Flow cytometry assay for apoptosis was used to detect the effect of knockdown of TEAD4 on BC cell apoptosis. (**C**) Western blotting was used to detect the effect of TEAD4 knockdown on the expression of cell apoptosis related molecules at protein level in BC cells. (**D**) MTT assay was performed to determine the impact of cell viability treated with NC + Ctrl, NC + Over-RBM8A, siTEAD4 + Ctrl, siTEAD4 + Over-RBM8A in BC cells. (**E**) Flow cytometry assay for apoptosis was performed to determine the impact of cell apoptosis treated with NC + Ctrl, NC + Over-RBM8A, siTEAD4 + Ctrl, siTEAD4 + Over-RBM8A in BC cells. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

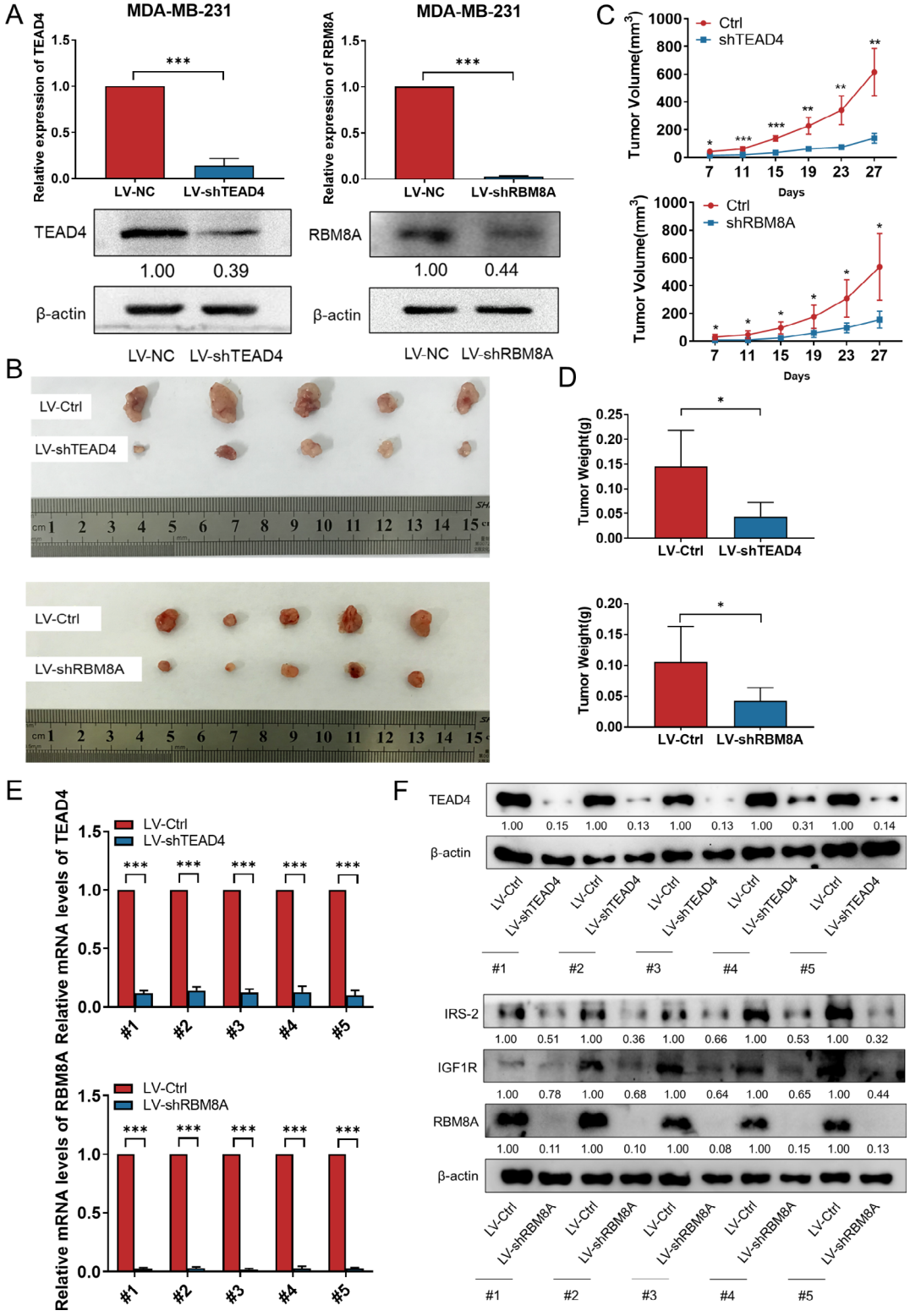

Fig. 7 TEAD4 and RBM8A promote BC tumor growth in vivo. (**A**) The mRNA and protein expression of TEAD4/RBM8A in LV-shTEAD4, LV-shRBM8A4 and LV-Ctrl cells were detected by qRT-PCR and western blotting. (**B**) Size of xenograft tumors 27 days after injection of LV-shTEAD4, LV-shRBM8A and LV-Ctrl cells. (**C**) Growth curves of tumor volume were generated every 4 d for 27 d. (**D**) Weighing and statistical mapping of xenografted tumors. (**E**) The mRNA level of TEAD4/RBM8A in xenograft tumors were analyzed by qRT-PCR. (**F**) The protein level of TEAD4/RBM8A in xenograft tumors were analyzed by western blotting. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

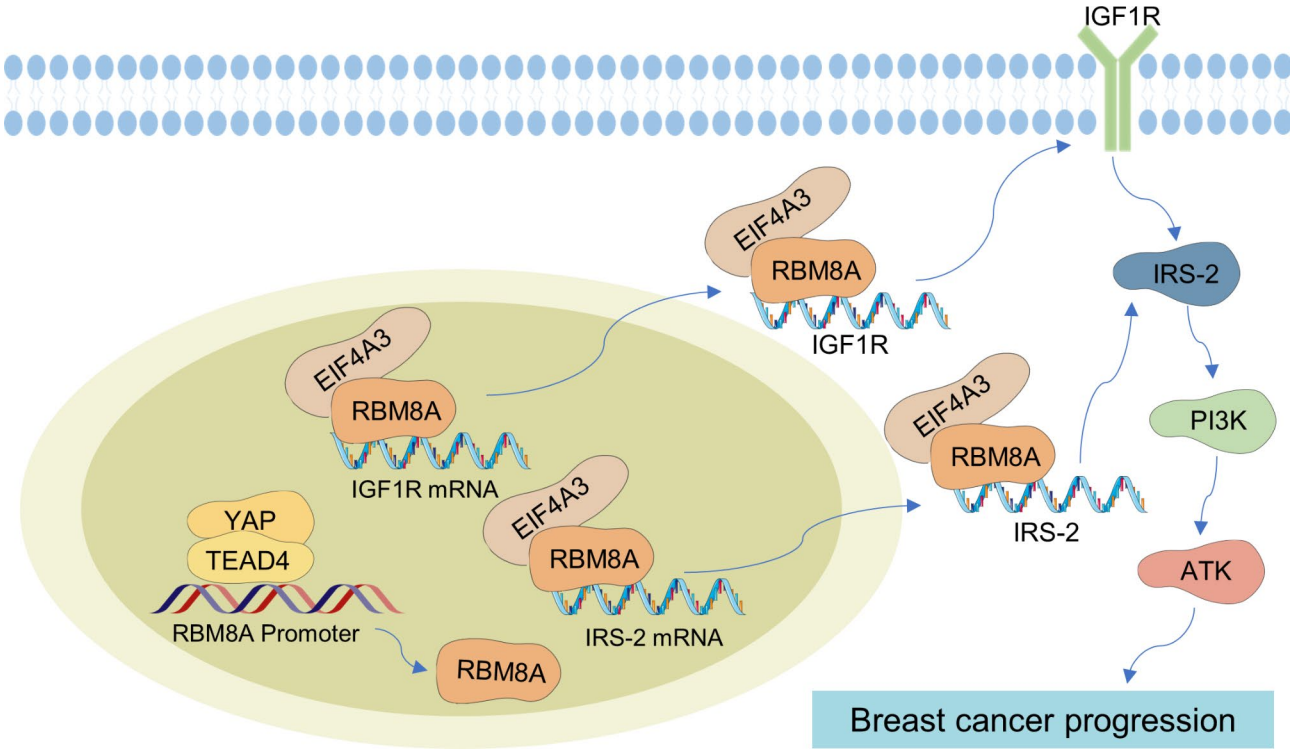

Fig. 8 Schematic diagram. TEAD4 novel target RBM8A forming a complex with EIF4A3 to promote BC progression by regulating IGF1R and IRS-2

effectively inhibit the proliferation of BC cells, which is consistent with the results of studies in other tumors.

To further explore the molecular mechanism by which RBM8A promotes BC, we performed RIP-seq to search for RNAs that bind to RBM8A protein, and found IGFIR and IRS-2. IGFIR activates PI3K/AKT/mTOR and ras/raf/MEK signaling pathways to promote cell proliferation, while inhibiting programmed cell death via Bcl2 antagonists that activate the cell death pathway, thereby causing cancer [27]. IGFIR has emerged as an attractive molecular target for cancer therapy because it is expressed in a wide range of tumors, including BC. Several studies have shown that IGFIR is abnormally expressed in BC and its activation is associated with BC growth, invasion and metastasis [28, 29]. Expression of active IGFIR in BC leads to tumor development, and silencing of IGFIR with small molecules inhibits BC cell growth, a result that was also validated in a xenograft model [30]. IRS proteins are cytoplasmic connexins that mediate the functional outcome of IGF1R signaling [31]. Our study showed that knockdown of RBM8A in BC reduced protein levels but not mRNA levels of IGF1R and IRS-2. Further RIP-PCR experiments confirmed that RBM8A can bind to the mRNAs of IGF1R and IRS-2. Thus, our findings suggest that RBM8A can promote BC progression by regulating IGF1R and IRS-2.

Since studies have reported that RBM8A and EIF4A3 play important roles in regulating gene expression as important components of the EJC complex [12], we next investigated whether EIF4A3 and RBM8A have similar biological functions in BC. Surprisingly, we found that, similar to RBM8A, silencing EIF4A3 in BC cells significantly inhibited cell proliferation. Meanwhile, we demonstrated by Co-IP that RBM8A and EIF4A3 also form a complex in BC. In addition to this, EIF4A3 can also bind to the RNAs of IGF1R and IRS-2, promoting their translation. Therefore, our experimental results confirmed that RBM8A and EIF4A3 act as a complex in BC to jointly regulate the translation of IGF1R and IRS-2 to promote the development of BC.

To investigate the reason for the upregulation of RBM8A in BC, we found that the transcription factor TEAD4 might bind to the promoter region of RBM8A and activate its transcription. TEAD4 has been reported to play an important role in the development of various malignancies [32–34]. Previous studies have shown that TEAD4 is significantly overexpressed in a variety of cancers, including BC, and that abnormal expression of TEAD4 is closely related to the development of these tumors [35–38]. Consistent with these findings, our experimental results also confirmed the cancer-promoting role of TEAD4 in BC. In addition, TEAD4 has been reported to participate as a transcription factor in the transcription of multiple oncogenes and tumor suppressor genes to promote tumor progression. YAP/TEAD4 complex directly inhibits ACADL at the transcriptional

level in HCC [39]. YAP/TEAD4 promotes progression and metastasis of anaplastic thyroid cancer by binding to the UCHL3 promoter and activating UCHL3 transcription [40]. We also found that TEAD4 as a transcription factor can bind to the promoter region of RBM8A to promote its transcription. Therefore, our current study shows that TEAD4 promotes BC progression by activating the transcription of RBM8A in BC. Finally, we validated the oncogenic roles of RBM8A and TEAD4 in animal models.

Abbreviations

BC	Breast cancer
RBM8A	RNA Binding Motif Protein 8 A
RIP	RNA immunoprecipitation
Co-IP	co-immunoprecipitation
Ch-IP	Chromatin immunoprecipitation
RBP	RNA-binding protein
EJC	exon junction complex
MAGOH	Mago homologue
EIF4A3	eukaryotic initiation factor 4A3
GBM	glioblastoma
HCC	hepatocellular carcinoma
TCGA	The Cancer Genome Atlas
GTEx	Genotype-Tissue Expression
FBS	fetal bovine serum
siRNA	Small interfering RNA
PVDF	polyvinylidene fluoride
DAPI	4'6-diamidino-2-phenylindole
SD	standard deviation
PFI	progression free interval
OS	overall survival

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Not applicable.

Authors' contributions
FL, XW, DT and CH made substantial contributions to the conception and design of the experiment. FL, XW, JZ, JZ, XJ and QJ performed the in vitro and in vivo experiments. FL, XW, JZ, JZ, LC and HP made acquisition, analysis, and interpretation of data. FL, XW, DT and CH wrote and reviewed the manuscript. All authors reviewed and approved the final manuscript.

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Data availability
The data and supporting materials regarding the findings of this study are accessible in the main document as well as in additional information.

Declartions

Ethics approval and consent to participate
This study was approved by the Medical Ethics Committee of Xi'an Jiaotong University (No. 2022 – 1149).

Consent for publication
All authors have read the manuscript and provided their consent for the submission.

Competing interests
The authors declare no competing interests.

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