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Hypoxia-induced RBM47 regulates the alternative splicing of FN1 in glioblastoma via intracellular and exosome-mediated mechanisms to promote invasiveness

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Abstract

The tumor microenvironment is the dynamic ecosystem surrounding the cancer cells and contains factors that collectively promote cancer cell progression. In solid cancers, such as glioblastoma (GBM), the formation of hypoxic niches promotes cancer cells' survival, growth, and, eventually, metastasis and is correlated with worse outcomes. Hypoxic TME not only affects cells but also alters exosome cargo, hence augmenting intercellular communication. In this study, we demonstrate that under hypoxia, RNA-binding motif protein 47 (RBM47), an RNA-binding protein, is transcriptionally induced by HIF1 α (Hypoxia-inducible factor 1-alpha) and is packaged into hypoxia-derived exosomes. When these hypoxia-derived exosomes are internalized by normoxic cells, they increase RBM47 abundance, thereby promoting invasive capabilities. Our RNA-seq analysis revealed that RBM47, when upregulated by hypoxia or hypoxia-derived exosome treatment, globally alters the alternative splicing landscape and promotes invasiveness by mediating the alternative splicing of fibronectin 1 (FN1), a key ECM glycoprotein that plays a multifaceted role in tumor progression. RBM47, when upregulated, favors the generation of the pro-invasive FN1 EDB+ isoform, frequently upregulated in aggressive cancers, driving invasiveness. Furthermore, we utilized the dCasRx system to modulate the FN1 splicing and observed a substantial reduction in cell invasiveness. Hence, this study reveals that RBM47 under hypoxia promotes GBM invasiveness by facilitating FN1 alternative splicing intracellularly and by exosomal transfer, eventually fostering invasiveness via cellular communication.

Key words: Hypoxia, Glioblastoma, Alternative splicing, Exosome, FN1, Invasiveness

Abbreviations

GBM – Glioblastoma; HIF1 α - Hypoxia-Inducible Factor 1-alpha; RBM47- RNA binding motif protein 47; FN1- Fibronectin 1; EDB- Extra Domain B; EDA- Extra Domain A; ECM- Extracellular matrix; TME- Tumor microenvironment; dCasRx- deactivated CasRx; gRNA- guide RNA; PBS- Phosphate buffer saline; EMT- Epithelial to Mesenchymal transition; TCGA- The Cancer Genome Atlas; HRE- Hypoxia response elements; CD63- Cluster of Differentiation 63; TSG101- Tumor Susceptibility Gene 101; qRT-PCR- quantitative RT-PCR; rMATs- replicate Multivariate Analysis of Transcript Splicing; PAR-CLIP- Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation

Introduction

Glioblastoma remains one of the most lethal and treatment-resistant malignancies of the central nervous system, characterized by its highly aggressive nature and profound cellular heterogeneity [1]. Despite the recent advancements in treatment, the median survival remains bleak at 12-15 months [2]. The current clinical standard of care, established by the Stupp protocol, involves maximal safe surgical resection followed by concurrent temozolomide and radiotherapy [3]. However, the therapeutic efficacy of this regimen is frequently undermined by the tumor's capacity for invasive spread into the surrounding healthy brain parenchyma, dynamic tumor microenvironment, and presence of glioma stem cells, accounting for frequent recurrences [4, 5]

A defining hallmark of the GBM landscape is its severely hypoxic microenvironment, formed as an outcome of extremely disorganized neovasculature, resulting in low oxygen tension [6]. The hypoxic tumor microenvironment is not merely a byproduct of rapid proliferation but a primary driver of malignant progression, therapeutic resistance, and maintenance of glioma stem cells [7]. Among the many aspects of hypoxia-mediated tumor pathogenesis, alternative splicing has recently garnered attention as a major source of proteome complexity and functional diversity. Alternative splicing is orchestrated by the spliceosome, which recognizes specific cis-acting sequences to precisely remove introns and join selected exons from a single pre-mRNA. This complex process is dynamically regulated by RNA-binding proteins that interact with splicing enhancers or silencers to either recruit or block spliceosomal assembly, thus altering the splicing output [8]. It is well-established that hypoxia-induced alternative splicing serves as a critical pathway that tumor cells leverage to drive phenotypic plasticity, aiding tumor growth and survival [9].

Beyond intracellular regulation, the GBM landscape is shaped by the constant exchange of bioactive cargo via extracellular vesicles, specifically exosomes. These small (30-200 nm), lipid bilayer-delimited vesicles originate from endosomes and are eventually released into the extracellular space following fusion of the multivesicular body with the plasma membrane. Enriched in bioactive cargo, these vesicles alter the recipient cell function upon uptake [10]. Exosome biogenesis is impacted by various external stressors, most notably hypoxia, which stimulates exosome biogenesis and affects its cargo composition. The tumor-derived exosome, particularly from hypoxic regions, is known to facilitate the intercellular transfer of pro-tumorigenic factors, which impact cancer progression by affecting various processes, including proliferation, angiogenesis, epithelial-to-mesenchymal transition, and immune evasion [11, 12].

Researchers have independently studied the impact of hypoxia-mediated aberrant alternative splicing and exosome-mediated intercellular crosstalk on cancer progression; however, their convergence offers a new conceptual framework. Since hypoxia reshapes the alternative splicing landscape, facilitating cellular adaptation [9], and given that exosomes serve as key mediators of cellular crosstalk within TME, the potential for hypoxia-derived exosomes to transport splicing-related cargo and modulate the alternative splicing landscape of recipient cells offers a previously unexplored perspective.

In this study, we report that RNA-binding motif 47 (RBM47), an RNA-binding protein, is upregulated under hypoxia via HIF1 α and contributes to the invasiveness in hypoxic GBM cells. We also observed that hypoxia-derived exosomes are enriched in RBM47, which, upon transfer, upregulates RBM47 protein levels, ultimately conferring enhanced invasive capabilities to the recipient cells. Globally, we observed that alterations in RBM47 expression lead to significant changes in the alternative splicing landscape, particularly in genes regulating epithelial-to-mesenchymal pathways. We have identified Fibronectin 1 (FN1) as a novel alternative splicing target of RBM47, in which it favors the inclusion of exon 25, forming the pro-tumorigenic isoform FN1 EDB⁺. This variant, also known as “oncofetal fibronectin,” contains the extra domain B, a highly conserved region that is usually absent in normal adult tissues but abundantly expressed during embryogenesis, wound healing, and cancer progression. FN1-EDB⁺ acts as a critical driver of malignancy by facilitating α v β 3 and α 5 β 1 integrin clustering, promoting vascular morphogenesis via endothelial proliferation, and serving as a scaffold for metastatic seeding [13]. Further, we have modulated this splicing event using an unconjugated dCasRx system, targeting it to a critical intronic regulatory region, thereby decreasing exon 25 inclusion and functionally inhibiting invasiveness.

Thus, our study demonstrates a novel HIF1 α -RBM47-FN1 axis in mediating GBM invasiveness. Furthermore, we show that RBM47 is a critical constituent of hypoxia-induced exosomes, facilitating the spread of an invasive phenotype through exosomal transfer. Collectively, our findings identify RBM47 as a mobile oncogenic factor that links the hypoxic microenvironment to functional changes in the GBM alternative splicing landscape, offering new avenues for targeted therapeutic intervention.

Materials and methods

Cell culture

Human glioblastoma cell lines U87MG and LN18 were procured from the American Type Culture Collection (ATCC) and were maintained in MEM (Invitrogen, 61100061) and DMEM (Invitrogen, 12800017), respectively. HEK293T was cultured in DMEM. The cell culture media were supplemented with 10% fetal bovine serum

(Sigma, F7524), 100 units/ml of penicillin and streptomycin (Invitrogen, 15140122), and 2 mmol/l L-glutamine (Invitrogen, 25030081). All the cells were cultured at 37°C with 5% CO₂ in a humidified incubator. For hypoxia treatment, cells were incubated in a Ruskinn InvivoO₂ 400 hypoxia chamber under 1% O₂. The cell lines used in the current study were authenticated by short tandem repeats (STR) profiling at a national cell repository facility and were routinely tested for mycoplasma contamination.

Plasmids

The RBM47 overexpression construct was generated by amplifying the RBM47 CDS region using U87MG cDNA as a template and was cloned between the *Hind*III and *Xho*I sites of the pCMV-3Tag-1A (Agilent, 240195) mammalian expression vector. For the construction of overexpression vectors of FN1 EDB+ and FN1 EDB-isoforms, insert fragments were amplified using U87MG cDNA and were cloned into the pMcherry-N1 vector using Gibson assembly (NEB, E2611S) following the manufacturer's protocol. Details of all primers used are provided in the Supplementary Table S2

Luciferase assay

To generate the reporter construct, the RBM47 promoter region was amplified from U87MG genomic DNA and cloned into the pGL3 basic luciferase reporter vector (Promega, E1751) using the *Kpn*I and *Hind*III sites. The details of primers used are provided in the Supplementary Table S2. The cells were seeded in a 12-well plate and allowed to attach overnight, followed by transfection with the RBM47 promoter construct along with the pRL-TK renilla luciferase plasmid (Promega, E2231). After 12 h, the cells were supplemented with fresh medium, subjected to normoxia or hypoxia, and lysed using passive lysis buffer. The Firefly and Renilla luciferase activity was assessed using the substrate buffers containing D-Luciferin and Coelenterazine, respectively, on the Cytation Multiplate reader (Agilent Biotek). The relative luciferase activity was calculated by normalizing Firefly luciferase activity to Renilla luciferase activity and to the empty pGL3 basic luciferase plasmid.

Site-directed mutagenesis

Site-directed mutagenesis was performed using primers harboring the desired mutations in HREs, listed in the Supplementary Table S2. The wild-type pGL3-RBM47pro construct was used as a template to generate a mutant construct. *Dpn*I digestion was performed to eliminate wild-type template DNA. Sanger sequencing was used to verify the sequence of the site-directed mutagenesis (SDM) construct. These constructs were then used to perform a luciferase assay.

Exosome isolation

Exosome-depleted media were used for all exosome-related experiments. The residual exosomes from FBS were removed by ultracentrifugation of the appropriate cell culture medium containing 20% FBS for 16 h at 100,000 × g at 4°C. The FBS concentration was made up 10% by adding serum-free media. The cells were seeded at 30-40% confluency and allowed to attach overnight. The medium was then replaced with exosome-depleted medium, and the cells were subjected to normoxic or hypoxic treatment for 48 h. For exosome isolation from conditioned media, the ExoEnrich™ (ExoCAN, PEC-50) kit was used as per the manufacturer's instructions. Briefly, conditioned media were subjected to differential centrifugation and filtration using a 0.22 µm syringe filter to remove cellular debris and larger particles. Following the manufacturer's protocol, solutions A and B were added, and the conditioned media were centrifuged at 4000 rpm for 20 min. The obtained exosome pellet was washed twice in PBS. For immunoblotting, the exosomes were lysed in ExoLyseP (ExoCAN, PEL-25P), and the concentration was determined using the Bradford assay. For exosome treatment, the pellet was resuspended in PBS, and a small aliquot was separately lysed and estimated for protein concentration. Using the estimated protein concentration, an equal concentration of exosomes was used to treat the cells.

Transmission electron microscopy

Isolated exosomes were resuspended in filtered 1X PBS and diluted to an appropriate concentration. A 10 µl aliquot of resuspended exosome was absorbed onto a Formvar carbon-coated copper grid for 10 min. The grid was gently rinsed with autoclaved distilled water and immediately incubated in 2% uranyl acetate solution for 5 min. The grid was gently washed twice with autoclaved distilled water to remove excess stain, then air-dried prior to imaging. The imaging was performed using a High-Resolution Transmission Electron Microscope (Talos F200S, Thermo Fisher Scientific) at 200 kV.

Dynamic light scattering

The size distribution of isolated exosomes was measured by dynamic light scattering using a Delsa™ Nano C, Beckman Coulter. The exosome pellet was resuspended in 1× cold PBS that had been pre-filtered through a 0.22 µm membrane, and measurements were performed at 25°C.

Exosome uptake assay

U87MG and LN18 cells were seeded onto glass coverslips in a 12-well plate and allowed to attach overnight. For the uptake assays, cells were incubated with labeled exosomes derived from the same cell line under either normoxic or hypoxic conditions. Exosome labeling was performed using PKH67 dye as previously described [14]. Briefly, the isolated exosome pellet was resuspended in 1 mL of diluent C. Separately in another tube, 1 mL of diluent C was mixed with 2 μ L of PKH67 dye. The exosome suspension and dye solution were mixed and incubated for 5 min. The labeling was quenched by adding an equal volume of cell culture medium supplemented with 2% exosome-depleted FBS. The labeled exosomes were reisolated using the ExoEnrich™ by centrifuging at 4000 RPM for 20 mins, followed by two washes with 1X PBS to remove the excess dye. The protein concentration of labeled exosomes was measured using the Bradford assay. Equal amounts of labeled exosomes from normoxic and hypoxic conditions were incubated with U87MG and LN18 cells for 6 h. After incubation, the cells were washed 3 times with 1X PBS and fixed with 4% formaldehyde for 10 mins. The cells were permeabilized using 0.25% Triton X-100 and were counterstained with DAPI to visualize the nuclei. Imaging was performed using a FLUOVIEW FV4000 confocal microscope equipped with a 60 $\times$ oil-immersion objective (numerical aperture 1.40).

Serum exosome isolation

Blood samples for serum isolation were collected from glioma patients and healthy donors, with informed consent obtained. After collection, the blood samples were left upright and undisturbed for 45–60 min to allow RBCs to clot. The sample was then centrifuged at 1,200g for 10 min at 4°C to separate the serum. The supernatant was carefully aspirated and stored at -80°C until further use. For exosome isolation from serum, the ExoQuick® ULTRA EV (Cat. no. EQUltra-20A-1) kit was used according to the manufacturer's guidelines. Briefly, the serum sample was centrifuged at 3000 g for 15 min, followed by 12,000 g for 10 min at 4°C to remove debris. 250 μ L of clarified serum was incubated with 67 μ L of ExoQuick on ice for 30 min, then centrifuged at 3,000 g for 10 min at 4°C. The obtained pellet was resuspended and purified according to instructions. The obtained exosome solution was lysed using 5 $\times$ RIPA buffer (250 mM Tris HCl pH 7.4, 750 mM NaCl, 2.5% sodium deoxycholate, 5% NP-40, and 0.5% SDS) supplemented with protease inhibitor cocktail (leupeptin 0.5 mM, pepstatin 1 μ M, 50 mM EDTA), and 1 mM PMSF. Protein quantification was performed using the BCA protein assay, and equal protein concentrations were used for immunoblotting.

Minigene cloning

For the construction of FN1 minigene, the region spanning exon 24, intron 24, exon 25, intron 25, and exon 26 of the FN1 gene was amplified using U87MG genomic DNA. The entire insert segment was divided into 3 overlapping fragments to facilitate amplification: fragment 1 encompassed exons 24 and 25, along with the middle intronic region, and was amplified using exon 24 forward and exon 25-intron 25 reverse primers. Fragment 2 stretched from the 3' end of exon 25 to the 5' end of exon 26 and was amplified with the exon 25-intron 25 forward primer and intron 25-exon 26 reverse primers. Fragment 3 consisted of exon 26 and was amplified using intron 25-exon 26 forward and exon 26 reverse primers. Each of the three fragments was amplified independently using Q5® High-Fidelity 2X Master Mix and purified by gel extraction. The fragments were then sequentially joined by overlap-extension PCR, comprising initial 15 cycles without primers to facilitate annealing of the fragments, followed by 20 cycles with flanking primers to amplify the full-length minigene. The primers were designed with appropriate overhangs to ensure 30-35 bp terminal overlaps and are listed in the Supplementary Table S2. The construct was designed with consideration to couple the alternative splicing inclusion of exon 25 to the production of functional mCherry fluorescent protein, ensuring a direct and quantifiable readout of the splicing outcome under different conditions. The FN1 exon 25 is 273 bp in length, which is a multiple of 3; therefore, its inclusion or exclusion does not inherently alter the reading frame of the downstream sequence. In order to generate a minigene reporter system specific to the included isoform, the 3' end of exon 25 and the 5' end of exon 26 were intentionally altered by insertion of one (G) and two (CT) nucleotides, respectively. These nucleotide additions were incorporated into the two sets of overlapping primers during primer design. Thus, this net +3 nucleotide insertion ensures that a functional mCherry reporter is produced only upon the inclusion of exon 25. The nucleotide additions were done while ensuring that the highly conserved GU/AG splice junction and the wild-type amino acid sequence were preserved to prevent any disruption of splicing recognition. The amplified minigene insert was cloned between the *NheI* and *BamHI* sites of the mCherry-N1 vector. The confirmed minigene was then transfected using Lipofectamine 2000, and imaging was performed using FLUOVIEW FV4000 confocal microscope equipped with 60 $\times$ oil-immersion objective (numerical aperture 1.40).

dCasRx-RBM47 plasmid cloning, guide RNA design, and guide RNA cloning

The RBM47 effector domain sequence was generated by removing the three RRM domains retrieved from the Uniprot database and was amplified using U87MG cDNA. The insert was cloned between the *NheI* site of the plasmid pXR002: EF1a-dCasRx-2A-EGFP (Addgene Plasmid #109050). A flexible Gly-Ser linker sequence was added to the 3' end of the forward primer to impart flexibility of movement to the bulky construct. The primer details are provided in Supplementary Table S2.

The gRNAs to be used with the dCas13Rx system were designed using the web-based platforms “TIGER Online Tool for Cas13 Efficacy Prediction” and “cas13design” as previously discussed [15]. The gRNA sequences with a score above 0.5 on TIGER or Q3 or Q4 on cas13design were considered. While designing the guide RNA sequences, the length was kept at approximately 30 bp, and the GC content was maintained around the ideal range of 40-60%, to ensure a balance between efficient specific targeting with minimal off-target binding and structural stability. For efficient ligation into pXR003: CasRx gRNA cloning backbone (Addgene Plasmid #109053), *BbsI*-compatible overhangs were added to the ends of the oligo pair. The oligos were annealed and cloned into the vector backbone between the *BbsI* restriction site. The cloned guide RNAs were verified by sequencing using the U6 Forward primer. Additionally, a non-targeted control gRNA sequence was cloned and used throughout the study. The oligo details are provided in Supplementary Table S3.

For dCasRx experiments, cells were seeded in a 6-well plate the day before transfection. Subsequently, dCasRx and gRNA plasmids were co-transfected at a 1500 ng:200 ng ratio using a 1:3 DNA: PEI ratio. The cells were harvested 24 h post-transfection and subjected to downstream analysis.

PCR-based fragment deletion from the plasmid

A specific region of interest (~200 bp) was deleted from the FN1 minigene plasmid using a previously described method [16] with some modifications. A set of divergent, overlapping primers was designed in a back-to-back orientation, with each primer half complementary to one of the flanking regions of the sequence to be deleted, as mentioned in the Supplementary Table S2. A high-fidelity Phusion polymerase was used to amplify the entire plasmid, excluding the specific region to be deleted. The PCR was performed in 2 steps: for the first 10 PCR cycles, both forward and reverse primers were added to separate reactions to prevent primer self-hybridization and maximize amplification of the desired deletion constructs. Then, the two reactions were combined, and the mixture was subjected to 15 additional PCR cycles. The PCR product was subjected to *DpnI* digestion, removing the methylated template plasmid and leaving only the unmethylated PCR product intact. The resulting fragments were then gel-extracted and transformed into competent *E. coli* cells.

Retrieval of TCGA-GBM RNA-seq data and their analysis

The TCGA-GBM RNA-seq data were retrieved from UCSC Xena, containing gene expression profiles for 172 primary GBM tumor samples generated on the Illumina HiSeq 2000 platform. Epithelial-mesenchymal transition and hypoxia gene signatures were obtained from the MSigDB Hallmark collection. Gene identifiers were extracted using the GSEABase package, and only genes present in the TCGA GBM expression matrix were retained for downstream analyses. For each tumor sample, a hypoxia score was calculated as the arithmetic mean expression of all genes belonging to the HALLMARK_HYPOXIA gene set [17]. Tumors were stratified into hypoxia-high and hypoxia-low groups using a quartile-based approach. Samples with hypoxia scores at or above the 75th percentile (Q3) were classified as hypoxia-high, while those at or below the 25th percentile (Q1) were classified as hypoxia-low. EMT pathway activity was quantified using single-sample Gene Set Enrichment Analysis (ssGSEA) via the GSVA R package. Samples were categorized into EMT-high and EMT-low groups based on a median split of the ssGSEA enrichment scores. Differential expression analysis was performed for both EMT and hypoxia comparisons using the limma package in R. Gene-wise linear models were fitted using lmFit, followed by empirical Bayes variance moderation with eBayes. The Benjamini-Hochberg false discovery rate (FDR) was used to adjust the raw p-values from both analyses. Genes with adjusted p-value < 0.05 were considered statistically significant. The hypoxia and EMT gene rankings were integrated using a combined rank, which was the sum of both the individual ranks. The list of genes obtained from the above analysis is mentioned in the Supplementary Table S6.

RNA-seq analysis

Total RNA was isolated from U87MG cells across four experimental conditions: hypoxic (control and RBM47-knockdown) and normoxic (treated with either normoxia- or hypoxia-derived exosomes). All extractions were performed using the PureLink™ RNA Mini Kit (Invitrogen, 12,183,025) as suggested by the manufacturer. 1 µg of total RNA was used to make cDNA libraries. mRNA enrichment was carried out prior to library preparation using NEBNext Poly(A) mRNA Magnetic Isolation Module (E7490S). The libraries were generated using the NEBNext Ultra II RNA Library Prep Kit for Illumina (E7770S) and sequenced on an Illumina NovaSeq

sequencing system (Neuberg Center for Genomic Medicine, Ahmedabad, India). Raw sequencing reads were aligned to the human reference genome (hg38 assembly) using STAR Aligner (version 2.7.1a) [18], and alternative splicing analysis was performed using rMATS 4.0.2 [19]. Differential alternative splicing events exhibiting an absolute Percent Spliced In difference (Δ PSI $\geq$ 0.1) and a False Discovery Rate $<$ 0.05 were considered significant. The list of genes undergoing exon skipping upon RBM47 knockdown under hypoxia, or hypoxia-derived exosome treatment, is provided in Supplementary Table S5.

Transwell invasion assay

Transwell inserts (Corning, Cat. no. 3422) were coated with 100 μ L Matrigel (Corning, Cat. no. 356230) at a concentration of 0.25% and were left undisturbed for 3 h at 37 °C. Then, about 7×10^3 cells for U87MG or 2×10^4 cells for LN18 were resuspended in serum-free medium, seeded onto the Matrigel layer of the insert, and placed in a well containing complete medium. The cells were incubated in normoxic or hypoxic conditions for 9–24 h. The cells were then gently washed with PBS, fixed with 4% formaldehyde for 10 min, and stained with 0.05% crystal violet (made in 10% methanol in PBS). After drying, the inserts were imaged on an inverted microscope (Olympus CKX41) and analyzed using ImageJ. The percentage of invaded cells was represented as mean $\pm$ SD of triplicates.

Chromatin immunoprecipitation assay

The ChIP assay was performed as previously mentioned [20]. Briefly, 10 million cells were crosslinked using 1% formaldehyde, scraped in PBS, lysed, and sonicated. Approximately 10 μ g of sheared chromatin was incubated overnight at 4°C with an antibody of interest, followed by immunoprecipitation using Dynabeads Protein G (Invitrogen, Cat. No. 10004D). The immunoprecipitated protein-DNA complexes and 5% input were analyzed by qRT-PCR using KAPA SYBR FAST master mix (Sigma, KK4618). Enrichment was performed using the formula $[2(\text{Ct}_{\text{input}} - \text{Ct}_{\text{immunoprecipitation}})]$. The obtained values were further normalized relative to rabbit IgG and control IP values. Significance between the two groups was assessed using Student's t-test, with p-values $<$ 0.05 considered statistically significant. The detailed list of primers is provided in the Supplementary Table S1.

Quantitative RT-PCR

Total RNA was extracted using TRIzol reagent (Ambion, 15596018) according to the manufacturer's instructions. 2 μ g of total RNA was reverse transcribed using the Invitrogen SuperScript® III First-Strand Synthesis System (18080–051). Amplifications were carried out using KAPA SYBR FAST QPCR master mix (Sigma, KK4618) in a qTOWER3-G (Analytik Jena) qPCR machine according to the manufacturer's protocol. Primers were designed using the IDT PrimerQuest tool (<https://www.idtdna.com>) and are listed in the Supplementary Table S1. Using the formula $2^{-(\text{Ct}_{\text{control}} - \text{Ct}_{\text{target}})}$, the average cycle thresholds from three independent biological replicates were computed and normalized to the housekeeping control gene RPS16. Student's t-test was performed to compare gene/exon expression levels between the two groups. $P <$ 0.05 was considered statistically significant.

Immunoblotting

Cell lysis was performed using Urea lysis buffer (8 M urea, 2 M thiourea, 2% CHAPS, 1% DTT) supplemented with $1 \times$ protease inhibitor cocktail (PIC; leupeptin 10–100 μ M, pepstatin 1 μ M, EDTA 1–10 mM, AEBSF $<$ 1 mM) and 10 mM PMSF at 4°C for 30 min followed by centrifugation at 14,000g for 1 h. The Bradford assay was used to determine the protein concentration, and equal amounts of protein were loaded. After separation, the proteins were electrotransferred to a PVDF membrane and incubated overnight with primary antibodies, followed by 1 h incubation with a secondary antibody. Imaging was performed with the Odyssey membrane scanning system, and band quantification was performed with ImageJ.

RNA interference

HEK293T cells were transfected with a lentiviral plasmid expressing shRNA against the target genes, along with the packaging plasmid, using PEI. The shRNA sequences are provided in the Supplementary Table S4. The lentivirus was collected 24 and 48 hours post-transfection. U87MG and LN18 cells were seeded at a density of 1.5×10^5 cells and 2.5×10^5 cells, respectively, in 6-well plates. The lentivirus-containing media obtained from HEK293T cells were inoculated with cells in the presence of 8 μ g/mL polybrene (Sigma, H9268, lot no. SLBH5907V). After 12 h, the cells were supplemented with fresh medium and selected for 72 h with 1 μ g/mL puromycin (Sigma, P9620), then used for various experiments.

PAR-CLIP qRT-PCR

PAR-CLIP was performed as described previously [21] with slight modifications. Briefly, 14–16 h before crosslinking, 100 μ M 4-thiouridine (4SU; Cayman Chemical, T4509) was added to the cell culture medium. To crosslink, cells were then irradiated with 365-nm UV light at a total energy of 0.15 J cm⁻² using a UV crosslinker.

The cells were scraped in PBS and pelleted by centrifugation at 500g for 5 min at 4°C. The pellet was resuspended in NP40 lysis buffer (50 mM HEPES-KOH, pH 7.5; 150 mM KCl; 2 mM EDTA-NaOH, pH 8; 1 mM NaF; 0.5% v/v NP-40), supplemented with protease inhibitor cocktail (PIC), 10 mM PMSF, 0.5 mM DTT, and 40 U/mL RNaseOUT (Invitrogen, 10777019). The cells were incubated on ice for 10 min to allow lysis and centrifuged at 13,000 g for 15 min at 4°C.

The Flag and control rabbit antibodies were individually conjugated to Dynabeads Protein G (Invitrogen, Cat# 10004D) for 1 h at room temperature. The clarified lysate was incubated overnight at 4 °C with Dynabead-antibody conjugates. The immunoprecipitated complex was washed sequentially with IP wash buffer (50 mM HEPES-KOH, pH 7.5, 300 mM KCl, 0.05% v/v NP40, 0.5 mM DTT (fresh), 1× PIC, 10 mM PMSF, 40 U/mL RNaseOUT) and high salt wash buffer (50 mM HEPES-KOH, pH 7.5, 500 mM KCl, 0.05% v/v NP40, 0.5 mM DTT (fresh), 1× PIC, 10 mM PMSF, 40 U/mL RNaseOUT). A 20-μL aliquot of the immunoprecipitated complex was kept aside for immunoblotting while the remainder was decrosslinked in Proteinase K buffer (100 mM Tris-HCl, pH 7.5; 12.5 mM EDTA-NaOH, pH 8; 150 mM NaCl; 2% v/v SDS; 1.2 mg/ml Proteinase K) at 55 °C for 30 min. TRIzol was added to the sample, and RNA was isolated as per the manufacturer's guidelines, including 1 μL of Glycoblue coprecipitant (Invitrogen, AM9515). The isolated RNA was reverse transcribed with random hexamers using Invitrogen SuperScript® III First-Strand Synthesis System (18080-051), and qRT-PCR was performed using KAPA SYBR FAST qPCR master mix (Sigma, KK4618). The enrichment of IP was normalized to its respective input using $(2^{Ct\ IP} - Ct\ Input)$. Fold change was calculated relative to IgG enrichment for representation.

Statistical analysis

Statistical analyses of all data are presented as mean values ± SD, derived from n = 3 biological replicates unless otherwise stated. The difference between the two groups was evaluated using a two-tailed Student's t-test, and, for comparisons involving more than two groups, a one-way ANOVA with Dunnett's multiple comparisons test was performed. All statistical analyses were conducted using GraphPad Prism 8, and P < 0.05 was considered statistically significant. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ns = not significant.

Results

RBM47 is overexpressed in hypoxic GBM

RNA-binding proteins (RBPs) are the central regulators of the alternative splicing landscape and are frequently deregulated in various malignancies [22]. To identify RNA-binding proteins that modulate alternative splicing under hypoxia and are pertinent to GBM, we classified the TCGA-GBM dataset into hypoxia-high and hypoxia-low groups based on hypoxia hallmark genes acquired from the mSigDB database (Fig. 1A). In addition to hypoxia, the mesenchymal phenotype significantly contributes to GBM severity, exhibiting a robust correlation with EMT-high states and unfavorable outcomes [23]. To address factors influencing illness severity, we categorized the TCGA-GBM dataset into EMT-high and EMT-low groups based on EMT hallmark genes obtained from the mSigDB database (Fig. 1B).

We subsequently compiled a library of approximately 300 genes from the Gene Ontology database, widely recognized as regulators of the splicing machinery. In order to identify hypoxia-driven splicing regulators in glioblastoma, we overlapped the RNA splicing-related library with the hypoxia-high and EMT-high genes from the TCGA-GBM dataset. This multi-layered screening approach identified several candidates, with RBM47 emerging as the most significant hit in our analysis (Fig. 1C, D).

To validate our computational findings, we analyzed RBM47 expression in several GBM cell lines (U87MG, LN18, and LN229) and in a glioma patient-derived cell line subjected to normoxia (21% O₂) or hypoxia (1% O₂) treatment. Consistent with our initial screen, the immunoblot showed upregulation in RBM47 protein levels under hypoxic conditions across all tested cell lines (Fig. 1E). qRT-PCR in U87MG and LN18 cells supported these findings, revealing a significant increase in RBM47 mRNA levels in response to hypoxia (Fig. 1F).

To further ascertain the clinical significance of RBM47, we queried the TCGA (transcriptomic) and CPTAC (proteomic) databases, revealing that RBM47 is overexpressed in GBM relative to healthy brain tissue (Fig. S1A, S1B). Crucially, elevated RBM47 expression served as a strong indicator of poor prognosis, correlating with significantly reduced disease-free survival (Fig. S1C).

RBM47 is a highly conserved member of the RNA-binding motif family, harboring three RNA recognition motifs and an alanine-rich C-terminus. While RBM47 is essential for vertebrate embryonic development [24], its role in malignancy appears to be context-dependent. Previous reports have highlighted its tumor suppressor function in breast, thyroid, and lung cancers, often downregulated during EMT progression in colorectal cancer [25–28]. However, emerging evidence underscores its oncogenic potential in nasopharyngeal carcinoma and GBM, where it may actively promote the epithelial-to-mesenchymal transition and tumor progression [29, 30]

Fig. 1 RBM47 is overexpressed in the hypoxic GBM cells

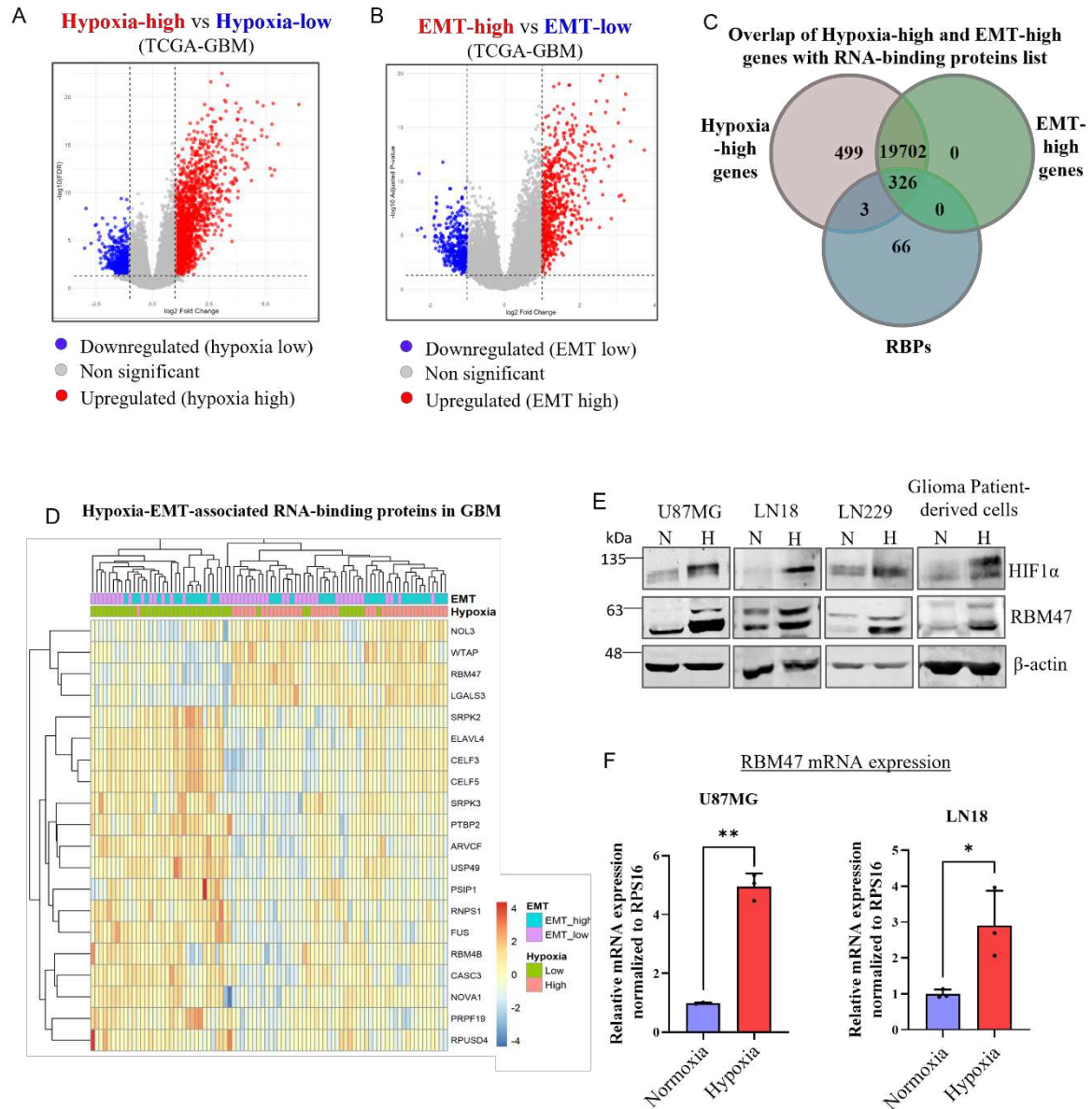


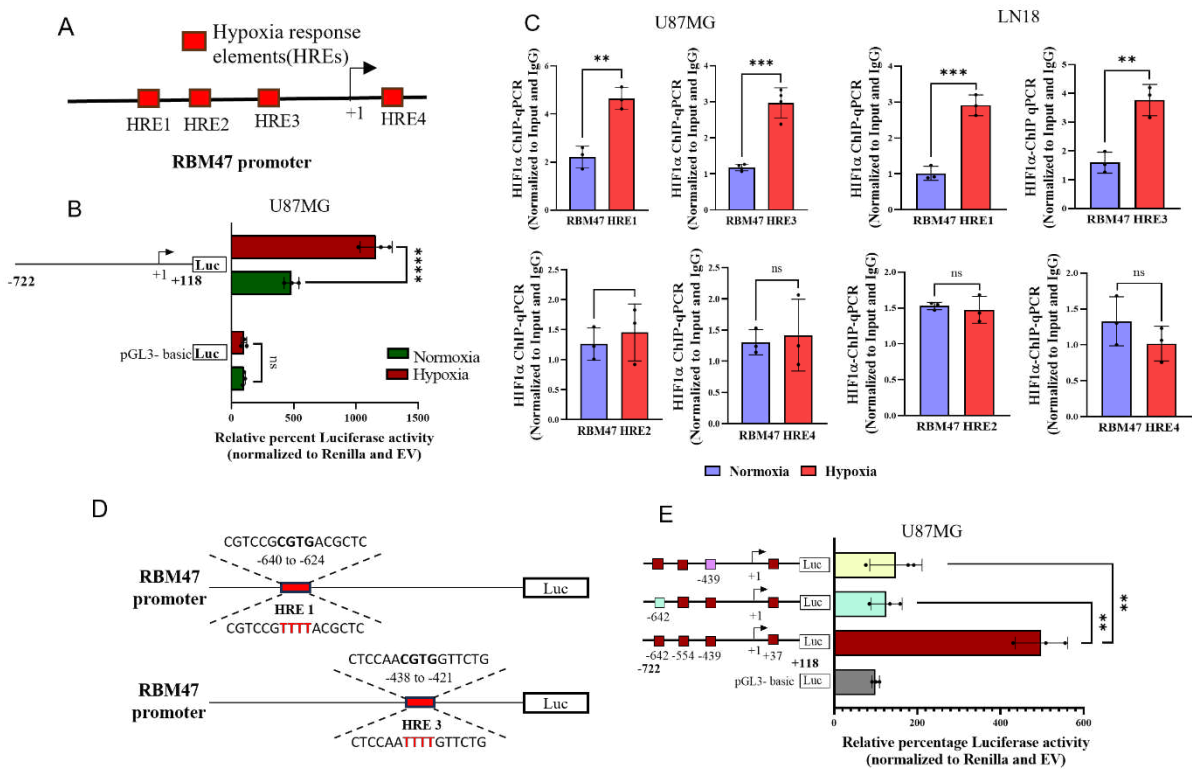
Fig.1 **A** Heat map of TCGA-GBM data segregated into hypoxia-high and hypoxia-low, and **B** EMT-high and EMT-low groups. **C** A Venn diagram showing overlap between hypoxia-high and EMT-high genes with RBPs. **D** Heat map of the top 20 common targets from Fig. C. **E** Immunoblot analysis of RBM47 under 24 h normoxia and hypoxia in U87MG, LN229, LN18, and glioma patient-derived cells. **F** qRT-PCR analysis of RBM47 normalized to RPS16 in U87MG and LN18 cells. Data represent mean $\pm$ SD (n = 3 biological replicates); two-tailed Student's t-test. *P $\leq$ 0.05, **P $\leq$ 0.01, ***P $\leq$ 0.001, ns = not significant.

RBM47 expression is regulated via HIF1 α and enhances invasiveness under hypoxia

Despite the dynamic, cancer-specific expression pattern of RBM47, its regulation in GBM remains largely unknown. Since we observed an increase in RBM47 mRNA levels, we speculated that RBM47 is transcriptionally upregulated under hypoxic conditions. To discover promoter elements responsible for this upregulation, we looked at the RBM47 promoter and found multiple putative hypoxia response elements (HREs) (Fig. 2A). The HREs are occupied by HIF1 α , a transcription factor well known for mediating cellular hypoxic responses. To assess the promoter activity under hypoxia, we cloned the RBM47 promoter fragment spanning -722 to +118 bp, encompassing all 4 HREs, upstream of the luciferase reporter in the pGL3-basic vector. We performed a dual luciferase assay using the pGL3_RBM47pro construct and observed enhanced promoter activity under hypoxia (Fig. 2B, S2A). To validate the binding of HIF1 α , we performed ChIP-qPCR analysis and observed increased HIF1 α occupancy at HRE1 and HRE3 (-624 and -439) under hypoxic stress (Fig. 2C). Subsequent site-directed mutagenesis of the HRE1 and HRE3 in the pGL3_RBM47pro construct dampened the promoter activity as compared to its wildtype counterpart (Fig. 2D, E). Further, we validated the role of HIF1 α in RBM47 hypoxic induction at the protein level by performing shRNA-mediated knockdown and observed diminished RBM47 expression in HIF1 α knockdown (Fig. 2F). We also overexpressed a mutant HIF1 α that stabilizes under normoxia and found that RBM47 expression increased (Fig. 2G). Thus, these results establish that HIF1 α is both necessary and sufficient for the induction of RBM47 expression.

Building upon the observed correlation between high RBM47 expression and poor clinical outcomes in large-scale dataset analysis, we investigated the functional contribution of RBM47 to the GBM invasive phenotype. We overexpressed RBM47, cloned into the pCMV-3Tag-1A vector, and performed a transwell invasion assay. Under normoxic conditions, we observed increased invasion in RBM47-overexpressing cells compared with empty vector controls (Fig. 2H, S2B). Furthermore, upon RBM47 knockdown, the cells' invasive potential decreased (Fig. 2I, S2C). Collectively, these findings establish RBM47 as a direct transcriptional target of HIF1 α and a key mediator of the invasive behaviour, a characteristic of hypoxic GBM tumors.

Fig. 2 HIF1 α transcriptionally upregulates the expression of RBM47 in hypoxic GBM cells



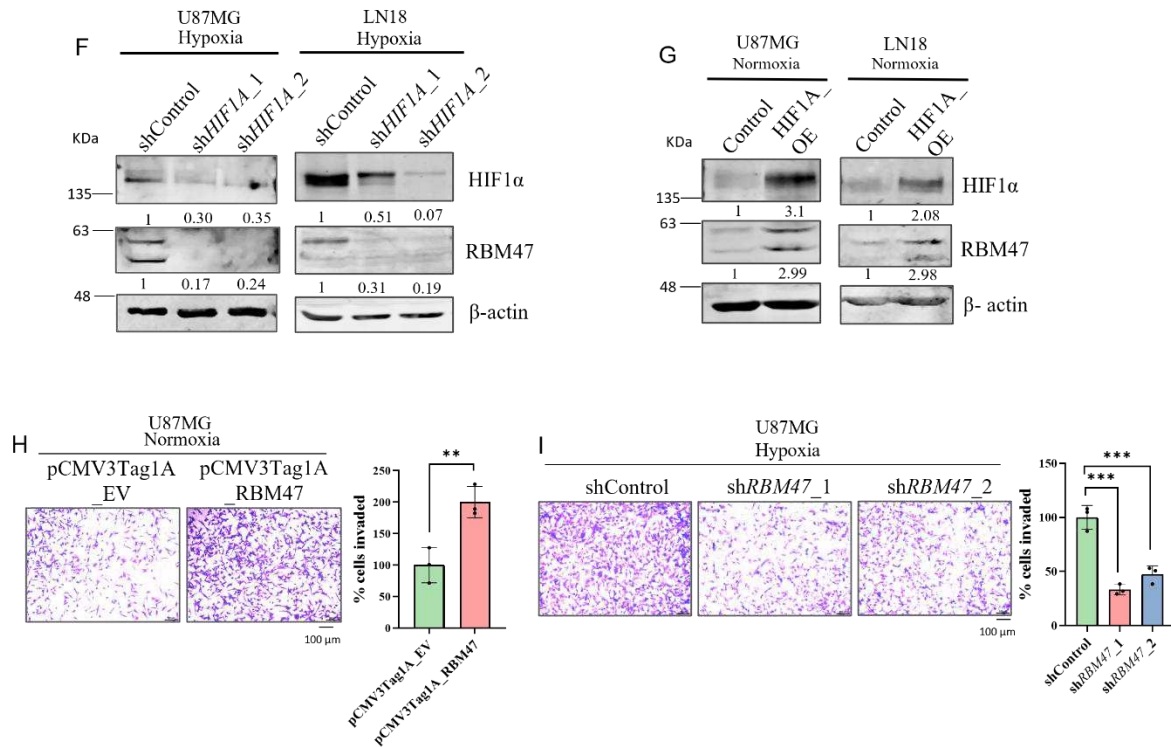


Fig.2 **A** Schematic representation of the RBM47 promoter displaying HREs. **B** Dual luciferase assay performed using RBM47 promoter construct under normoxia and hypoxia for 24h in U87MG cells. **C** HIF1α ChIP-qPCR performed at RBM47 promoter under normoxia and hypoxia in U87MG and LN18, showing increased occupancy at HRE1 and HRE3 under hypoxia. **D** A schematic of the RBM47 promoter construct with mutated HREs. **E** Dual luciferase assay comparing wildtype RBM47 promoter to HRE mutant RBM47 promoter activity under hypoxia in U87MG cells. **F** Immunoblot analysis demonstrating the effect of HIF1α knockdown and **G** HIF1α overexpression on RBM47 expression in U87MG and LN18. **H** Invasion assay performed in RBM47 overexpressing U87MG cells under normoxic conditions. **I** Invasion assay performed in control and RBM47 knockdown U87MG cells under hypoxic conditions. Data represent mean $\pm$ SD (n = 3 biological replicates); two-tailed Student's t-test. *P $\leq$ 0.05, **P $\leq$ 0.01, ***P $\leq$ 0.001, ns = not significant.

RBM47 is enriched in hypoxia-derived exosomes and can be transferred to normoxic cells by exosome uptake

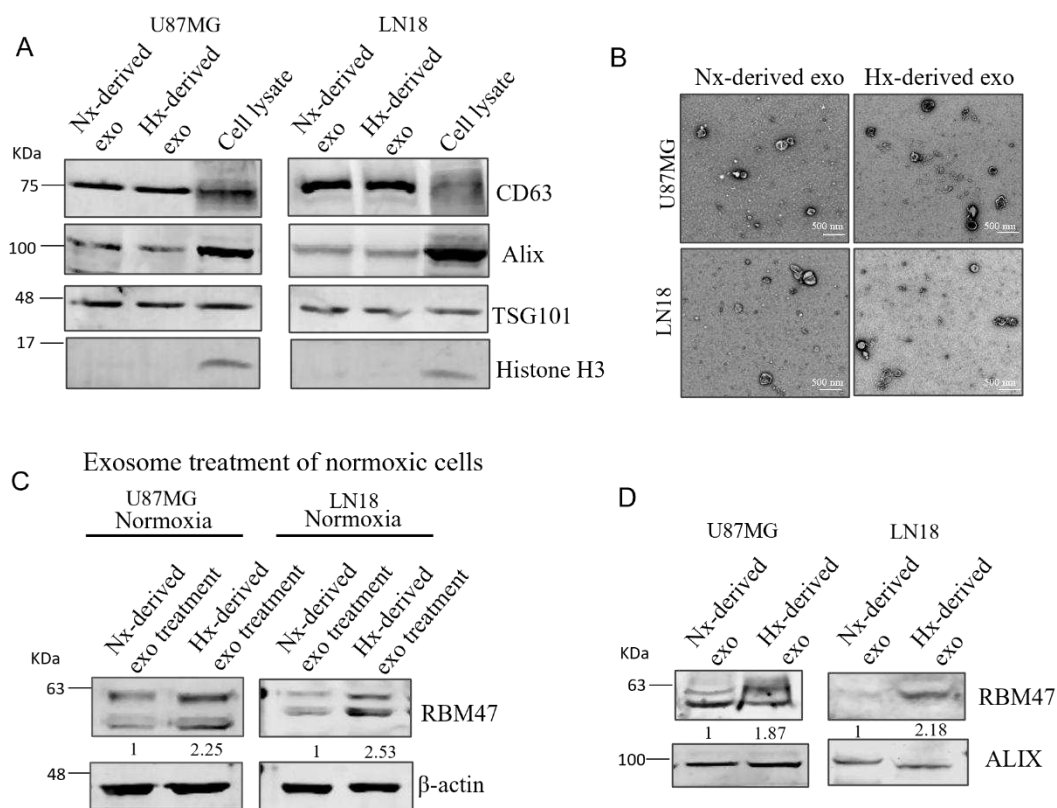
Based on our finding that HIF1α directly orchestrates RBM47 expression, we aimed to determine how this hypoxia-induced protein might influence the broader tumor microenvironment through intercellular communication. Exosomes are widely acknowledged as the principal facilitator of such crosstalk, promoting GBM progression by transferring bioactive cargo between cells [31, 32]. As the molecular composition of these vesicles often mirrors the physiological profile of the originating cell [11, 33], we hypothesized that hypoxia-derived exosomes could modulate RBM47 expression in the recipient cells. To investigate this, we isolated exosomes from normoxic and hypoxic conditioned media using a polymer-based precipitation method. We validated effective purification by the enrichment of canonical exosomal markers CD63, Alix, and TSG101, and by the absence of the negative marker Histone H3 (Fig. 3A). We analyzed the morphology and size distribution using transmission electron microscopy and dynamic light scattering, respectively, which confirmed predominant enrichment of the exosome fraction (Fig. 3B, S3A). Fluorescence imaging further confirmed the internalization of PKH67-labeled exosomes by the recipient cells within 6 hours of treatment (Fig. S3B). We then treated normoxic cells with equal concentrations of exosomes derived from normoxic or hypoxic conditions. Interestingly, normoxic GBM cells treated with hypoxia-derived exosomes exhibited a substantial increase in RBM47 protein as compared to those treated with normoxia-derived exosomes (Fig. 3C). Subsequent immunoblot

analysis revealed that RBM47 itself is preferentially enriched within the cargo of hypoxia-derived exosomes (Fig. 3D). To determine whether this increase resulted from *de novo* synthesis, we performed qRT-PCR and luciferase promoter assay following the treatment with normoxia- and hypoxia-derived exosomes, which demonstrated insignificant change in RBM47 transcription or promoter activity (Fig. S3C, S3D). These findings indicate that the observed increase in RBM47 protein in recipient cells is mediated by a paracrine mechanism involving direct exosomal transfer of protein rather than transcriptional induction. Further, to validate the horizontal transfer, we overexpressed the RBM47-Flag fusion protein in the donor cells. Exosomes were subsequently isolated from the conditioned media of these donor cells and were used to treat the recipient cells. The presence of the RBM47-Flag fusion protein was confirmed by immunoblotting in both isolated exosomes and whole-cell lysates of recipient cells, reinforcing that RBM47 is actively secreted via exosomes and can be internalized by recipient cells (Fig. S3E, S3F, and S3G). Immunofluorescence performed in recipient cells also confirmed the presence of RBM47-Flag fusion protein, thus supporting the previous observations (Fig. S3H).

To validate the clinical relevance of these findings, we analyzed exosomes isolated from the serum of glioma patients and age- and sex-matched healthy controls (n=16), and validated the isolation using TEM and DLS (Fig. S3I, S3J). Immunoblotting demonstrated a marked elevation of RBM47 in patient-derived exosomes, reinforcing its potential function in GBM progression (Fig. 3E).

Finally, we investigated whether this exosomal transfer of RBM47 functionally drives the aggressive GBM phenotype by performing transwell invasion assays. We observed that while hypoxia-derived exosomes significantly promoted the invasive capacity of GBM cells (Fig. 3F, S3K), this effect was substantially diminished when RBM47 was depleted in the donor cells (Fig. S3L, 3G, S3M). These results indicate that RBM47 not only contributes to invasiveness within the hypoxic TME but is a functional component of the hypoxia-induced secretome that actively promotes the invasive spread of glioblastoma.

Fig. 3 Hypoxia-derived exosomes transfer RBM47 to normoxic cells



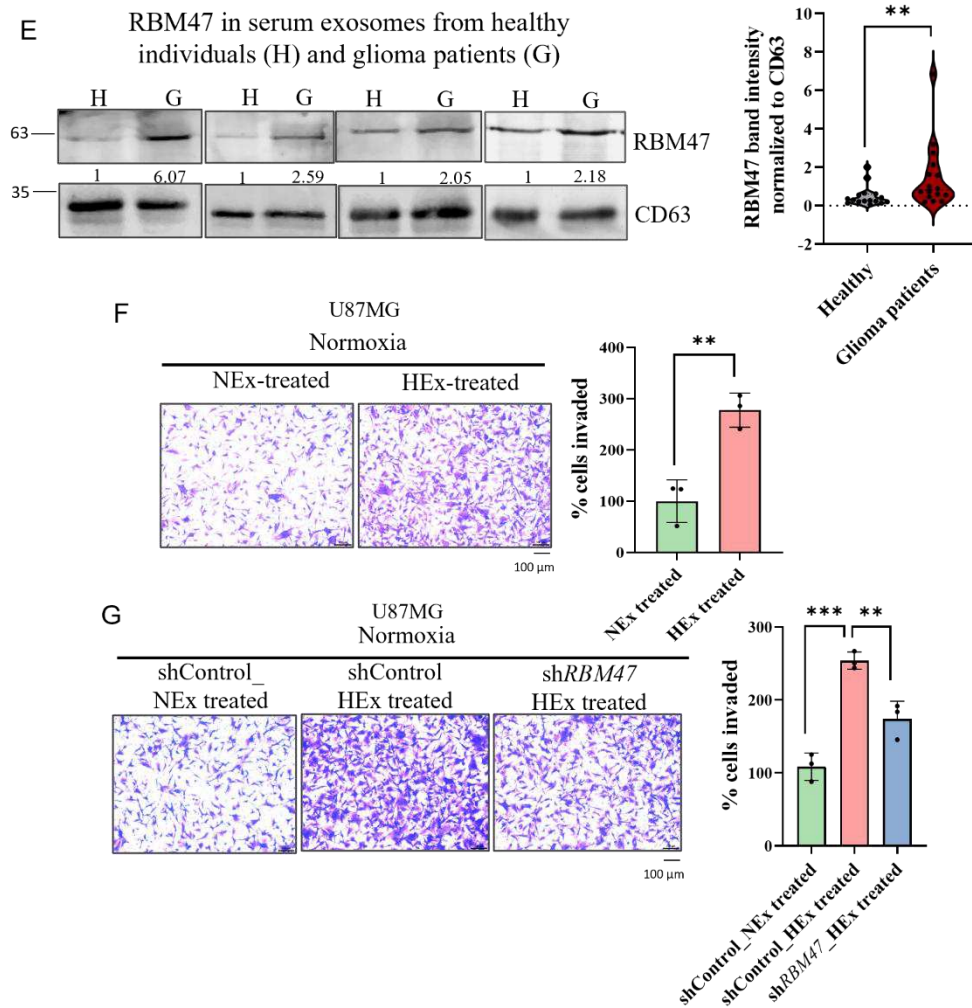


Fig.3 **A** Immunoblot depicting detection of exosome markers (CD63, Alix, and TSG101) in normoxia and hypoxia-derived exosomes isolated from U87MG and LN18 cells, and absence of Histone H3 (negative marker). **B** Transmission electron microscopy (TEM) images of normoxia and hypoxia-derived exosomes isolated from U87MG and LN18 cells. **C** Immunoblot of normoxic cells treated with normoxia and hypoxia-derived exosomes, showing increased RBM47 upon hypoxia-derived exosome treatment. **D** Immunoblot of normoxia- and hypoxia-derived exosomes showing RBM47 enrichment in hypoxia-derived exosomes. **E** Representative images of immunoblotting of exosomes isolated from glioma patients and healthy individuals with quantification. **F** Invasion assay performed following the treatment with normoxia and hypoxia-derived exosomes in U87MG cells. **G** Invasion assay performed in cells treated with exosomes isolated from control and RBM47 knockdown U87MG cells. Data represent mean $\pm$ SD (n = 3 biological replicates); two-tailed Student's t-test. *P $\leq$ 0.05, **P $\leq$ 0.01, ***P $\leq$ 0.001, ns = not significant.

RBM47 mediated the alternative splicing of FN1, favoring the generation of the pro-invasive isoform

Previous reports have suggested that RBM47 plays diverse roles in RNA metabolism, including alternative splicing. To understand the possible consequences of RBM47 upregulation on the alternative splicing landscape, either through hypoxia-mediated transcriptional upregulation or via exosomal transfer, we performed bulk RNA-seq after RBM47 knockdown and after treatment with normoxia- or hypoxia-derived exosomes. We analyzed RNA-seq data using rMATS to detect differential alternative splicing and found that collectively, skipped exons were the dominant AS events (Fig. 4A, B). Upon performing gene ontology analysis of genes undergoing exon skipping in both datasets, we found that the epithelial-to-mesenchymal transition pathway was the most enriched

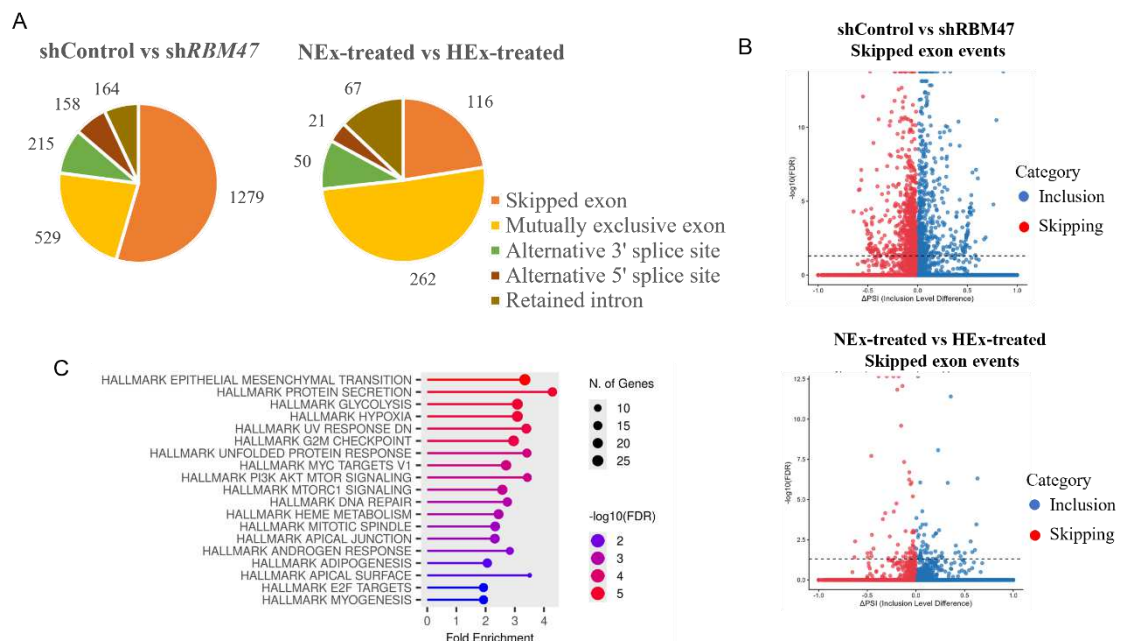
category (Fig. 4C). Additionally, we identified overlapping genes exhibiting exon skipping in both datasets and discovered several shared targets (Fig. 4D). To identify the common splicing events that exhibited opposite regulatory patterns, such that exon inclusion was favored in one dataset while exclusion was favored in another, a discordance analysis was performed, which revealed FN1 as one of the top targets (Fig. 4E). Previous studies have suggested that FN1 splicing is a significant factor in GBM progression; however, its regulation in GBM remains unknown, prompting us to investigate this splicing event.

FN1 is an ECM glycoprotein that frequently exhibits cancer-specific alternative splicing to support cancer cell growth and survival. FN1 is known to undergo alternative splicing at exon 25 and exon 33, encoding an extra domain B (EDB) and an extra domain A (EDA), respectively, giving rise to cancer-specific isoforms. In our RNA-seq data, we observed that knockdown of RBM47 reduced the inclusion of exon 25. Subsequently, we observed an increase in exon 25 inclusion upon treatment with hypoxia-derived exosomes (Fig. 4F). Exon 25 of FN1 encodes for extra domain B (EDB), a type III repeat, which is typically absent in adult tissues; however, it is frequently re-expressed during embryogenesis, wound healing, and cancer progression [34]. To study the splicing event, we designed primers flanking exon 25 and performed semi-quantitative PCR after RBM47 knockdown, observing a decrease in the FN1 EDB+ isoform, whereas the FN1 EDB- isoform remained unchanged (Fig. 5A). Subsequently, we also performed semi-quantitative PCR to assess the effect of RBM47 knockdown on FN1 exon 33 splicing and observed minimal and inconsistent differences (Fig. S4A). Furthermore, we performed qRT-PCR using primers specifically designed to amplify the FN1 EDB+ isoform. Concomitant with the semi-quantitative PCR data, we observed a decrease in the FN1 EDB+ isoform in RBM47 knockdown and a concomitant increase upon RBM47 overexpression and hypoxia-derived exosome treatment (Fig. 5B, C, D).

To investigate alternative splicing, we constructed an FN1 minigene reporter spanning exons 24-26, including introns, ensuring that inclusion of exon 25 results in an in-frame protein with the mCherry reporter (Fig. 5E). Consistent with our qRT-PCR data, RBM47 knockdown markedly attenuated mCherry fluorescence, whereas RBM47 overexpression and hypoxia-derived exosome treatment enhanced the reporter activity (Fig. 5F, G, H).

Collectively, these results establish that RBM47, whether cell-intrinsic or exosome-delivered, orchestrates the oncogenic FN1 EDB+ isoform switch to drive GBM progression. Further, to determine the functional contribution of the FN1 EDB+ isoform, we overexpressed both isoforms in RBM47-depleted cells and performed a transwell invasion assay. The cells overexpressing the FN1 EDB+ isoform exhibited significantly higher invasive potential, validating that RBM47 promotes GBM cell invasion by mediating FN1 alternative splicing, favoring the formation of the FN1 EDB+ isoform (Fig. 5I, J, and S4B, S4C).

Fig. 4 RBM47 regulates the alternative splicing landscape in GBM



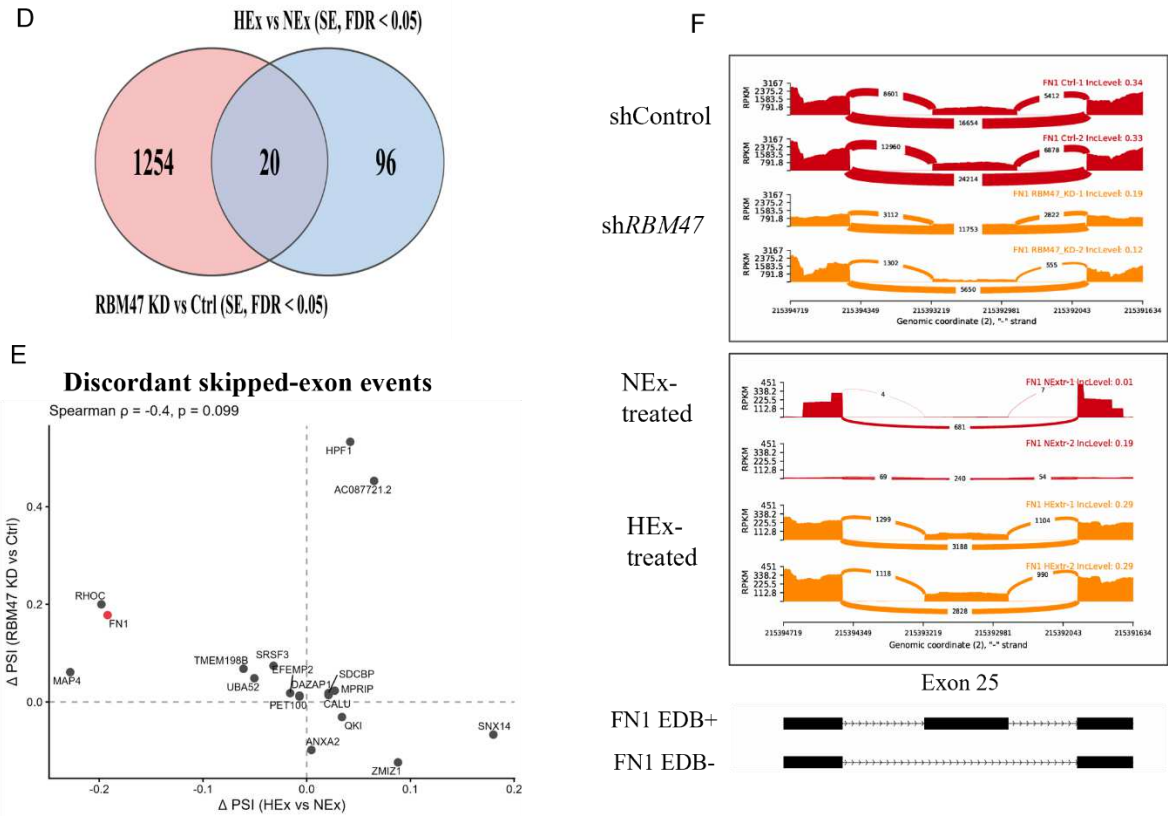
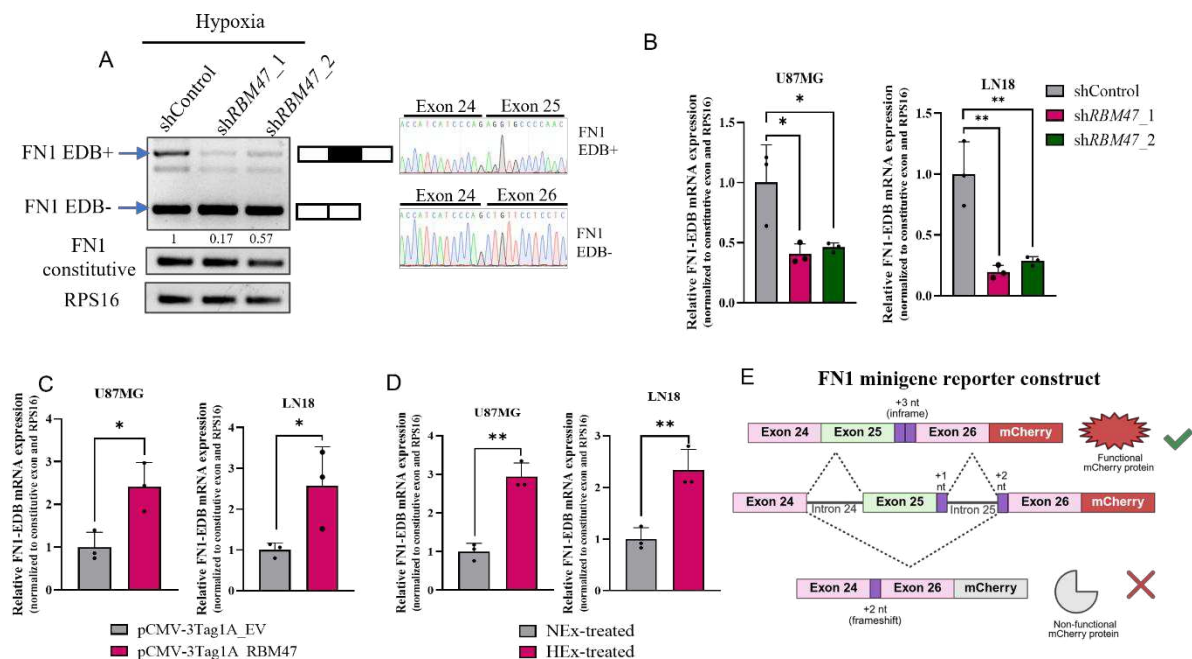


Fig.4 A RNA-seq analysis of alternative splicing events in control vs RBM47 knockdown and in normoxia-derived exosome-treated and hypoxia-derived exosome-treated cells using rMATS at FDR value 0.05. Pie chart showing the proportion of alternative splicing events. **B** Volcano plots of skipped exon events from RNA-seq analysis. **C** Gene Ontology (GO) analysis of skipped exon events in both RNA-seq datasets. **D** A Venn diagram showing overlapping skipped exon events between the two datasets. **E** Graph representing exon skipping events exhibiting a significant discordance pattern between the two RNA-seq datasets. **F** Sashimi plot depicting the FN1 alternative splicing event. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, ns = not significant.

Fig. 5 RBM47 mediates the alternative splicing of FN1, promoting invasiveness



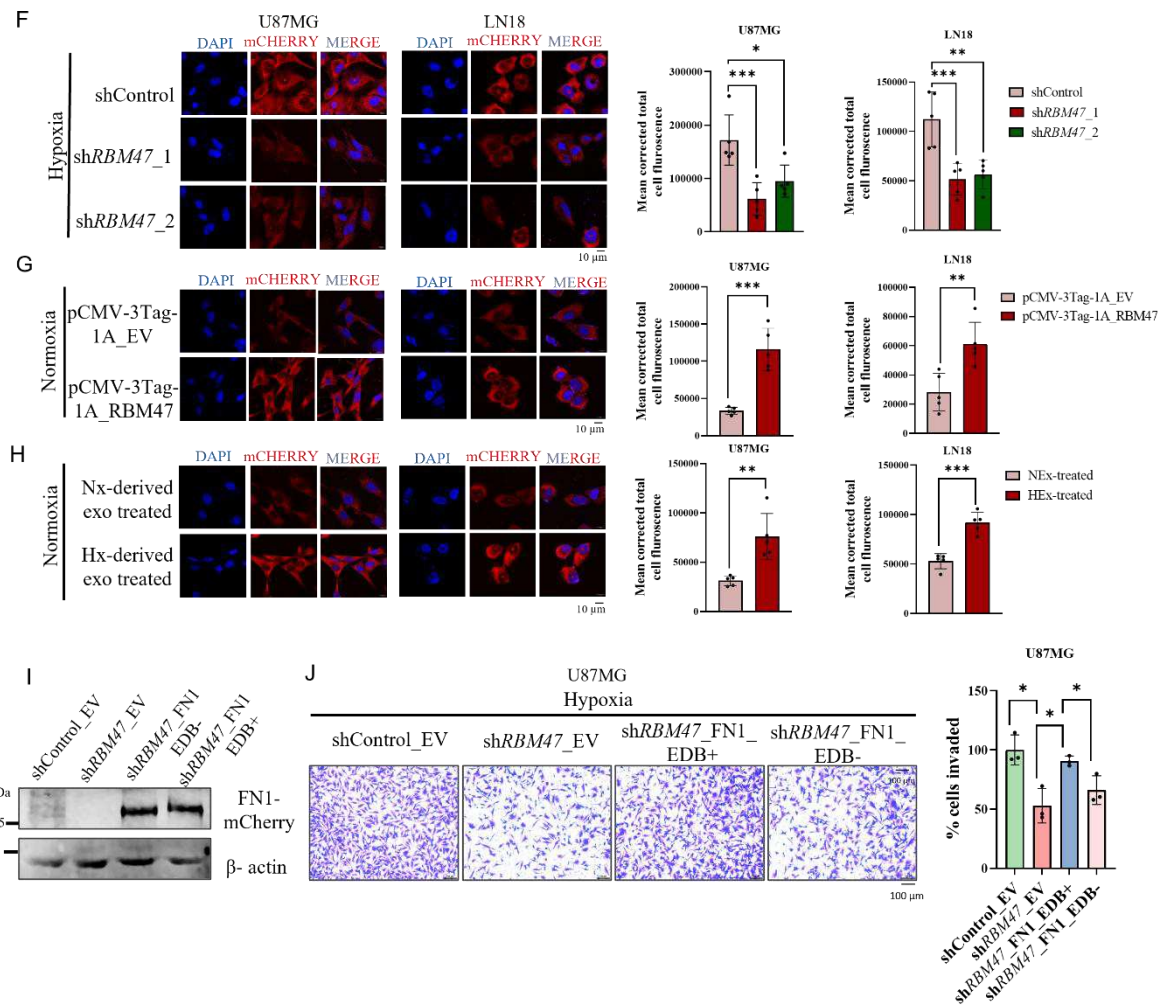


Fig.5 **A** Semi-quantitative PCR showing decreased inclusion of FN1 exon 25 (EDB) under RBM47 knockdown in U87MG cells and a chromatogram for Sanger sequencing of the PCR products obtained. **B** qRT-PCR depicting decreased FN1 exon 25 expression in RBM47 knockdown, **C** increased expression upon RBM47 overexpression, **D**, and upon treatment with hypoxia-derived exosomes in U87MG and LN18 cells. **E** A schematic of the FN1 minigene reporter construct. **F** Representative confocal microscopy images showing mCherry reporter expression using FN1 minigene reporter. mCherry signal, indicating exon 25 inclusion, is decreased upon RBM47 knockdown in hypoxia and is **G** enhanced upon RBM47 overexpression under normoxia. **H** mCherry signal showing increased inclusion of exon 25 under treatment with hypoxia-derived exosomes. **I** Immunoblot showing overexpression of FN1 EDB+ or EDB- isoform in RBM47 knockdown U87MG cells, and **J** invasion assay performed under normoxia. Data represent mean $\pm$ SD (n = 3 biological replicates); two-tailed Student's t-test. *P $\leq$ 0.05, **P $\leq$ 0.01, ***P $\leq$ 0.001, ns = not significant.

RBM47 binds to the intronic region of FN1 and regulates its splicing

RNA-binding proteins frequently orchestrate splicing transitions through their conserved RNA recognition motifs, which recognize specific nucleotide sequences within the intronic or exonic region of their target pre-mRNA [35]. To elucidate the mechanism governing RBM47-mediated alternative splicing of FN1 exon 25, we focused on the sequence-specific interactions between RBM47 and its target pre-mRNA. To identify potential recruitment sites, we utilized the Homer motif discovery tool, which predicted three candidate RBM47-binding sequences, among which one (UGCAUG) was previously validated in literature (Fig. S4D). Consistent with previous reports identifying UGCAUG as a high-affinity binding motif for RBM47 [36], we identified multiple clusters of this sequence within the intron immediately downstream of FN1 exon 25 (Fig. 6A). To confirm a direct physical association, we performed PAR-CLIP-qPCR following RBM47 overexpression. This analysis revealed

significant enrichment of RBM47 on the FN1 pre-mRNA relative to control conditions, with no change in GAPDH, which served as a negative control, thereby validating FN1 as a direct RBM47 target in GBM (Fig. 6B, S4E).

To further map the cis-regulatory architecture required for this interaction, we generated a series of FN1 minigene deletion constructs targeting three distinct downstream intronic regions: +41 to +250 (harboring two binding sites), +564 to +786 (harboring four sites), and +939 to +1088 (harboring two sites) (Fig. 6C). Reporter assays demonstrated that the deletion of the +564 to +786 region most significantly attenuated mCherry expression, indicating a marked decrease in exon 25 inclusion (Fig. 6D). To investigate the splicing mechanism further, we employed the CRISPR-dCas13 (dCasRx) RNA editing system, which allows for precise manipulation of alternative splicing [37]. As our previous results identified the intronic region +564 to +786 downstream of exon 25 as a critical regulatory region, we designed guide RNAs (gRNAs) targeting the RBM47 binding motif (UGCAUG) within this region and cloned them into the pXR003: CasRx gRNA cloning backbone. Simultaneously, we cloned the effector domain of RBM47 downstream to dCasRx in the EF1a-dCasRx-2A-EGFP plasmid. We co-transfected this construct with candidate gRNAs, followed by qRT-PCR analysis, which revealed recruitment of dCasRx-RBM47 to binding site 4 significantly increased exon 25 inclusion (Fig. 6E). To evaluate the functional impact of this targeted splicing modulation, we conducted invasion assays following the recruitment of dCasRx-RBM47 to binding site 4. As predicted by our qRT-PCR results, the induction of exon 25 inclusion significantly enhanced cellular invasiveness (Fig. 6F). Interestingly, recent studies have shown that unconjugated dCasRx can itself modulate splicing in a position-dependent manner, through steric hindrance, effectively blocking splicing factors or the spliceosomal machinery from accessing specific motifs [38]. To test this effect in our target region, we transfected cells with unconjugated dCasRx directed at binding site 4. In contrast to its RBM47-conjugated counterpart, targeting unconjugated dCasRx resulted in a significant decrease in exon 25 inclusion (Fig. 6G). To confirm the effect of dCasRx on the binding of RBM47 at FN1 pre-mRNA, we performed PAR-CLIP in RBM47-overexpressing cells, transfected with dCasRx and either gRNA_4 (targeting FN1) or gControl (non-targeting control). As expected, the qRT-PCR revealed a decrease in RBM47 binding to the FN1 pre-mRNA in the presence of gRNA_4 (Fig. 6H, S4F). Subsequent invasion assays revealed that the reduction in exon 25 inclusion, when targeted by dCasRx + gRNA_4, significantly diminished cell invasiveness (Fig. 6I). These findings suggest that RBM47 functions as a splicing activator for FN1 exon 25 by binding to UGCAUG at +739 bp downstream of exon 25, promoting exon inclusion, and forming pro-invasive isoform FN1 EDB+. Additionally, blocking this site with unconjugated dCasRx reduced exon 25 inclusion, thereby reducing the invasive properties (Fig. 6I). Collectively, these results define a robust HIF1 α -RBM47-FN1 axis that drives the expression of oncogenic fibronectin isoforms in the hypoxic GBM microenvironment.

Discussion

Hypoxia is a well-known driver of malignancy and therapeutic resistance in glioblastoma, nurturing a tumor microenvironment that feeds almost all major hallmarks of cancer progression, ensuring survival and eventually invasion into healthy brain parenchyma [39]. This hypoxia-driven regulation of biological pathways offers cells the means to adapt and thrive. One of the multiple ways in which hypoxia regulates cellular pathways is by regulating alternative splicing, which offers flexibility to shift the balance toward the production of pro-oncogenic isoforms [9]. While intracellular changes aid survival, they are often accompanied by complex mechanisms of cellular crosstalk, facilitating tumor progression. One such key mediator is exosomes. While previous studies have delved into the impact of hypoxia-mediated alternative splicing in GBM, this process, being mediated through the transfer of RNA-binding proteins, remains unexplored.

In this study, we demonstrate that RBM47 is a direct target of HIF1 α and is also a critical orchestrator of the oncogenic alternative splicing landscape in hypoxic GBM. We also observed RBM47 as a significant contributor of invasiveness in hypoxic GBM cells. As several reports have suggested in other solid tumors, such as lung adenocarcinoma and colorectal cancer, RBM47 is documented as a tumor suppressor that inhibits growth and limits EMT progression. In contrast, our study, supported by recent independent reports, reveals that RBM47 functions as an oncogene in GBM, pointing towards its highly tissue-specific function. We further report that RBM47 is actively secreted and transferred via hypoxia-derived exosomes, adding a significant layer of

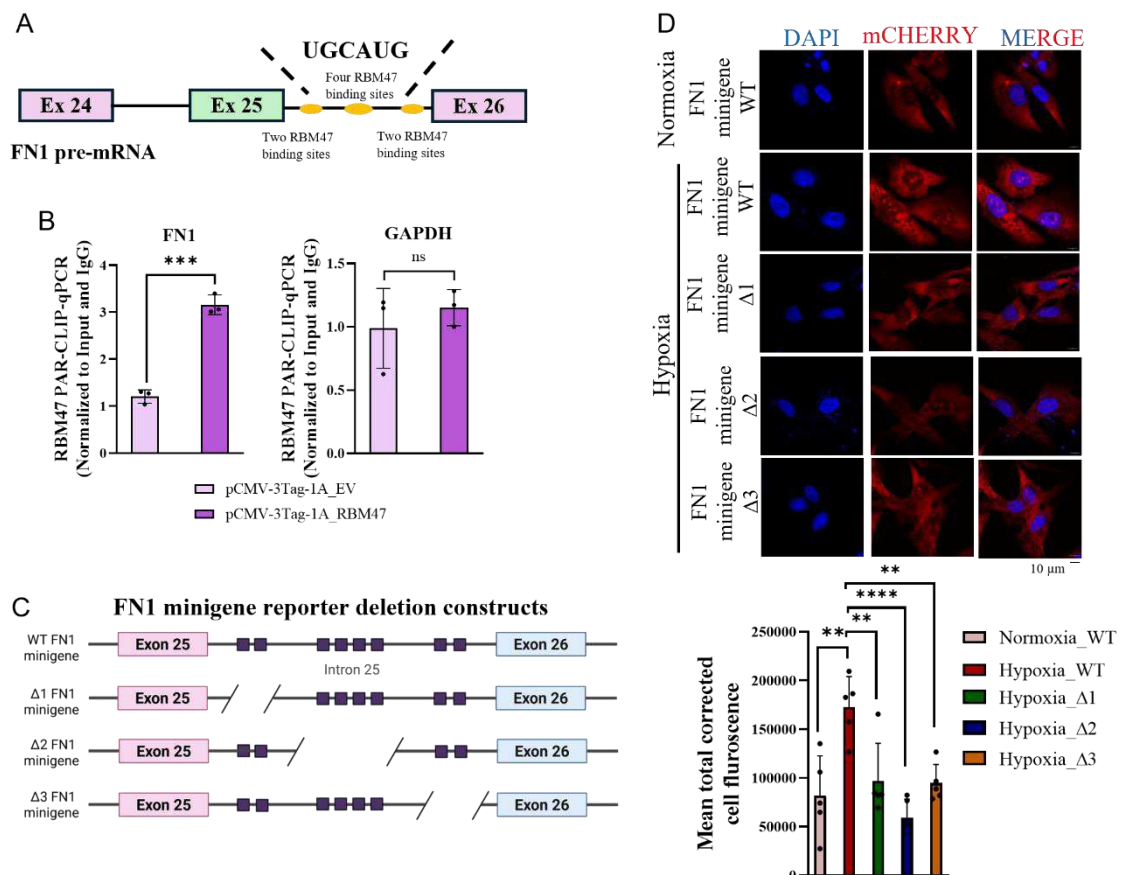
complexity to its role in the tumor microenvironment, effectively expanding the invasive phenotype to better-oxygenated regions of the tumor.

Mechanistically, our study provides evidence that RBM47 modulates the alternative splicing of FN1 in glioblastoma, a critical downstream event that influences the invasive properties of cells. We identified that RBM47 binds to the intronic UGCAUG motif downstream of FN1 exon 25 (+739), facilitating an isoform switch towards the formation of pro-invasive isoform FN1 EDB+. The identification of this binding site aligns with the previous studies that established the importance of these specific repeats in the alternative splicing of FN1 exon 25. These evolutionarily conserved cis-acting hexanucleotide elements are established cell-type-specific regulators that, when present in multiple copies within the downstream intron, significantly enhance exon inclusion [40]. By identifying RBM47 as the specific trans-acting factor that exploits these cis-acting elements, we provide a definitive mechanistic basis for the pro-invasive isoform switch toward FN1 EDB+ in GBM. We further validated the site-specific influence of RBM47 on FN1 exon 25 by utilizing a dCasRx-RBM47 fusion protein, which endogenously increased exon 25 inclusion and promoted invasion.

The correlation between high FN1 EDB+ expression and poor progression of glioblastoma underscores its importance as a biomarker as well as therapeutic target [41]. To explore the therapeutic potential of targeting this axis, we employed unconjugated dCasRx to reverse the splicing. This approach leverages the steric hindrance introduced by a bulky molecule, dCas13, to block the binding of endogenous splicing factors, such as RBM47, to the target pre-mRNA. We observed that targeting the RBM47 binding region with dCasRx effectively reduced exon 25 inclusion, leading to a significant decrease in the invasive phenotype in GBM cells.

Therefore, our study demonstrates a robust HIF1 α -RBM47-FN1 signaling axis that links the hypoxic microenvironment to systemic secretome changes and alternative splicing alterations (Fig. 7). Given that high RBM47 expression correlates with poor clinical outcomes, targeting this axis, either by inhibiting RBM47's RNA-binding activity or disrupting the exosomal transfer of its cargo, may offer a promising therapeutic strategy for mitigating the invasive spread of glioblastoma. Additionally, as the FN1 EDB+ isoform is expressed in malignant cancer cells and recognized as a potential therapeutic target in malignant gliomas, targeting this splicing event by using the dCasRx system can potentially serve as an appealing target for further GBM therapies.

Fig. 6 dCasRx-mediated modulation of FN1 alternative splicing regulates invasive phenotype



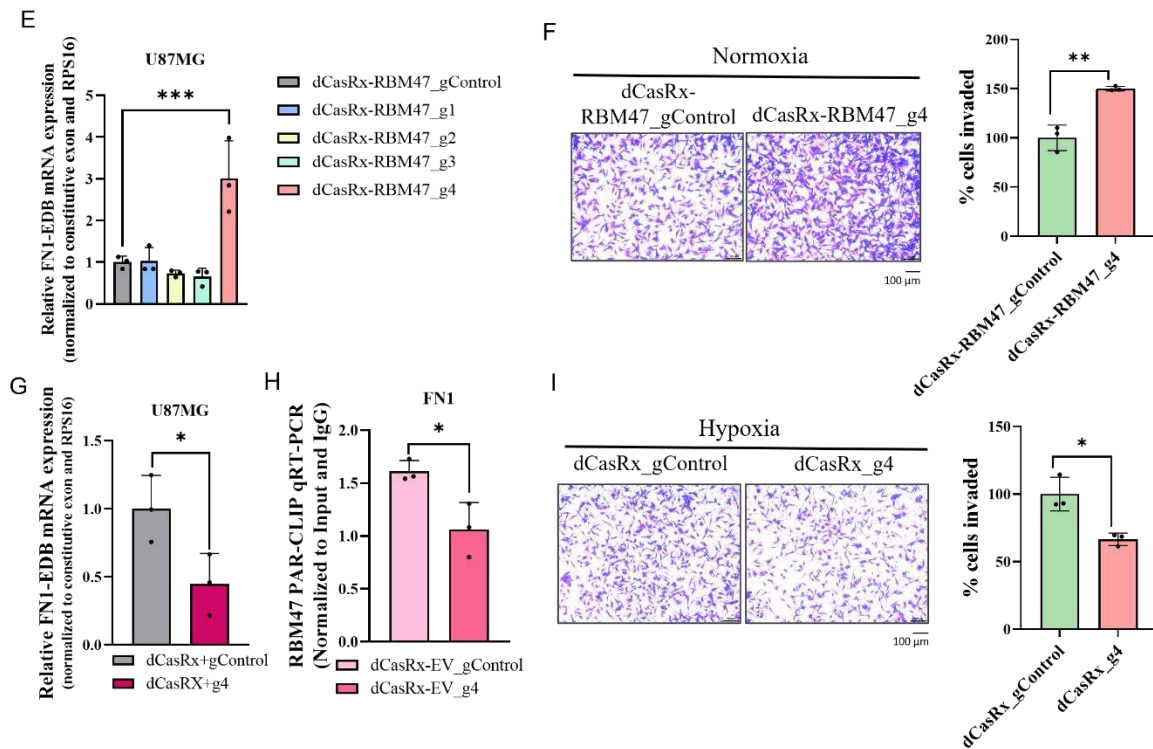


Fig.6 **A** Schematic showing the RBM47-binding site sequence on FN1 pre-mRNA. **B** PAR-CLIP-qPCR performed in cells overexpressing Flag-RBM47, validating its binding to FN1 pre-mRNA. **C** Schematic of FN1 minigene deletion constructs. **D** Representative confocal microscopy images of mCherry signal in RBM47-binding site deletion constructs as compared to the wildtype. **E** qRT-PCR of FN1 exon 25 expression in U87MG cells transfected with dCasRx-RBM47 along with gControl or gRNA targeted to FN1 pre-mRNA. **F** Invasion assay performed in normoxic U87MG cells, showing increased invasion following transfection with dCasRx-RBM47 + gRNA_4 compared to gControl. **G** qRT-PCR depicting decreased FN1 exon 25 expression in U87MG cells transfected with unconjugated dCasRx along with gRNA_4 as compared to gControl. **H** PAR-CLIP-qPCR depicting decreased Flag-RBM47 recruitment to FN1 pre-mRNA upon transfection of dCasRx with gRNA_4. **I** Invasion assay performed in hypoxic U87MG cells shows decreased invasion following transfection with dCasRx + gRNA_4 compared with gControl. Data represent mean $\pm$ SD ($n = 3$ biological replicates); two-tailed Student's t-test. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, ns = not significant.

Fig. 7 Graphical summary

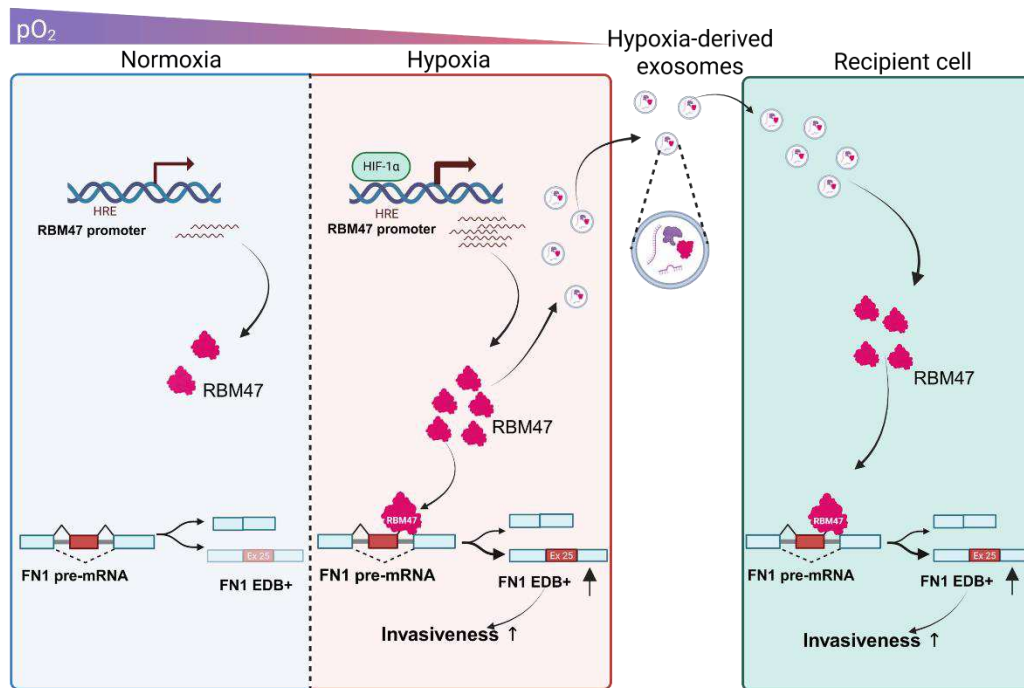


Fig.7 Schematic model illustrating hypoxia-induced RBM47 mediating the alternative splicing of FN1 exon 25 in hypoxic conditions and through its exosomal transfer. Created in <https://BioRender.com>.

Statements & Declarations

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Competing interests

The authors declare no competing interests.

Ethics approval

The study was conducted with approval from the Institute Ethics Committee, Indian Institute of Science Education and Research, Bhopal, India (IISERB/IEC/certificate/2021-1/10) and the Institutional Human Ethics Committee, All India Institute of Medical Sciences, Bhopal, India (LOP/2023/EL042).

Author Contributions

The study was designed and conceived by Sanjeev Shukla and Sommya Sinha. Experiments were designed, conducted, and analyzed by Sommya Sinha and Nirlipta Khandai. Rakesh Kumar, Sumit Raj, Pradeep Chouksey, and Adesh Shrivastava provided the clinical input and resources. The first draft of the manuscript was written by Sommya Sinha with input from Sanjeev Shukla. The funding was acquired by Sanjeev Shukla.

Data availability

The data generated or analyzed during the current study are available in the Supporting Information section. The RNA sequencing data generated in this study are deposited in the GEO database under accession number GSE338398. This study does not report any original code.

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