CPEB2 m6A methylation regulates blood–tumor barrier permeability by regulating splicing factor SRSF5 stability

Mengyang Zhang1,2,3, Chunqing Yang4,5,6, Xuelei Ruan1,2,3, Xiaobai Liu4,5,6, Di Wang4,5,6, Libo Liu1,2,3, Lianqi Shao1,2,3, Ping Wang1,2,3, Weiwei Dong4,5,6 & Yixue Xue1,2,3✉

The blood–tumor barrier (BTB) contributes to poor therapeutic efficacy by limiting drug uptake; therefore, elevating BTB permeability is essential for glioma treatment. Here, we prepared astrocyte microvascular endothelial cells (ECs) and glioma microvascular ECs (GECs) as in vitro blood–brain barrier (BBB) and BTB models. Upregulation of METTL3 and IGF2BP3 in GECs increased the stability of CPEB2 mRNA through its m6A methylation. CPEB2 bound to and increased SRSF5 mRNA stability, which promoted the ETS1 exon inclusion. P51-ETS1 promoted the expression of ZO-1, occludin, and claudin-5 transcriptionally, thus regulating BTB permeability. Subsequent in vivo knockdown of these molecules in glioblastoma xenograft mice elevated BTB permeability, promoted doxorubicin penetration, and improved glioma-specific chemotherapeutic effects. These results provide a theoretical and experimental basis for epigenetic regulation of the BTB, as well as insight into comprehensive glioma treatment.
Gloma is the most common primary tumor of the central nervous system. Currently, the main treatment method is surgery-assisted radiotherapy and chemotherapy. Due to the blood–brain barrier (BBB), it is difficult for macromolecular chemotherapeutics to reach tumor tissues and exert therapeutic effects. The BBB comprises endothelial cells (ECs) with continuous tight junctions and efflux pumps, the basement membrane of the parenchyma (astrocytes), and pericytes. Primary or metastatic tumors in the brain change the BBB to form a reconstructed structure [the blood–tumor barrier (BTB)]. Therefore, selective opening of the BTB is an effective strategy to improve the chemotherapeutic efficacy in brain glioma. Tight junctions comprising tight-junction-related proteins (TJPs) are the main targets for regulating BTB permeability. Down-regulation of TJPs, such as the transmembrane proteins occludin, claudin-5, and cytoplasmic plaque protein zona occludens-1 (ZO-1), can increase BTB permeability. Doxorubicin (Dox), an anthracycline, represses DNA replication, interrupts the cell cycle, and facilitates the generation of intracellular reactive oxygen species to induce tumor cell death. Dox is unable to penetrate the BBB by itself but exhibits potent cytotoxicity when cultured with glioma cells. In the present study, we examined the leakage of Dox in glioblastoma multiforme (GBM) orthotopic xenograft nude mice, as well as the size of the grafted tumors in nude mice, to evaluate BTB permeability.

N6-methyladenosine (m6A), which mainly occurs on the adenine of the highly conserved RRACH (R: purine; A: m6A; H: nonguanine) sequence, is the most abundant form of methylation modification in eukaryotic mRNAs, and its function is determined by the methyltransferase (encoder), demethylase (decoy), and binding protein (code reader). Increasing studies have shown that m6A can functionally regulate eukaryotic transcriptome functions, such as mRNA splicing, nucleation, localization, translation, and stabilization. Moreover, m6A can determine the fate of hematopoietic stem cells during vertebrate embryo development. Methyltransferase 3 (METTL3), among the first identified m6A methyltransferases, is highly expressed in a variety of tumor tissues, where it promotes mRNA translation and regulates tumor cell proliferation by regulation of the methylation of target genes. Insulin-like growth factor 2 mRNA-binding protein 3 (IGF2BP3), a member of the m6A-reading family of IGF2BPs, enhances mRNA stability and translation by recognizing the GG (m6A) C sequence shared by mRNAs. Interestingly, IGF2BP3 is highly expressed in lung and breast cancers, where it regulates tumor occurrence and development. However, few studies have focused on the roles of METTL3 and IGF2BP3 in the regulation of the vascular endothelial cell.

In this study, we revealed mechanisms associated with METTL3-mediated m6A modification and its role in regulating BTB permeability. Moreover, we identified methods for selectively opening the BTB, increasing drug penetration into tumor tissues, and improving chemotherapeutic efficacy.

**Results**

**Knockdown of Upregulated METTL3 and IGF2BP3 Levels in GECs Increases BTB Permeability.** We first analyzed the differentially expressed m6A-related genes in glioma and non-tumor brain tissue using the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) (Figs. 1a and S1a). To evaluate the BTB at the molecular level, we used LCM to capture brain microvessels in NBT, LGG, and HGG from resected human brain specimens (Fig. 1b) and determining levels of METTL3 and METTL14 mRNA using qRT-PCR. We found that compared with that in NBT, METTL3 mRNA level increased significantly in LGG and HGG microvessels, whereas METTL14 mRNA level showed no significant difference (Fig. 1c). We then co-cultured three cell lines (U87, U373, and U251) with ECs to establish in vitro BTB models (GECs), with NHAs co-cultured with ECs (AECs) used as controls. Western blot analysis showed that METTL3 levels in GECs from the co-culture of ECs with U251 increased significantly relative to those in controls (Fig. S1b); therefore, we used GECs from co-cultured ECs and U251 for subsequent experiments. Figure S1c shows a simple schematic of the in vitro BBB and BTB models. We then determined the TEER value and used FITC–dextran tracers and HRP flux to evaluate BBB and BTB permeability, with the results revealing BTB permeability as higher than that of the BBB (Fig. S1d, e). We then knocked down METTL3 levels in GECs using shRNA [sh-METTL3 and sh-negative control (sh-NC)] and measured the TEER value, FITC-dextran, and HRP flux to analyze BBB integrity and permeability. We found that compared with the sh-NC group (cells transfected with empty plasmids), the TEER value of the sh-METTL3 group was significantly reduced, whereas FITC–dextran and HRP-flux signals increased significantly (Fig. 1d, e). Additionally, western blot analysis indicated significant decreases in ZO-1, occludin, and claudin-5 levels in the sh-METTL3 group (Fig. 1f). Moreover, IF assays revealed the continuous distribution of TJPs in the control and sh-NC groups, whereas this was not the case in the sh-METTL3 group (Fig. 1g).

As shown in Figs. 1a and S1a, we noted significantly higher expression of the m6A-binding protein IGF2BP in GBM tissues relative to NBT. Therefore, upon investigating the expression levels of IGF2BP1, IGF2BP2, and IGF2BP3 in AECs and GECs, we found IGF2BP3 expression levels to be significantly elevated in GECs relative to those in AECs (Fig. 1h). Notably, after IGF2BP3 knockdown, the TEER values, expression levels, and continuous distribution of ZO-1, occludin, and claudin-5 decreased significantly, whereas the FITC-dextran and HRP-flux signals increased significantly, relative to observations in the sh-NC group (Fig. 1i–l).

**Knockdown of elevated CPEB2 expression in GECs increases BTB permeability.** We analyzed the RNA-Seq and MeRIP-Seq data from the GSE115850, GSE157544, and GSE182607 datasets from the GEO database and TCGA for molecules associated with glioma prognosis. We detected 28 factors that were differentially expressed in the sh-METTL3 group (Fig. 2a), with correlation analysis of the expression profiles of these factors in ECs performed using the BBomics database (http://bioinformaticstools.mayo.edu/bbomics/). The correlation heatmap shown in Fig. 2b revealed correlations of three molecules with METTL3 and IGF2BP3 (Fig. 2c). We then detected mRNA levels in AECs and GECs, finding that CPEB2 mRNA level was significantly elevated in GECs (Fig. 2d) and significantly decreased in the sh-METTL3 group (Fig. 2e). Additionally, RIP experiments showed that CPEB2 mRNA level was significantly enriched by the introduction of the IGF2BP3 antibody (Fig. 2f). Further analysis of TCGA data indicated that CPEB2 was significantly associated with poor prognosis in glioma (Fig. 2g). Moreover, we observed that CPEB2 protein level significantly higher in GECs than in AECs (Fig. 2h), as well as that the sh-CPEB2 group exhibited a significant decrease in the TEER value and significant increases in FITC–dextran and HRP-flux signals relative to the sh-NC group (Fig. 2i–j). Furthermore, western blotting and IF assays confirmed significant reductions in ZO-1, occludin, and claudin-5 levels in the sh-CPEB2 group relative to levels in the sh-NC group (Fig. 2k, l).

METTL3-mediated m6A modification maintains CPEB2 levels via IGF2BP3-dependent regulation of CPEB2 mRNA stability and is essential for regulating BTB permeability. To determine
the molecular mechanism underlying inhibition of BTB permeability by Mettl3, we measured the overall m6A level in the sh-Metttl3 and sh-Metttl4 groups using an m6A dot blotting assay. The results showed significant reductions in m6A levels in the sh-Metttl3 group (Fig. 3a). To determine whether the m6A modification of CPEB2 is mediated by Mettl3, we performed MeRIP-qPCR analysis in sh-Metttl3 GECs, revealing significant reductions in m6A-modified CPEB2 mRNA following Mettl3 knockdown (Fig. 3b).

Subsequently, we used mRNA half-life and nascent RNA assays to investigate the effect of Mettl3 and IGF2BP3 on CPEB2 mRNA stability. Although the half-life of CPEB2 mRNA was significantly shorter following Mettl3 knockdown (Fig. 3c), we detected no significant change in nascent CPEB2 mRNA levels in the sh-Metttl3 group relative to the sh-NC group (Fig. 3d), with similar results obtained in the sh-IGF2BP3 group (Fig. 3e, f). Using the RMBase database (https://rna.sysu.edu.cn/rmbase/index.php), we found that m6A modification occurs in CPEB2 mRNA in the 3′ untranslated region (UTR) (Fig. 3g, h).

To determine whether CPEB2 modifies BTB permeability according to the levels of m6A modification to CPEB2 mRNA, we mutated the m6A-methylation site and stably transfected GECs with either wild-type (WT) or mutant (Mut) constructs (Fig. 3i). The results showed significant reductions in methylation levels in the Mut group according to MeRIP-qPCR analysis (Fig. 3j). Additionally, RIP assay revealed that significantly lower levels of CPEB2-Mut overexpression significantly reversed the effects of sh-Metttl3 (Fig. 3k). Notably, we observed that CPEB2 protein level in the Mut group was significantly reduced, whereas the FITC-dextran and HRP-flux signals and BTB permeability increased significantly (Fig. 3l–r). We subsequently overexpressed CPEB2-Wt and CPEB2-Mut in sh-Metttl3 GECs, with the results showing that CPEB2-Wt overexpression significantly reversed the effects of sh-Metttl3 on the TEER value, HRP flux, and the status of TJs, whereas CPEB2-Mut overexpression had no effect on these parameters (Fig. 3s–u).
CPEB2 enhances SRSF5 mRNA stability and regulates BTB permeability. We then performed a single-gene differential analysis of CPEB2 through TCGA and analyzed the splicing factors retrieved from the SpliceAid 2 database (http://193.206.120.249/splicing_tissue). The Venn diagram showed six splicing factors in the intersection (Fig. 4a). Single-gene co-expression analysis of CPEB2 with these molecules identified SRSF1, SRSF2, SRSF5, SRSF6, and SRSF7 as showing a significant positive correlation with CPEB2 expression (Fig. 4b). We further examined their respective mRNA levels in AECs and GECs, finding that SRSF5 mRNA level was significantly elevated in GECs (Fig. 4c). Subsequent detection of SRSF5 mRNA level in GECs revealed a significant decrease in the sh-CPEB2 group (Fig. 4d), with confirmation that SRSF5 protein level was significantly higher in GECs than in AECs (Fig. 4e). Additionally, compared with the sh-NC group, the TEER value and protein levels of ZO-1, occludin, and claudin-5 were significantly reduced, whereas FITC–dextran and HRP-flux signals increased significantly in the sh-SRSF5 group (Fig. 4f–i). Moreover, these proteins were discontinuously distributed in the sh-SRSF5 group according to IF staining (Fig. 4j, l). Further investigation of the mechanism associated with SRSF5 regulation by CPEB2 using sh-CPEB2 GECs and RNA-stability and nascent RNA assays revealed that the half-life of SRSF5 mRNA was significantly shorter in sh-CPEB2 GECs relative to that in sh-NC GECs (Fig. 4m), whereas the nascent SRSF5 mRNA level remained unchanged (Fig. 4n).

CPEB2 regulates BTB permeability via SRSF5. We then performed western blot analysis to detect protein levels of SRSF5 in CPEB2-knockdown and -overexpressing GECs, with the results showing significant decreases and increases in SRSF5 expression in these groups, respectively (Fig. 5a). Additionally, we showed that the TEER value and TJP levels were significantly decreased, whereas FITC–dextran and HRP-flux signals increased significantly in the sh-
CPEB2, sh-SRSF5, and sh-CPEB2 groups. However, in the sh-CPEB2 + SRSF5-overexpression [SRSF5(+) group, we observed a reversal of the effects caused by CPEB2 knockdown (Fig. 5b–e).

SRSF5 induces ETS1 exon 7 inclusion to regulate BTB permeability. We predicted the transcription factors that bind to the promoter regions of ZO-1, occludin, and claudin-5, respectively, using the JASPAR database (https://jaspar.genereg.net/) and predicted SRSF5-specific RNA-binding motifs using the catRAPID database (http://service.tartaglialab.com/page/catrapid_group). Additionally, we used TCGA to identify co-interaction networks involving SRSF5 (Fig. 6a) and GTEx and TCGA to evaluate the expression of these factors in NBT and glioma tissues (Fig. 6b), with the five molecules showing the highest expression selected for qRT-PCR verification. We found that ETS1 mRNA level was significantly increased in GECs (Fig. 6c), and that these levels were significantly decreased following SRSF5 knockdown (Fig. 6d). Interestingly, ETS1 contains multiple splice variants (Fig. 6e), and we found that levels of the P51-ETS1 protein were significantly higher in GECs than in AECs, whereas P42-ETS1 level was significantly lower in GECs than in AECs (Fig. 6f). RIP assays subsequently confirmed that SRSF5 binds to ETS1 mRNA (Fig. 6g). We then performed ETS1 exon 7 minigene assays. Figure 6h shows that expression of EGFP signaled the retention of the entire open reading frame (ORF) after skipping...
P51-ETS1 regulates BTB permeability by promoting the transcription of ZO-1, occludin, and claudin-5. We then established GECs stably expressing sh-SRSF5 + P51-ETS1(+) and sh-SRSF5 + P42-ETS1(+), revealing significant increases in the TEER value and TJP levels and significant reductions in the FITC–dextran and HRP-flux signals in the sh-SRSF5 + P51-ETS1 group and no change in the sh-SRSF5 + P42-ETS1(+) group (Fig. 7a–d). Additionally, dual-luciferase reporter assays confirmed significant increases in the activities of the ZO-1, occludin, and claudin-5 promoters in the pEX3–P51-ETS1 group relative to that in the empty vector group (Fig. 7e). Furthermore, ChIP assays demonstrated that P51-ETS1 directly bound to the promoter regions of ZO-1, occludin, and claudin-5 (Fig. 7f).

**BTB permeability in Dox-treated GBM xenograft mouse models.** We injected U251-LUC cells into nude mice to establish a model of in situ GBM orthotopically transplanted tumors. After 20 days, we injected sh-METTL3, sh-IGF2BP3, CPEB2-Mut, sh-SRSF5, and/or sh-P51-ETS1 constructs in the tail vein of each mouse, respectively, using the AAV2/9 serotype to enable transfection into mouse cerebral microvessels. After 3 weeks, the mice were treated with Dox once every week. Observation of in situ tumor size using BLI on the treatment day and days 15 and 30 after treatment revealed that Dox treatment after knocking down the target molecules in GECs significantly delayed tumor growth and induced a longer survival time as compared with that in the control group (Fig. 8a–c). Additionally, evaluation of Dox distribution in frozen tumor tissue sections using fluorescence microscopy indicated increased red fluorescence in the sh-METTL3, sh-IGF2BP3, CPEB2-Mut, sh-SRSF5, and sh-P51-ETS1 group, indicating that knockdown of these molecules increased the amount of Dox crossing the BTB into the glioma (Fig. 8d). To quantitatively measure Dox in U251 tumors and normal brain parenchyma, we harvested tumor-bearing and contralateral brain
Fig. 5 CPEB2 Regulates BTB Permeability via SRSF5. a The expressions of SRSF5 in sh-CPEB2 and CPEB2(+) BTB model were detected by western blot analysis. Data are represented as mean ± SD (n = 3). **P < 0.01 vs. sh-CPEB2 group. b, c The permeability and integrity of the sh-CPEB2, sh-SRSF5, sh-CPEB2 + sh-SRSF5, sh-CPEB2 + SRSF5(-) BTB model in vitro were detected by TEER values, FITC-dextran, and HRP flux. Data are represented as mean ± SD (n = 5). **P < 0.01 vs. sh-CPEB2 group. d The expressions of ZO-1, occludin, and claudin-5 in sh-CPEB2, sh-SRSF5, sh-CPEB2 + sh-SRSF5, sh-CPEB2 + SRSF5(-) BTB model detected by western blot analysis. Data are represented as mean ± SD (n = 3). ##P < 0.01 vs. sh-NC group. e The distributions of ZO-1, occludin and claudin-5 in sh-CPEB2, sh-SRSF5, sh-CPEB2 + sh-SRSF5, sh-CPEB2 + SRSF5(-) BTB model were determined by IF staining. Scale bar represents 50 μm.

Discussion

The BTB partially or fully inhibits the transport of macromolecular chemotherapeutic drugs to glioma tissue to exert their therapeutic effects. Therefore, regulating the selective permeability of the BTB represents an effective strategy for improving chemotherapy efficacy in glioma. In this study, we identified significant upregulation of METTL3, IGF2BP3, CPEB2, SRSF5, and P51-ETS1 group was significantly greater than that in the control group. Furthermore, IF staining of ZO-1, occludin, and claudin-5 revealed significant reductions in the TJPs in the sh-METTL3, sh-IGF2BP3, CPEB2-Mut, sh-SRSF5, and sh-P51-ETS1 group (Fig. 8f). Figure 8g provides a diagram of the proposed mechanism in which CPEB2 m6A methylation regulates BTB permeability by regulating SRSF5 mRNA stability.

M6A methyltransferases include METTL3, METTL14, and WT1-associated protein16–18. In the present study, we evaluated three NBTs and 10 glioma tissue samples to analyze the differential expression of m6A-related genes and further verified them in the BTB model. We identified METTL3 as highly expressed in GECs, and that its knockdown increased BTB permeability, suggesting that METTL3 participates in regulating BTB permeability. Recent significant progress has been made in elucidating the role of m6A modifications in various stages of the RNA life cycle19. For example, m6A reportedly participates in the progression of various cancers20, with METTL3 expression identified as upregulated in a variety of tumor tissues and playing both carcinogenic roles and increases in levels of m6A modification. Additionally, high levels of METTL3 have been found in cancers associated with the liver and lungs21. To further clarify the role of METTL3, we explored the role of CPEB2, which is targeted by METTL3, using RNA-Seq and MeRIP-seq data from the GEO database, followed by MeRIP-qPCR analysis upon METTL3 knockdown to examine the mechanism of METTL3-mediated m6A modification of CPEB2 mRNA. CPEB2 binds to the cytoplasmic polyadenylation element in the 3′ UTR to regulate the mRNA translation of target genes and reportedly plays an important role in the development of triple-negative breast cancer22. In the present study, we found that CPEB2 was highly expressed in GECs, and that its knockdown increased BTB permeability. Moreover, we found that METTL3 enhanced CPEB2 mRNA stability. Similar studies in gastric cancer cells showed that METTL3 promotes m6A modification of the mRNA of recombinant protein hepatoma-derived growth factor (HDGF), whereas IGF2BP3 directly recognizes and binds to the m6A site on HDGF mRNA, enhancing its stability23.

The IGF2BP family includes IGF2BP1, IGF2BP2, and IGF2BP324, with studies reporting IGF2BP3 as highly expressed in a variety of tumors, thereby suggesting it as a potential therapeutic target25–27; however, its function in blood vessels has not been reported. In the present study, we found significantly higher IGF2BP3 levels in GECs relative to IGF2BP1 and IGF2BP2 levels.
Moreover, after IGF2BP3 knockdown, we identified increases in BTB permeability, suggesting its regulatory role in this process. In eukaryotes, IGF2BPs are recognized as m6A readers, and reports indicate that IGF2BPs regulate the expression of MYC proteins in an m6A-dependent manner in hepatocarcinoma cells. Furthermore, IGF2BP1 promotes serum response factor-dependent transcription in cancer in an m6A-dependent manner, whereas IGF2BP2 targets m6A-containing differentiation-antagonizing non-protein-coding RNA (DANCR) to enhance its translation, with IGF2BP2 and DANCR jointly promoting the occurrence of pancreatic cancer. In the present study, we demonstrated that IGF2BP3 binds and enhances the stability of CPEB2 mRNA. Interestingly, mutating the CPEB2 m6A site increased BTB permeability and promoted Dox entry into tumor cells.

Alternative splicing is among the most common gene-regulation mechanisms and plays an important role in the complex regulation of protein functions. Splicing dysregulations are closely related to the occurrence and development of many tumors in humans. Additionally, alternative splicing of pre-mRNA is ubiquitous in mammalian cells, with ~95% of human...
mRNA being formed by alternative splicing31–33, which helps regulate gene expression and expand proteome diversity. SR proteins are widely regarded as active splicing regulators and promote exon inclusion34. Among them, SRSF1 promotes the inclusion of CD33 exons in Alzheimer’s disease to enhance the transcription and expression of full-length CD33 and regulates their specific interaction with CD33 pre-mRNA, thus altering the protein levels on the cell surface35. Moreover, SRSF6 reportedly increases the inclusion of OGDHL exon 3, thereby affecting pancreatic cancer cell metastasis36. SRSF5 contains two N-terminal RNA-recognition motifs and an arginine/serine-enrichment domain and plays an important regulatory role in RNA splicing and translation37. Abnormal expression of SRSF5 was reported in breast, renal, and lung cancers38, and studies identified SRSF5 involvement in the alternative splicing of HSD17B2 mRNA in prostate cancer and its regulation of tumor growth via alternative splicing of CCA1 pre-mRNA in lung cancer39–41. In the present study, we found significant decreases in SRSF5 levels following CPEB2 knockdown, suggesting that SRSF5 mRNA might be a CPEB2 target. Additionally, we identified elevated SRSF5 levels in GECs and noted that BTB permeability increased upon SRSF5 knockdown, suggesting the involvement of SRSF5 in regulating BTB permeability. We further confirmed the binding affinity of CPEB2 with SRSF5 mRNA, and that CPEB2 knockdown significantly reduced SRSF5 levels and shortened the SRSF5 mRNA half-life (although levels of nascent SRSF5 mRNA remained unchanged), suggesting that CPEB2 might regulate BTB permeability by binding to SRSF5 mRNA and increasing its stability.

We detected possible SRSF5 targets through correlation analysis using TCGA data and subsequently showed that ETS1 level was significantly decreased following SRSF5 knockdown, suggesting ETS1 as an SRSF5 target. ETS1 is involved in regulating tumor cell proliferation, development, apoptosis, metastasis, invasion, and angiogenesis42, and its ETS domain (transcription-activation domain) and helical DNA-binding domain are involved in regulating the maturation of vascular ECs and endothelial barrier function43. ETS1 is highly expressed in a variety of tumor tissues, and inhibiting ETS1 can block tumor proliferation, migration, and invasion in vivo44,45. Moreover, expression of ETS-family transcription factors is essential for EC differentiation, with ETS1 and ETS2 affecting tumor angiogenesis and metastasis in the tumor microenvironment, especially in ECs46,47. Among the splice variants of mouse Ets1, P42-ETS1 and P51-ETS1 were identified as transcription factors with different targets and activities48. For example, MDA-MB-231 breast cancer cells express P51-ETS1 but not P42-ETS1, with only 10% of primary breast cancer cells simultaneously expressing P51-ETS1

Fig. 7 P51-ETS1 Regulates BTB Permeability by Promoting the Transcription of ZO-1, Occludin, and Claudin-5. a, b The permeability and integrity of the sh-SRSF5, sh-SRSF5 + P51-ETS1(+) and sh-SRSF5 + P42-ETS1(+) BTB model in vitro were detected by TEER values, FITC-dextran, and HRP flux. Data are represented as mean ± SD (n = 5). ***P < 0.01 vs. sh-NC group, ###P < 0.01 vs. sh-SRSF5 group. c, d Effects of sh-SRSF5, sh-SRSF5 + P51-ETS1(+) and sh-SRSF5 + P42-ETS1(+) on the expressions of ZO-1, occludin and claudin-5 were analyzed by western blot assays. Data are represented as mean ± SD (n = 3). ##P < 0.01 vs. sh-NC group. e, f Dual luciferase reporter assays were performed to determine the binding sites of ETS1 and ZO-1, occludin and claudin-5 in HEK293T cells. **P < 0.01 vs. sh-NC group, ##P < 0.01 vs. sh-SRSF5 group.
and P42-ETS1. In the present study, we found that in contrast to P42-ETS1, P51-ETS1 was highly expressed in GECs, and that its knockdown increased BTB permeability and overexpression reversed this effect. Conversely, P42-ETS1 overexpression had no effect on BTB permeability, suggesting that P51-ETS1 participates in regulating BTB permeability. We further verified that P51-ETS1 binds to the promoter regions of ZO-1, occludin, and claudin-5 to increase their transcription levels. Furthermore, we identified that SRSF5 promotes the inclusion of ETS1 exon 7 and regulates BTB permeability in GECs, which further elucidated the function of SR proteins in alternative splicing.

Dox is an anthracycline antitumor antibiotic used in the clinical treatment of malignant tumors. However, Dox has difficulties entering the brain parenchyma and reaching an effective concentration. In this study, we demonstrated that Dox can be effectively delivered to the brain through GECs, which may provide a new strategy for the treatment of brain tumors.
Fig. 8 BTB Permeability in Dox-treated GBM Xenograft Mouse Models. a BLI of intracranial tumor in mice bearing U251-LUC derived GBM after treatment with Dox on indicated days after GBM implantation. b Statistical analyses for the BLI signal intensity of U251 tumors. Data are presented as the mean ± SD (n = 5, *P < 0.01 vs. control group). c Kaplan–Meier survival curves of mice bearing GBM xenografts with indicated treatments, compared with sh-NC group (n = 5 mice/group; two tailed log-rank test, *P < 0.01). d Fluorescence microscope images showing the distribution of DOX in the glioma. DOX distribution is in red and cell nuclei were stained with DAPI (blue). Scale bar, 50 μm. e Quantitative analysis of DOX in excised mouse brains. Data are presented as the mean ± SD (n = 3, *P < 0.01). f Immunofluorescent analyses of CD31 (green) and ZO-1, occludin and claudin-5 (red) in GBM xenografts from mice with indicated treatments. Scale bar, 50 μm. g The schematic diagram of the mechanism with which CPEB2 m6A methylation regulates BTB permeability by regulating splicing factor SRSF5 mRNA stability.

therapeutic concentration for the treatment of glioma due to the BBB29. A previous study showed that the combined application of KHDRBS3 can promote the transmembrane transport of Dox and induce apoptosis of glioma cells39. To further evaluate the regulatory effects of these factors on BTB permeability, we used GBM orthotopic xenograft mouse to demonstrate that mutation of m6A sites in CPEB2 mRNA and METTL3, SRSF5, and P51-ETS1 knockdown in GECs combined with Dox administration significantly increased BTB permeability, significantly reduced the size of GBM-transplanted tumors, prolonged survival, and increased the amount of Dox crossing the BTB into tumors. Low passive paracellular permeability and high expression levels of active efflux drug transporters in BBB together limit the exposure of many anticancer drugs to the brain52,54. Increased passive permeability does not always equate to increased drug accumulation, as P-glycoprotein efflux transporters are still active in tumors despite disrupted vasculature52,55. Thus, the link between METTL3 or IGF2BP3 and efflux transporters deserves further investigation.

In summary, we found that upregulated levels of METTL3 and IGF2BP3 in GECs increase the stability and expression of CPEB2 mRNA via m6A methylation. This enables CPEB2-mediated increases in the stability of SRSF5 mRNA to promote ETS1 exon-7 inclusion and formation of the P51-ETS1 spliceosome, which stimulates transcription of ZO-1, occludin, and claudin-5 to regulate BTB permeability. Furthermore, in vivo knockdown of these proteins in GBM xenograft mice enhanced the entry of Dox through the BTB and promoted the apoptosis of glioma cells. These findings provide a theoretical and experimental basis for the epigenetic regulation of the BTB, as well as strategies for the comprehensive treatment of gliomas.

Methods
The Cancer Genome Atlas (TCGA) and Genotype–Tissue Expression Project (GTEx) database analysis. Data from glioma patients concerning gene expression, correlation, and prognosis were obtained from TCGA (https://portal.gdc.cancer.gov/). Normal brain tissue (NBT) data were obtained from the GTEx database (https://www.gtexportal.org/home/). All analyses were performed in R (https://www.r-project.org/).

Laser capture microdissection (LCM). All human glioma specimens and NBT were obtained from the Department of Neurosurgery of Shengjing Hospital, China Medical University. All participants signed and provided informed consent, and this study was approved by the Institutional Review Board of Shengjing Hospital of China Medical University. Surgical human brain specimens of tissues, low-grade glioma (LGG), and high-grade glioma (HGG) were frozen and sectioned at 10-μm thickness using a microtome/cryostat (Microm International, Walldorf, Germany). Subsequently, LCM was performed, as previously described37. Sections of vessels in glioma tissue (or NBT) were stained using the Ulex europaeus agglutinin I (UEA-I) fluorescent dye-tagged lectin (Vector Laboratories, Burlington, ON, Canada) according to manufacturer instructions. LCM was then conducted using the ArcturusXT microdissection instrument. Captured microvessels were transferred into CapSure LCM caps (Applied Biosystems, Foster City, CA, USA) and further processed for RNA isolation.

Cell lines and cell culture. The hCMEC/D3 cells (ECs) immortalized human brain endothelial cell line was provided by Dr. Couraud (Cochin Institute, Paris, France) and cultured on culture inserts (0.4-μm pore size; Corning, Lowell, MA, USA) coated with 150 μg/mL Cultrex rat collagen I (R&D Systems, Minneapolis, MN, USA). Cells were cultured in endothelial base medium (EBM-2; Lonza, Walkersville, MD, USA) containing 5% fetal bovine serum (FBS) “Gold” (PAA Laboratories, Pasching, Austria), 1% penicillin–streptomycin (Life Technologies, Paisley, UK), 1.4 mmol/L hydrocortisone (Sigma-Aldrich, St Louis, MO, USA), 1% lipid concentrate (Life Technologies), 5 g/mL ascorbic acid (Sigma-Aldrich, St Louis, MO, USA), 10 mmol/L HEPES (PAA Laboratories GmbH), and 1 ng/mL human basic fibroblast growth factor (Sigma-Aldrich). ECs were maintained for no more than 30 passages. The U251 human glioblastoma and HER293T cell lines was purchased from the Cell Resource Center of Shanghai Institute of Biological Sciences (Shanghai, China) and stored in Dulbecco’s modified Eagle medium containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Life Technologies). Normal human astrocytes (NHAs) were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA) and cultured in the astrocyte culture medium (Dulbecco’s Modified Eagle Medium (DMEM) (ScienCell Research Laboratories). The U251 cell line was previously tested for mycoplasma contamination and authenticated by short tandem-repeat DNA profiling. All cells were cultured in a humidified incubator at 37 °C and 5% CO2.

Establishment of an in vitro BTB and Blood–Brain Barrier (BBB) model. The in vitro BBB model was established by co-cultivation of ECs and U251 cells, as previously described47. U251 cells were seeded in 6-well plates at a density of 2 × 104 cells/well and cultured for 2 days. ECs were seeded at a density of 2 × 105 cells/well on an insert coated with Cultrex rat collagen I (R&D Systems), which was then placed in the 6-well plates. After co-culturing for 4 days to reach confluence, gloma microvascular ECs (GECs) were obtained. Both ECs and U251 cells were cultured with the prepared EBM-2 medium, which was changed every 2 days. The in vitro BBB model used the same method of co-culturing ECs with NHAs to obtain microvascular ECs (AECs).

Real-time PCR (qRT-PCR) assays. Total RNA was extracted using TRIzol reagent (Life Technologies), and a SYBR PrimeScript primary RT-PCR kit (Takara Bio, Beijing, China) was used to evaluate RNA-expression levels using the 7500 Fast RT-PCR system (Applied Biosystems), with GAPDH used as an endogenous control. Relative expression was calculated using the 2−ΔΔCt method. The primers are provided in Supplementary Table 1.

Western blot assays. Western blot assays were performed as previously described48. For details of the experiment, please refer to the Supplementary Information. The primary antibodies used for western blotting were as follows: METTL3 (15073-1-AP; 1:500 dilution; Proteintech), IGF2BP3 (14642-1-AP; 1:500 dilution; Proteintech), CPEB2 (ab51069; 1:500 dilution; Abcam), SRSF5 (ab67175; 1:500 dilution; Abcam), ETS1 (sc-55381; 1:500 dilution; Santa Cruz Biotechnology), ZO-1 (1:67300; 1:500 dilution; Thermo Fisher Scientific), occludin (71-1500; 1:500 dilution; Thermo Fisher Scientific), claudin-5 (35-2500; 1:500 dilution; Thermo Fisher Scientific), GAPDH (60004-1-lg; 1:10000 dilution; Proteintech). The antibody used are provided in Supplementary Table 3.

Cell Transfection. Cell transfections were performed as previously described14. ECs were seeded in 24-well plates and transfected using Opti-MEM I and Lipofectamine LTX reagents (Life Technologies) under fusion conditions of ~80% according to manufacturer instructions. Stable cell lines were selected using geneticin (G418) or puromycin. After 4 weeks of application, G418-resistant (or puromycin-resistant) clones were obtained. Plasmids and corresponding empty vectors were constructed using GenePharma (Shanghai, China). Protein knockdowns were confirmed using western blot (Fig. S2). The CPEB2 m6A site mutation was introduced using the QuikChange site-directed mutagenesis kit (Agilent Technologies) according to manufacturer instructions. The target sequences and plasmid vectors are shown in Supplementary Table 2.

Transendothelial electrical resistance (TEER) assays. The TEER value was measured using a Millicell-ERS instrument (Millipore, Billerica, MA, USA) after establishing the in vitro BTB model. The media in the upper and lower chambers of the Transwell were replaced with fresh media, and the TEER value was measured after 12 h. Low serum (0.5%) was used to keep the cell viability at ~90% 25 °C. We obtained the final resistance (GΩm) by subtracting the background resistance from the measured blocking resistance and then multiplying it by the effective surface area of the filter.
Horseradish peroxidase (HRP) flux assays. After establishing the in vitro BTB model, 0.5 μmol/L HRP (Sigma-Aldrich) was added to the upper chamber of the Transwell, and incubated for 1 h at 37 °C. Then 200 μL TMB color developing solution and 5 μL small chamber culture medium were added to a 96-well plate and incubated at ambient temperature for 30 min. The OD value of each 96-well sample was measured using a microplate reader to calculate the HRP content of the lower chamber.

Fluorescein isothiocyanate (FITC)-dextran permeability assays. FITC–dextran (4 kDa; Sigma-Aldrich) was added to the upper chamber of the Transwell insert (2 mg/mL) to assess in vitro BTB permeability. The medium was collected from the lower chamber of the Transwell insert after 1 h of incubation, and a multi-function microplate reader was used to measure the FITC–dextran content.

Immunofluorescence (IF) assays. Cell slides were fixed with paraformaldehyde for 30 min and washed three times with phosphate-buffered saline with Tween 20 (PBST). After penetrating the membrane with Triton X-100 for 10 min, the slides were washed again with PBST. After blocking with 5% bovine serum albumin for 2 h, the slides were incubated with the corresponding antibodies for ZO-1, occludin, and claudin-5 overnight. After reheating at 25 °C for 30 min, the primary antibody was washed with PBST, and the corresponding secondary antibody was applied at 25 °C for 2 h. Three washes with PBST for 10 min were then performed. The nuclei were stained with DAPI for 5 min. After the staining was completed, the PBST wash was performed three times, and the slides were sealed with 50% glycerol. Slides were then observed and photographed under a confocal microscope.

Methylated RNA immunoprecipitation (MeRIP)-qPCR assays. Cells were incubated with an anti-m6A antibody (ab208577; Abcam) at 4 °C for 1 h and then mixed with pre-washed Pierce protein A/G magnetic beads (88,803; Thermo Fisher Scientific). The m6A antibody was digested with proteinase K digestion buffer, and methylated RNA was purified for qRT-PCR analysis.

M6A dot blot assays. PolyA(+) RNA was first denatured by heating at 65 °C for 5 min and then transferred to a cellulose nitrate membrane (Achemers, GE Healthcare, USA) using a Bio-dot device (Bio-Rad, USA). The membranes were then UV-crosslinked, sealed, and incubated overnight at 4 °C with m6A antibody (1:1000, Abcam) and then incubated with HRP-conjugated goat anti-mouse IgG (1:300, Proteintech, USA). The membranes were then visualized by enhanced chemiluminescence (Bio-Rad). The membrane was stained with 0.02% Methylene Blue (MB) in 0.3 M sodium acetate (pH 5.2) to ensure consistency across groups.

RNA immunoprecipitation (RIP) and RNA pull-down assays. For details on these experiments, please refer to the Supplementary Methods.

Nascent RNA capture. Nascent RNA was detected using the Click-IT nascent RNA capture kit (Thermo Fisher Scientific). 5-Ethynyl uridine (EU) was incorporated into nascent RNAs, followed by RNA capture using streptavidin magnetic beads and qRT-PCR analysis.

RNA stability measurement. Cells were cultured in medium containing 5 μg/mL actinomycin D (Act D, Nobletryder, China). Subsequently, total RNA was extracted at different time points and detected via qRT-PCR. Compared with the zero time point, the half-life of RNA was determined according to the level of RNA reduced to 50% at a set time point.

Minigene assays. The ETS proto-oncogene 1 (ETS1) exon7 minigene was constructed by inserting the exons and flanking intron region of ETS1 into the pGient Vector. The pGient Vector. The ETS1 exon7 and its flanking intron region were amplified from genomic DNA using the ETS1 minigene-F and minigene-R primers. The specific primers for complete EGFP RNA were used to determine the splicing efficiency by qRT-PCR. Primers are shown in Supplementary Table 1.

Chromatin immunoprecipitation (ChIP) assays. ChIP assays were performed using the Simple ChIP enzymatic chromatin IP kit (Cell Signaling Technology, Danvers, MA, USA) according to manufacturer instructions. For details on this experiment, please refer to the Supplementary Methods. Primers used for ChIP PCR are shown in Supplementary Table 4.

Establishment of the orthotopic brain glioblastoma xenograft model. For the animal studies, a protocol detailing experimental procedures following the China Medical University guidelines was submitted to and approved by Ethics Committee of China Medical University. Female Balb/c nude mice (8-weeks old) were purchased from Shanghai Experimental Animal Science & Technology Co., Ltd. (Shanghai, China). U87 and U251 cells stably expressing luciferase constructs (1 x 106 cells/mouse) were injected into the caudate nucleus of the right brain hemisphere of nude mice. Recombinant AAV2/9 was used to repress gene expression in mice cerebral microvascular ECs. Short-hairpin (sh) RNA sequences were ligated into pA2K-CMV-bGlobin-eGFP-H1-shRNA (Obio Technology, Beijing, China). The sequences are shown in Supplementary Table 2. For details on this experiment, please refer to the Supplementary Methods.

Bioluminescence in vivo imaging (BLI). Mice were intraperitoneally injected with 200 μL of 150 mg/kg D-fluorescein (Promega, Madison, WI, USA) and then anesthetized with isoflurane after 5 min. Animals were imaged using the IVIS Lumina II imaging system (Xenogen, Alameda, CA, USA).

Dox uptake by tumors. After 2 h of Dox treatment on day 30, brain tissue was collected after anesthesia and perfusion and divided into left (control) and right (tumor-tissue-containing) brain hemispheres. Brain samples were then homogenized and soaked in acidic ethanol (50% ethanol in 0.3 N hydrochloric acid) for 24 h at 4 °C to completely extract Dox. A freezing centrifuge was then used to centrifuge samples at 14,000 rpm for 10 min. Dox concentration in the clear supernatant was analyzed by high-performance liquid chromatography (HPLC) and expressed as Dox per gram of tissue.

Histological analysis. At the end of treatment, mice were sacrificed, and the tissues were fixed for histologic analysis. Brain tissue was collected, prepared, and sectioned according to standard procedures. The tissue was embedded in OCT compound and cut into 8-µm-thick sections for IP staining. Fluorescence microscopy was used to qualitatively assess the permeability to Dox.

Statistics and reproducibility. Statistical analysis was performed using Student’s t test or one-way ANOVA in GraphPad Prism7, and data are presented as the mean ± standard deviation (SD). P < 0.05 was considered significant. The number of samples per independent experiment is described in the legends.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. All data that support the findings of this study are available from the corresponding author upon reasonable request. Full-length uncropped original western blots used in the manuscript are shown in Supplementary Information. The numerical data that make up the all graphs in the paper are shown in Supplementary Data 1.

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Author contributions

Y.X.X. and Y.H.L. contributed to conceive and design the project. M.Y.Z., C.Q.Y., and J.W. carried out the experiments. Y.X.X. and Y.H.L. contributed to analyze data and write the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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