

N⁶-Methyladenosine Modulates Nonsense-Mediated mRNA Decay in Human Glioblastoma



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Abstract

The N⁶-methyladenosine (m⁶A) modification influences various mRNA metabolic events and tumorigenesis, however, its functions in nonsense-mediated mRNA decay (NMD) and whether NMD detects induced carcinogenesis pathways remain undefined. Here, we showed that the m⁶A methyltransferase METTL3 sustained its oncogenic role by modulating NMD of splicing factors and alternative splicing isoform switches in glioblastoma (GBM). Methylated RNA immunoprecipitation-seq (MeRIP-seq) analyses showed that m⁶A modification peaks were enriched at metabolic pathway-related transcripts in glioma stem cells (GSC) compared with neural progenitor cells. In addition, the clinical aggressiveness of malignant gliomas was associated with elevated expression of METTL3. Furthermore, silencing *METTL3* or overexpressing dominant-negative mutant METTL3 suppressed the growth

and self-renewal of GSCs. Integrated transcriptome and MeRIP-seq analyses revealed that downregulating the expression of METTL3 decreased m⁶A modification levels of serine- and arginine-rich splicing factors (SRSF), which led to YTHDC1-dependent NMD of *SRSF* transcripts and decreased SRSF protein expression. Reduced expression of SRSFs led to larger changes in alternative splicing isoform switches. Importantly, the phenotypes mediated by METTL3 deficiency could be rescued by downregulating *BCL-X* or *NCOR2* isoforms. Overall, these results establish a novel function of m⁶A in modulating NMD and uncover the mechanism by which METTL3 promotes GBM tumor growth and progression.

Significance: These findings establish the oncogenic role of m⁶A writer METTL3 in glioblastoma stem cells.

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Introduction

Nonsense-mediated mRNA decay (NMD) contributes to mRNA surveillance pathways that affect a broad spectrum of cellular functions and maintain homeostasis. Although the primary function of NMD in reducing errors in gene expression by eliminating mRNA transcripts that contain premature termination codons (PTC) is well known, the mechanisms of target mRNA selection for NMD are still not well understood.

RNA methylation is a reversible modification of mRNA and has been linked to many types of cancer. N⁶-Methyladenosine (m⁶A) represents the most abundant methylation modification of mRNAs in eukaryotes (1–3), and it regulates almost every aspect of mRNA metabolism, including RNA processing (4, 5), transport from the nucleus to cytoplasm (6, 7), translation (8, 9), and decay (10, 11). The m⁶A methylation marks on mRNA are dynamically regulated in mammals through the methyltransferase complex, composed of the catalytic subunit METTL3, and demethylases (e.g., FTO and ALKBH5; refs. 7, 11–13) and are detected by "m⁶A readers." YTH domain-containing proteins, including YTHDF1-3, YTHDC1, and YTHDC2, act directly as "m⁶A readers" and can interact with distinct subsets of m⁶A sites to produce different effects on RNA processing (9, 10, 14–16). The function of m⁶A RNA methylation is highly variable and context-dependent, and its underlying mechanisms in the recognition of NMD targets are not well understood.

Recent studies have revealed that m⁶A methylation of mRNA results in diverse regulatory functions in cancer initiation and

progression. In addition, dysregulated m⁶A methylation is closely related to various types of cancers. It has been reported that the m⁶A methyltransferase METTL3 is required for the growth, survival, and invasion of cancer cells (17–19). The m⁶A demethylase FTO was found to play a critical oncogenic role in promoting acute myeloid leukemia (AML; ref. 20). Although evidence is emerging, linking m⁶A modulators and tumorigenesis, it remains to be determined whether m⁶A modifications on different regions of mRNA, recognized by distinct readers, will lead to different cell fates. METTL3 elevates m⁶A methylation modification to promote glioma stem cells (GSC) stemness by enhancing *SOX2* stability in glioblastoma (GBM; ref. 21). Controversially, another research group found that knockdown (KD) of *METTL3* dramatically promoted GSC self-renewal and tumorigenesis (22). Moreover, *ALKBH5*, which decreases m⁶A modification in GSCs, exerts an important tumorigenic role in the progression of GBM through regulation of *FOXM1* expression (23). These findings have raised questions about whether m⁶A methylation modifications that affect GBM progression are dependent on the RNA sequence and are dynamically regulated.

Here, we observed preferential distribution of m⁶A peaks in GBM cells. Elevated METTL3 in clinical specimens correlated with higher grades of gliomas, increased tumor recurrence, and worse clinical outcomes. Moreover, we found that silencing of *METTL3* led to reduced aggressive and tumorigenic capabilities, as well as diminished GSC phenotypes in GBM cells. Methylated RNA immunoprecipitation-seq (MeRIP-seq) and RNA-seq analyses revealed that KD of *METTL3* led to downregulation of NMD-targeted splicing factor mRNA transcripts that was dependent on the m⁶A reader YTHDC1. Importantly, splice alterations of targeted mRNAs were critical for tumor growth inhibition and suppression of stemness due to *METTL3* KD. Together, our study identifies m⁶A methyltransferase METTL3 as a modulator of NMD to sustain malignancy in GBM.

Materials and Methods

Glioma specimens and brain tissue collection

Both GBM and normal brain tissue surgical specimens were collected in The First People's Hospital of Changzhou and Xiangya Hospital of Central South University, in accordance with institution-approved protocols. Written informed consent was obtained from each study participant after a thorough explanation of the procedure and its risk, in compliance with the Declaration of Helsinki. Collected specimens were further split into two parts for RNA extraction and protein isolation. If only a limited amount of specimens was obtained, only an RNA extraction assay was performed. Three freshly obtained specimens were specifically used for primary cell establishment (see section below). All specimens were examined by neuropathologists to verify tumor types and grades.

Cell culture and reagents

The human GBM cell lines of U251 and U87MG were provided as a gift from Dr. Jun Cui's laboratory at Sun Yat-sen University and were grown in Gibco DMEM containing 10% FBS (Gibco) at 37°C in a humidified atmosphere containing 5% CO₂.

For the culture of primary GBM cells, surgically removed GBM specimens were washed with and minced in sterile PBS. Next, a single-cell suspension was obtained by pressing the minced tissues through 40-µm cell strainers (Falcon). Dissociated cells

were cultured in DMEM supplemented with 15% FBS (Gibco), 1 × B27 (Invitrogen), 20 ng/mL epidermal growth factor (CantonBIO), and 20 ng/mL FGF (CantonBIO) at 37°C in a humidified atmosphere containing 5% CO₂. All primary cells were passaged every 7 days.

Subcutaneous tumor model and intracranial GBM xenograft model

Five-week-old female Balb/c athymic nude mice were purchased from Model Animal Research Center of Nanjing University and housed in individually ventilated microisolator cages. Nude mice were divided into three groups of 6 mice each.

For subcutaneous tumor model, each mouse was injected subcutaneously in the right flank with 2 × 10⁶ U87MG cells (*METTL3*-KD or control) in 100-µL PBS. Tumor sizes were determined with calipers every 5 days by measuring the length and width. Tumor volumes were calculated according to the following formula: volume (mm³) = (length × width × width)/2. Fifty-eight days after the tumor cell injection, the mice were sacrificed and tumor xenografts were removed, weighted, fixed in formalin, and stored at 4°C.

For intracranial GBM Xenograft Model, each mouse was intracranially injected with 5 × 10⁵ luciferase-transduced U87MG cells (*METTL3*-KD or control) in 10-µL PBS solution as described previously (24). Tumor growth was monitored by using a Xenogene IVIS Spectrum system (Caliper Life Sciences) weekly.

Animal experiments were approved by the Animal Care and Use Committee of Sun Yat-sen University.

Measurements of total m⁶A mRNA levels

Total m⁶A content was measured in 200-ng aliquots of total RNA extracted from *METTL3*-KD or scrambled, control U87MG, or U251 GBM cells using an m⁶A RNA methylation quantification kit (EpiGentek), according to the manufacturer's instructions.

MeRIP-seq

Total RNA was isolated from *METTL3*-KD or scrambled control U87MG GBM cells, as mentioned above, and the mRNA was further separated using Dynabeads mRNA Purification Kit (Invitrogen, 61006). After fragmentation, using RNA fragmentation reagent (Invitrogen, AM8740), the obtained mRNA was immunoprecipitated with anti-m⁶A antibody (Synaptic Systems, 202003), and then washed and eluted by competition with m⁶A sodium salt (Sigma-Aldrich, M2780). Both input samples and immunoprecipitation (IP) eluates were used for preparing the sequencing libraries using NEBNext Ultra RNA Library Prep Kit for Illumina and submitted for sequencing using Illumina HiSeq 2500. Reads, mapping and m⁶A peak calling, were performed as previously described (25). The m⁶A peaks of sh*METTL3* U87MG cells were from the overlapped peaks of sh*METTL3*-1 and sh*METTL3*-2.

RNA immunoprecipitation-qPCR analysis

In YTHDC1 RNA immunoprecipitation (RIP)-qPCR experiments, U87MG cells were harvested and lysed in IP lysis buffer (150 mmol/L KCL, 0.5 mmol/L DTT, 5 mmol/L EDTA, 0.5% NP-40, 25 mmol/L Tris, pH 7.4). Each lysate was further divided into three groups for anti-YTHDC1, anti-IgG (negative control), and input (positive control). Either YTHDC1 antibody (Abcam) or IgG was added to each sample to enrich RNA-binding protein (RBP). Subsequently, the RBP of interest, together with the bound RNA, was collected using Dynabeads (Thermo Fisher Scientific).

After washing off unbound material, the RBP was digested by proteinase K, and the RNA bound to immunoprecipitated RBP was purified and reverse transcribed into cDNA. Then, qPCR assay was performed to measure the %Input of *SRSFs* mRNAs in each group. The primer sequences used for RIP-qPCR analysis were provided in Supplementary Table S2.

Statistical analysis

All analyses were performed using GraphPad Prism version 5.0 (GraphPad Software). The survival curves for combined expression of *METTL3* and splicing factors were plotted according to the Kaplan–Meier method, using PROGene V2 software online (<http://watson.compbio.iupui.edu/chirayu/proggene/>). The association among the expression levels of *METTL3* and splicing factors was analyzed using Spearman rank correlation. Data were presented as the mean ± SD, and the significance levels of all tests were two-sided. The *P* value of less than 0.05 was considered statistically significant and marked as *; a *P* value less than 0.01 or 0.001 was marked as ** and ***, respectively.

Results

The m⁶A methylome in GSCs is distinct from that of normal neural progenitor cells

Previous studies have suggested that GSCs are derived from mutated neural progenitor cells (NPC), which are critical for GBM tumorigenesis (26). Using MeRIP-seq data from GSE87515 (23) and GSE54365 (27), we first compared the m⁶A peaks at each locus between GSCs and NPCs, respectively (Fig. 1A) and then divided the peaks into three categories comprising gene loci with m⁶A peak enrichment in (i) both GSCs and NPCs; (ii) GSCs, or (iii) NPCs (denoted as "shared," "GSC," and "NPC," respectively; Fig. 1B; Supplementary Fig. S1A). We found that 9,627 loci in GSCs had elevated levels of m⁶A modifications that were initially unmodified in NPCs (Fig. 1B). We further analyzed the signaling pathways of the three categories and found that loci with elevated levels of m⁶A modifications were associated with metabolic pathways (Fig. 1C; Supplementary Fig. S1B). Specifically, the cancer metabolism–associated loci *TGFB2*, *TGFB3*, and *TEAD2* were highly enriched with m⁶A modifications in GSCs (Fig. 1D).

The m⁶A methyltransferase METTL3 is elevated in GBM clinical specimens

To determine the expression of m⁶A modulators in GBM, we first queried the Repository for Molecular Brain Neoplasia Data (REMBRANDT) datasets. Compared with normal brain controls, GBM specimens displayed increased levels of *METTL3*, *METTL14*, *YTHDF1*, and *YTHDF2* (Supplementary Fig. S2A). To confirm these findings, we examined the mRNA expression of m⁶A modulators in 36 GBM specimens from the First People's Hospital of Changzhou. *METTL3* and *YTHDF2* were markedly elevated in GBM patient samples as compared with normal brain tissues (Fig. 2A). Western blot analysis also showed higher *METTL3* protein levels in GBMs compared with that in normal brain tissues (Supplementary Fig. S2B). Next, we examined *METTL3* expression in a paraffin-embedded human glioma tissue array by IHC. As expected, there were remarkably more *METTL3*-positive cells in GBMs (grade 4) than in normal brain tissues or in lower grade gliomas (Fig. 2B). Intriguingly, significantly higher levels of

METTL3 were found in recurrent tumors compared with paired, original tumors (Fig. 2C).

Next, we designed a screen based on a CRISPR/Cas9 library of sgRNAs to identify potential m⁶A modulators regulating GBM cell proliferation and GSC sphere formation (Supplementary Table S1; Supplementary Fig. S2C). We transduced U87MG cells with 16 sgRNA lentiviral supernatants (one sgRNA per well), containing a puromycin selection cassette to eliminate uninfected cells. Infected cells were grown in a two-dimensional monolayer and three-dimensional Matrigel for 7 days. After three rounds of screening, we found that *METTL3* and *YTHDF2* were involved in the maintenance of GBM cell proliferation (Supplementary Fig. S2D) and sphere-forming capacity (Fig. 2D). Notably, high *METTL3* levels in GBMs predicted poorer patient survival [GBM patient data from REMBRANDT, GSE7696 (28), and GSE43378 (Fig. 2E; ref. 29)]. However, the expression of other modulators, except *YTHDF2*, did not significantly correlate with the time of GBM patient survival (Supplementary Fig. S2E). Collectively, *METTL3* was upregulated in GBMs and may be critical for tumor growth.

The m⁶A methylation catalytic domain of METTL3 is essential for its function in GBM

To test whether *METTL3* was essential for GBM cell growth, we suppressed the expression of *METTL3* in GBM cells through shRNA-expressing lentiviruses. Both sh*METTL3*-1 and sh*METTL3*-2 could downregulate *METTL3* expression in GBM cells (Supplementary Fig. S3A and S3B). As expected, depletion of *METTL3* also led to significantly reduced m⁶A modification levels of mRNAs in both GBM cell lines (Supplementary Fig. S3C). Compared with cells expressing control shRNAs, both *METTL3*-KD GBM cell lines (U87MG and U251) showed significantly reduced cell proliferation (Fig. 3A). Similar results were also obtained in *METTL3*-KD primary GBM cells derived from 3 patients with GBM at Xiangya Hospital (Fig. 3A). Moreover, overexpression of the m⁶A catalytic inactive mutant *METTL3* acted in a dominant-negative manner to suppress cell growth and m⁶A modification levels of mRNAs in U87MG and U251 cells (Fig. 3B; Supplementary Fig. S3D and S3E). Consistent with previous reports, *METTL3* KD increased the proportion of apoptotic cells in GBMs (Fig. 3C; Supplementary Fig. S3F). Furthermore, *METTL3* KD resulted in significantly decreased migration and invasiveness of GBM cells (Supplementary Fig. S3G and S3H). Conversely, overexpression of the *METTL3* dominant-negative mutant in GBMs inhibited cell migration and invasion (Supplementary Fig. S3I). These data support an important role of the m⁶A catalytic domain of *METTL3* in controlling GBM cell growth, survival, and invasion *in vitro*.

We further investigated whether *METTL3* inhibits the tumorigenic capacity of GBM cells *in vivo*. When U87MG cells, transduced with sh*METTL3* lentiviruses, were inoculated into nude mice, the cells produced much smaller xenograft tumors than cells expressing scrambled, control shRNAs (Supplementary Fig. S3J and S3K). Subsequently, the excised xenograft tumors were processed for IHC and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay (Supplementary Fig. S3L). Significantly reduced ratios of KI67-positive cells to apoptotic cells were observed in *METTL3* KD cell-derived tumors (Supplementary Fig. S3L).

Compared with the control shRNA, both sh*METTL3* sequences significantly decreased stem cell size (Supplementary Fig. S4A),

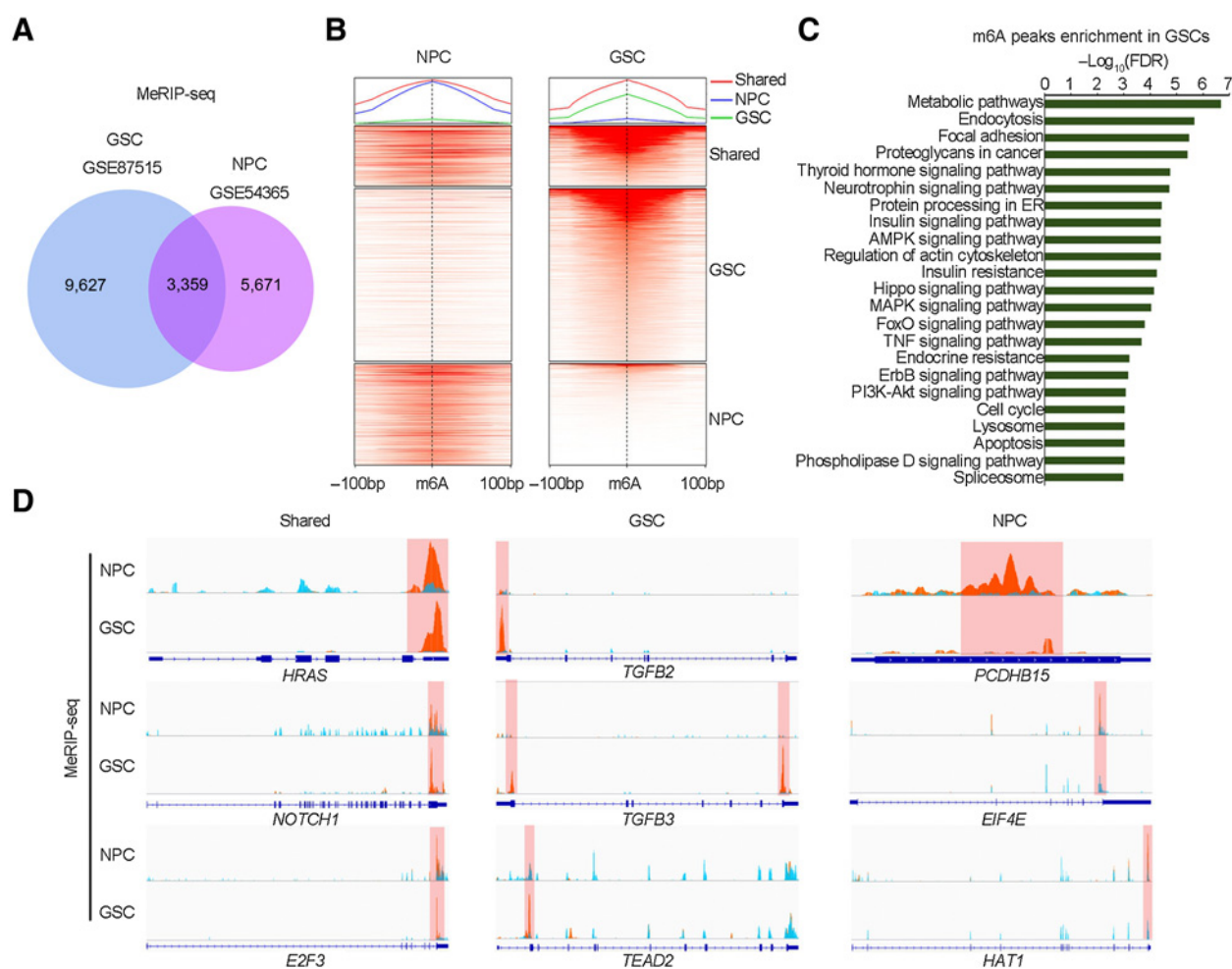


Figure 1.

The m⁶A methylome in NPCs and GSCs. **A**, Venn diagrams of m⁶A modification peaks between NPCs and GSCs. **B**, Heatmap and overlaps of m⁶A MeRIP-seq signals for NPCs (GSE54365) and GSCs (GSE87515). The global m⁶A modification status was arranged into three groups according to m⁶A modification enrichment (enrichment score >1.5): shared (genes with m⁶A modification in both GSCs and NPCs), GSC (genes with m⁶A modification in GSCs but not in NPCs), and NPC (genes with m⁶A modification in NPCs but not in GSCs). **C**, Kyoto Encyclopedia of Genes and Genomes analyses of genes with increased m⁶A modifications in GSCs. **D**, The m⁶A modification status of the represented genes from three groups.

number (Fig. 3D), and frequency (Fig. 3E) in GSC-derived tumor neurospheres. Whereas overexpression of WT METTL3 promoted U87MG and U251 neurosphere formation and stem cell frequency, the METTL3 mutant inhibited these phenotypes (Fig. 3F; Supplementary Fig. S4B and S4C). Moreover, shMETTL3 decreased the CD133-positive populations in U251- and U87MG-derived neurospheres (Supplementary Fig. S4D). Compared with the mice injected with control U87MG spheroid-derived GSCs, those injected with shMETTL3 spheroid-derived GSCs displayed impaired tumor growth and a lower rate of tumor formation (Fig. 3G).

The m⁶A modifications of splicing factor mRNAs are regulated by METTL3

To map m⁶A modification sites and unveil potential METTL3 functions in GBM, we performed MeRIP-seq on U87MG cells with silenced METTL3 (Supplementary Fig. S5A and S5B). Consistent with previous studies, we demonstrated that m⁶A

peaks in GBMs were enriched in the RGACH motif (R = G/A; H = A/C/U; Fig. 4A), and abundant in coding sequences (CDS) and untranslated regions (UTR) of mRNAs (Fig. 4B). Compared with the control GBMs, the m⁶A peaks across entire gene bodies and 3'UTRs were markedly decreased in shMETTL3 GBMs (Supplementary Fig. S5C). Furthermore, 6,444 genes with significantly decreased m⁶A levels in shMETTL3 GBMs were identified as potential m⁶A-regulated genes (Fig. 4C). Moreover, carcinogenesis pathways were significantly enriched in these m⁶A-regulated genes (Fig. 4D), suggesting a role for METTL3-mediated m⁶A modifications in GBM tumorigenesis.

We further characterized the molecular signaling pathways regulated by METTL3 using RNA-seq (Fig. 4E; Supplementary Fig. S5D and S5E). Transcripts encoding apoptotic signaling pathways and glial cell differentiation genes were enriched in both METTL3-KD GBM cells (Fig. 4F and G). The upregulated expression of apoptotic and differentiation genes was confirmed by RT-qPCR (Fig. 4H). Interestingly, a large number of genes were

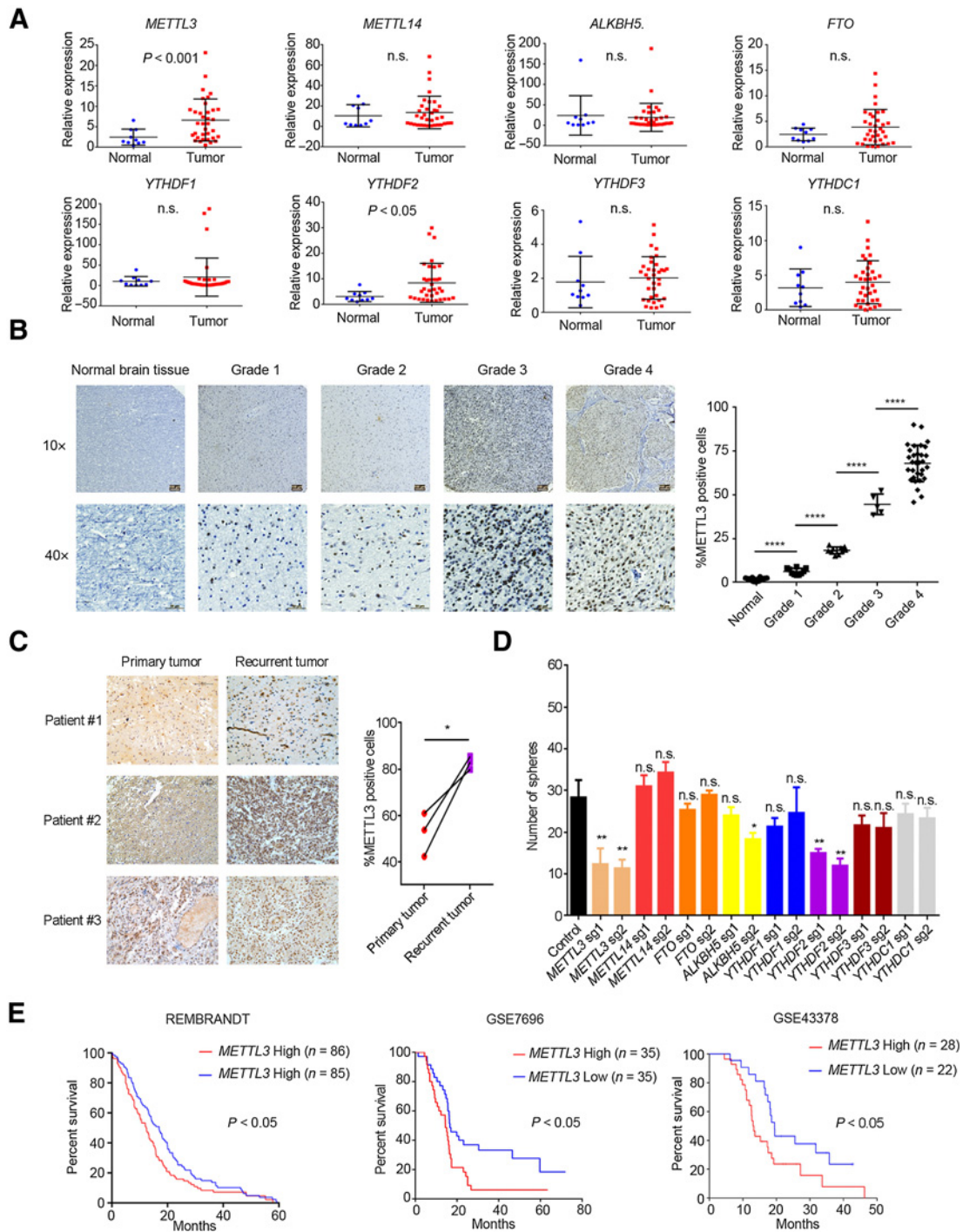


Figure 2. Increased expression of METTL3 in GBM cells and in classical tumors. **A**, Expression of m⁶A modulators was measured by qPCR in GBM specimens ($n = 35$) and compared with normal brain tissues ($n = 10$). **B**, IHC staining of METTL3 in patients with gliomas (grades 1–4) and comparison with normal brain tissue. The statistical results showed the proportion of METTL3-positive cells in each group. **C**, IHC staining of METTL3 in primary and recurrent GBM tumors from three patients with GBM. The statistical results showed the proportion of METTL3-positive cells in each group. **D**, The sphere-forming efficiency was plotted postinhibition of m⁶A modulators using two different sgRNAs in U87MG cells. The number of spheres formed after 7 days were counted using ImageJ software. **E**, The association between METTL3 expression in GBM and overall survival time of the selected patients was analyzed by Kaplan–Meier analysis. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$ is based on the Student *t* test. All results are from three independent experiments. Values are mean \pm SD. n.s., no significant difference.

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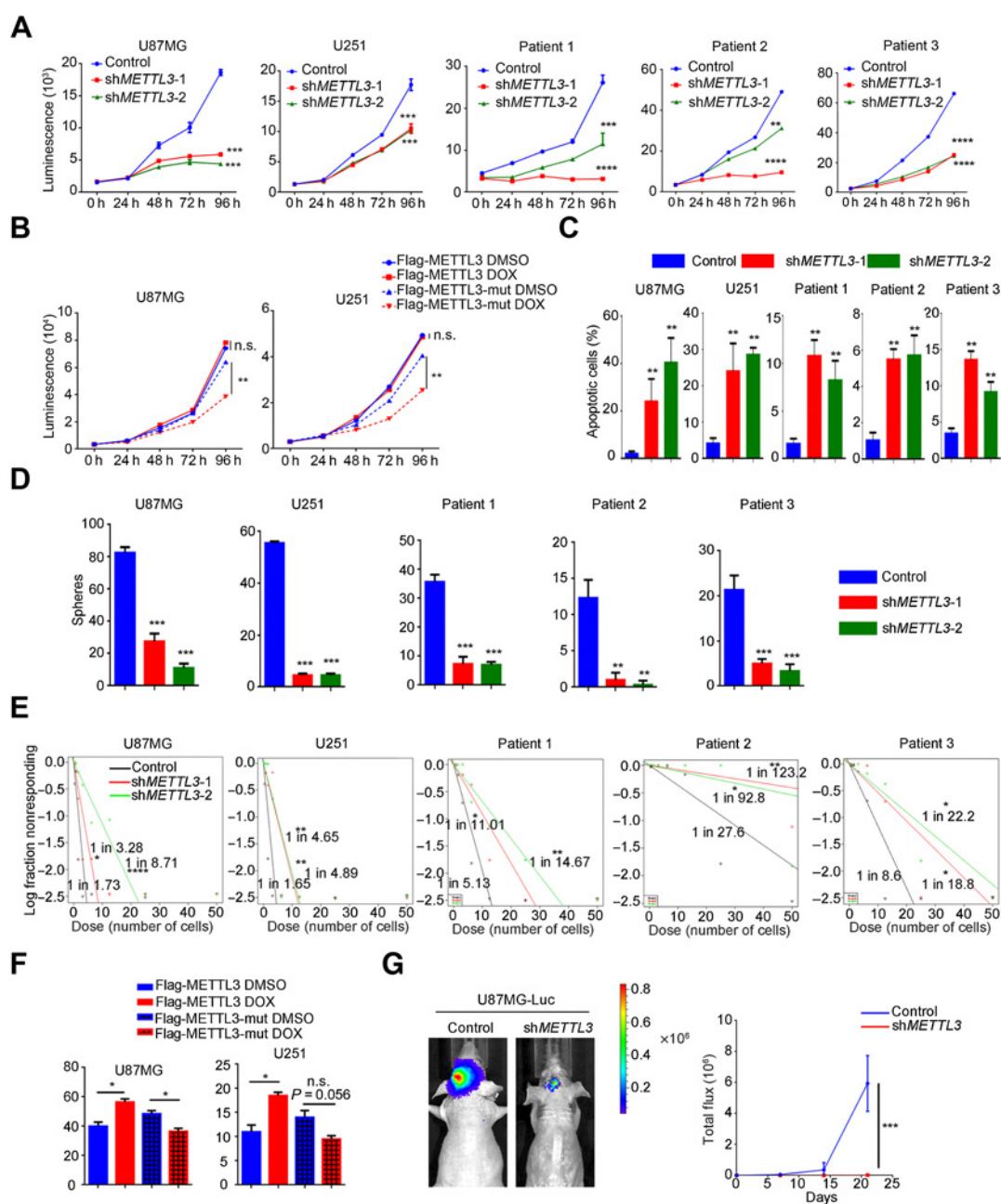


Figure 3. Impairment of GBM proliferation and tumorigenicity by METTL3 inhibition. **A**, The cell viability tests of U87MG, U251, and primary GBM cells transduced with shMETTL3 were performed using CellTiter-Glo. **B**, The cell viability tests of U87MG and U251 cells overexpressing wild-type (WT) METTL3 or METTL3 with a mutated catalytic domain (METTL3-MUT) were performed using CellTiter-Glo. **C**, The proportion of apoptotic cells in METTL3-KD and control GBM cells was evaluated by flow cytometry. The statistical results showed the proportion of Annexin V⁺ PI⁻ cells, which indicate the amount of apoptotic cells in each group. **D**, Sphere-forming assay after METTL3 silencing in U87MG cells compared with control cells. The number of spheres formed was counted after transferring spheres to stem cell culture conditions for 7 days. **E**, Limiting dilution assay of GSCs transduced with control shRNA or METTL3 shRNAs. **F**, U87MG and U251 cells were transduced with flag-tagged WT METTL3 or METTL3 with a mutated catalytic domain (METTL3-mut). The number of spheres formed was counted after transferring spheres to stem cell culture condition for 7 days. Representative images of the spheres shown at $\times 10$ magnification. Scale bar, 100 μ m. **G**, Xenograft images of brain tumors in GSC-grafted nude mice ($n = 4$) transplanted with U87MG sphere cells that were transduced with control shRNA or METTL3 shRNA. The scale bar for bioluminescence intensity is shown on the right. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ is based on Student t test. Values are mean \pm SD of three independent experiments. n.s., no significant difference.

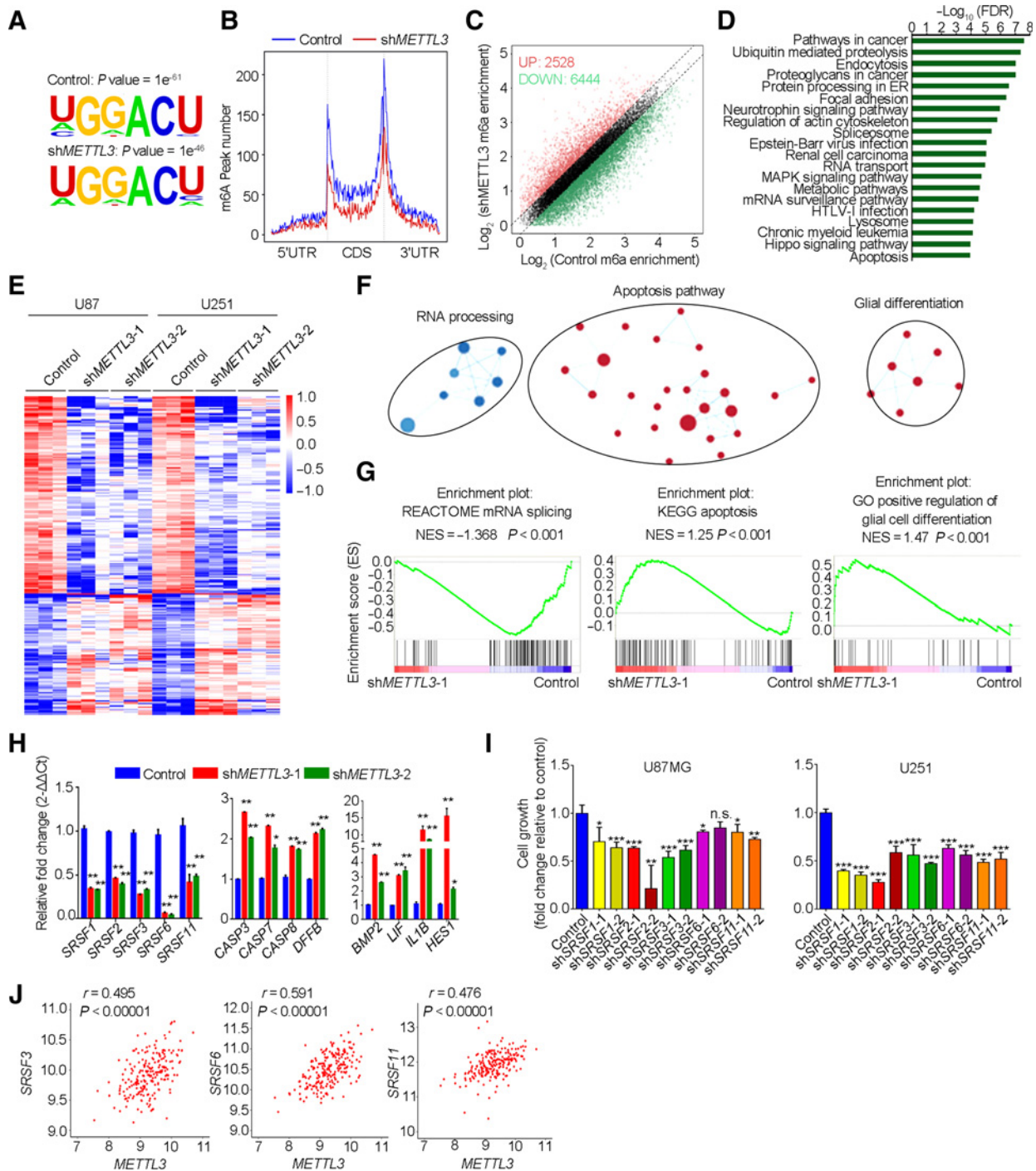


Figure 4.

Splicing factors are critical target genes of METTL3 in GBM. **A**, Motif analysis of m⁶A modification peaks in control and METTL3-KD MeRIP-seq data. **B**, Distribution of m⁶A modification peak reads across all mRNAs in control and METTL3-KD U87MG cells. **C**, Scatter plots showing the increased (red) and decreased (green) m⁶A modification enrichment in mRNAs from control and METTL3-KD U87MG cells. **D**, Gene ontology analysis of mRNAs with decreased m⁶A modification in METTL3-KD U87MG cells. **E**, Heatmap showing the mRNA expression changes in GBM cells depleted of METTL3. **F**, Gene ontology (GO) analyses of the genes differentially regulating genes between METTL3-KD and control cells. **G**, Gene set enrichment analysis enrichment plots of differentially regulated genes between METTL3-KD (shMETTL3-1) and control cells. Data of shMETTL3-2 drew same conclusion (data not shown). **H**, A qRT-PCR analysis. The mRNA levels were first normalized to the level of β -actin mRNA. The relative ratio (fold change) obtained in the presence of control shRNA was set to 1. **I**, U87MG or U251 cells transduced with indicated shRNAs were plated in a 96-well plate for 72 hours. Cell viability was assayed using CellTiter-Glo. **J**, Pearson correlation analysis of METTL3 with SRSF3, SRSF6, or SRSF11 based on REMBRANDT data. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ is based on Student t test. Values are mean \pm SD of three independent experiments. n.s., no significant difference. KEGG, Kyoto Encyclopedia of Genes and Genomes.

also significantly downregulated in *METTL3*-KD cells. Gene ontology (GO) and gene set enrichment analysis analyses revealed that these downregulated genes were mainly involved in RNA processing and mRNA splicing (Fig. 4F and G). The downregulated expression of these splicing factors was also confirmed by RT-qPCR (Fig. 4H; Supplementary Fig. S5F). Next, we randomly selected several splicing factors regulated by *METTL3* and found that KD of these genes impaired the proliferation of GBM cells (Fig. 4I; Supplementary Fig. S5G). Consistently, we found that *METTL3* expression positively correlated with splicing factors, especially *SRSF3*, *SRSF6*, and *SRSF11* (Fig. 4J; Supplementary Fig. S5H). GBM specimens displayed increased levels of *SRSF3/6/11* compared with normal brain controls (Supplementary Fig. S5I). Kaplan–Meier survival analysis revealed that patients with elevated expression of *METTL3* and *SRSF3/6/11* had shorter overall survival time (Supplementary Fig. S5J). Together, these results identify SRSFs as downstream targets regulated by *METTL3*, which are dysregulated in GBM.

The m⁶A modification of splicing factor transcripts mediates mRNA selection for NMD

Expression of splicing factors is regulated via alternative splicing of the conserved regions to yield mRNAs, which are degraded by NMD. However, whether a significant proportion of splicing factor mRNA transcripts occur via reduced levels of m⁶A modifications and are ultimately degraded by NMD is not known. NMD analyses using the *METTL3*-KD RNA-seq data revealed that the mRNA transcripts that undergo NMD were significantly enriched in genes associated with RNA splicing (Supplementary Fig. S6A). By comparing the m⁶A modification of SRSFs in NPCs and GSCs, we found that m⁶A modifications around start codon of splicing factor transcripts (e.g., *SRSFs*) were elevated (Fig. 5A; Supplementary Fig. S6B). The specific m⁶A modification sites of the mRNAs of SRSFs were identified by miCLIP-seq analyses (Supplementary Fig. S6C; ref. 30). Importantly, on the basis of the markedly decreased m⁶A modification peaks, premature termination (i.e., stop) codons (PTCs) in the mRNAs of SRSFs were generated via exon inclusion or skipping upon *METTL3* depletion (Fig. 5B; Supplementary Fig. S6D and S6E). Therefore, these mRNAs with PTCs were predicted to be subject to NMD, and the predicted protein products were not physiologically relevant. A similar phenotype was also found in other splicing factors (Supplementary Fig. S6E). Silencing *METTL3* significantly reduced protein-coding mRNAs (mRNAs without PTCs) of SRSFs (Fig. 5C). In contrast, an inhibitory effect on mRNAs with PTCs was also observed upon overexpression of WT *METTL3* (Fig. 5D). Moreover, protein-coding mRNAs of SRSFs were significantly decreased, upon overexpression of mutant *METTL3* (Fig. 5E).

To investigate whether the mRNAs of SRSFs with PTCs were substrates for NMD, we analyzed steady-state levels of these mRNAs via inhibition of NMD. We used shRNA targeted at *UPF1* (Supplementary Fig. S6F), which is the central component of the NMD pathway. Treatment with shRNA against *UPF1* greatly increased the steady-state levels of the mRNAs with PTCs and total mRNAs of SRSFs (Fig. 5F). Similar increases in the levels of the mRNAs with PTCs were also observed after inhibition of NMD by treatment with cycloheximide (Fig. 5G). In addition, we found that the expression levels of SRSFs NMD variants were decreased in clinical GBM samples (Supplementary Fig. S6G).

The removal of m⁶A modification around start codon of splicing factors is required for NMD in a YTHDC1-dependent manner

To analyze m⁶A modification levels around start codon that modulate NMD signaling, we used a previously described (31) *in vitro* luciferase reporter systems (Fig. 6A). The reporter systems were derived from *SRSF6*, including the pre-mRNA sequence from transcription start site up to exon 3. The PTC in the intron 2 of *SRSF6* is maintained. In addition, we mutated the A with G to inactivate the m⁶A modification-mediated exon inclusion. We observed that the *SRSF6*-Renilla-fused mRNA is m⁶A modified (Supplementary Fig. S7A). Consistent with previous results, KD *METTL3* efficiently promoted the formation of the mRNA with PTC in the WT reporter, as indicated by reduced luciferase activity (Fig. 6B). However, no significant change of luciferase activity occurred in the reporter with the mutation in m⁶A modification (Fig. 6B). Similarly, a lack of luciferase activity increase has also been observed with addition of the *METTL3* mutant in GBM cells, compared with addition of the WT *METTL3* (Fig. 6C). RT-qPCR analyses showed higher NMD RNA (RNA with PTC) levels and lower protein-coding mRNA (mRNA without PTC) levels in mutated *SRSF6* reporter compared with WT reporter, further suggesting that the m⁶A modification is critical for inhibition of NMD (Supplementary Fig. S7B). Inhibition of NMD with sh*UPF1* greatly increased the Renilla-*SRSF6* NMD RNA and total mRNA of mutated reporter (Supplementary Fig. S7C). To further analyze the effects of m⁶A modifications on NMD, a reporter was constructed consisting of exon 2, exon 3, and flanking intron sequences of *SRSF3* without m⁶A modification site (Supplementary Fig. S7D). In contrast to the m⁶A modification around start codon reporter system data, this reporter was greatly resistant to KD of *METTL3*-induced NMD (Supplementary Fig. S7E). Consistently, WT *METTL3* cannot increase the luciferase activity of *SRSF3* minigene reporter constructs without m⁶A modification site (Supplementary Fig. S7F).

Adenine base editing is a novel genome-editing approach to convert a target T•A to C•G without requiring homology-directed repair or introducing double-stranded DNA breaks (32). To generate m⁶A site mutation in the SRSFs gene of interest in U87MG cells, we transduced the base editor construct and guide RNA targeting the *SRSF3* m⁶A modification site around start codon (Fig. 6D). A total of 14 cell clones were obtained by limited dilution and Sanger sequencing indicated that nine carried the expected mutation (Fig. 6E). We next found that the NMD RNA of *SRSF3* significantly increased in m⁶A-mutant U87MG cells compared with WT control (Fig. 6F). These results support that m⁶A modifications around start codon mediate repression of NMD in GBM.

YTHDC1 has been reported as an m⁶A modification reader that mediates mRNA splicing, and mutating either W377 or W428 to alanine completely disrupts its binding to m⁶A RNA (15). KO of *YTHDC1* reduced sphere number substantially in *METTL3* overexpression cells but not in control cells (Supplementary Fig. S7G). Overexpressing mutant *YTHDC1* (m⁶A binding activity loss) failed to enhance the sphere formation capacity of U87MG cells (Supplementary Fig. S7H), suggesting that *YTHDC1* contributes to the GBM phenotype (e.g., sphere formation) dependently on its m⁶A-binding activity. Moreover, RIP-qPCR assay results showed that *YTHDC1* binds to *SRSF3*, *SRSF6*, and *SRSF11* mRNA when compared with negative control (Fig. 6G; Supplementary Fig. S7I). In addition, by analyzing PAR-CLIP-seq data from

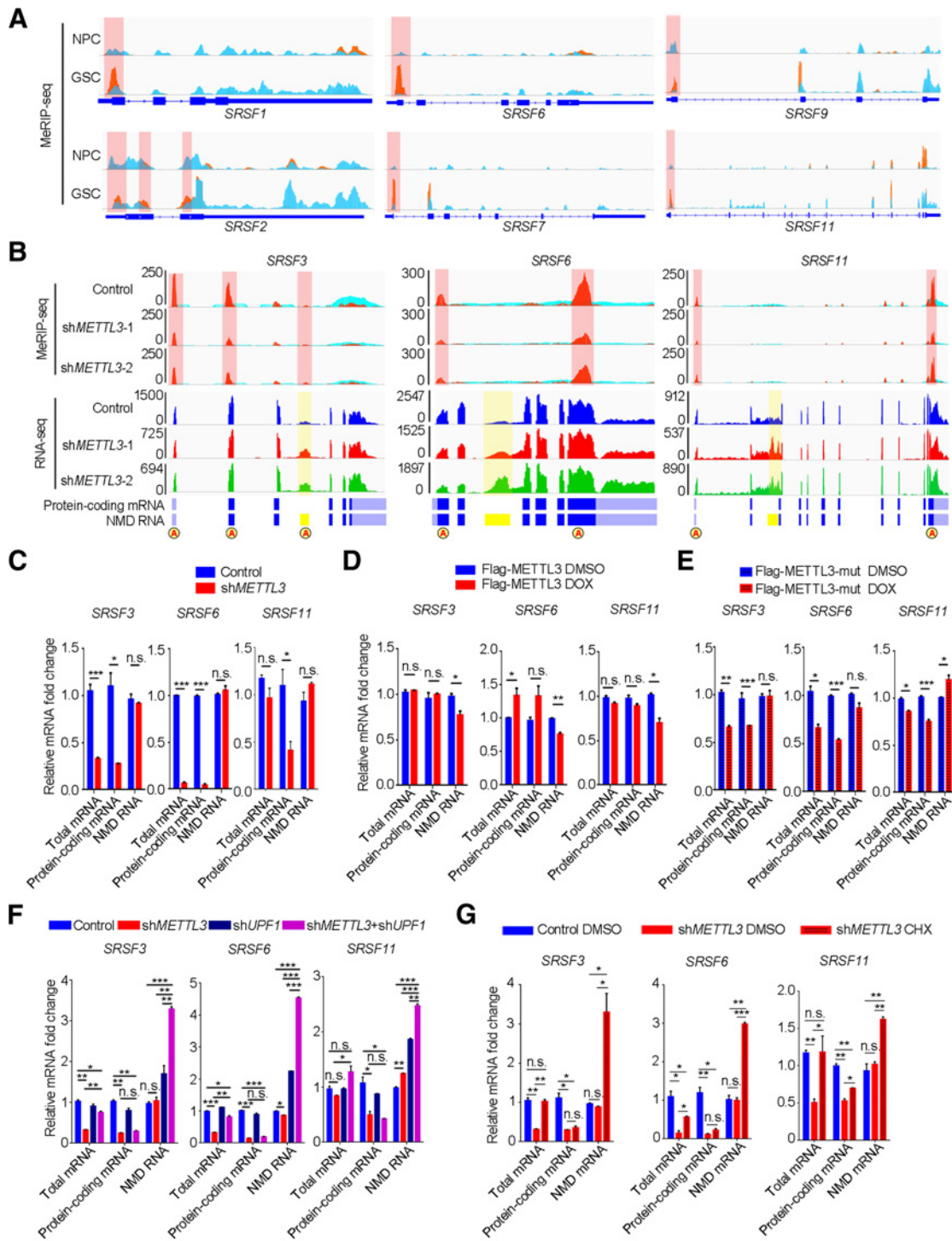


Figure 5.

METTL3-mediated NMD of *SRSFs* mRNAs rely on its m⁶A methyltransferase activity. **A**, Genomic visualization of the m⁶A immunoprecipitation-normalized signal in NPCs and GSCs of the *SRSFs*. The x-axis represents the genomic position. The y-axis shows normalized reads per million (rpm). **B**, Integrative Genomics Viewer plots of m⁶A peaks and RNA-seq peaks at *SRSFs* mRNAs. The y-axis shows the sequence read number, blue boxes represent protein-coding exons, and yellow boxes represent NMD exons. RT-qPCR analysis of the total, protein-coding, or NMD RNA levels of *SRSFs* in U87MG cells transduced with shMETTL3 (**C**) or METTL3 (**D**). **E**, RT-qPCR analysis of the total, protein-coding, or NMD RNA levels of *SRSFs* in U87MG cells transduced with a mutated catalytic domain (METTL3-mut). **F**, RT-qPCR analysis of the total, protein coding, or NMD RNA levels of *SRSFs* in U87MG cells treated with indicated shRNA(s). **G**, RT-qPCR analysis of the total, protein coding, or NMD RNA levels of *SRSF3*, *SRSF6*, and *SRSF11* in METTL3-KD U87MG cells treated with with 10 μg/mL cycloheximide (CHX) or DMSO during an 8 hour time course. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001 is based on Student *t* test. Values are mean ± SD of three independent experiments. n.s., no significant difference.

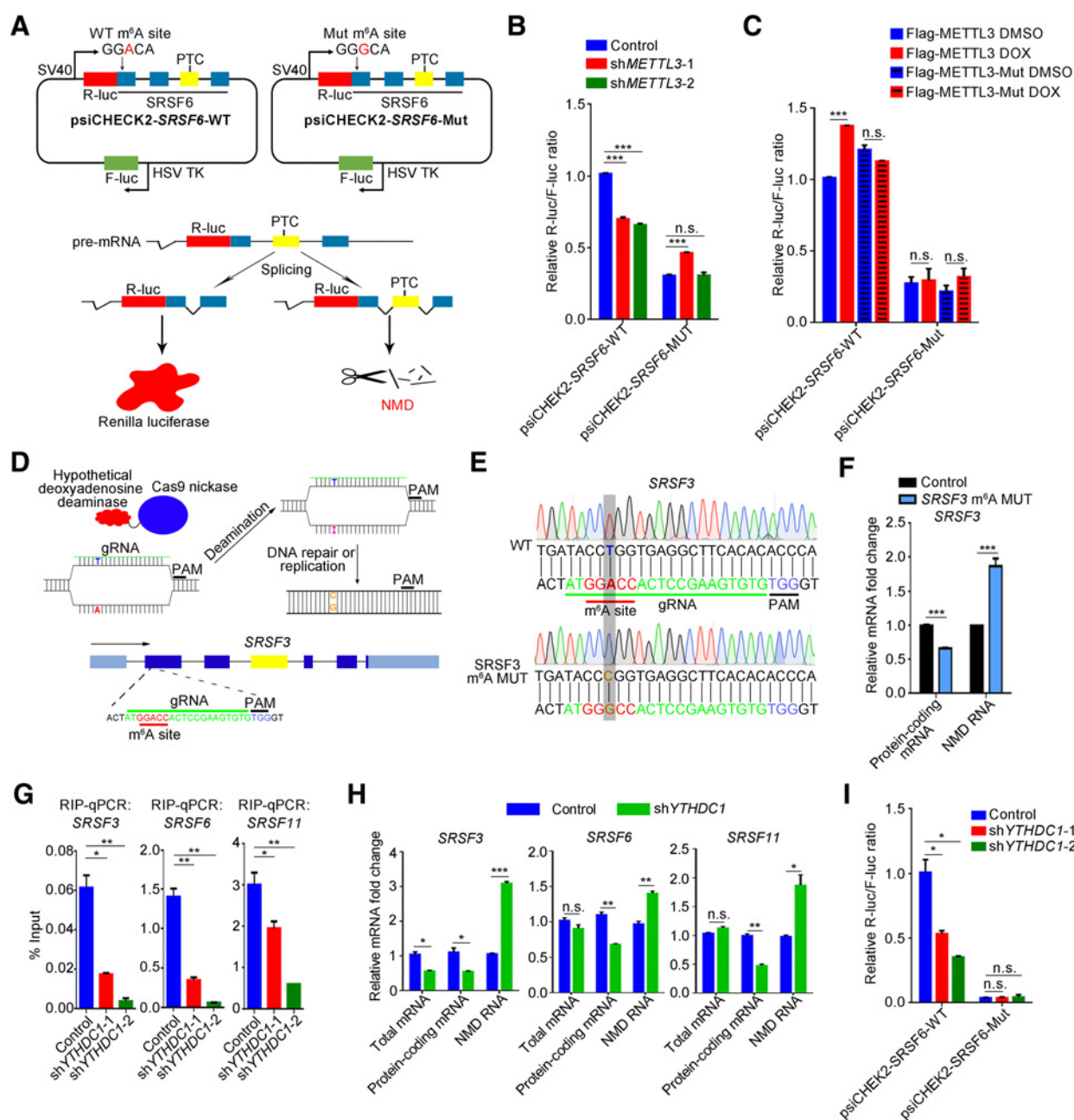


Figure 6.

The m⁶A modification around start codon of splicing factors modulates NMD through *YTHDC1*. **A**, Schematic illustration of WT *SRSF6* minigene or m⁶A consensus sequence mutant (A-to-G mutation) reporter constructs. *SRSF6* minigene was fused with *Renilla* luciferase reporter. NMD splicing of *SRSF6* minigene reporter cannot generate *Renilla* luciferase. F-luc, firefly luciferase; R-luc, *Renilla* luciferase. **B**, Relative luciferase activity of *SRSF6* minigene with wild-type or mutated m⁶A sites after cotransfection with control shRNA, or *METTL3* shRNA in U87MG cells. *Renilla* luciferase activity was measured and normalized to firefly luciferase activity. **C**, Relative luciferase activity of *SRSF6* minigene with wild-type or mutated m⁶A sites after cotransfection with WT *METTL3* or *METTL3*-mut in U87MG cells. **D**, Schematic illustration of base editing system. **E**, Sequence of *SRSF3* in WT and m⁶A site-edited U87MG cells. **F**, RT-qPCR analysis of the total or NMD RNA levels of *SRSF3* in WT and m⁶A site-edited U87MG cells. **G**, RIP-qPCR analysis of *YTHDC1* in U87MG cells and negative control cells (*YTHDC1*-KD U87MG cells). **H**, RT-qPCR analysis of the total, protein-coding, or NMD RNA levels of *SRSFs* in U87MG cells transduced with sh*YTHDC1*. **I**, Relative luciferase activity of *SRSF6* minigene with wild-type or mutated m⁶A sites or *SRSF3* minigene after cotransfection with control shRNA, or *YTHDC1* shRNA in U87MG cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ is based on Student *t* test. Values are mean \pm SD of three independent experiments. n.s., no significant difference.

GSE74397 (7) and GSE58352 (2), we found that *YTHDC1* binds near the start codon region of *SRSFs* mRNAs (Supplementary Fig. S7). KD *YTHDC1* led to the accumulation of NMD of *SRSF3*,

SRSF6, and *SRSF11* mRNAs in GBM cells (Fig. 6H). Moreover, KD *YTHDC1* could affect the luciferase activity of the WT *SRSF6* minigene reporter but not the *SRSF6* reporter with the mutated

m⁶A site (Fig. 6I). Together, these data suggest that *YTHDC1* KD-mediated NMD is dependent on m⁶A around start codon of the mRNA in GBM cells.

KD of the m⁶A methyltransferase *METTL3* results in dysregulation of alternative splicing events in GBM

We compared the alternative splicing events in *METTL3* KD U87MG cells compared with U87MG control cells. Using the rMATS tool, a total number of alternative splicing events was identified with an obvious change of exon inclusion levels (ψ , $\psi \geq 0.1$). We found that downregulation of splicing factors can affect various types of alternative splicing, including skipped exon (SE), alternative 5' splice exon (A5SS), alternative 3' splice exon (A3SS), retained intron (RI), and mutually exclusive exons (MXE), and, in particular, SE events in sh*METTL3* U87MG cells were most affected (Fig. 7A). Subsequent analysis indicated that the SE type of alternative splicing was negatively regulated by *METTL3*-KD, whereas A3SS, MXE, and RI were positively regulated by *METTL3*-KD (estimated by changes of ψ after *METTL3*-KD; Fig. 7B). Next, we compared genes that were differentially spliced in *METTL3*-KD U87MG cells to cells with scrambled control shRNA. When analyzing the cellular functions of *METTL3*-regulated alternative events using GO, we found that *METTL3* affected alternative splicing of a number of genes, such as *BCL-X* and *NCOR2*, with functions in cancer cell death and motility (Fig. 7C; Supplementary Fig. S8A).

BCL-X is a well-known example of genes critical for cancer that has splicing variants that can function as cancer biomarkers and therapeutic targets. The *BCL-XL* isoform is antiapoptotic in various cancer types, whereas the *BCL-XS* isoform is proapoptotic in cancer. Using semiquantitative RT-PCR and qPCR, we confirmed that KD of *METTL3* significantly shifted the transcription of *BCL-XL* into *BCL-XS* (Fig. 7D and E; Supplementary Fig. S8B). The protein levels of *BCL-XL* were also reduced in *METTL3*-KD GBM cells (Supplementary Fig. S8C). To examine whether the splicing alteration of *BCL-X* was responsible for the *METTL3*-KD phenotypes, we designed shRNAs to specifically target *BCL-XS* to inhibit the expression of *BCL-XS* but not *BCL-XL* (Supplementary Fig. S8D). As shown in Fig. 7F and G, *METTL3* and *BCL-XS* double-KD GBM cells grew significantly faster with reduced apoptosis than cells with the *METTL3*-KD alone. This phenotypic rescue suggests that *METTL3* maintains the tumorigenicity of GBM cells, at least partially, through the splicing of *BCL-X*.

NCOR2 (also known as *SMRT*) exists in two major splicing isoforms, α and τ , which have different roles in preserving cellular identity and tissue homeostasis. Little is known about potential functional differences between these two isoforms in GBM. Using semiquantitative RT-PCR and qPCR, we revealed that KD of *METTL3* significantly increased the transcription of isoform α of *NCOR2* (Fig. 7H and I; Supplementary Fig. S8E). RT-PCR analysis showed that shRNAs targeting isoform α specifically inhibited its expression but not the τ isoform of *NCOR2* in U87MG cells (Supplementary Fig. S8F). Furthermore, we demonstrated that KD of the *NCOR2* α isoform partially rescued *METTL3* KD-induced inhibition of U87MG cell growth (Fig. 7J). In addition, our neurosphere formation data show that KD of the *NCOR2* α isoform significantly increased the neurosphere formation capacity and stem cell frequency in *METTL3*-KD U87MG cells (Fig. 7K and L), and together these data suggest that the *NCOR2* α isoform may play an important role in the regulation of GSC self-renewal.

Discussion

In this study, we established a novel mechanism for m⁶A modifications around start codon of mRNA splicing factors in modulating NMD of these splicing factors. In addition, we found that *METTL3* modulated alternative splicing of *BCLX* and *NCOR2*, which leads to GBM tumor outgrowth and self-renewal. Unlike previous studies, reporting that m⁶A modifications at 3'-terminal ends are destabilizers of mRNA, our study revealed that m⁶A modifications around start codon stabilize the mRNAs of *SRSFs* by preventing NMD. By preventing NMD and promoting mRNA degradation, m⁶A modifications act as a molecular rheostat to finely adjust the transcript levels of *SRSFs* to influence alternative splicing events.

The role that *METTL3* plays in cancer is complex. Two research teams reported opposite conclusions on the role that *METTL3* plays in the self-renewal and tumorigenesis of GSCs. The reasons for these opposite conclusions may depend on the patients from whom the GBM cells originated and other compensatory genetic mutations and epigenetic changes in GBM cells. In this study, we chose clinical GBM samples from different stages, knocked down *METTL3* expression using both sgRNAs and shRNAs in primary GBM cells, and validated our findings using catalytic inactive mutants of *METTL3*. All of these results consistently demonstrated that *METTL3* promotes proliferation and self-renewal of GBM cells. Notably, the oncogenic ability of *METTL3* is dependent upon the m⁶A methyltransferase catalytic domain. Interestingly, although KD *METTL14* expression reduced mRNA m⁶A levels in GSCs (22), KO of *METTL14* has no effect on GBM oncogenicity. A recent study in AML cells showed that *METTL3* bound the promoter regions of active genes (about 80 genes) independent of *METTL14* (33). They also showed that *CEBPZ* is required for recruitment of *METTL3* to the promoters. Their results indicate that specialized partner proteins might exist at splicing factor loci in GBM cells that give clues to decipher *METTL14*-independent *METTL3*'s functions in GBM.

m⁶A modifications may play different roles at different developmental stages of GBM tumors. In glioma, the mutation of isocitrate dehydrogenase 1 (*IDH1*) occurs frequently, which results in accumulation of the metabolic by-product 2-hydroxyglutarate (2-HG). The 2-HG could inhibit *FTO* activity, thereby increasing global m⁶A modifications and contributing to cancer initiation. In the late stage of glioma, high m⁶A modification levels may increase epigenetic reprogramming of non-GSCs into GSCs, whereas KD *METTL3* may reduce the proportion of GSCs in GBM. This hypothesis was indirectly verified by the observation that KD of *METTL3* in pluripotent stem cells, at naive or primed states, resulted in different cell fate transitions (34). Thus, strategies designed to reduce levels of m⁶A modifications may provide a means to target malignant GBM and to develop more effective therapies.

The prevailing goal of understanding the regulatory roles of m⁶A modifications in RNA processing has been mainly focused on the regulation of mRNA translation or mRNA stability. Indeed, we found that *METTL3* regulates the stability of a specific set of transcripts, such as apoptosis pathways and glial differentiation genes, in GBM. The m⁶A-binding protein *YTHDF2* may recognize these methylated mRNAs, leading to their decay and subsequently to decreased cell apoptosis and differentiation, thereby promoting GBM tumor growth and dedifferentiation. Another m⁶A modification reader, *YTHDC1*, is involved in the process of

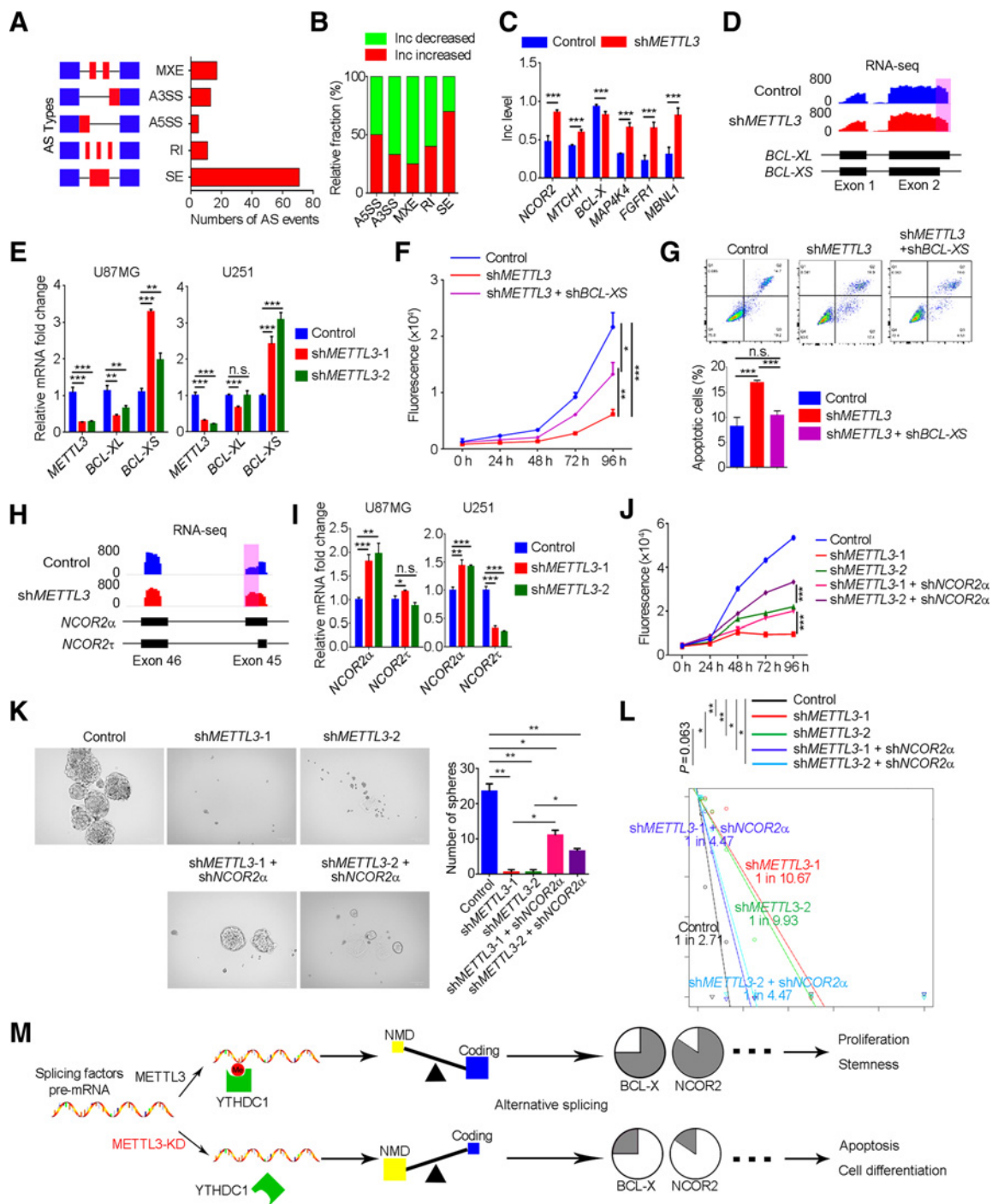


Figure 7. METTL3 regulates *BCL-X* and *NCOR2* alternative splicing in GBM cells. **A**, The amount of changed alternative splicing events in U87MG cells transduced with *shMETTL3*. **B**, The relative fraction of each alternative splicing event affected either positively or negatively by METTL3. **C**, Representative genes with significantly changed alternative splicing events. **D**, Integrative Genomics Viewer plot illustrating the splicing changes of *BCL-X* promoted by METTL3. **E**, RT-qPCR analysis of splicing changes of *BCL-X* after *METTL3* KD. **F**, The cell viability tests of U87MG cells transduced with *shMETTL3* and/or *shBCL-XS* were performed using CellTiter-Glo. **G**, The proportion of apoptotic cells in U87MG cells transduced with *shMETTL3* and/or *shBCL-XS* was evaluated by flow cytometry. **H**, Integrative Genomics Viewer plot illustrating the splicing changes of *NCOR2* promoted by METTL3. **I**, RT-qPCR analysis of splicing changes of *NCOR2* after *METTL3* KD. **J**, The cell viability tests of U87MG cells transduced with *shMETTL3* and/or *shNCOR2* were performed using CellTiter-Glo. **K**, U87MG cells were transduced with *shMETTL3* and/or *shNCOR2*. The number of spheres formed was counted after transferring to stem cell culture condition for 7 days. **L**, Limiting dilution assay of GSCs transduced with *shMETTL3* and/or *shNCOR2*. **M**, Schematic illustration of the working model. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ is based on Student *t* test. Values are mean \pm SD of three independent experiments. n.s., no significant difference.

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alternative splicing through recruitment and modulation of splicing factors to their targeted RNAs (35). We have presented a number of findings supporting the notion that m⁶A modifications modulate the NMD of splicing factors. The NMD pathway protects eukaryotic cells by reducing the production of harmful truncated proteins translated from PTC-bearing mRNA transcripts. In our study, the reduced m⁶A modifications by KD *METTL3* consequently led to the degradation of *SRSFs* transcripts via triggering NMD and thus control of GBM initiation and progression. It should be noted that KD *METTL3* affected not only *YTHDC1*-mediated NMD but also the m⁶A-mediated mRNA degradation. Therefore, the effect of KO *YTHDC1* on GBM phenotype was not as significant as that of KD/KO *METTL3*.

SRSFs proteins are known for their ability to promote exon inclusion and exon-skipping events, suggesting the regulatory role of *SRSFs* proteins in alternative events. Brain tissue has been found to have particularly high levels of alternative splicing (36, 37). Consistently, a large number of cancer-relevant genes have undergone splice alterations in GBM (38–40). The importance of alternative splicing in the development of GBM was further reinforced by the findings that a large number of splicing factors were overexpressed in GBM (41, 42), yet the mechanisms responsible for this upregulation and its clinical relevance remain to be fully addressed. Our results support a model where *METTL3* controls the cancer-relevant phenotypes of GBM cells by promoting the expression of *SRSFs*. This subsequently results in the creation of cancer-specific alternative splicing patterns, such as the preferential expression of the antiapoptotic transcript variant of *BCL-X* and the GSC-promoting transcript variant of *NCOR2* (Fig. 7M). It is worth noting that analysis of clinical outcomes revealed significant relationships between combined expression of *METTL3* and splicing factors with GBM patient prognosis.

Taken together, this study provides an important mechanistic insight into how m⁶A methyltransferase *METTL3* serves as an NMD regulator of splicing factors with potential clinical implications of alternative splicing events of *BCL-XS* and *NCOR2*. Our

study also demonstrates that expression of *METTL3* can be used to dissect the molecular differences between histologically similar GBM entities and to help predict GBM prognosis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Miao, W. Long, C. Zou, K. Zhu, Q. Cao, W. Zhao
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Li, Y. Yi, Y. Miao, T. Long, Y. Zheng, X. Wu, J. Ding, Q. Cao, W. Zhao

Writing, review, and/or revision of the manuscript: F. Li, Y. Yi, Y. Miao, Q. Cao, W. Zhao

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): F. Li, Y. Miao, W. Long, T. Long, S. Chen, W. Cheng, Q. Xu, J. Wang, Q. Liu, F. Zhi, J. Ren, Q. Cao, W. Zhao

Study supervision: Q. Cao, W. Zhao

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