ORIGINAL ARTICLE

Essential role of METTL3-mediated m⁶A modification in glioma stem-like cells maintenance and radioresistanceA Visvanathan¹, V Patil¹, A Arora¹, AS Hegde², A Arivazhagan³, V Santosh⁴ and K Somasundaram¹

Despite advances in biology and therapeutic modalities, existence of highly tumorigenic glioma stem-like cells (GSCs) makes glioblastomas (GBMs) invincible. N⁶-methyl adenosine (m⁶A), one of the abundant mRNA modifications catalyzed by methyltransferase-like 3 and 14 (METTL3/14), influences various events in RNA metabolism. Here, we report the crucial role of METTL3-mediated m⁶A modification in GSC (neurosphere) maintenance and dedifferentiation of glioma cells. METTL3 expression is elevated in GSC and attenuated during differentiation. RNA immunoprecipitation studies identified SOX2 as a bona fide m⁶A target of METTL3 and the m⁶A modification of SOX2 mRNA by METTL3 enhanced its stability. The exogenous overexpression of 3'UTR-less SOX2 significantly alleviated the inhibition of neurosphere formation observed in METTL3 silenced GSCs. METTL3 binding and m⁶A modification *in vivo* required intact three METTL3/m⁶A sites present in the SOX2-3'UTR. Further, we found that the recruitment of Human antigen R (HuR) to m⁶A-modified RNA is essential for SOX2 mRNA stabilization by METTL3. In addition, we found a preferential binding by HuR to the m⁶A-modified transcripts globally. METTL3 silenced GSCs showed enhanced sensitivity to γ -irradiation and reduced DNA repair as evidenced from the accumulation of γ -H2AX. Exogenous overexpression of 3'UTR-less SOX2 in METTL3 silenced GSCs showed efficient DNA repair and also resulted in the significant rescue of neurosphere formation from METTL3 silencing induced radiosensitivity. Silencing METTL3 inhibited RasV12 mediated transformation of mouse immortalized astrocytes. GBM tumors have elevated levels of METTL3 transcripts and silencing METTL3 in U87/TIC inhibited tumor growth in an intracranial orthotopic mouse model with prolonged mice survival. METTL3 transcript levels predicted poor survival in GBMs which are enriched for GSC-specific signature. Thus our study reports the importance of m⁶A modification in GSCs and uncovers METTL3 as a potential molecular target in GBM therapy.

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INTRODUCTION

Glioblastoma (GBM) is an aggressive malignancy of astrocytes which occurs commonly in adults. Despite advances in therapy over the past few decades, prognosis remains poor with a median survival of 12–15 months.¹ GBM exhibits heterogeneity among which a small entity known as glioma stem-like cells (GSCs) reconstitute the milieu, elicit recurrence and resistance for standard therapy.² The bidirectional inter-conversion between differentiation and dedifferentiation provides survival advantage to tumor cells over various stress conditions. Recent studies by the Bernstein group coined four developmental transcription factors OLIG2, SALL2, SOX2 and POU3F2, which are sufficient to reprogram differentiated glioma cells to induced GSCs.³ Since poorly differentiated GBM tumors elicit poor prognosis and therapy resistance, further insight into molecular mechanisms which enhance GSC pool is required.

Analogous to DNA methylation, mRNA methylation occurs at N⁶-position of adenosine (m⁶A) on poly-A transcripts and it causes context-dependent perturbations in RNA duplex structure. METTL3 and METTL14 (methyltransferase-like) make the core heterodimeric complex of m⁶Ase along with scaffold protein WTAP (Wilm's tumor associated protein) whereas FTO and ALKBH5 reverse the methylation. Recent co-crystallization studies of METTL3/14 complex have shown that solely METTL3 contains

the catalytic core which accepts S-adenosyl methionine, a donor for methylation and emphasizes the indispensability of METTL3 in m⁶A modification.⁴ Further, they show point mutations in METTL14 had little effect on the activity of m⁶A addition and it acts as a scaffold to enhance the activity.⁴ Addition of m⁶A influences different aspects of mRNA metabolism such as half-life,^{5,6} translatability,^{7–9} splicing and nuclear export.¹⁰ Outcome of the modification in mRNA stability is governed by m⁶A switches/readers that are recruited to m⁶A and the binding affinity is context dependent.⁶

As m⁶A is indispensable for resolution of naïve ESCs¹¹ and aberrant differentiation of ESCs can produce cells with properties of cancer stem cells,¹² we investigated the role of m⁶A modification in the context of cancer stem cells. We identified that m⁶A modification is higher in GSCs and METTL3-dependent m⁶A modification is crucial for GSC maintenance. We identified SOX2 as one of downstream target and further recruitment of HuR onto m⁶A-modified sites in SOX2 is essential for SOX2 mRNA stabilization. Additional studies revealed that METTL3 alters the DNA repair efficiency and radiation sensitivity partially via SOX2 in GSCs. To our knowledge, we report for the first time the involvement of METTL3 in GSC maintenance and it might serve as a better target to overcome stem-like phenotype and radioresistance in GBM.

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RESULTS

Elevated METTL3 levels observed in GSCs is essential for GSC maintenance

Experimental investigation of the significance of m⁶A in GSC maintenance and reprogramming of tumor cells revealed the following. Northwestern blot using m⁶A-specific antibody showed higher m⁶A occurrence in GSC total RNA compared with that of the matched differentiated glioma cells (DGCs) (Figure 1a). Transcriptome analysis of m⁶A modification associated proteins

from published data³ revealed that the METTL3 transcript level solely was elevated in GSCs—MGG4, MGG6 and MGG8—compared with DGCs whereas METTL14 and ALKBH5 were downregulated (Figure 1b). FTO remains unregulated in GSCs compared with DGCs (data not shown). Specific upregulation of METTL3 in GSCs is also independently validated by quantitative reverse transcriptase-PCR in three neurospheres MGG4, MGG6, MGG8, obtained from other laboratory¹³ and in GB1, a neurosphere established in our laboratory (Figure 1c). METTL3 silencing

Figure 1. METTL3 is upregulated in GSC over DGC and essential for stem cell maintenance. (a) m⁶A northwestern blot assay was performed using an equal amount of RNA (1 μg) isolated from GSCs MGG6, MGG8 and GB1 with respective matched DGCs. (b) Heat map depicting the transcript level variation of m⁶A members METTL3, METTL14, ALKBH5 between GSCs and DGCs (MGG4, MGG6 and MGG8). Normalized reads were represented from RNA-Seq data.³ Brown denotes higher expression and blue for lower expression. (c) Validation of high METTL3 transcripts in MGG4, MGG6, MGG8 and GB1 GSCs compared with matched DGCs. Real-time PCR was performed and the ratio of dCt values normalized to DGC was plotted. (d) m⁶A northwestern blot assay was performed using an equal amount of RNA (1 μg) isolated from siNT and pooled siMETTL3-transfected MGG8 cells. (e) Gene Set Enrichment Analysis enrichment plots of differentially regulated genes between METTL3 high and low (>/< median) expressing patients from Agilent (TCGA) cohort. Pre-ranked genes that are differentially regulated were compared against the gene sets specific for GSC or DGC³ for enrichment. (f) The sphere formation efficiency was plotted post inhibition of METTL3 using five different shRNAs in MGG8 and GB1 spheres. Number of spheres formed after 7 days were counted using Image J software and shNT condition of each GSC was depicted as 100%. Representative images of the sphere lines after 7 days were showed for shNT and shMETTL3 condition at ×10. Scale bar=100 μm. (g) Limited dilution assay with 1, 10 and 50 cells of shNT/shMETTL3-transfected MGG8 and GB1 were plated in triplicates. The number of wells with no sphere was plotted as percentage. (h) METTL3 expression was measured by real-time PCR in TIC (tumor-initiating cell) derived from U87-MG and LN229 post 10 days of dedifferentiation in neural basal medium. The ratio of dCt was normalized to cell lines and plotted. (i) Sphere formation assay post METTL3 silencing in U87-MG cell line was plotted as percentage compared with shNT. Cell line was transiently transfected with shNT or shMETTL3 and transferred to stem culture condition. Number of spheres formed was counted after 10 days using Image J software and representative images at ×10 were shown. Scale bar=100 μm. (j) Sphere formation assay post METTL3 silencing in LN229 cell line was plotted as percentage compared with shNT. Cell line was transiently transfected with shMETTL3 and transferred to stem culture condition. Number of spheres formed was counted after 10 days using Image J software and representative images at ×10 were shown. Scale bar=100 μm. P-value was analyzed by Student's T-test. ***P-value < 0.001, **P-value < 0.01, *P-value < 0.05. Error bars represents standard deviation.

Figure 2. m⁶A methylation by METTL3 regulates SOX2 mRNA stability. **(a)** RIP-PCR (RNA immunoprecipitation-PCR) with anti-DDK antibody was performed using GB1 cell lysate overexpressing pCMV-METTL3-DDK. Enrichment of each transcript was measured by real-time PCR. Precipitated RNA of each gene was normalized to IgG RIP and plotted as log2. **(b)** RIP with anti-m⁶A antibody was performed in GB1 using RNA (2 µg) and enrichment was measured for each gene by real-time PCR. The Ct value of m⁶A RIP was normalized to IgG RIP and plotted as log2. **(c)** RIP with anti-m⁶A antibody was performed in GB1 using RNA (2 µg) and SOX2 mRNA was measured by real-time PCR. The Ct value of m⁶A RIP was normalized to IgG RIP and plotted as ratio. **(d)** RIP with anti-m⁶A antibody was performed in METTL3 silenced GB1 cells using an equal amount of RNA (2 µg) and SOX2 transcript was measured by real-time PCR. Differential enrichment for siMET condition was determined by normalizing enrichment of siNT condition (m⁶A RIP-IgG RIP) to that of siMET condition (m⁶A RIP-IgG RIP) and plotted as ratio. Pooled siRNA was used for METTL3 silencing **(e)**. Transcript level of SOX2 post METTL3 silencing in MGG4, MGG6, MGG8 and GB1 was measured by real-time PCR and dCts of siMET condition was normalized with siNT and shown as ratio. **(f)** SOX2 protein level after 72 h of METTL3 silencing in MGG4, MGG6, MGG8 and in GB1 was shown by SDS-PAGE. Actin was used as a loading control. **(g)** Expression of METTL3 and SOX2 were quantified by SDS-PAGE in eight GBM samples and five neurosphere lines (*R* value = 0.72; *P* = 0.0048). **(h)** SOX2 transcript was measured at indicated time points post Actinomycin D (5 µg/ml) treatment in siNT and pooled siMETTL3-transfected GB1 cells by real-time PCR. Log2 ratio of remaining SOX2 was plotted using linear regression after normalizing to 0th hour of respective condition. **(i, j)** Sphere formation was assessed in METTL3 silenced MGG8 and GB1 cells followed by SOX2 (3'UTR-less) overexpression by viral transduction. Number of spheres formed post 7 days were counted and analyzed by Image J software. Number of spheres formed in shNT condition was plotted as 100%. *P*-value was analyzed by Student's *T*-test. METTL3 and SOX2 expression were measured in all three conditions for MGG8 and GB1 by SDS-PAGE. ****P*-value < 0.001, ***P*-value < 0.01, **P*-value < 0.05. Error bars represent standard deviation.

in MGG8 GSCs reduced global m⁶A modification (Figure 1d), suggesting that METTL3 is a key mediator of m⁶A modification in GSCs. Gene Set Enrichment Analysis revealed that GSC-specific upregulated genes compared with DGCs (GSC vs DGC_up) were positively enriched while GSC-specific downregulated genes compared with DGCs (GSC Vs. DGC_down) were negatively enriched in GBMs with high levels of METTL3 (Figure 1e and Supplementary Tables S1 and S2). GB1 neurosphere cells were confirmed for the higher expression of various specific markers of GSCs (Supplementary Figures S1A–C). METTL3 silencing by pooled siRNA (Supplementary Figures S1D and E) inhibited GSC (MGG4, MGG6, MGG8 and GB1) growth examined by neurosphere formation assay and limiting dilution assay (Supplementary

Figures S1F and G). METTL3 silencing resulted in reduced expression of stem cell-specific marker SSEA1 (Supplementary Figure S1H). In addition, METTL3 silencing in GB1 and MGG8 cells attenuated the expression of glioma reprogramming factors POU3F2, SOX2, SALL2 and OLIG2 (Supplementary Figures S1I and SJ, respectively). As a confirmation, we also show that silencing METTL3 by five different shRNAs resulted in the inhibition of GSC (MGG8 and GB1) growth by neurosphere formation assay and limiting dilution assay (Figures 1f and g and Supplementary Figure S2A). Further, we also found that the proportion of live cells was reduced significantly in METTL3 silenced condition (Supplementary Figure S2B), while there was an increase in the proportion of apoptotic cells as seen by increased

Figure 3. METTL3 interacts with SOX2 mRNA *in vivo* and *in vitro* via 3'UTR. **(a)** Top: Schematic representation of human SOX2 transcript and three putative binding sites which contain m⁶A consensus sites 'GGACH' present in 3'UTR was denoted in boxes with respective mRNA coordinates. Line denotes *in vitro*-transcribed biotin-labeled (body labeling) 3'UTR region (636 bp) that was used for binding assays. Below: Schematic representation of human SOX2-3'UTR-driven firefly luciferase reporter (pMIR-SOX2-3'UTR). Consensus sites chose for studies were showed in gray boxes. The reporters with point mutation of A to G at three individual sites within 3'UTR were shown by red letter. **(b)** *In vitro* biotin pull-down assay was performed by incubating increasing concentrations (2, 4, 8 μg) of biotin-labeled SOX2-3'UTR Wt/Individual mutants with whole-cell lysate of LN229 and elutions were quantified by SDS-PAGE using anti-METTL3 antibody. **(c)** Competition pull-down assay was performed by addition of labeled Wt (4 μg) with increasing amounts of unlabeled Wt (8, 16, 32 μg)/Mut A (32 μg)/Mut B (32 μg)/Mut C (32 μg) SOX2-3'UTR fragments. Elutes were resolved in SDS-PAGE using anti-METTL3. **(d)** Activity of 3'UTR reporter 72 h post silencing of METTL3 in MGG8. An equal amount of protein was taken based on Bradford assay. RLU (relative luminescence unit) values were normalized to pMIR-REPORT basal activity and β-Gal assay. **(e)** Individual mutants were transiently transfected in METTL3 overexpressing LN229 cells and post 72 h the luminescence activity of the equal amount of protein was measured. RLU values were normalized to pMIR-REPORT basal activity and β-Gal assay. EV-pMIR-REPORT empty vector; VC-pCMV-Vector control. **(f)** Schematic representation of SOX2 and respective primer pairs FP1/RP1 which amplifies endogenous SOX2 segment (top) and FP2/RP2 which amplifies exogenous 3'UTR reporter-derived SOX2 (below) were indicated. **(g, h)** m⁶A RIP-PCR and DDK METTL3 RIP-PCR were performed in LN229 cells co-transfected with LUC-SOX2-3'UTR Wt or mutants with pCMV-DDK-METTL3. RNA immunoprecipitated by anti-m⁶A antibody/anti-DDK (METTL3) antibody was analyzed by real-time PCR for exogenous LUC-3'UTR-derived SOX2 RNA (measured by FP2/RP2). Values were plotted as the ratio of enrichment after normalizing with IgG RIP and Input. **(i, j)** m⁶A RIP-PCR and DDK/METTL3 RIP-PCR were performed in LN229 cells co-transfected with LUC-SOX2-3'UTR Wt or mutants with pCMV-DDK-METTL3. RNA immunoprecipitated by anti-m⁶A antibody/anti-DDK (METTL3) antibody was analyzed by real-time PCR for endogenous SOX2 RNA (measured by FP1/RP1). Values were plotted as the ratio of enrichment after normalizing with IgG RIP and Input. *P*-value was analyzed by Student's *T*-test. ****P*-value < 0.001, ***P*-value < 0.01, **P*-value < 0.05. Error bars represent standard deviation.

Annexin V-positive cells (Supplementary Figure S2C). The METTL3 transcript level was elevated in neurospheres derived from glioma cell lines LN229 and U87 through reprogramming (Figure 1h). LN229- and U87-derived neurospheres expressed high levels of four glioma reprogramming factors (Supplementary Figures S2D and E). Further, METTL3 silencing reduced the neurosphere formation significantly in U87 and LN229 cells (Figures 1i

and j, respectively). The reduced neurosphere formation in METTL3 silenced condition was accompanied by reduced expression of glioma reprogramming factors (Supplementary Figure S2F). Taken together, these results indicate m⁶A modification and METTL3 level are high in GSCs and METTL3 is required for GSC maintenance and reprogramming of established glioma cell lines.

SOX2 mediates the METTL3-dependent GSC maintenance

A panel of GSC-specific transcription factors identified by Bernstein group was investigated as the potential target³ of METTL3 in GSC maintenance. RNA immune precipitation (RIP) was performed using DDK tag (METTL3) antibody in GB1 neurospheres overexpressing METTL3-DDK and the binding affinity was calculated (Figure 2a). The genes SOX2 and SOX21 showed high binding affinity. We also carried out m⁶A RIP in GB1 neurospheres and the binding affinity was calculated (Figure 2b). Though OLIG1, HESE and ASCL1 showed maximum m⁶A modification followed by SOX2, we chose SOX2 for in-depth studies as SOX2 showed higher binding affinity to METTL3. Another reason is that SOX2 is one of the essential reprogramming factors, besides OLIG2, SALL2 and POU3F2 in GSCs.³ Further, analysis of anti-METTL3 PAR-CLIP-Seq data set (GSE46705) identified a peak in SOX2 gene but none in other reprogramming factors (Supplementary Figure S3A). Anti-m⁶A RIP brought down SOX2 mRNA efficiently (Figure 2c), but was diminished upon METTL3 silencing in GB1 neurospheres (Figure 2d). METTL3 silencing repressed the SOX2 transcript and protein levels in MGG4, MGG6, MGG8 and GB1 neurospheres (Figures 2e and f). SOX2 and METTL3 showed significant positive correlation in four different GBM transcriptome data sets and in our cohort at mRNA level (Supplementary Figures S3B–F) as well as at protein level in our cohort of GBM tissue sample (Supplementary Figure S3G). Further, immunohistochemistry staining of METTL3 and SOX2 proteins on serial sections of GBM tumors showed positive staining of both proteins in the same tumor cell population. (Supplementary Figure S3H). The protein levels of METTL3 and SOX2 in GBM samples and in GSCs measured by western blot also showed a significant positive correlation (Figure 2g). In actinomycin D-treated cells, METTL3 silencing reduced the SOX2 mRNA half-life (1.48 h) compared with control cells (4.66 h) (Figure 2h). Exogenous SOX2 overexpression from a 3'UTR-less construct rescued significantly the neurosphere growth in METTL3 silenced MGG8 and GB1 GSCs (Figures 2i and j compare bar 3 with 2). From these results, we conclude that GSC maintenance by METTL3 is mediated by SOX2 mRNA stabilization.

METTL3 interacts and methylates specific sites of SOX2-3'UTR

Since the published data sets revealed an enrichment of m⁶A sites around stop codon and 3'UTR,^{6,14–16} a search for METTL3 binding motif (GGACH) across SOX2-3'UTR identified six sites (Supplementary Figure S3I). Further, the analysis on m⁶A RIP-Seq data set (GSE29714) of mouse brain (SOX2-3'UTR of mouse and human are highly similar with 86% identity—data not shown) identified three METTL3 binding motifs (site# 1, 2 and 3) close to stop codon in the SOX2-3'UTR as putative sites for METTL3 binding (Supplementary Figure S3I; see green shade; see Supplementary Data for more details). METTL3 protein from LN229 cell extract interacted with a 636 bp *in vitro*-transcribed biotinylated RNA fragment, which includes the three putative m⁶A sites (Figure 3a) in a concentration-dependent manner (Figure 3b). However, RNA carrying mutation in any one of the m⁶A sites ('A' converted to 'G') failed to bind METTL3 (Figure 3b; compare Mut A, Mut B and Mut C RNA with Wt RNA). Competition experiments revealed that the binding by the wild-type biotinylated RNA to METTL3 was competed efficiently by unlabeled wild-type RNA but not by the unlabeled mutant RNAs (Figure 3c).

METTL3 silencing in MGG8 cells inhibited the activity significantly measured from a luciferase-SOX2-3'UTR construct (Figure 3d). Exogenous overexpression of METTL3 induced luciferase activity from reporter with Wt sites (Figure 3e; compare bar 2 with 3) but not from the reporter with mutated sites (Figure 3e; compare bars 4–9 with 3). To relate the influence of METTL3 levels on reporter activity to m⁶A modification, the pull down efficiency of endogenous SOX2 transcript (Figure 3f; primer pair FP1/RP1 which amplifies exon region of SOX2) and SOX2-3'

UTR reporter derived transcript (Figure 3f; primer pair FP2/RP2 which amplifies fusion region of Luc-SOX2-3'UTR) by anti-m⁶A and anti-DDK (METTL3) antibodies in cells transfected with METTL3 construct and Wt or mutated luciferase-SOX2-3'UTR constructs were measured. We found that the interaction between anti-m⁶A or anti-DDK (METTL3) with SOX2-3'UTR reporter-derived transcript increased upon METTL3 overexpression in cells transfected with WT SOX2-3'UTR construct, but failed to interact with mutated SOX2-3'UTR constructs (Figures 3g and h; compare bar 2 with 1 and bars 3–5 with 2). In contrast to this observation, both anti-m⁶A and anti-DDK (METTL3) interaction with endogenous SOX2 transcript increased upon METTL3 overexpression in both wild-type and mutant conditions (Figures 3i and j; compare bars 2–5 with 1).

It was intriguing that METTL3 failed to bind and methylate adenine of point mutated constructs that had other two sites intact, which suggested the possibility of non-redundant function of three m⁶A sites in METTL3 binding. The secondary structure of 500 base fragments of Wt, Mut A, Mut B and Mut C RNA derived from SOX2-3' UTR was predicted by RNAfold Vienna tool and was further subjected to tertiary structure construction by RNAComposer. A–G conversion of any one of the sites altered the tertiary conformation of the RNA fragment carrying any one of the mutations compared with wild type (Supplementary Figure S4A). Upon superimposition of the wild type and mutant tertiary structures, by using Chimera tool, revealed a significant structural alteration of mutant fragments as revealed by a high (95–104 Å) RMSD (root-mean-square deviation) value (Supplementary Figure S4B). Further, the Pearson correlation coefficient of local secondary structural changes that occur within a window of 300 bases between Wt and mutant RNA fragments by using RNAsnp web tool showed a significant *P*-value (< 0.2) for all three mutants (Supplementary Figure S4C). Thus, we conclude that METTL3 binds and methylates specific adenines in the SOX2-3'UTR and this process requires a specific conformation created by three Wt METTL3 consensus sequences. Further, we also demonstrate that SOX2 mRNA stabilization by METTL3 requires METTL3 binding and m⁶A modification to SOX2 mRNA.

Recruitment of HuR to m⁶A-modified sites is essential for SOX2 mRNA stabilization

Next, we chose to investigate the potential role of HuR in METTL3-dependent stabilization of SOX2 mRNA since HuR is one of m⁶A-modified RNA-bound proteins as per mass spectrometry studies and HuR binding affects RNA stability and translation. HuR is also essential for glioma growth and chemoresistance.^{9,14,17,18} We found that the transcript level of HuR is higher in GBMs compared with control brain samples (Supplementary Figure S5A) and further analysis showed high HuR transcripts levels in GSCs compared with DGCs (Supplementary Figure S5B). We found that METTL3 failed to stabilize SOX2 transcripts in HuR silenced MGG8 GSCs (Figure 4a). In addition, RIP-PCR using anti-HuR showed significantly reduced binding of HuR to SOX2 mRNA in METTL3 silenced GSCs (Figures 4b and c).

Next, we tested the requirement of m⁶A modification for HuR binding to RNAs by measuring the HuR association to the total RNA in METTL3 silenced cells. METTL3 silencing reduced the global m⁶A modification as seen by northwestern blotting using anti-m⁶A (Figure 4d; compare shMETTL3 with shNT in input control). In shNT GSCs, HuR binding to total RNA was threefold more than control IgG condition (Figure 4e; compare anti-HuR with anti-IgG in shNT GSCs). However, the binding of HuR to the total RNA is abolished in METTL3 silenced GSCs (Figure 4e; compare anti-HuR with anti-IgG in shMETTL3 GSCs). Confocal imaging using anti-m⁶A and anti-HuR antibodies confirmed their nuclear colocalization (Figure 4f; Pearson's *R* value=0.85). However, the co-immunoprecipitation experiments revealed that there

Figure 4. HuR is essential in METTL3-dependent SOX2 mRNA stabilization. **(a)** Relative SOX2 and HuR transcript levels were determined by real-time PCR post 72 h of METTL3 transient overexpression followed by silencing of HuR by shRNA in MGG8. dCts were normalized to shNT condition and plotted as ratio. **(b)** Semi-qPCR of SOX2 measured by intensity of PCR products followed by HuR RIP performed in METTL3 silenced MGG8 cells. **(c)** Graph plotted for affinity of SOX2 RNA to HuR in siNT and siMETTL3 condition as intensity ratio normalized to respective IgG RIP. Pooled siMETTL3 was used for silencing. **(d)** m⁶A northwestern blot of input and RNA elutes from HuR/IgG RIP performed in MGG8 shNT and shMETTL3 condition was shown. **(e)** Normalized dot intensities calculated to respective IgG RIP by Image J were plotted for shNT and shMETTL3 condition. **(f)** Colocalization of m⁶A (green) and HuR (red) in LN229 nuclear compartment was showed under ×63 magnification (Pearson's *R* value for colocalization = 0.85). Scale bar = 25 µm. **(g)** Cumulative expression distribution of m⁶A mRNAs with HuR consensus within 50 bp was compared with m⁶A genes without HuR sites across METTL3 high/low patients (Agilent; TCGA). *P*-value was calculated using Mann–Whitney *T*-test. **(h)** Cumulative expression distribution of common targets with both METTL3 and HuR binding sites within 50 bp vicinity was compared with HuR bound genes without METTL3 consensus across HuR high/low patients (Agilent; TCGA). *P*-value was calculated using Mann–Whitney *T*-test.

is no direct interaction of these to proteins and hence the interaction might occur via m⁶A (Supplementary Figure S5C). Next, we tested the global effect of preferential binding of HuR in m⁶A-modified RNA on gene expression. The cumulative distribution function (cdf) of the expression of m⁶A modified transcripts (in log2) with or without HuR consensus in the vicinity was plotted. First, we identified genes with m⁶A peaks from m⁶A RIP-Seq data (GSE46705), which were then divided into two groups based on the presence of consensus sequence derived from HuR PAR CLIP data (GSE29780) within ± 50 bps of METTL3 consensus sequence. Next, we tested the variation of log2 fold changes among these two groups of genes between METTL3 high and METTL3 low GBMs (derived from TCGA data). We found that the group with

both sites (red line) showed higher log2 fold change compared with the group containing only METTL3 sites (black line) (Figure 4g; *P* = 0.0001). Since HuR binding has been shown to regulate mRNA stability, it is of our interest to test the importance of METTL3 sites in the vicinity of HuR consensus sequence for HuR-dependent mRNA stabilization. For this analysis, we identified genes with HuR peaks from HuR PAR-CLIP data (GSE29780), which were then divided into two groups based on the presence of METTL3 consensus sequence derived from METTL3 PAR CLIP data (GSE46705) within ± 50 bps of HuR consensus sequence. Interestingly, we found that the group with both sites (red line) showed higher log2 fold change between HuR high and HuR low GBMs (derived from TCGA data) compared with the genes containing

Figure 5. Silencing METTL3 radiosensitizes GSCs by interruption of SOX2-dependent DNA repair. **(a)** Sphere formation assay post METTL3 silencing and irradiation (4 Gy) in MGG8 cells. Single-cell suspension was irradiated and equal number of cells was plated. Number of spheres formed after 7 days was plotted as percentage and untreated condition of siNT was considered as 100%. RS index (radiosensitivity index = unirradiated - radiated (each condition) / unirradiated - radiated (siNT)) was calculated for siNT and siMETTL3 condition. Pooled siMETTL3 was used for silencing. **(b)** Sphere formation assay was performed in MGG8 cells. Post 24 h of shMETTL3 transfection, cells were infected with lenti viral SOX2 lacking 3'UTR and after 24 h cells were irradiated at 3, 6, 9 and 12 Gy. shNT unirradiated condition was considered as 100% and plotted by nonlinear curve fit. RS index (radiosensitivity index = unirradiated - radiated (each condition) / unirradiated - radiated (shNT)) was calculated for shNT, shMETTL3 and rescue conditions. **(c)** Sphere formation efficiency was analyzed after SOX2 silencing followed by irradiation (4 Gy) in MGG8. Number of spheres formed after 7 days was counted and spheres formed in siNT untreated condition was plotted as 100%. RS index (radiosensitivity index = unirradiated - radiated (each condition) / unirradiated - radiated (siNT)) was calculated for siNT and siSOX2 condition. **(d)** shNT or shMETTL3 vector containing MGG8 cells were transfected with SOX2-3'UTR-less vector. Radiation of 4 Gy was given to cells and post 4 h, expression of SOX2, METTL3, γ -H2AX was quantified in SDS-PAGE. **(e)** γ -H2AX in SOX2 silenced MGG8 cells under irradiated condition (4 Gy) was measured post 4 h by SDS-PAGE. **(f)** GFP-positive cells were analyzed by flow cytometry post 48 h of transient transfection of shMETTL3/SOX2-3'UTR-less constructs in DR-GFP-MGG8 stable cells. Double-strand breaks were induced by I-Sce overexpression. Percentile of GFP-positive cells in shNT was plotted as 100%. METTL3 and SOX2 expression was measured in all four conditions by SDS-PAGE. **(g)** Expression of METTL3 and SOX2 post irradiation (4 Gy) was measured in shNT/shMETTL3-transfected MGG8 cells at different time points by SDS-PAGE. **(h)** m^6A modification post irradiation (4 Gy) was shown by m^6A northwestern blot at indicated time points for total RNA (1 μ g) isolated from MGG8 cells. *** P -value < 0.001, ** P -value < 0.01, * P -value < 0.05. Error bars represent standard deviation. Tubulin was used as a loading control in SDS-PAGE.

only HuR sites (black line) (Figure 4h; $P=0.0001$). From these results, we show that HuR recruitment is essential for m^6A -mediated SOX2 mRNA stabilization. Further, we also show that HuR and m^6A modification work in a complementary fashion in stabilizing mRNAs and the circuit might exist in various cancer cells.

METTL3 imparts radioresistance in GSCs through SOX2-dependent enhanced DNA repair

Cancer stem cells are known for their resistance to most treatment modalities and hence associated with cancer recurrence.¹⁹ Since

this study finds METTL3 contributes to stem cell maintenance, we tested the importance of METTL3 and m^6A modification in radiation sensitivity of GSCs. METTL3 silenced MGG8 GSCs were irradiated (4 Gy was chosen based on kill curves with different doses of irradiation) and plated for sphere formation. As expected, γ -irradiation and METTL3 silencing independently attenuated the sphere formation by 26% and 35%, respectively (Figure 5a, compare bar 2 and 3 with 1). Interestingly, γ -irradiation in METTL3 silenced condition decreased the sphere formation of GSCs much more efficiently by 66% (Figure 5a compare bar 4 with 1). The radiosensitivity index increased to 1.82-fold in irradiated siMETTL3

Figure 6. METTL3 silencing diminishes *in vivo* tumor propagation. **(a)** RasV12-induced transformation of IMA2.1 immortalized astrocytes was performed with shNT/shMETTL3 and the colonies formed in soft agar were counted. Number of colonies formed in shNT condition was plotted as 100%. **(b)** 50000 U87-TIC-Luc-derived cells were deposited in immune-deficient nude mice by intracranial injection and images of bioluminescence with days were shown for shNT and shMETTL3 condition. **(c)** Expression of METTL3 and SOX2 were analyzed in lysates of normal mouse brain and of tumor (shNT) by SDS-PAGE. The decreased expression of METTL3 and SOX2 in tumors grown in shNT and shMETTL3 condition was shown. **(d)** Average total photon flux of bioluminescence for shNT ($n=3$) and shMETTL3 ($n=3$) groups was plotted and P-value was calculated by Student's *T*-test. **(e)** Kaplan–Meier survival fraction plot for animals in the shNT ($n=6$) and shMETTL3 ($n=6$) groups. P-value was analyzed by Mantel–Cox test. **(f)** Representative H/E stained sections ($\times 10$) for depicting comparative cellularity in shNT and shMETTL3 tumor were shown. Histopathology of GBM-like features of U87-TIC/shNT tumors was shown at $\times 20$. Qualitative representation of blood vessels (yellow arrow) and necrotic areas (white arrow) were denoted in the image and enlarged regions of the same were given below at $\times 50$. **(g)** The Kaplan–Meier survival curve of METTL3 was plotted for subset of GBM patients with high GSC score (above median) from Agilent (TCGA) Rembrandt data and from GSE7696 data. P-value was analyzed by Mantel–Cox test. ***P-value < 0.001, **P-value < 0.01, *P-value < 0.05. Error bars represent standard deviation.

GSCs compared with irradiated siNT GSCs (Figure 5a, see inset above). This observation supports the hypothesis that high levels of METTL3 seen in GSCs contribute to radioresistance in neurospheres.

Towards validating one of the mediators of METTL3 which ensue radioresistance, we chose SOX2 since our work finds it to be the target of METTL3 in GSCs maintenance. In addition, SOX2 has been shown to be associated with radiation resistance in various cancers.^{20–22} Exogenous overexpression of SOX2 from a 3'UTR-less construct in METTL3 silenced GSCs rescued the sphere formation in irradiated METTL3 silenced GSCs significantly (Figure 5b, compare blue line with red line). The increased radiosensitivity index in irradiated shMETTL3 GSCs (1.40- and 1.25-fold at 3 and 6 Gy doses) is significantly reduced (1.05- and 0.98-fold at 3 and 6 Gy doses) in SOX2 overexpressing irradiated shMETTL3 GSCs (Figure 5b, see inset above). Furthermore, SOX2 downregulation independently reduced sphere formation by 26% (Figure 5c, compare bar 3 with 1). γ -Irradiation of SOX2 silenced GSCs reduced the sphere formation more effectively (71%) than that in siNT condition (52%) (Figure 5c, compare bar 4 with 1 and 2 with 1). The radiosensitivity index increased to 1.26-fold in irradiated siSOX2 GSCs compared with irradiated siNT GSCs (Figure 5c, see inset above).

One of the mechanisms through which GSCs display radioresistance is by efficient and preferential activation of DNA repair pathways.²³ Since METTL3 enhances the pool of GSC, we investigated whether radiosensitization post METTL3 inhibition is attributed to METTL3 regulation of DNA repair genes with the resultant modulation of DNA repair activity. We subjected m⁶A RIP-Seq data from GSE37001¹⁴ to Gene Set Enrichment Analysis in order to check the possible enrichment of DNA repair pathways. We found gene set- DNA_REPAIR_CHAE_ET_AL related to DNA repair pathway was significantly enriched in METTL3 silenced cells with negative score (Supplementary Figure S5D and Supplementary Table S3). Since above results suggest DNA repair genes are positively regulated by METTL3 and SOX2 partially mediates METTL3-dependent radioresistance, we tested the regulation of DNA repair governed by METTL3–SOX2 cascade in GSCs. We checked for the γ -H2AX levels upon irradiation of METTL3 silenced cells and also upon SOX2-mediated rescue. We observed elevated γ -H2AX in irradiated shMETTL3 GSCs compared with irradiated shNT GSCs (Figure 5d, compare lane 4 with 2). Upon exogenous overexpression of SOX2, the induction of γ -H2AX seen in irradiated shMETTL3 GSCs is significantly reduced (Figure 5d, compare lane 8 with 4). In support to the above data, increased radiation sensitivity of SOX2 silenced GSCs is found to

be due to decreased DNA repair as seen by high levels of γ -H2AX (Figure 5e, compare lane 4 with 2).

Homologous recombination (HR) and non-homologous end joining repair pathways play important role in repair of irradiation induced double-strand breaks.²⁴ Next, we investigated the potential possibility of SOX2 activating transcription of DNA repair genes, thus resulting in the enhanced DNA repair in GSCs. To address this question, we examined the enrichment of gene sets related to HR, non-homologous end joining, nucleotide excision repair and mismatch repair in SOX2-ChIP-Seq data reported from MGG8 GSC cells (GSE54047) using Gene Set Enrichment Analysis. This analysis revealed a significant enrichment for the HR pathway alone (Supplementary Figure S5E and Supplementary Table S4). Next, we investigated the regulation of HR-dependent repair by the METTL3–SOX2 axis using DR-GFP/I-Sce reporter assay.²⁵ The DR-GFP substrate contains a rare restriction site I-Sce site within GFP (referred as SceGFP) and downstream of which, iGFP, a truncated GFP gene, is located. iGFP sequence acts as a wild-type sequence template to the broken SceGFP during HR which results in active GFP gene (Supplementary Figure S5F). We found significantly reduced repair of double-strand DNA breaks in METTL3 silenced GSCs and the repair efficiency was significantly restored with SOX2-3'UTR-less overexpression (Figure 5f, compare bar 2 with 1 and 4 with 2, respectively). In addition, METTL3 and SOX2 protein levels were found elevated as early as 1 h of γ -irradiation in shNT transfected, but not in shMETTL3 transfected, MGG8 GSCs, which further explains the substantial requirement of METTL3 during radiation-induced DNA repair (Figure 5g, compare lanes 2 and 3 with 1, compare lanes 5 and 6 with 2 and 3). METTL3 protein upregulation upon irradiation is also well correlated with a time-dependent increase in total m⁶A modification in GSCs (Figure 5h, compare lanes 3–5 with 1). Taken together from these results, we conclude that METTL3–SOX2 cascade plays a major role in imparting radioresistance to GSCs through enhanced DNA repair. Increased METTL3, SOX2 and m⁶A modification upon irradiation further emphasizes the essential role of METTL3 in radioresistance.

METTL3 is upregulated in GBM and its silencing in GSCs suppresses tumor growth *in vivo*

Towards delineating the importance of METTL3 in GBM whole tumor, we found METTL3 transcripts are upregulated in multiple GBM data sets and also in lower grade astrocytomas (Supplementary Figures S6A and B, respectively). Increased METTL3 transcript level seen in GBM was also validated in our cohort by quantitative reverse transcriptase–PCR (Supplementary Figure S6C). Immunohistochemical staining showed significantly higher cytoplasmic levels of METTL3 protein in GBMs compared with control brain (Supplementary Figure S6D). Glioma-derived cell lines had a higher level of METTL3 transcript compared with control brain (Supplementary Figure S6E). Silencing METTL3 inhibited proliferation, increased apoptosis and increased temozolomide sensitivity in LN229 glioma cells (Supplementary Figures S6F–H). These results imply an oncogenic role for METTL3 in glioma. To confirm this notion, we tested the effect of METTL3 silencing on the ability of Ras (V12Ras) to transform SV40 immortalized IMA2.1 mouse astrocytes²⁶ *in vitro*. Silencing METTL3 efficiently inhibited Ras-mediated transformation of IMA2.1 cells (Figure 6a compare bar 2 with 1). Further, in an orthotopic intracranial mouse model, while the tumor-initiating cells (TIC) derived from U87/shNT formed tumors very efficiently, the tumor size was significantly reduced in U87/shMETTL3 injected mice with diminished expression of SOX2 (Figures 6b–d). The median survival of mice injected with U87-TIC-Luc/shMETTL3 cells is significantly higher (71 days) compared with those injected with U87-TIC-Luc/shNT cells (38.5 days) (Figure 6e). U87-TIC-Luc/shNT cell-derived tumors showed high cellularity, vasculature and

necrosis unlike tumors derived from U87-TIC-Luc/shMETTL3 cells (Figure 6f). High METTL3 levels predicted poor survival in GBMs (GBM patient data from Agilent-TCGA, REMBRANDT and GSE7696) with high GSC signature (Figure 6g) but not with low GSC-specific signature (data not shown). Collectively from these experiments, we conclude that METTL3 is upregulated in GBMs and is essential for tumor growth.

DISCUSSION

The current treatment for GBM that includes surgery, irradiation and adjuvant temozolomide chemotherapy fails to provide great benefit. Recent reports demonstrate a fraction of cells in GBM referred as GSCs impart resistance to therapy. New paradigm of treatment should target the network of genes essential for the stabilization of the GSC pool. Being a reversible epitranscriptome modifier, m⁶A writer METTL3 has been shown to play wide spectrum of functions in embryonic stem cell development in context-dependent manner. Though previous reports support elevated m⁶A levels during transformation,²⁷ the role of METTL3 in cancer stem cells remains obscure. Our findings show high levels of global m⁶A modification and METTL3 in GSCs. Though METTL14 dimerizes with METTL3, the literature evidence suggests that METTL3 is the rate-limiting enzyme and METTL14 act as a scaffold for the m⁶A methylation process.⁴ Further, knockdown of METTL3 alone in various types of cells significantly reduced m⁶A modification followed by drastic effects on cell survival, stem cell maintenance and lineage determination,^{14,16,28} which provides support to the fact that METTL3 can play a crucial role independent of METTL14 availability. Our results also demonstrate that METTL3 is essential for GSC maintenance and dedifferentiation as it augments the GSC fraction by sustaining the stem cell characteristics.

Dedifferentiation process in GBM cells plays a critical role in aggressiveness and in incident of recurrence.^{29,30} Circuit of transcription factors crucial for GSC maintenance has been identified.³ In this study, RIP–PCR experiments identified SOX2 as the potential target of METTL3 in GSC maintenance. METTL3-mediated m⁶A modification of SOX2 mRNA resulted in mRNA stabilization with the subsequent increase in SOX2 protein levels. We also demonstrate exogenous overexpression of SOX2 rescued the neurosphere growth in METTL3 silenced condition, thus confirming that SOX2 is an essential mediator of METTL3 functions. Further, we provide evidence that METTL3 binds to the SOX2-3'UTR. The binding and m⁶A modification by METTL3 requires the presence of all three METTL3/m⁶A sites. The importance of each site complemented by tertiary structure prediction supports that the combinatorial availability of three METTL3 binding sites might hold importance in preferential inter-spatial conformation for METTL3 binding on SOX2-3'UTR. Though it is intriguing to observe significant structural reorganization of 3' UTR post point mutations, the previous reports on SNPs support the fact that they are sufficient to induce global conformational changes in mRNAs.^{31–33} Our experiments demonstrate that HuR binds to SOX2 mRNA in an m⁶A modification-dependent manner. We also show that METTL3-mediated SOX2 mRNA stabilization is HuR-dependent. We found that HuR bound more efficiently to m⁶A-modified RNA over unmethylated RNA and there was a colocalization of HuR and m⁶A modification. In addition, our study revealed that HuR and m⁶A modification might have mutual interdependency in stabilizing mRNA globally which might co-ordinate molecular events in GBM.

GSCs are refractory to radiotherapy and partly it is due to efficient DNA repair.²³ We found that METTL3 is important for radioresistance shown by GSC. Indeed, previous reports show that inhibition of m⁶A complex partners METTL3 or WTAP leads to apoptosis in cancer cells.¹⁶ The radiosensitivity seen in low METTL3 GSC was partially rescued by SOX2 overexpression, which

Figure 7. METTL3 imparts stemness and radioresistance in GBM. GSCs show elevated expression of METTL3 compared with DGCs and the METTL3 levels further increases with γ -irradiation. In GSCs, METTL3 methylates SOX2 mRNA. The methylation recruits HuR to the modified SOX2 and stabilizes the mRNA which enhances the stem-like properties. In irradiated GSCs, further enhanced expression of METTL3 leads to additional induction of SOX2 transcripts which support the radioresistant nature of GSCs.

supports SOX2 as a key effector of METTL3 signaling. In agreement, we found an increase in METTL3 and SOX2 protein levels with the simultaneous increase in global m⁶A modification post irradiation, suggesting the possible existence of a coping mechanism for irradiation stress. HR repair plays a crucial role in radioresistance of GSC compared with non-homologous end joining and abrogating HR key regulators sensitizes GSCs to radiation.³⁴ Our studies support METTL3-mediated m⁶A modification assists in efficient DNA repair. In addition, the METTL3–SOX2 axis augments the HR pathway and there is a convergence between radioresistance phenotype of METTL3 and SOX2-dependent transcriptional activation of DNA repair pathway genes in GSC. Further studies are required to substantiate direct contribution of SOX2 in DNA repair.

During the revision of this work, two reports showed that METTL3 and the associated m⁶A modification inhibited tumorigenicity of GSCs while high levels of ALKBH5 maintained tumorigenicity of GSCs.^{35,36} In contrast, we found METTL3 and the associated m⁶A modification is essential for GSC maintenance. The phenotypic differences seen could possibly be explained by different reliance on m⁶A-modified RNAs in different cell types and the genetic heterogeneity that exists in GBMs. Since the fate of m⁶A-modified mRNAs is mainly determined by reader proteins, their cell-specific abundance between subtypes of GBM, RNA affinity and cumulative binding of various readers on m⁶A-modified mRNAs will eventually determine the functional significance of m⁶A modification.

Contrary to few reports suggesting a positive association between reduced m⁶A methylation and increased tumorigenicity of cancer stem cells,^{35–38} majority of the other studies including our current work implicate an oncogenic role for METTL3.^{14,27,39–45} (<https://books.google.co.in/books?id=4UFUDgAAQ>

BAJ&pg=PT1528&dq=hcc+mettl3+in+proliferation&hl=en&sa=X&ved=0ahUKEwjupL6Ay6vUAhVFKo8KHQoMDVvKQ6AEIJzAA) To add further insight, a comparison of expression levels of two methylases—METTL3/METTL14 and two demethylases—FTO/ALKBH5 in multiple GBM transcriptome data sets was made. Interestingly, METTL3 transcript alone showed a significantly elevated level in all data sets analyzed (Supplementary Figure S7). In contrast, demethylases are not upregulated in any of the data sets and in fact mostly they are either significantly downregulated or unregulated in few data sets (Supplementary Figure S7). We further extended this observation to pan cancer ($n=28$) study derived from TCGA transcriptome data. Similar to GBM data, this analysis also revealed a significant upregulation of METTL3 alone in majority of tumors (64%) compared with METTL14, FTO and ALKBH5 (7, 10 and 7% respectively). Moreover, FTO, ALKBH5 and METTL14 were significantly downregulated in majority of tumors (46, 71 and 64%, respectively) compared with METTL3 (7%) (Supplementary Figures S8A–D). These results as well as other reports and our current study implicate rather an oncogenic role for m⁶A modification. Conflicting results about the role of m⁶A modification with respect to mouse embryonic stem cells prevail in literature.^{28,46–48} The discrepancy was explained by Guela *et al.* using naïve ESCs and Primed epiblast stem cells (Primed EpiSC) as experimental system. They show that while m⁶A preserves the stability of EpiSCs, which are primed for differentiation, it assists naïve ESCs to undergo epiblast transition by degrading stem specific factors.¹¹ Hence, it appears that m⁶A plays conflicting roles according to the cell state and the dominant RNA species. However, additional studies are essential to explain these discrepancies by carrying out context and compartment dependent functions of m⁶A and the inter-play among reader proteins.

In summary, we provide evidence for a vital role for METTL3 in GSC maintenance and radioresistance (Figure 7). We propose mRNA stabilization of m⁶A-modified SOX2 via HuR as the underlying mechanism through which METTL3 preserves stem-like phenotype and we further demonstrate SOX2 salvage GSCs from radiation-induced cytotoxicity by promoting efficient HR repair. Concurrently, impeded transformation efficiency of immortalized astrocytes *in vitro* and orthotopic tumor progression *in vivo* upon silencing of METTL3 reaffirm the crucial role of METTL3 in tumorigenesis. In nutshell, by exploiting METTL3–SOX2 driven defense of GSCs, the self-renewal and tolerance for radiation can be overcome.

MATERIALS AND METHODS

Tumor samples and establishing neurosphere lines from primary GBM tissue

The GBM tissue specimens were procured with an informed, written consent from all the patients, prior to the initiation of the study, in accordance with the institutional ethics committee (IEC) guidelines and approval. All the patients underwent surgical resection of GBM (WHO Grade IV) either at Sri Sathya Sai Institute of Higher Medical Sciences (SSIHMS) or at National Institute of Mental Health and Neurosciences (NIMHANS), Bangalore, India. Freshly collected GBM tissue samples were subjected to neurosphere establishment as described before⁴⁹ (see details in Supplementary Information).

GSCs culture

Primary tumor GSCs MGG4, MGG6, MGG8 and MGG23 were kindly gifted by Dr Wakimoto H (Massachusetts General Hospital, Boston, MA, USA) and GB1 generated in our laboratory were cultured as described previously.¹³

Other experimental methods

Details are provided in Supplementary Information.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on the Oncogene website (<http://www.nature.com/onc>)