**The RNA m6A reader YTHDF2 maintains oncogene expression and is a targetable dependency in glioblastoma stem cells**

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**Supplementary Figure S1.** Comparative RNA m6A profile of GSCs and NSCs. Associated with Figure 1. **A,** Schematic representing the methylation specific RNA immunoprecipitation followed by sequencing (meRIP-seq) discovery approach in GSCs and NSCs. **B,** Unsupervised correlation heatmap of genome-wide localization of m6A peaks in two replicates each of GSC387, GSC4121, NSC11 and HNP1. Spearman correlation was calculated for each pairwise comparison. **C,** Genomic localization heatmap and profile plot of GSC-specific and NSC-specific m6A peaks. Peak regions are scaled and m6A signal is shown 5 kb upstream and downstream of each peak. **D,** Histogram of log2 fold change in m6A signal comparing all m6A peaks in GSCs vs. NSCs. **E,** Quantification of m6A RNA modification levels in NSCs (n = 4) and GSCs (n = 6). **F,** Pie chart demonstrating the m6A peak distribution across transcripts genome-wide. **G,** Metagene plot showing average scaled m6A signal over gene bodies genome-wide in two GSCs and two NSCs. **H,** mRNA expression (log2 transcripts per million, TPM) of genes based on the presence or absence of an m6A peak within the gene in GSCs. **I,** mRNA expression (log2 transcripts per million, TPM) of genes based on the presence or absence of an m6A peak within the gene in NSCs. **J,** Genes with GSC-specific m6A peaks (Fold change greater than 1), ranked based on patient survival significance in HGU133A GBM dataset (median cutoff). X-axis indicates the survival difference between patients with high vs low expression of the indicated gene in days. Y-axis indicates the significance of the survival difference with positive values indicating poor prognosis with high expression. **K,** Genes with GSC-specific m6A peaks (fold change > 1), ranked based on essentiality Z-score in GSCs. Data was derived from Macleod et al. **L,** Comparison of total expression for all transcripts stratified by m6A peak locations in GSCs. Transcripts with peaks in more than one location were included in both groups. **M,** Comparison of total expression for all transcripts stratified by m6A peak locations in NSCs. Transcripts with peaks in more than one location were included in both groups.



**Supplementary Figure S2.** Expression of m6A regulators in TCGA glioblastoma tumors and function of METTL14 in GSCs. Associated with Figure 2. **A,** mRNA expression of selected m6A regulatory genes in the TCGA GBM-LGG dataset classified based on the 2016 WHO criteria. **B,** Analysis of TCGA patient tumor data showing mRNA expression pattern of multiple RNA m6A regulators across grades of patient glioma samples. **C,** YTHDF1, YTHDF2 and YTHDF3 expression in non-tumor and tumor specimens in TCGA glioblastoma HG-U133A microarray data. **D,** METTL14 expression in non-tumor and tumor specimens in TCGA glioblastoma RNA-seq. **E,** Representative immunoblot showing METTL14 levels after transduction of 4121 GSC with a control non-targeting sgRNA sequence (sgCONT) or three independent sgRNAs targeting METTL14 (sgMETTL14 #1, sgMETTL14 #2 and sgMETTL14 #3). **F,** Cell viability of 4121 GSCs expressing a control non-targeting sgRNA sequence (sgCONT) or two independent sgRNAs targeting METTL14 (sgMETTL14 #2 and sgMETTL14 #3). \*\*, p < 0.01. Error bars show standard deviation. **G,** Kaplan-Meier survival curves of immunocompromised mice bearing intracranial 4121 GSCs transduced with either a control sgRNA sequence (sgCONT) or one of two sgRNAs targeting METTL14 (sgMETTL14 #2 or sgMETTL14 #3). **H,** Relative mRNA expression of stemness associated genes normalized to 18S mRNA level in 387 GSCs. \*, p < 0.05, \*\*, p < 0.01. Error bars show standard deviation. **I,** Relative mRNA expression of stemness associated genes normalized to 18S mRNA level in 4121 GSCs. \*, p < 0.05, \*\*, p < 0.01. Error bars show standard deviation. **J,** Cell viability of two patient-derived GSCs (387 and 4121) following transduction with either a non-targeting control sgRNA (sgCONT) or one of two independent, non-overlapping sgRNAs targeting YTHDF2. \*\*, p < 0.01, \*\*\*, p < 0.001. Error bars show standard deviation.  **K,** Cell viability of DGCs, differentiated from two patient-derived GSCs (387 and 4121) following transduction with either a non-targeting control sgRNA (sgCONT) or one of two independent, non-overlapping sgRNAs targeting YTHDF2. \*\*, p < 0.01. Error bars show standard deviation.

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**Supplementary Figure S3.** YTHDF2 target genes and pathways in GSCs and glioblastoma. Associated with Figure 3. **A,** Gene set enrichment analysis of Hallmark gene sets using a pre-ranked gene list weighted by gene expression in YTHDF2 knockdown vs. control in 387 and 4121 GSCs. **B,** Representative western blots showing protein levels normalized to GAPDH level in 387 and 4121 GSCs transduced with sgCONT, sgYTHDF2#1 or sgYTHDF2 #2. **C,** Correlation between YTHDF2 and MYC mRNA expression in all glioblastoma specimens in the Chinese Glioma Genome Atlas dataset. **D,** Correlation between YTHDF2 and MYC mRNA expression in all Classical glioblastoma specimens in the Chinese Glioma Genome Atlas dataset. **E,** Correlation between YTHDF2 and MYC mRNA expression in all Mesenchymal glioblastoma specimens in the Chinese Glioma Genome Atlas dataset. **F,** Correlation between YTHDF2 and MYC mRNA expression in all Proneural glioblastoma specimens in the Chinese Glioma Genome Atlas dataset.

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**Supplementary Figure S4.** YTHDF2 direct targets in GSCs. Associated with Figure 4. **A,** YTHDF2 iCLIP autoradiographs showing pulldown efficiency by YTHDF2 antibody **B,** Top RNA motif enriched in YTHDF2 iClIP peaks in different cell lines **C,** Metagene plot showing average scaled YTHDF2 iClIP signal over gene bodies genome-wide in different cell lines. **D,** Overlap between mRNAs upregulated upon YTHDF2 knockout in GSCs (498 mRNAs) with mRNAs obtained from iCLIP using anti-YTHDF2 antibody in GSCs **E,** Overlap between mRNAs upregulated upon YTHDF2 knockout in GSCs (498 mRNAs) with m6A-tagged mRNAs (m6A IP) which also bind to YTHDF2 (CLIP) in GSCs.



**Supplementary Figure S5.** YTHDF2 binds to and regulates MYC mRNA expression. Associated with Figure 4. **A,** Left panel shows overlap between mRNAs downregulated upon YTHDF2 knockout in GSCs (pink circle, 512 mRNAs) with mRNAs obtained from cross-linking immunoprecipitation (CLIP) using an anti-YTHDF2 antibody in HeLa cell extracts ([27](#_ENREF_27)) (blue circle, 3178 mRNAs). Right panel shows ClueGO analysis using Cytoscape to identify enriched pathways using 108 genes from Venn diagram intersection (p < 0.05). **B,** Left panel shows overlap between mRNAs downregulated in GSCs upon YTHDF2 knockout (pink circle, 512 mRNAs) with mRNAs from HeLa cells that contain m6A modifications (m6A IP) and also bind to YTHDF2 (CLIP; blue circle, 1253 mRNAs). Right panel shows pathway enrichment bubble plot of gene sets enriched among genes (18) obtained from the intersection of two datasets obtained from ClueGO analysis. **C,** Metascape bar graph illustrating top, non-redundant enrichment clusters, one per cluster, using a discrete color scale to represent statistical significance, generated from the genes downregulated upon YTHDF2 knockout in GSCs that were also YTHDF2-bound (108 genes) in HeLa cells obtained using cross-linking immunoprecipitation (CLIP). **D,** Graphs showing enrichment of CBP mRNA in the YTHDF2 immunoprecipitated RNA fraction. CBP was used as a positive control for YTHDF2 binding in the RNA immunoprecipitation experiment. Error bars show standard deviation. **E,** Representative western blots showing METTL3 level normalized to GAPDH level in 4121 GSCs transduced with sgCONT or sgMETTL3. **F,** MYC mRNA levels assessed by quantitative RT-PCR following METTL3 knockout in 387 and 4121 GSCs. \*, p < 0.05. Error bars show standard deviation. **G, H,** MYC mRNA levels assessed by quantitative RT-PCR following transduction with YTHDF2 shRNAs (shYTHDF2) or control, non-targeting shRNA (shCONT), with or without exogenous overexpression of wild type (WT) or m6A binding mutant (W432A and W486A) YTHDF2 in 387 (**G**) and 4121 (**H**) GSCs. \*, p < 0.05, \*\*, p < 0.01 and \*\*\*, p < 0.001. Error bars show standard deviation. **I,** MYC mRNA levels assessed by quantitative RT-PCR following overexpression of WT or m6A binding mutant YTHDF2 clones (W432A and W486A) or a control vector in 387 and 4121 GSCs. Error bars show standard deviation.

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**Supplementary Figure S6.** YTHDF2 does not affect the stemness of NSCs. Associated with Figure 5. **A, B,** Sphere formation using an extreme limiting dilution assay (ELDA) was performed with NSC11 and HNP1 NSCs expressing siCONT orsiYTHDF2.

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**Supplementary Figure S7.** IGFBP3 regulates Glucose metabolism-associated genes. Associated with Figure 6. **A,** Graphs showing enrichment of IGFBP3 mRNAs in the YTHDF2 immunoprecipitated RNA fraction. \*, p < 0.05. Error bars show standard deviation. **B,** Graphs showing changes in the mRNA levels of IGFBP3, at different time points following actinomycin-D treatment in GSCs transduced with sgYTHDF2 #1, sgYTHDF2 #2 or sgCONT. **C,** Representative western blots showing the protein levels of MYC and IGFBP3 normalized to GAPDH level in GSCs transduced with shRNAs against MYC (shMYC.1657 or shMYC.1377) or control shRNA (shCONT). **D,** Growth of HNP1 and NSC11 NSCs expressing shCONT, shIGFBP3.1095 or shIGFBP3.770 as measured by direct cell count (fold change). Error bars show standard deviation. **E,** Cell viability of HNP1 and NSC11 NSCs over 5 days post-transduction with shCONT, shIGFBP3.1095 or shIGFBP3.770, as measured by CellTiter-Glo reagent. Error bars show standard deviation. **F,** Heatmaps of TCGA glioma samples displaying mRNA levels of IGFBP3 and several glucose metabolism-associated genes along with clinical and genetic variant information for each sample. **G,** Relative mRNA level of IGFBP3 target genes normalized to 18S mRNA levels in GSCs transduced with shRNAs against IGFBP3 (shIGFBP3.1095 or shIGFBP3.770) or control shRNA (shCONT) in 387 and 4121 GSCS. \*, p < 0.05, \*\*, p < 0.01. Error bars show standard deviation. **H,** Relative mRNA level of IGFBP3 target genes normalized to 18S mRNA levels in GSCs transduced with sgYTHDF2 #2 or sgCONT, in the presence or absence of IGFBP3 overexpression in 387 and 4121 GSCs. \*, p < 0.05, \*\*, p < 0.01. Error bars show standard deviation. **I,** Representative western blots showing protein levels normalized to GAPDH level in 4121 GSCs transduced with sgCONT, sgYTHDF2 or IGFBP3 overexpression construct. **J,** mRNA levels of MYC assessed by quantitative RT-PCR following IGFBP3 knockdown with two, non-overlapping shRNAs or a non-targeting control sequence in 4121 and 387 GSCs. Error bars show standard deviation. **K,** Relative mRNA level of MYC normalized to 18S mRNA levels in GSCs transduced with sgYTHDF2 or sgCONT, in the presence or absence of IGFBP3 overexpression in 387 and 4121 GSCs. \*\*, p < 0.01. Error bars show standard deviation.

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**Supplementary Figure S8.** Clinical significance of YTHDF2. Associated with Figure 7. **A,** YTHDF2 dependency score in a whole genome CRISPR-Cas9 screen across 558 cancer cell lines. A lower score indicates that a gene is more likely to be a dependency in a given cell line. A score of 0 is equivalent to a gene that is non-essential whereas a score of -1 corresponds to the median of all common essential genes. Data were derived from the Cancer Dependency Map (www.depmap.org). Dependency score was calculated using the CERES algorithm. **B, C,** Kaplan-Meier survival analysis based on YTHDF2 expression stratified by the median of TCGA glioblastoma (**B**) and CGGA (**C**) datasets



**Supplementary Figure S9.** Pharmacodynamics markers of Linsitinib treatment in GSCs. Associated with Figure 7.Representative western blots showing protein levels normalized to GAPDH level in 387 and 4121 GSCs treated with DMSO (0) or indicated concentrations of linsitinib for 3 hours.