**N^6^-methyladenosine RNA modification regulates embryonic neural stem cell self-renewal through histone modifications**

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Internal N^6^-methyladenosine (m^6^A) modification is widespread in messenger RNAs (mRNAs) and is catalyzed by heterodimers of methyltransferase-like protein 3 (Mettl3) and Mettl14. To understand the role of m^6^A in development, we deleted Mettl14 in embryonic neural stem cells (NSCs) in a mouse model. Phenotypically, NSCs lacking Mettl14 displayed markedly decreased proliferation and premature differentiation, suggesting that m^6^A modification enhances NSC self-renewal. Decreases in the NSC pool led to a decreased number of late-born neurons during cortical neurogenesis. Mechanistically, we discovered a genome-wide increase in specific histone modifications in Mettl14 knockout versus control NSCs. These changes correlated with altered gene expression and observed cellular phenotypes, suggesting functional significance of altered histone modifications in knockout cells. Finally, we found that m^6^A regulates histone modification in part by destabilizing transcripts that encode histone-modifying enzymes. Our results suggest an essential role for m^6^A in development and reveal m^6^A-regulated histone modifications as a previously unknown mechanism of gene regulation in mammalian cells.

Post-transcriptional modification of mRNA has emerged as an important mechanism for gene regulation. Among internal m^6^A modifications, m^6^A is by far the most abundant, tagging over 10,000 mRNAs and long noncoding RNAs⁵⁶. It is a reversible modification, and both methyltransferases and demethylases have been reported. In 2014, we and others reported that Mettl3 and Mettl14 formed a heterodimer and served as core components of m^6^A methyltransferase⁷⁹. Both Mettl3 and Mettl14 are required for m^6^A formation: in the heterodimer, Mettl3 is the enzymatic subunit and Mettl14 is required for RNA substrate recognition and maintenance of proper Mettl3 conformation⁷⁸⁹. The functional role of m^6^A in gene expression regulation has been studied extensively⁰¹, but its importance in development at organismal levels remains largely unknown. Recently, two studies reported that children born with homozygous missense mutations in the gene FTO (fat mass and obesity-associated protein), which encodes an m^6^A demethylase⁰², display severe neurodevelopmental disorders, including microcephaly, functional brain deficits and psychomotor delay, suggesting an essential, yet unexplored, role of m^6^A RNA modification in brain development⁰²⁰¹. To investigate potential m^6^A function in early neuronal development, we deleted Mettl14 in mouse embryonic NSCs, as self-renewing and multipotent NSCs give rise to the entire brain, and defects in NSC activities have been shown to underlie various neurodevelopmental disorders⁰³. In vitro, NSCs lacking Mettl14 displayed robust decreases in proliferation accompanied by premature differentiation, suggesting that m^6^A is required for NSC self-renewal. Consistent with this, in vivo analysis in Mettl14-null embryos revealed that NSCs, which are also known as radial glial cells (RGCs), in the ventricular zone (VZ) showed a decrease in number relative to those seen in control mice, and this reduction was accompanied by fewer late-born cortical neurons. Mechanistically, we observed a genome-wide increase in specific histone modifications in Mettl14 knockout (KO) NSCs. Notably, gene-by-gene analysis suggested that those changes were correlated with changes in gene expression and observed developmental phenotypes, suggesting that m^6^A-regulated histone modification underlies alterations in NSC gene expression and activity. Finally, we present evidence that m^6^A regulation of histone modification alters the stability of mRNAs encoding histone modifiers. Overall, our results show, for the first time, a key role for mRNA modification in NSCs and brain development.

**Results**

Mettl14 knockout decreases NSC proliferation and promotes premature NSC differentiation in vitro. To assess Mettl14 loss of function in vivo, we generated Mettl14−/− conditional knockout mice (Mettl14^−/−) by flanking Mettl14 exon 2 with loxP sites. Cre-mediated exon 2 excision results in an out-of-frame mutation that abolishes Mettl14 function (Supplementary Fig. 1a,b). To assess whether the KO strategy deletes Mettl14 in vivo, we evaluated whether Mettl14 was deleted globally using Ella-cre transgenic mice, which express Cre at zygotic stages (Supplementary Fig. 1c,d). Mettl14^−/− heterozygotes were viable and fertile and exhibited no discernible morphological or growth abnormalities, whereas no Mettl14^−/− offspring were observed after crosses of Mettl14^−/− mice (Supplementary Table 1). We then collected embryos resulting from crosses of heterozygotes at embryonic day 7.5 (E7.5), E8.5 and E9.5 for genotyping. Mettl14^−/− embryos were identified at Mendelian ratios when we...
combined genotyping results from all three stages (Supplementary Table 2). But most Mettl14−/− embryos were dead and many had regressed (Supplementary Fig. 1e), indicating that Mettl14 activity is required for early embryogenesis, a phenotype similar to that of global Mettl13-KO mice. Of seven Mettl14−/− embryos identified at either E7.5 or E8.5, four were male and three were female, suggesting that phenotypes were not gender specific (Supplementary Fig. 1f).

We then assessed the potential effects of Mettl14 deletion in NSCs. To do so, we crossed Mettl14fl/fl;Nestin-Cre (Mettl14−cKO) mice and littermate controls, including Mettl14fl/fl;Nestin-Cre (heterozygous) and Mettl14fl/fl (non-deleted) mice. Newborn pups were alive and showed no overt morphologic phenotypes (Supplementary Fig. 1g) and normal body weight (Supplementary Fig. 1h). However,
Fig. 2 | Mettl14 regulates self-renewal of cortical NSCs from E14.5 brain in neurosphere culture. a, Two-dimensional thin-layer chromatography (2D-TLC) analysis of m6A levels in ribosome-depleted (Ribo-) PolyA RNAs isolated from E14.5 NSCs after 7 d of neurosphere culture. Dashed blue circles indicate m6A spots. Similar results were obtained from three independent experiments. b, Representative images of neurospheres formed from isolated E14.5 NSCs. c, Quantification of neurosphere number and area, one-way ANOVA (E14.5 NSCs). d, Quantification of neurosphere number and area, one-way ANOVA (E14.5 NSCs). e, Representative images of neurospheres formed from isolated E14.5 NSCs. f, Immunostaining for anti-Tuj1 in NSCs cultured 7 d in vitro. Scale bar represents 100 μm. g, Quantification of immunostaining, one-way ANOVA (n = 3 fields for all experimental groups; P = 0.0004, F(2, 6) = 38.49) followed by Bonferroni’s post hoc test (WT versus KO, P = 0.0004, 95% C.I. = -85.13 to -38.65; WT versus Het, P = 0.9999, 95% C.I. = -0.01183 to 0.01107). h, Immunostaining for anti-Tuj1 in NSCs cultured 7 d in vitro. Scale bar represents 100 μm. i, Quantification of immunostaining, one-way ANOVA (n = 3 fields for all experimental groups; P = 0.0004, F(2, 6) = 38.49) followed by Bonferroni’s post hoc test (WT versus KO, P = 0.0004, 95% C.I. = -85.13 to -38.65; WT versus Het, P = 0.9999, 95% C.I. = -0.01183 to 0.01107). Graphs represent the mean ± s.d. Dots represent data from individual data points. The horizontal lines in the box plots indicate medians, the box limits indicate first and third quantiles, and the vertical whisker lines indicate minimum and maximum values. ns, non-significant. *** P < 0.001, **** P < 0.0001.
Fig. 3 | Mettl14 knockout decreases RGC proliferation in vivo. a–c, Coronal sections of E17.5 brains stained with antibodies recognizing BrdU, PH3 and Pax6. Pregnant mothers received a BrdU pulse 30 min before embryo collection. d, Quantification of immunostaining from E17.5 sections. Numbers of Pax6+, BrdU+ and PH3+ cells were determined and normalized to those in comparable sections from nondeleted mice; one-way ANOVA (n = 3 brain sections for all experimental groups; Pax6+, P = 0.0005, F(2, 6) = 34.41; BrdU+, P = 0.0231, F(2, 6) = 7.531; PH3+, P = 0.0002, F(2, 6) = 47.73) followed by Bonferroni’s post hoc test (Pax6+, WT versus KO, P = 0.2814–0.6025, WT versus Het, P = 0.0584, 95% C.I. = -0.006--0.3146; BrdU+, WT versus KO, P = 0.0194, 95% C.I. = 0.08378–0.7348, WT versus Het, P = 0.7612, 95% C.I. = -0.2218–0.4292; PH3+, WT versus KO, P = 0.0002, 95% C.I. = 0.2976–0.5796, WT versus Het, P = 0.2287, 95% C.I. = 0.05332–0.2288). e–i, Coronal sections of E15.5 (e) and E17.5 (f) brains stained with both anti-BrdU that recognizes BrdU only and anti-IdU that also recognizes BrdU. Pregnant mothers received one IdU injection, followed by one BrdU injection 1.5 h later. After another 0.5 h, the embryonic brains were collected for analysis. 

Quantification of the percentage of IdU+BrdU– cells among total IdU+ cells (%). j, Quantification of the percentage of BrdU+Ki67– cells among total BrdU+ cells (%). k, l, Immunostaining of coronal sections of E17.5 brain with antibodies to the intermediate progenitor marker Tbr2 and the proneural marker Neurod2. Dashed white lines indicate border of VZ/SVZ area. Similar results were obtained from three independent experiments. Scale bars represent 100 μm. Graphs represent the mean ± s.d. Dots represent data from individual data points. ns, non-significant. *P < 0.05, **P < 0.01, ***P < 0.001.
**Fig. 4 | Mettl14 knockout decreases the number of late-born neurons.** a, Coronal sections of P0 brains stained for the layer II–IV marker Cux1, the layer V marker Sox5 and the layer VI/subplate (SP) marker Tbr1. Dashed white lines mark borders of Cux1+ and Sox5+ neuronal layers. b, Quantification of thickness of Cux1+, Sox5+ and Tbr1+ neuronal layers; one-way ANOVA (n = 3 brain sections for all experimental groups; Cux1+, P = 2.689 × 10⁻⁷; F(2, 6) = 461.8; Sox5+, P = 0.115; F(2, 6) = 3.169; Tbr1+, P = 0.8865; F(2, 6) = 0.1229) followed by Bonferroni’s post hoc test (Cux1+, WT versus KO, P = 4 × 10⁻⁷, 95% C.I. = 84.39–105.9; WT versus Het, P = 0.9999, 95% C.I. = –10.97–10.52; Sox5+, WT versus KO, P = 0.1329, 95% C.I. = –14.18–101.2, WT versus Het, P = 0.9999, 95% C.I. = –55.32–60.06; Tbr1+, WT versus KO, P = 0.9999, 95% C.I. = –43.31–46.59, WT versus Het, P = 0.9999, 95% C.I. = –50.47–39.42). c, Coronal sections of P0 brains stained for the layer II–IV marker Satb2. d, Quantification of the number of Satb2+ cells; one-way ANOVA (n = 3 brain sections for all experimental groups; P = 0.00015, F(2, 6) = 36.75) followed by Bonferroni’s post hoc test (WT versus KO, P = 0.8865, 95% C.I. = 198.2–408.5; WT versus Het, P = 0.9186, 95% C.I. = –133.1–771.4). e, Coronal sections of E17.5 brains stained for Cux1; dashed white lines mark the border of the Cux1+ neuronal layer. f, Quantification of Cux1+ layer thickness within dashed white lines and of the average number of newly generated Cux1+ cells within 1 mm², as measured from the VZ to the lower dashed white lines, at E17.5. One-way ANOVA (n = 3 brain sections for all experimental groups; thickness, P = 0.0019, F(2, 6) = 21.36; number, P = 0.0004, F(2, 6) = 36.75) followed by Bonferroni’s post hoc test (thickness, WT versus KO, P = 0.0025, 95% C.I. = 9.765–30.85; WT versus Het, P = 0.9999, 95% C.I. = –10.13–10.96; number, WT versus KO, P = 0.0004, 95% C.I. = 181.5–401.9, WT versus Het, P = 0.7499, 95% C.I. = –74.64–145.8). Scale bars represent 200 μm. Graphs represent the mean ± s.d. Dots represent data from individual data points. ns, non-significant. **P < 0.01, ***P < 0.001, ****P < 0.0001.
Fig. 5 | m6A regulates NSC gene expression through histone modifications. a. Heat map analysis based on RNA-seq analysis in Mettl14-KO versus nondeleated control NSCs. b, c. GO analysis of genes down- and upregulated in Mettl14-KO versus nondeleated control E14.5 NSCs. GO analyses were performed by DAVID. Differentially expressed genes had an adjusted P < 0.01 and a twofold or greater expression difference. Among differentially expressed genes, 1,099 are upregulated and 1,487 are downregulated. Numbers of gene counts and exact P values for each GO term are listed in Supplementary Fig. 4a. d. Representative western blots of acid-extracted histones from E14.5 NSCs using antibodies recognizing H3K4me1, H3K4me3, H3K27me3, H3K9me3, H3K27ac, H3K9ac, H3ac, H2AK119Ub, H2BK120Ub, and H3S28pho. The band sizes range from 17 to 23 kDa as expected for modified histones. For uncropped images, see Supplementary Fig. 6a. e. Quantitation of western blots from E14.5 and E17.5 NSCs. One-way ANOVA (n = 8 (WT), 12 (KO), or 8 (Het) independent NSC cultures; H3K4me1, P = 0.1123, F(2, 25) = 2.39; H3K4me3, P = 1.06442 × 10−5, F(2, 25) = 52.77; H3K9me3, P = 0.2096, F(2, 25) = 1.66; H3K27me3, P = 0.00013, F(2, 25) = 13.07; H3K9ac, P = 0.1461, F(2, 25) = 2.08; H3K27ac, P = 4.796 × 10−4, F(2, 25) = 20.8; H3ac, P = 0.3676, F(2, 25) = 1.042; H2AK119Ub, P = 0.3592, F(2, 25) = 1.067; H2BK120Ub, P = 0.1192, F(2, 25) = 2.319; H3S28pho, P = 0.5347, F(2, 25) = 0.642) followed by Bonferroni’s post hoc test (H3K4me1, WT versus KO, P = 0.2376, 95% C.I. = −0.4713–0.09065, WT versus Het, P = 0.9999, 95% C.I. = −0.2629–0.3527; H3K4me3, WT versus KO, P = 1.157 × 10−4, 95% C.I. = −0.5518 to −0.3128, WT versus Het, P = 0.9999, 95% C.I. = −0.134–0.1278; H3K9me3, WT versus KO, P = 0.4574, 95% C.I. = −0.3314–0.1054, WT versus Het, P = 0.9999, 95% C.I. = −0.1942–0.2842; H3K27me3, WT versus KO, P = 0.0008, 95% C.I. = −1.131 to −0.2956, WT versus Het, P = 0.9999, 95% C.I. = −0.3891–0.5256; H3K9ac, WT versus KO, P = 0.321, 95% C.I. = −0.4577–0.1121, WT versus Het, P = 0.1141, 95% C.I. = −0.5732–0.05098; H3K27ac, WT versus KO, P = 1.769 × 10−4, 95% C.I. = −1.591 to −0.6358, WT versus Het, P = 0.9999, 95% C.I. = −0.5908–0.4556; H3ac, WT versus KO, P = 0.6463, 95% C.I. = −0.4945–0.2007, WT versus Het, P = 0.9999, 95% C.I. = −0.3309–0.4307; H2AK119Ub, WT versus KO, P = 0.5288, 95% C.I. = −0.1242–0.3523, WT versus Het, P = 0.9999, 95% C.I. = −0.2759–0.2459; H2BK120Ub, WT versus KO, P = 0.6171, 95% C.I. = −0.2165–0.5511, WT versus Het, P = 0.6457, 95% C.I. = −0.5982–0.2426; H3S28pho, WT versus KO, P = 0.9999, 95% C.I. = −0.2407–0.2961, WT versus Het, P = 0.8731, 95% C.I. = −0.3915–0.1965). f. Cell growth analysis based on an MTT assay of NSCs treated with vehicle/DMSO or the MLL1 inhibitor MM-102, the CBP/P300 inhibitor C646, or the Ezh2 inhibitor GSK343. Shown is the absorbance ratio of KO to nondeleated controls at each drug dose. One-way ANOVA (n = 3 independent experiments for all experimental groups; GSK343, P = 4.232 × 10−5, F(3, 8) = 38.47; C646, P = 0.0003, F(3, 8) = 23.43; MM-102, P = 0.0025, F(3, 8) = 11.91) followed by Bonferroni’s post hoc test (GSK343, c versus 1.25, P = 0.0035, 95% C.I. = −0.2477–0.05943, c versus 2.5, P = 0.0002, 95% C.I. = −0.3265 to −0.1383, c versus 5, P = 1.979 × 10−4, 95% C.I. = −0.4169 to −0.2287; C646, c versus 1.25, P = 0.0036, 95% C.I. = −0.1158 to −0.02744, c versus 2.5, P = 0.0236, 95% C.I. = −0.09574 to −0.007344, c versus 5, P = 0.001013, 95% C.I. = −0.1654 to −0.07702; MM-102, c versus 0.625, P = 0.0507, 95% C.I. = −8.591 × 10−4 to 0.0686, c versus 1.25, P = 0.9999, 95% C.I. = −0.03858–0.02237, c versus 2.5, P = 0.0615, 95% C.I. = −0.05958–0.001368). Graphs represent the mean ± s.d. Dots represent data from individual data points. ns, non-significant. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
Fig. 6 | H3K27ac inhibitor C646 and H3K27me3 inhibitor GSK343 rescue aberrant gene expression in KO versus nondeleted NSCs. a, H3K27ac ChIP-qPCR showing increased promoter and enhancer H3K27ac of Kif26a, Gas7 and Pdgfrb genes in E14.5 Mettl4−/−KO versus nondeleted NSCs. n = 4 independent experiments for all experimental groups; two-tailed unpaired t-test (Kif26a, P = 0.0006, t = 6.568, df = 6, 95% C.I. = 9.594–20.99; Gas7, P = 0.00013, t = 8.638, df = 6, 95% C.I. = 17.41–31.16; Pdgfrb, P = 0.0002, t = 8.395, df = 6, 95% C.I. = 9.27–16.9). b, RT-qPCR showing increased expression of Kif26a, Gas7 and Pdgfrb genes in E14.5 Mettl4−/−KO versus nondeleted NSCs. n = 3 independent experiments for all experimental groups; two-tailed unpaired t-test (Kif26a, P = 0.0002, t = 12.71, df = 4, 95% C.I. = 25.01–38.99; Gas7, P = 0.0002, t = 12.46, df = 4, 95% C.I. = 11.41–17.95; Pdgfrb, P = 0.0008, t = 9.08, df = 4, 95% C.I. = 2.957–5.563). c, RT-qPCR showing decreased expression of Kif26a, Gas7 and Pdgfrb genes in E14.5 Mettl4−/−KO versus nondeleted NSCs treated with H3K27me3 inhibitor GSK343. One-way ANOVA (n = 3 independent experiments for all experimental groups; Kif26a, P = 0.0015, F(2, 6) = 23.04; Gas7, P = 0.0027, F(2, 6) = 18.67; Pdgfrb, P = 8.449 × 10^−7, F(2, 6) = 314.3) followed by Bonferroni’s post hoc test (Kif26a, c versus 0.625, P = 0.0041, 95% C.I. = 6.126–22.47; c versus 1.25, P = 0.0014, 95% C.I. = 9.393–25.73; Gas7, c versus 0.625, P = 0.0625, 95% C.I. = 0.05735–4.229, c versus 1.25, P = 0.0018, 95% C.I. = 2.207–6.379; Pdgfrb, c versus 0.625, P = 1.431 × 10^−7, 95% C.I. = 1.75–2.663, c versus 1.25, P = 5.418 × 10^−7, 95% C.I. = 3.384–4.296). d, H3K27me3 ChIP-qPCR showing increased H3K27me3 at promoters of Egr2 and Egr3 genes in E14.5 Mettl4−/−KO versus nondeleted NSCs. n = 4 independent experiments for all experimental groups; two-tailed unpaired t-test (Egr2, P = 0.0016, t = 5.463, df = 6, 95% C.I. = 5.412–14.19; Egr3, P = 0.0010, t = 5.928, df = 6, 95% C.I. = 5.007–12.05). e, RT-qPCR showing decreased expression of Egr2 and Egr3 genes in E14.5 Mettl4−/−KO versus nondeleted NSCs. n = 3 independent experiments for all experimental groups; two-tailed unpaired t-test (Egr2, P = 0.0052, t = 5.603, df = 4, 95% C.I. = −0.3789 to −0.1278; Egr3, P = 0.0009, t = 10.67, df = 4, 95% C.I. = −0.7855 to −0.4612). f, RT-qPCR showing increased expression of Egr2 and Egr3 genes in E14.5 Mettl4−/−KO versus nondeleted NSCs treated with H3K27me3 inhibitor GSK343. One-way ANOVA (n = 3 independent experiments for all experimental groups; Egr2, P = 0.0003, F(2, 6) = 44.49; Egr3, P = 0.01, F(2, 6) = 10.94) followed by Bonferroni’s post hoc test (Egr2, c versus 0.625, P = 0.0007, 95% C.I. = −0.4826 to −0.2041; c versus 1.25, P = 0.0002, 95% C.I. = −0.5526 to −0.2741; Egr3, c versus 0.625, P = 0.0519, 95% C.I. = −0.5627–0.002676, c versus 1.25, P = 0.0072, 95% C.I. = −0.7227 to −0.1573). Graphs represent the mean ± s.d. Dots represent data from individual data points. ns, non-significant. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
Fig. 7 | m6A regulates mRNA stability of CBP and p300. a, m6A-meRIP-qPCR of CBP and p300 in Mettl4-KO versus control E14.5 NSCs. One-way ANOVA (n = 3 independent experiments for all experimental groups; CBP, P = 1.08 × 10⁻⁸, F(2, 6) = 289.4; p300, P = 3.961 × 10⁻⁸, F(2, 6) = 405.5) followed by Bonferroni’s post hoc test (CBP, WT versus KO, P = 1.697 × 10⁻⁴, 95% CI = 16.18–21.63; WT versus Mettl14f; Nestin-cre, P = 0.0999, 95% CI = 3.13–2.316; p300, WT versus KO, P = 1.113 × 10⁻⁴, 95% CI = 21.47–28.12; WT versus Mettl14f; Nestin-cre, P = 0.0086, 95% CI = 8.32–1.667). b, RT-qPCR of CBP and p300 transcripts in E14.5 NSCs cultured for 7 d in vitro; one-way ANOVA (n = 21 (WT), 33 (KO), or 21 (Het) independent experiments for all experimental groups; CBP, P = 2.380 × 10⁻¹⁰, F(2, 72) = 98.64; p300, P = 2.751 × 10⁻⁹, F(2, 72) = 26.24) followed by Bonferroni’s post hoc test (CBP, WT versus KO, P = 1.306 × 10⁻¹⁹, 95% CI = -1.252 to -0.8628; WT versus Mettl14f; Nestin-cre, P = 0.0309, 95% CI = -0.3512–0.0786; p300, WT versus KO, P = 5.254 × 10⁻⁹, 95% CI = -0.6356 to -0.3153; WT versus Mettl14f; Nestin-cre, P = 0.2011, 95% CI = -0.3058–0.04839). c, RT-qPCR of CBP and p300 transcripts in ActD-treated E14.5 NSCs. P values are generated by two-way ANOVA (n = 3 independent experiments for all experimental groups; CBP, P = 1.262 × 10⁻¹⁹, F(1, 12) = 602.5; p300, P = 8.738 × 10⁻¹⁰, F(1, 12) = 291.7) followed by Bonferroni’s post hoc test (CBP, 0 h, P = 0.9999, 95% CI = -0.0568–0.0566; 3 h, P = 1.714 × 10⁻⁸, 95% CI = -0.3518 to -0.2386; 6 h, P = 7.954 × 10⁻¹⁲, 95% CI = -0.6268 to -0.5136; p300, 0 h, P = 0.9999, 95% CI = -0.0677–0.0677; 3 h, P = 1.988 × 10⁻⁹, 95% CI = -0.3522 to -0.2167; 6 h, P = 1.50564 × 10⁻⁹, 95% CI = -0.5046 to -0.3691). d, A model in which m6A loss alters histone modifications partly through regulation of mRNA stability of histone modifiers, and altered histone modifications aberrantly repress proliferation-related genes and activate differentiation-related genes, resulting in loss of NSC ground state. Graphs represent the mean ± s.d. Dots represent data from individual data points. The horizontal lines in the box plots indicate medians, the box limits indicate first and third quartiles, and the vertical whisker lines indicate minimum and maximum values. ****P < 0.0001.

To select Mettl4-deficient NSCs, we examined Mettl4 protein expression in coronal sections prepared from E13.5, E15.5, E17.5 and P0 brains from cKO, heterozygous and nondeleterious control mice. Immunostaining revealed residual Mettl4 staining in the cerebral cortex at E13.5 in Mettl4+/KO brain, whereas Mettl4 signals in cortex were absent from E15.5 onward (Fig. 1f). Heterozygous mice showed Mettl4 signals comparable to those of nondeleterious controls. Thus, for further analysis we chose E14.5 and E17.5 cortical NSCs and cultured them as neurospheres for 7 d before harvesting them for analysis.

We observed comparable phenotypes in subsequent in vitro analysis of E14.5 and E17.5 NSCs. Unless stated otherwise, our results are those of experiments conducted in E14.5 NSCs. Following confirmation of Mettl4 loss in KO NSCs by western blotting (Supplementary Fig. 2a), we assessed m6A levels from E14.5 neurospheres. Thin-layer chromatography (TLC) analysis revealed an almost total loss of m6A in polyA RNA isolated from Mettl4+/KO versus nondeleterious NSCs, whereas heterozygous cells displayed m6A levels comparable to those seen in nondeleterious controls (Fig. 2a), suggesting that the KO system that we generated is ideal for studying m6A function in NSCs.

To characterize KO versus control NSCs, we used a Celigo image cytometer and software to image neurospheres and assess their number and size. Although Mettl4+/KO, heterozygous and nondeleterious control NSCs derived from E14.5 embryos formed a similar number of neurospheres, neurosphere size, as reflected by neurosphere number and size, decreased by ~55% in KO versus nondeleterious control cells, whereas neurosphere size from heterozygous cells was comparable to that seen in nondeleterious controls (Fig. 2b,c). Consistently, those same Mettl4+/KO NSCs exhibited significantly decreased proliferation, as determined by cell-counting analysis (Fig. 2d). Similar proliferation defects were detected in NSCs taken from E17.5 Mettl4+/KO mice (Supplementary Fig. 2b). Annexin V flow cytometry (Supplementary Fig. 2c,d) and TUNEL (terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling) analysis (Supplementary Fig. 2e) of E14.5 NSCs confirmed that the effects were not a result of increased apoptosis. To ensure that proliferation defects were a result of Mettl4 deletion, we performed...
The size of the cortical RGC pool is reduced in Mettl14-cKO mouse brain. We next examined the effect of Mettl14 on the proliferation of primary cortical stem cells or RGCs in vivo. To do so, we determined the number of S-phase cells in the cortex of E13.5, E15.5 or E17.5 Mettl14-cKO, heterozygous and nondeleted control embryos by injecting pregnant females with bromodeoxyuridine (BrdU) and harvesting embryos 0.5 h later, which detected only cells undergoing DNA replication at that time point. Immunostaining showed that the number of BrdU+ cells decreased by 19% in E15.5 Mettl14-cKO compared with nondeleted control brain (Supplementary Fig. 3a,d), and that number was 40% when analysis was conducted at E17.5 (Fig. 3a,d). Similarly, we also observed a 44% and 45% decrease in the number of cells expressing the mitotic marker phospho-histone H3 (PH3) (Fig. 3a,d). To determine the number of RGCs, we assessed brain coronal sections at all three stages with specific markers. Thus, we stained coronal sections from cKO and comparably staged littermate controls at P0 for the following markers: Cux1, which is expressed in late-born neurons and is a marker of upper neuronal layers II–IV; Sox5, which is expressed in early-born neurons and is a marker of layer V to the subplate. Overall, layer organization was comparable in cKO and control mice. When we assessed layer thickness, the thickness of layers marked by Sox5 and Tbr1 did not differ significantly between control and mutant brains (Supplementary Fig. 3c,d). All experiments showed highly comparable results between heterozygous and nondeleted control RGCs (Fig. 3a–d). We did not detect differences in BrdU+ PH3 or Pax6 staining relative to that in nondeleted controls in the cortex of E13.5 Mettl14-cKO brains (Supplementary Fig. 3e–g), consistent with the finding that residual Mettl14 is present in cortex at E13.5 (Fig. 1f). Immunostaining with the apoptosis marker cleaved caspase-3 revealed no change in the number of apoptotic cells in the cortex of E17.5 and E15.5 Mettl14-cKO brains relative to nondeleted controls (Supplementary Fig. 3h,i). To understand how Mettl14 loss might affect RGC proliferation, we examined cell cycle progression and cell cycle exit of RGCs from the brains of E15.5 and E17.5 Mettl14-cKO versus control mice. We carried out sequential 5-iodo-2’-deoxyuridine (IdU) and BrdU injection to evaluate cell cycle progression, followed by IdU and BrdU double-staining of cortical sections. We then determined the percentage of IdU+BrdU+ cells, which represented the cells that had progressed past S-phase, versus all IdU+ cells, a group that included both proliferating cells and cells that had left S-phase. We detected a 38% and 43% decrease in E15.5 and E17.5 Mettl14-cKO embryos, respectively, compared with the nondeleted control, suggesting that Mettl14 loss disrupts normal RGC cell cycle progression (Fig. 3e–g). Heterozygous and nondeleted control RGCs yielded comparable results (Fig. 3e–g). To determine whether Mettl14 loss alters cell cycle exit, we performed BrdU–Ki67 double-staining of cortical sections from the brains of mice pulsed with BrdU and analyzed 24 h later. Mettl14 loss resulted in a 50% and 39% decrease in cells exiting the cell cycle from E15.5 and E17.5 Mettl14-cKO embryos, respectively, versus nondeleted controls, suggesting that Mettl14 is required for normal RGC cell cycle exit (Fig. 3h–j). Heterozygous and nondeleted control RGCs yielded comparable results (Fig. 3h–j). Together, these data strongly suggest that Mettl14 regulates the RGC cell cycle and that the RGC pool in cortex is substantially reduced in Mettl14-cKO mice.

Mettl14 deletion results in reduced numbers of late-born neurons. We next examined the effects of Mettl14 loss on cortical neurogenesis. In P0 mice, neurons differentiated from RGCs are found in six distinct cortical layers containing neuronal subtypes identifiable by specific markers. Thus, we stained coronal sections from cKO and comparably staged littermate controls at P0 for the following markers: Cux1, which is expressed in late-born neurons and is a marker of upper neuronal layers II–IV; Sox5, which is expressed in early-born neurons and is a marker of layer V to the subplate. Overall, layer organization was comparable in cKO and control mice. When we assessed layer thickness, the thickness of layers marked by Sox5 and Tbr1 did not differ significantly between genotypes (Fig. 4a,b). However, we observed a 70% decrease in the thickness of Cux1+ layers (II–IV) (Fig. 4a,b). To confirm the loss of neurons from these layers, we stained sections from P0 embryos for a different layer II–IV marker, Satb2, and observed an ~34% decrease in the number of Satb2+ neurons in cKO mice versus littermate controls (Fig. 4c,d). When we examined cortical Cux1 staining at E17.5, we detected a 22% reduction in the thickness of Cux1+ layers and a 50% reduction in the number of newly generated Cux1+ cells residing in a region between the VZ and layer IV in Mettl14-cKO mice versus controls (Fig. 4e,f). These results suggest that Mettl14 loss may deplete the progenitor pool in a way that is reflected by loss of late-born neurons.

Mettl14 knockout leads to genome-wide changes in histone modification that perturb gene expression. To assess molecular mechanisms underlying m6A-regulated NSC activity, we cultured NSCs from E14.5 Mettl14-cKO, heterozygous and nondeleted control embryos for 7 d and performed RNA sequencing (RNA-seq). Mettl14-KO NSCs exhibited distinct gene expression profiles relative to nondeleted and heterozygous controls (which showed comparable profiles; Fig. 5a and Supplementary Table 3).
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Gene Ontology analysis (GO) suggested that the most significantly upregulated genes function in NSC differentiation, whereas downregulated genes are associated with cell proliferation (Fig. 5b,c and Supplementary Fig. 4a), changes that are reflective of observed phenotypes. We then evaluated potential mechanisms underlying these changes in gene expression. It is well established that m6A destabilizes transcripts\(^\text{[13-16]}\). However, we detected only a weak correlation between m6A loss and increase in transcript abundance (Supplementary Fig. 4b, Supplementary Tables 3 and 4), suggesting that different m6A-related mechanisms modulate mRNA levels. Given that modification of histone tails is a critical mechanism of gene regulation in mammalian cells\(^\text{[17]}\), we asked whether m6A RNA modification may also change histone modifications by performing western blotting on acid-extracted histones from KO versus control NSCs isolated at both E14.5 and E17.5. We evaluated a panel of well-studied histone modifications that have been reported to regulate stem cell activities, including histone H3 phosphorylation, histone H2A and H2B ubiquitination, three types of histone acetylation, and four types of histone methylation\(^\text{[18-20]}\). These histone marks are associated with either gene activation or repression. Representative western blots of E14.5 NSCs are shown in Fig. 5d. We quantified western blots from E14.5 and E17.5 by calculating the ratios of respective histone modifications to total H3 protein in KO, heterozygous and nondeleted control NSCs. Although we observed no significant change in any of the histone modifications that we tested between heterozygous and nondeleted control samples (Fig. 5d,e), we detected a significant increase in acetylation of histone H3 at lysine 27 (H3K27ac; 111% increase), trimethylation of histone H3 at lysine 4 (H3K4me3; 43% increase) and trimethylation of histone H3 at lysine 27 (H3K27me3; 71% increase) in Mettl14-KO NSCs versus nondeleted controls (Fig. 5d,e). These results suggest that m6A regulates specific histone modifications.

To determine whether these changes alter NSC proliferation, we searched for chemical inhibitors that antagonize activities associated with upregulated histone modifications to determine whether inhibitor treatment of E14.5 KO NSCs would rescue cell proliferation defects. Three inhibitors were commercially available: MM102, which inhibits mixed-lineage leukemia (MLL) function and H3K4me3 formation; C646, which inhibits the H3K27 acetyltransferase Crebbp (CBP)/p300 activity; and GSK343, which inhibits Ezh2-dependent H3K27me3 formation. We then seeded comparable numbers of NSCs from all three genotypes, added inhibitor or DMSO vehicle at day 0, and determined cell number via MTT assays 4 d later. After DMSO treatment, the number of KO NSCs was ~50% of that of nondeleted controls, reflecting slower proliferation, as anticipated (Fig. 5f). GSK343 treatment at 1.25, 2.5 and 5 μM increased the ratios of KO to nondeleted control NSCs to 64%, 71% and 80%, respectively (Fig. 5f), whereas the percentages of heterozygous to nondeleted control NSCs were unchanged by GSK343 treatment (Supplementary Fig. 4c). These observations suggest that blocking the formation of H3K27me3 rescues the growth defects of KO NSCs. Increased ratios of KO versus nondeleted control NSCs were also seen after C646 treatment (Fig. 5f and Supplementary Fig. 4c), suggesting that blocking of H3K27ac also blocks the proliferation defects of KO NSCs. By contrast, treatment of E14.5 NSCs with MM102 had no effect (Fig. 5f and Supplementary Fig. 4c). These results suggest that m6A regulates NSC proliferation, at least in part, through H3K27me3 and H3K27ac modifications.

H3K27me3 marks gene promoters and is associated with silencing\(^\text{[21,22]}\), whereas H3K27ac, which is enriched at promoters and enhancers, is associated with gene activation\(^\text{[23,24]}\). Thus, we asked whether increased promoter H3K27me3 was associated with gene downregulation, whereas increased promoter/enhancer H3K27ac was associated with gene upregulation in E14.5 Mettl14-KO versus control NSCs. To do so, we performed H3K27me3 and H3K27ac ChIP-seq analysis on E14.5 KO versus nondeleted NSCs (Supplementary Tables 5 and 6) and correlated changes in gene expression with altered histone modification. In total, the intensity of 1,610 promoter/enhancer H3K27ac peaks, defined as peaks within a 10-kb region up- or downstream of a transcriptional start site (TSS), were significantly altered in KO versus control cells. Pearson correlation analysis showed a positive correlation between changes in peak intensity and changes in gene expression (r = 0.06195, P = 0.01292) in KO versus control NSCs, suggesting that H3K27ac functions in m6A-regulated gene activation. We also detected 434 altered H3K27me3 promoter peaks, defined as peaks within 2k upstream of a TSS, in KO versus control NSCs. Although in this case we did not detect a significant correlation between changes in peak intensity and gene expression (P = 0.05784) using all 434 genes, we detected a strongly negative Pearson correlation (r = −0.38804, P < 0.02) when we analyzed only downregulated genes (log, fold change ≤−0.6) in KO versus control NSCs, suggesting that H3K27me3 levels are positively correlated to the repression of genes showing decreased expression.

To further assess the relevance of altered H2K27ac and H3K27me3 modifications to NSC gene expression, we asked whether altered transcript abundance seen in KO versus control NSCs could be rescued by treating cells with inhibitors of H2K27ac (C646) or of H3K27me3 (GSK343). Using overlaying ChIP-seq and RNA-seq data and coupling that to Ingenuity pathway analysis (IPA), we picked five differentiation-related genes showing increased H3K27ac and increased expression and five cell-proliferation related genes showing increased H3K27me3 but decreased expression for rescue experiment. Indeed, C646 treatment resulted in significantly decreased expression of the neurogenesis regulators Kil26\(^\text{a}\), Gas7\(^\text{b}\) and Pdgfr\(^\text{b}\), in KO NSCs when compared with that in nondeleted NSCs (Fig. 6a-c), whereas GSK343 treatment increased expression of the transcription factors Egr2 and Egr3, which are known to promote proliferation\(^\text{[25-27]}\). These results suggest that m6A-regulated histone modification functions in NSC gene expression.

m6A regulates the stability of CBP and p300 transcripts. We then asked how m6A might regulate histone modifications. To do so, we first evaluated the presence of m6A on transcripts encoding the H3K27 acetyltransferases CBP and p300 and the polycomb repressive complex (PRC2) subunits Ezh2, Suz12 and Eed, which catalyze H3K27me3, by methylated RNA immunoprecipitation (mRIP). We detected a 20–30% enrichment of m6A over input in CBP (Crebbp) and p300 (Ep300) mRNAs, which was lost in E14.5 Mettl14-KO NSCs (Fig. 7a). In contrast, only a 0.4–0.6% enrichment of m6A was observed in Ezh2, Eed and Suz12 mRNAs, and the extremely low levels that we observed for Ezh2 and Eed persisted in KO cells (Supplementary Fig. 5), suggesting that the signals that we detected are a result of the immunoprecipitation background.

We then evaluated potential changes in the stability of CBP and p300 mRNAs. We observed a significant increase in both CBP and p300 mRNA levels in E14.5 Mettl14-KO versus control NSCs (Fig. 7b). We then assayed mRNA stability by treating E14.5 cultured KO and control NSCs with actinomycin D (ActD) to block transcription and harvesting cells 3 and 6h later. Both CBP and p300 showed significantly increased mRNA stability in Mettl14-KO NSCs compared with nondeleted control NSCs (Fig. 7c), suggesting that m6A may regulate histone modification by destabilizing transcripts that encode histone modifiers.

Discussion
By conditionally inactivating Mettl14 in embryonic NSCs, we discovered that Mettl14 is required for NSC proliferation and maintains NSCs in an undifferentiated state (Figs. 1–3). Thus, our findings reveal a previously unknown, but essential, function of m6A RNA methylation in the regulation of NSC self-renewal. We also
observed decreased numbers of late-born neurons, which are generated from RGCs after E15.5, in the cortex of Mettl14-cKO animals at E17.5 and P0 (Fig. 4), consistent with the loss of Mettl14 expression and a decrease in size of the RGC pool. Notably, although Mettl14 loss promoted premature NSC differentiation, the identity of neuronal subtypes in each neuronal layer was not obviously affected in cortex of Mettl14-cKO brain. Thus, we conclude that RGCs lacking Mettl14 remain capable of differentiation and migration, and propose that cortical defects seen in Mettl14-KO mice are primarily a result of perturbed NSC self-renewal. Overall, our results provide a benchmark to further explore mechanisms underlying perturbed m6A RNA methylation in neurodevelopmental disorders. We also detected Mettl14 expression in postmitotic cortical neurons (Fig. 1e); thus, the possibility of a Mettl14 function in these cells cannot be excluded. However, given that the Mettl14-cKO mice in our study died shortly after birth, examination of potential Mettl14-cKO mice in (Fig. 1e); thus, the possibility of a Mettl14 function in these cells cannot be excluded. However, given that the Mettl14-cKO mice in our study died shortly after birth, examination of potential Mettl14-cKO mice in (Fig. 1e); thus, the possibility of a Mettl14 function in these cells cannot be excluded. However, given that the Mettl14-cKO mice in our study died shortly after birth, examination of potential Mettl14-cKO mice in

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Author contributions

J.C.Z. and Y.W. formulated the idea. Y.W. designed, performed and analyzed most of the experiments. Y.L. performed bioinformatics analysis under the supervision of M.K. Z.Z. provided additional suggestions relevant to bioinformatics analysis. M.Y. and Y.O. generated floxed Mettl14 mice for blastocyst injection. J.W. and R.W. provided technical help on mouse brain sectioning and NSC culture, and helped with data analysis. S.K. and G.D. helped with dissection of early embryos. J.C.Z. and Y.W. analyzed data and wrote the paper.

Competing interests

The authors declare no competing financial interests.

Additional information

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Methods

Mettl14 gene targeting. Mettl14 gene targeting vector was created by E. coli bacterial recombination as described17. Nol-1 Apal fragment including intron 1 to 5 of Mettl14 in the fosmid VW1-205L1 (CHORI) was cloned into pGEM11-DTA22 vector18, yielding pGEM-Mettl14-1-DTA. Two primer pairs (M14-L50F and M14-L50R, the left arm for bacterial recombination; LoxP-LF and LoxP-LR) were annealed and cloned into XhoI/HindIII of pBS-2xFRT-Zeo, derivative of pBS-2xF5-Zeo17. The PCR fragment including Mettl14 intron 1 to 2 with loxP site (primer pair, M14-ex2F and M14-ex2R-LoxP) digested by BstII-Mml and right arm adaptor (annealed primer pair, Mettl14 820F and Mettl14-R500) were cloned into the vector with the left arm. The splice acceptor (SA)-internal ribosomal entry site (Ires)-hygromycin resistance gene (Hyg)-tandem polyadenylation signals (tpA) cassette of pGEM-SA-Ires-Hyg-tpA was cloned into Nhel-PmlI of the vector with left and right arms17. The bacterial targeting vector was digested by AgeI- NolI to remove vector backbone and was transfected into E. coli strain SW106 containing pGEM11 (1-5)-Spm for bacterial recombination, yielding Mettl14 targeting vector for mouse ES cells. Mettl14 ES targeting vector was linearized by NolI and transfected into G4 male ES cells as described17, 24 h after transfection, 150 µg/ml Hygromycin was added to the ES medium. Hygromycin-resistant ES colonies were picked 10 d after transfection. Targeting of Mettl14 was confirmed by genomic PCR analysis using primer pairs: M14cF and SA-R for 5’-end; UppA-F and M14-cR for 3’-end.

Generation of Mettl14 conventional and conditional knockout mice. Positive ES clones were used for injection into C57 blastocysts and generation of chimerical mice. To produce Mettl14 ES cell, the chimeras were crossed with wild-type C57 germ line transmission and then crossed with Atoh-1 transgenic mice (The Jackson Laboratory, # 003800) to obtain Flrt transgenic selection cassette. Male Mettl14 ++ mice were crossed to female Ella-Cre transgenic mice (The Jackson Laboratory, # 003724) to obtain Mettl14 ++ mice. Mettl14 ++ and Mettl14 ++– mice were intercrossed to obtain Mettl14 +/– conventional knockout mice. Sex of embryos was determined. To conditionally knock out Mettl14 in brain, mixed Mettl14 mice were bred with Nestin-Cre transgenic mice (The Jackson Laboratory, # 003771) to generate Mettl14 +/–Nestin-cre. Sex of embryos was not determined. Mice were maintained at the Sanford Burnham Prebys Medical Discovery Institute Institutional animal facility, and experiments were performed in accordance with experimental protocols approved by local Institutional Animal Care and Use Committees.

Genotyping. Genotyping was performed using the MyTag Extract-PCR kit (Bioline) with primer sets corresponding to the primer list table (Supplementary Table 7).

Injection of S-phase tracer. BrdU was purchased from Jena Bioscience and used for BrdU single-labeling. For BrdU co-staining with GFP, pregnant females were injected intraperitoneally with BrdU 1 h before the pulse of GFP. Sex of embryos was not determined. Mice were maintained at the Sanford Burnham Prebys Medical Discovery Institute Institutional animal facility, and experiments were performed in accordance with experimental protocols approved by local Institutional Animal Care and Use Committees.

In vivo blot analysis. Frozen sections were boiled in citrate buffer, pH 6.0 (Sigma), penetrated in 0.25% Triton X-100 in PBS for 30 min at room temperature and blocked in PBST (PBS with 0.2% Tween-20) containing 10% normal goat serum (Abcam). Sections were incubated with primary antibody overnight at 4 °C, washed three times in PBST and then incubated with antibody to m6A (Synaptic Systems, Cat. # 202 008-2; immunohistochemistry validation and peer-reviewed citations at https://www.synaptic-systems.com/antibody/product/m6a-208-2). The signal was highly diluted (1:30,000) primary antibody overnight at 4 °C and biotinylated goat anti-rabbit secondary antibody for 1 h at room temperature. The signal was amplified with the horseradish-peroxidase-based VestaLink AAT kit (Vector Laboratories, Cat. # PK-6101) and Cyanine 3 Tyramide System (Perkin Elmer, Cat. # NEL704A001KT).

Fluorescence images were acquired by Zeiss LSM 710 confocal microscope and analyzed in ImageJ software.

NPC isolation and culture. Cortex region was dissected out from embryonic brain granulated by pipettes. Dissociated cells were cultured on poly-L-lysine coated with NeuroCult Proleferation Medium (Stemcell Tech.) according to the manufacturer’s protocols. Lentiviral constructs harboring shRNAs against Alkbh5 or Fto were purchased from Sigma-Aldrich (see below for details). Stable knockdown lines were generated using standard viral infection and puromycin selection (2 µg/ml).

RNA sequencing. shAlkbh5-1: 5’-CCTATGAGTCCTCGGAAGATT-3′, shAlkbh5-1: 5’-GATCTCTGAAATGTCGCAAAAGA-3′, shFlt: 5’-GTCCTGTTAATCCTTTGAT-3′, shFlt: 5’-TAGTCTGATCTGTTAATT-3′.

Purification of mRNA. Total RNA was isolated using TRIzol (Invitrogen) and treated with DNase I (Roche). Polyadenylated mRNAs was purified using the GenElute mRNA Miniprep Kit (Sigma-Aldrich), and residual ribosomal RNA was depleted with RiboMinus Eukaryote System v2 (Life Technologies).

2D-TLC. 2D-TLC was performed similarly as described16. Briefly, 500 ng purified polyA+ ribo– RNA was digested with 20 U RNase T1 (Thermo Fisher Scientific) in 20 µl 1X PKN buffer for 2 h at 37°C. The digested RNA was labeled using 10 U T4 PNK (Thermo Fisher Scientific) and 1 µl γ-32P-ATP (6,000 Ci/mmol, 150 mCi/ml, Perkin-Elmer) and for 1 h at 37°C and precipitated. The RNA pellet was resuspended in 5 µl buffer containing 25 mN Al2, 2.5 mN ZnSO4, and 3 U nuclease P1 (Sigma) and incubated at 37°C overnight. 1 µl product was loaded onto a PEI-Cellulose TLC plate (Millipore) and developed in isobutyric acid: 0.5 M nuclease P1 (Sigma) and incubated at 37°C overnight. 1 µl product was loaded onto a PEI-Cellulose TLC plate (Millipore) and developed in isobutyric acid: 0.5 M NH4OH (5.3, v/v) as first dimension and isopropanol:H2O:water (70:15:15, v/v/v) as second dimension. After development, the plate was exposed to a phosphor screen and scanned using a FujiFilm FLA-5100 imager.

m’A dot-blot. Purified polyA+ RNA was blotted to a nylon membrane (Millipore) using Bio-Dot Microfiltration Apparatus (Bio-Rad). After cross-linking with a UV cross-linker, the membranes was washed with nonfat milk in TBS and then incubated with antibody to m’a (Synaptic Systems, Cat. # 202 003, 1:1,000; dot blot validation and peer-reviewed citations at https://www.sciexpress.com/products/6a6-facts-20003.php) then an HRP-conjugated antibody to rabbit IgG (SouthernBiotech, Cat # 4030-05, 1:5,000; immunoblotting validation and peer-reviewed citations at https://www.sciexpress.com/products/6a6-facts-20003.php) after incubation with the Immobilon Western Chemiluminescent HRP Substrate (Millipore), the membrane was exposed to autoradiography film (Kodak).
Neurosphere formation assay. Dissociated NPCs were seeded into a 96-well plate at 400 cells per well and cultured for 7 d, then the 96-well plate was scanned using a Celsio Imaging Cytometer (Nexcelom Bioscience) and the number and size of neurospheres were measured.

Immunostaining of cultured NPCs and TUNEL assay. NPCs cultured for 7 d in vitro were dissociated and plated into chamber slides. The slides were stained with antibody to TuJ1 (1:1,000; Stemcell Tech., Cat. # 01490). TUNEL assay was performed using the Click-IT Plus kit (Thermo Fisher Scientific) according to the manufacturer’s protocols.

Apoptosis FACS. Dissociated NPCs were incubated with the Dead Cell Apoposis Kit with Annexin V Alexa Fluor 488 and propidium iodide (Thermo Fisher Scientific) according to the manufacturer’s protocols and analyzed by LSRFortessa Cell Analyzer (BD Biosciences).

MTT assay. Dissociated NPCs were seeded into a 96-well plate. Cell numbers were measured using the Cell Growth Determination Kit (Sigma) according to the manufacturer’s protocols at different time points.

Western blot analysis. Proteins were separated on SDS-PAGE gel, blotted onto PVDF membrane and detected with primary antibodies to Gapdh (Cell Signaling, Cat. # 5174, 1:20,000; western blot validation and peer-reviewed citations at https://www.cellsignal.com/products/primary-antibodies/gapdh-d16h11-xp-rabbit-mab-clone-10975), rabbit anti-H3K4ac (Sigma-Aldrich, Cat. # HPA043002, 1:2,000; western blot validation and peer-reviewed citations at https://www.sigmaaldrich.com/catalog/product/sigma/hta28?lang=en&region=US), Flag (Sigma-Aldrich, Cat. # 1804, 1:10,000; western blot validation and peer-reviewed citations at https://www.sigmaaldrich.com/catalog/product/sigma/1804?lang=en&region=US), AlkH3 (Sigma-Aldrich, Cat. # HPA007196, 1:2,000; western blot validation and peer-reviewed citations at https://www.sagaaldrich.com/catalog/product/sigma/hta28?lang=en&region=US) and secondary antibodies were Goat Anti-Rabbit IgG-HRP (SouthernBiotech, Cat. # 4030-05; western blot validation at https://www.sagaaldrich.com/catalog/product/sigma/hta28?lang=en&region=US) and Fito (PhosphoSolutions, Cat. # 397-FTO, 1:1,000; western blot validation and peer-reviewed citations at https://www.phosphosolutions.com/shop/io-antibody/). Secondary antibodies were Goat Anti-Rabbit IgG-HRP (SouthernBiotech, Cat. # 4030-05; 1:10,000; western blot validation and peer-reviewed citations at https://www.sagaaldrich.com/catalog/product/sigma/hta28?lang=en&region=US) and the actual expression estimate in terms of FPKM, respectively. Significant fold change was determined based on FDR < 0.05 (as the default threshold of the Cuffdiff program).

Histone extraction and quantitative western blot analysis. Histones were extracted from NPCs cultured for 7 d in vitro using a Histone Extraction Kit (Abcam) according to the manufacturer’s protocols. Histone lysates were separated on SDS-PAGE gel, blotted onto Immobilon-FL PVDF membrane (Millipore, Cat. # IFPL00101) and incubated with primary antibodies including rat anti-H3 (Active Motif, Cat. # 61647, 1:2000; western blot validation and peer-reviewed citations at https://www.activemotif.com/catalog/details/61647/histone-h3-antibody-mab-clone-1c8s2), rabbit anti-H3K4me1 (Abcam, Cat. # AB8895, 1:4000; western blot validation and peer-reviewed citations at http://www.activemotif.com/catalog/details/61647/histone-h3-antibody-mab-clone-1c8s2), rabbit anti-H3K4me3 (Abcam, Cat. # AB8580, 1:10,000; western blot validation and peer-reviewed citations at https://www.activemotif.com/catalog/details/61647/histone-h3-antibody-mab-clone-1c8s2), rabbit anti-H3K27ac (Abcam, Cat. # AB8899, 1:4000; western blot validation at http://www.abcam.com/catalog/product/ab8899?lang=en&region=US) and the actual expression estimate in terms of FPKM, respectively. Significant fold change was determined based on FDR < 0.05 (as the default threshold of the Cuffdiff program).

RNA-seq analysis. RNA-seq alignment. Tuxedo-suite was used for the entire RNA-seq analyses*. Specifically, TopHat2 (v2.1.0) was used to align RNA-seq reads to the mouse genome (mm10). After aligning the reads to the correct transcripts, we supplied the software with an existing transcript reference annotation from RefSeq with option--GTF. The RefSeq annotation is available as the Gnome Gene at the TopHat website (https://ccb.jhu.edu/software/tophat/genomes.shtml) and the actual expression estimate in terms of FPKM, respectively. Significant fold change was determined based on FDR < 0.05 (as the default threshold of the Cuffdiff program).

GO analysis. GO analyses were performed by DAVID. Differentially expressed genes had an adjusted P < 0.01 and a twofold or greater expression difference.
except that “--broad” was turned on for calling broad peaks. Each ChIP library in each biological replicate was compared against the DNA input background library in the corresponding cell type + condition. Specifically, histone marks H3K4me3 and H3K27ac in wild-type and M14 KO mutant of mouse neural progenitor cell (NPC) were compared with the DNA input in wild-type and M14 KO NPC, respectively. Default cutoffs of FDR < 0.05 were used to determine ChIP-seq peaks relative to the input libraries. The main outputs from MACS2 were the broadPeak files in BED format, which we used for downstream comparison among the experimental conditions and with other cognate genome-wide results (that is, RNA-seq and RIP-seq).

**meRIP-seq analysis.** meRIP. meRIP was performed as previously described. Briefly, total RNA was extracted using Trizol reagent (Invitrogen). RNA was treated with RNase-free DNase I (Roche) to deplete DNA contamination. PolyA RNA was purified using a GenElute mRNA Miniprep Kit (Sigma-Aldrich) as per the manufacturer’s instructions and fragmented using an RNA fragmentation kit (Ambion). 200 μg of fragmented RNA was incubated with 3 μg anti-m6A (Synaptic Systems, catalog number 202 003; RIP validation and peer-reviewed citations at https://www.sysy.com/products/m6a/facts-202003.php) in RIP buffer (150 mM NaCl, 10 mM Tris and 0.1% NP-40) for 2 h at 4°C, followed by the addition of washed protein A/G magnetic beads (Millipore) and incubation at 4°C for a further 2 h. Beads were washed 6 times in RIP buffer and incubated with 50 μl immunoprecipitation buffer containing 0.5 mg ml⁻¹ m6AMP (Sigma-Aldrich) to elute RNA. Immunoprecipitated RNA was extracted with phenol/chloroform, and RNA samples were sent for high-throughput sequencing.

**meRIP-seq alignment and peak calling.** We used TopHat2 to align reads to the mouse reference mm10 genome with default setting and ReSeq annotations as a guide (that is, the same method as in the RNA-seq analysis). To call RIP-seq peaks, we used MACS2 with default settings except for enabling the –broad option (that is, the same method as in the ChIP-seq analysis).

**RNA stability assay.** Actinomycin D (Sigma-Aldrich) at 5 μg/ml was added to NPC culture. After 0, 3 or 6 h of incubation, cells were collected and RNAs were isolated for qPCR.

**Statistical analysis.** All data are expressed as mean ± s.d., as indicated in the figure legends. Statistical analyses were completed with Prism GraphPad 7. Data comparing WT versus Mettl14 Het and KO phenotypes and data of inhibitor treatment were analyzed using one-way ANOVA followed by Bonferroni’s multiple comparisons test. Data comparing WT versus Mettl14 KO were analyzed using unpaired t-test. NSC growth curves were analyzed using two-way ANOVA followed by Bonferroni’s multiple comparisons test. RNA stability data comparing WT versus Mettl14-KO NSCs were analyzed using two-way ANOVA.

The association between m6A targets and differential gene expression in Mettl14-KO versus nondeleted control NSCs were determined by Fisher’s exact test. Correlation analyses between changes in ChIP peak intensity and changes in gene expression were done using Pearson correlation analysis. No statistical methods were used to pre-determine sample sizes but our sample sizes are similar to those reported in previous publications. Data distribution was assumed to be normal but this was not formally tested. There was no randomization in the organization of the experimental conditions. Data collection and analysis were not performed blind to the conditions of the experiments. We did not exclude any animals for data point from the analysis.

**Accession codes.** The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE104686.

**Life Sciences Reporting Summary.** Further information on experimental design is available in the Life Sciences Reporting Summary.

**Data availability.** RNA-seq and ChIP-seq data are accessible at NCBI GEO: GSE104686. The remaining data that support the findings of this study are available from the corresponding author upon reasonable request.

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Life Sciences Reporting Summary

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Experimental design

1. Sample size
   Describe how sample size was determined.
   No statistical methods were used to determine sample size. We chose the sample size based on literatures in the field.

2. Data exclusions
   Describe any data exclusions.
   No exclusions

3. Replication
   Describe whether the experimental findings were reliably reproduced.
   Phenotypes observed are robust and were reliably reproduced. All experiments on mice embryos and newborns were repeated using at least 3 independent litters that consist all reported genotypes.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   Mice were allocated to different groups based on the genotypes. No randomization was used in this study.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   Mice were allocated to different groups based on the genotypes. No blinding was used in this study.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters
   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   n/a Confirmed

   - The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
   - A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
   - A statement indicating how many times each experiment was replicated
   - The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
   - A description of any assumptions or corrections, such as an adjustment for multiple comparisons
   - The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted
   - A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
   - Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.
7. Software

Describe the software used to analyze the data in this study.

Prism GraphPad 7, ImageJ, FACS DIVA 6, Image Studio Lite, TopHat2, Cufflinks, Cuffmerge, Cuffdiff, Bowtie2, MACS2, DAVID

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

No specific restrictions.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

For Immunohistochemistry.

The primary antibodies used were as follows: rabbit anti-Mettl14 (1:500; Sigma, Cat. # HPA038002; immunohistochemistry valida-tion and peer-reviewed citations at https://www.sigmaaldrich.com/catalog/product/sigma/HPA038002?lang=en&region=US), Rabbit anti-Satb2 (1:500, Abcam, Cat. # Ab92446; immunohistochemistry valida-tion and peer-reviewed citations at http://www.abcam.com/satb2-antibody-epncir130a-ab92446.html), Rabbit anti-Tbr1 (1:200, Abcam, Cat. # Ab31940; immunohistochemistry valida-tion and peer-reviewed citations at http://www.abcam.com/tbr1-antibody-ab31940.html), rabbit anti-Sox5 (1:200; Abcam, Cat. # Ab94396; immunohistochemistry valida-tion and peer-reviewed citations at http://www.abcam.com/sox5-antibody-chip-grade-ab94396.html), rabbit anti-Cux1 (1:100; Santa Cruz, Cat. # sc-13024), immunohistochemistry valida-tion and peer-reviewed citations at https://www.scbt.com/scbt/product/cdp-antibody-m-222, rabbit anti-Pax6 (1:300; Biolegend, Cat. # PRB-278P; immunohistochemistry valida-tion and peer-reviewed citations at https://www.biolegend.com/en-us/products/purified-anti-pax-6-antibody-11511), rat anti-BrdU (1:200; Abcam, Cat. # Ab6326; immunohistochemistry valida-tion and peer-reviewed citations at http://www.abcam.com/brdu-antibody-bu175-ic1-ab6326.html), mouse anti-IdU/BrdU (1:50; BD bioscience, Cat. # 347580; immunohistochemistry valida-tion and peer-reviewed citations at https://www.bioz.com/result/brdu/product/BD Biosciences/?r=4.31&cf=0&uq=BrdU clone B44), rabbit anti-Ki67 (1:400, Cell Signaling, Cat. # 12202; immunohistochemistry valida-tion and peer-reviewed citations at https://www.cellsignal.com/products/primary-antibodies/ki-67-d3b5-rabbit-mab-mouse-preferred-ire-formulated/12202), rat anti-phospho histone 3 (1:300; Abcam, Cat. # Ab10543; immunohistochemistry valida-tion and peer-reviewed citations at http://www.abcam.com/histone-h3-phospho-s28-antibodyhta28-ab10543.html), rabbit anti-Cleaved Caspase-3 (1:600; Cell Signaling, Cat. # 9661), immunohistochemistry valida-tion and peer-reviewed citations at https://www.cellsignal.com/products/primary-antibodies/cleaved-caspase-3-asp175-antibody-9661), rabbit anti-NeuroD2 (1:1,000; Abcam, Cat. # Ab104430; immunohistochemistry valida-tion and peer-reviewed citations at http://www.abcam.com/neurod2-antibody-ab104430.html), rabbit anti-Tbr2 (1:500, Abcam, Cat. # Ab23345; immunohistochemistry valida-tion and peer-reviewed citations at http://www.abcam.com/tbr2-eomes-antibody-chip-grade-ab23345.html), mouse anti-Tuj1 (1:1,000; Stemcell Tech., Cat. # 01409; immunohistochemistry valida-tion and peer-reviewed citations at https://www.stemcell.com/anti-beta-tubulin-iii-antibody-cloned-tuj1.html and peer-reviewed citations at https://www.bioz.com/result/tuj1/product/STEMCELL Technologies Inc/?r=3.00&cf=0&uq=Stemcell Tech tuj1). Secondary antibodies were Alexa Fluor 488 Goat anti-Rabbit IgG (Thermo Fisher Scientific, Cat. # A-11008; 1:1,000; immunohistochemistry valida-tion and peer-reviewed citations at https://www.thermo.com/antibody/product/Goat-anti-Rabbit-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/ A-11008), Alexa Fluor 488 Goat anti-Mouse IgG (Thermo Fisher Scientific, Cat. # A-11001; 1:1,000; immunohistochemistry valida-tion and peer-reviewed citations at https://www.thermo.com/antibody/product/Goat-anti-Mouse-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-11001), Texas Red-X Goat anti-Rat IgG (Thermo Fisher Scientific, Cat. # T-6392; 1:1,000; immunohistochemistry valida-tion and peer-reviewed citations at https://www.thermo.com/antibody/product/Goat-anti-Rat-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-
10. Eukaryotic cell lines
   a. State the source of each eukaryotic cell line used. The only cell lines used in the study are primary cultured E14.5/E17.5/P0 mouse NSCs.
   b. Describe the method of cell line authentication used. NSC genotypes were determined by genomic PCR.
   c. Report whether the cell lines were tested for mycoplasma contamination. All NSC lines were tested negative for mycoplasma contamination.
   d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use. No commonly misidentified cell lines were used in this study.

11. Description of research animals
    Genetically modified C57 mice were used in this study. Both male and female embryos (E7.5, E8.5, E9.5, E13.5, E15.5, E17.5) or neonatal mice (P0 - P7) were used for analysis.

12. Description of human research participants
    This study did not involve human research participants.
ChIP-seq Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

Data deposition

1. For all ChIP-seq data:
   - a. Confirm that both raw and final processed data have been deposited in a public database such as GEO.
   - b. Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

2. Provide all necessary reviewer access links.
   *The entry may remain private before publication.*

3. Provide a list of all files available in the database submission.

4. If available, provide a link to an anonymized genome browser session (e.g. UCSC).

Methodological details

5. Describe the experimental replicates.

ChIP-seq were performed on 2 E14.5 Mettl14 KO NSC lines and 2 E14.5 nondeleted NSC lines.

6. Describe the sequencing depth for each experiment.

On average 20 million 76-bp single-end reads per library were mapped and 70% of the reads were uniquely mapped to the reference genome.

7. Describe the antibodies used for the ChIP-seq experiments.

The antibodies used were as follows: rabbit anti-H3K27ac (Active Motif, Cat. # Ab4729, Lot. # GR312658-1) and rabbit anti-H3K27me3 (Millipore, Cat. # 07-449, Lot. # 2686928). Both antibodies are ChIP grade and used in ENCODE project (https://genome.ucsc.edu/ENCODE/antibodies.html).

8. Describe the peak calling parameters.

To align ChIP-seq DNA reads to the reference mm10 genome, we used Bowtie2 (v2.3.1) (Langmead and Salzberg 2012) with the default options except that “--broad” was turned on for calling broad peaks. Each ChIP library in each biological replicate was compared against the DNA input background library in the corresponding cell type + condition. Specifically, histone marks H3K4me1, H3K4me3, H3K27ac, and H3K27me3 in wild-type and M14 knockout (KO) mutant of mouse neural progenitor cell (NPC) were compared with the DNA input in wild-type and M14 KO NPC, respectively. Default cutoff of FDR < 0.05 were used to determine ChIP-seq peaks relative to the input libraries.

9. Describe the methods used to ensure data quality.

Default MACS peak call threshold was used to obtain peaks at FDR < 0.05. On average, 52,299 peaks were retained per library and 6368 peaks with fold-enrichments greater than 5 over the 20 ChIP-seq libraries.

10. Describe the software used to collect and analyze

R environment and custom scripts were used to collect peaks and analyze.
the ChIP-seq data. Detailed methods are described in the manuscript. Scripts are available upon request.
Flow Cytometry Reporting Summary

For all flow cytometry data, confirm that:

1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
3. All plots are contour plots with outliers or pseudocolor plots.
4. A numerical value for number of cells or percentage (with statistics) is provided.

Mouse brain cortex were dissected out from E14.5 or E17.5 embryonic brains and triturated by pipetting. Dissociated cells were cultured as neurospheres with NeuroCult Proliferation Medium (Stemcell Tech.) before flow cytometry. Dissociated NSCs were incubated with Dead Cell Apoptosis Kit with Annexin V Alexa Fluor-488 & Propidium Iodide (Thermo Fisher Scientific) following manufacturer’s protocols. Briefly cells were diluted in 1× annexin-binding buffer to ~ 1×10^6 cells/mL, add 5 μL Alexa Fluor® 488 annexin V and 1 μL 100 μg/mL PI working solution to each 100 μL of cell suspension and incubate at room temperature for 15 minutes. After incubation add 400 μL 1× annexin-binding buffer and analyze the stained cells by flow cytometry.

LSRFortessa Cell Analyzer (BD Biosciences)

FACSDiva 6 (BD Biosciences)

No sorting was involved in this study.

Cell debris were excluded by FSC-A/SSC-A plot and singlets were gated by SSC-A/SSC-W plot. The gates for Propidium Iodide (PI) and AnnexinV-FITC were determined by non staining cells, PI single staining cells and AnnexinV-FITC single staining cells.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.