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Temporal Control of Mammalian Cortical Neurogenesis by m⁶A Methylation

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AUTHOR CONTRIBUTIONS

K-J. Y. led the project and contributed to all aspects of the study. F.R.R. performed m⁶A-seq, RNA-seq, and bioinformatics analysis. C.V. contributed to most aspects of the study. F.J. contributed to human NPC and organoid analysis. M.P. and S.R. contributed to time-lapse imaging analysis of cell cycle. D.J.-C., Y.S., N-S.K., Y.Z., L.Z., S.K., X.W. and P.J. contributed to additional data collection and analyses. L.C.D., X.Z. and C.H. contributed *Mett1^{fl/fl}* mice. S.C. contributed to bioinformatics analyses. G-I.M., H.S. and K-J.Y. conceived the project. K-J. Y., F.R.R., C.V., G-I. M., and H.S. wrote the manuscript.

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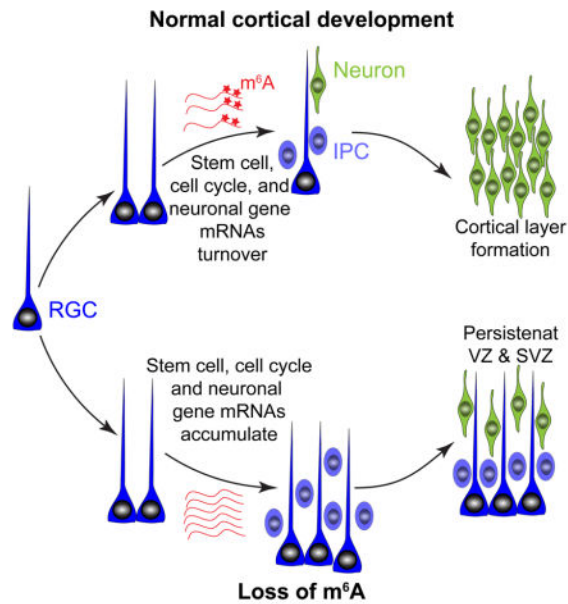
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SUMMARY

N⁶-methyladenosine (m⁶A), installed by the *Mettl3/Mettl14* methyltransferase complex, is the most prevalent internal mRNA modification. Whether m⁶A regulates mammalian brain development is unknown. Here we show that m⁶A depletion by *Mettl14* knockout in embryonic mouse brains prolongs cell cycle of radial glia cells and extends cortical neurogenesis into postnatal stages. m⁶A depletion by *Mettl3* knockdown also leads to prolonged cell cycle and maintenance of radial glia cells. m⁶A-sequencing of embryonic mouse cortex reveals enrichment of mRNAs related to transcription factors, neurogenesis, cell cycle and neuronal differentiation, and m⁶A-tagging promotes their decay. Further analysis uncovers previously unappreciated transcriptional pre-patterning in cortical neural stem cells. m⁶A signaling also regulates human cortical neurogenesis in forebrain organoids. Comparison of m⁶A-mRNA landscapes between mouse and human cortical neurogenesis reveals enrichment of human-specific m⁶A-tagging of transcripts related to brain disorder risk genes. Our study identifies an epitranscriptomic mechanism in heightened transcriptional coordination during mammalian cortical neurogenesis.

In brief

m⁶A-dependent mRNA decay is critical for transcriptional pre-patterning in mammalian cortical neurogenesis.



INTRODUCTION

Proper development of the nervous system is critical for its function, and deficits in neural development have been implicated in many brain disorders, such as microcephaly, autistic spectrum disorders, and schizophrenia (Jamuar and Walsh, 2015; Taverna et al., 2014). In the embryonic mouse cortex, radial glia cells (RGCs) function as neural stem cells, sequentially giving rise to neurons residing in different cortical layers and then switching to glial production before their depletion during early postnatal stages (Taverna et al., 2014). Such a precise and predictable developmental schedule requires a highly coordinated genetic program (Okano and Temple, 2009). Indeed, previous studies have revealed transcriptional cascades that orchestrate the dynamics of mammalian cortical neurogenesis (Martynoga et al., 2012; Miller et al., 2014; Nord et al., 2015). Recent discoveries of widespread mRNA chemical modifications (Zhao et al., 2017a) raise the question of whether this mechanism plays any regulatory role in cortical neurogenesis.

Modified nucleotides in mRNAs were initially discovered over 40 years ago, but little was known about the extent, transcript identities, and potential functions of various reversible chemical modifications until very recently (Zhao et al., 2017a). High-throughput sequencing approaches have revealed a dynamic “epitranscriptome” landscape for many mRNA modifications in various organisms, including N⁶-methyladenosine (m⁶A), N¹-methyladenosine (m¹A), 5-methylcytosine (m⁵C), 5-hydroxymethylcytosine (hm⁵C), and pseudouridine (ψ) (Li et al., 2016). Among these modifications, m⁶A is the most abundant internal modification in mRNAs of eukaryotic cells (Desrosiers et al., 1975). m⁶A profiling with cell lines has revealed m⁶A sites in over 25% of human transcripts, with enrichment in long exons, and near transcription start sites and stop codons (Meyer and Jaffrey, 2014). In mammals, m⁶A is installed by the methyltransferase complex consisting of Mettl3 (methyltransferase-like 3), Mettl14, Wtap (Wilms tumor 1-associated protein), KIAA1429,

RBM15 (RNA-binding motif protein 15) and its paralogue (RBM15B) (Patil et al., 2016), whereas its removal is mediated by demethylases Fto (fat mass and obesity-associated) and Alkbh5 (alkB homolog 5) (Zhao et al., 2017a). Recent in vitro studies have identified multiple functions of m⁶A in mRNA metabolism, from processing in the nucleus to translation and decay in the cytoplasm (Zhao et al., 2017a). The field has just started to investigate physiological functions of m⁶A. For example, *Mettl3* or *Mettl14* knockdown reduces m⁶A levels and decreases self-renewal of primed mouse embryonic stem cells (mESCs) (Wang et al., 2014a), whereas *Mettl3* knockout naïve mESCs exhibit improved self-renewal and impaired differentiation, due to dysregulated decay of m⁶A-tagged transcripts, such as *Nanog* (Batista et al., 2014; Geula et al., 2015).

Identification of the molecular machinery mediating m⁶A mRNA methylation provides an entry point to explore physiological functions of this pathway in vivo. Studies of *Drosophila* development showed that m⁶A methylation regulates sex determination and neuronal functions by modulating mRNA splicing (Hausmann et al., 2016; Lence et al., 2016). In *Zebrafish* embryos, m⁶A-tagging promotes clearance of maternal mRNAs and maternal-to-zygotic transition (Zhao et al., 2017e). In mice, germline *Mettl3* deletion results in early embryonic lethality (Geula et al., 2015). Nothing is known about the role of m⁶A signaling during mammalian embryonic brain development in vivo. Here we used the *Mettl14* conditional knockout mouse as a model to examine m⁶A function in embryonic cortical neurogenesis in vivo. We further investigated underlying cellular and molecular mechanisms. Finally, we extended our analysis to human embryonic cortical neurogenesis using induced pluripotent stem cell (iPSC)-derived forebrain organoids and compared m⁶A-mRNA landscapes between mouse and human cortical neurogenesis. Together, our results reveal critical epitranscriptomic control of mammalian cortical neurogenesis and provide insight into mechanisms underlying this highly coordinated developmental program.

RESULTS

Nervous system *Mettl14* deletion extends cortical neurogenesis into postnatal stages

We first investigated the expression pattern of molecular players mediating m⁶A signaling during mouse embryonic cortical neurogenesis. Mining the recently published single-cell RNA-seq dataset of RGCs and their progeny (Telley et al., 2016) revealed that highest *Mettl14* expression in RGCs and relatively constant levels of m⁶A methyltransferase components (*Mettl3*, *Wtap*), demethylases (*Fto*, *Alkbh5*) and m⁶A readers (*Ythdf2*, *Ythdf3*) during neurogenesis (Figure S1A). We next conditionally deleted *Mettl14* in the developing mouse nervous system starting at E11.5 using the *Nestin-Cre:Mettl14^{fl/fl}* conditional knockout (cKO) model (Figure S1B). We confirmed *Mettl14* deletion at the protein level with Western blot analysis of E17.5 brains (Figure S1B). The cKO animals were smaller in size by P5 compared to wildtype (WT) littermates, and all cKO animals died before P25 (Figure S1C–D). Thus, the function of m⁶A molecular machinery in the nervous system is indispensable for life in the mammalian system.

We then examined cortical structures at P5. cKO mice exhibited enlarged ventricles with an adjacent dense layer of cells that resembled the embryonic germinal zone (Figure 1A). Immunohistological analysis showed the presence of Pax6⁺ and Nestin⁺ cells with radial

fibers along the ventricle in cKO mice, but not in WT mice (Figure 1A–B). During mouse cortical development, Pax6⁺ RGCs are largely depleted by P5 (Dwyer et al., 2016). In contrast, a substantial number of Pax6⁺ cells were present in cKO mice at P5 (Figure 1C). Neurogenic Pax6⁺ RGCs give rise to intermediate progenitor cells (IPCs) expressing Tbr2/Eomes (Englund et al., 2005). The presence of Pax6⁺ cells in cKO mice was accompanied by Tbr2⁺ IPCs, which were absent in WT mice by P5 (Figure 1D–E). To confirm that cortical neurogenesis continued postnatally, we pulsed animals with EdU at P5 and analyzed 2 days later. Significant numbers of EdU⁺Pax6⁺ proliferating RGCs, EdU⁺Tbr2⁺ IPCs, and EdU⁺Tbr2⁺TuJ1⁺ neuroblasts were present in cKO mice, but very few in WT littermates (Figure 1F–G). These results indicate that cKO mice maintain neurogenic RGCs with extended cortical neurogenesis into postnatal stages.

To further characterize the impact of *Mettl14* deletion on cortical development, we examined neuronal subtype and glia production. We pulsed animals with EdU at E15.5 and examined them at P5. Compared to WT littermates, cKO mice exhibited a significantly decreased number of EdU⁺Satb2⁺ neurons, suggesting a deficit in producing late-born upper-layer neurons (Figure 1H–I). Direct measurement of the number of different cortical neuron subtypes also showed a reduced number of Satb2⁺ upper-layer neurons, but comparable numbers of Tbr1⁺ and Ctip2/Bcl11b⁺ lower-layer early-born neurons in P5 cKO mice (Figure S1E–F). On the other hand, Ctip2⁺ neurons were reduced in number in the E17.5 cKO cortex, suggesting a delay in the production of neuron subtypes of different cortical layers, rather than differentiation deficits (Figure S1G–H). In addition, we observed a significant decrease in the number of s100β⁺ astrocytes in cKO mice at P5 (Figure 1J–K). Together, these results indicate that *Mettl14* function is critical for proper temporal progression of neurogenesis and gliogenesis during mouse cortical development in vivo.

***Mettl14* deletion in neural progenitor cells leads to protracted cell cycle progression**

Given the well-defined temporal progression of cortical neurogenesis from RGCs (Okano and Temple, 2009), we suspected that there could be RGC deficits during embryonic stages in cKO mice. Interkinetic nuclear migration (INM), the periodic movement of the cell nucleus in phase with cell-cycle progression, is a common feature of developing neuroepithelial (Taverna et al., 2014). We pulsed animals with EdU at E17.5 to label cells in S-phase and followed positions of nuclei in EdU⁺Pax6⁺ RGCs (Figure 2A). While there was no difference at 0.5 hr after EdU labeling, nuclei of labeled RGCs were positioned further away from the ventricular surface at 6 hr in cKO mice compared to WT (Figure 2B), suggesting delayed INM and potential cell cycle deficits. To directly examine the S to M phase transition of the cell cycle, we analyzed expression of phospho-Histone 3 (pH3), an M phase marker, 2 hr after EdU labeling (Figure 2C). We found a significant decrease in the percentage of EdU⁺pH3⁺Pax6⁺ cells among all pH3⁺Pax6⁺ cells in cKO mice, suggesting a prolonged S to M phase transition of RGCs (Figure 2D). To examine cell cycle exit of proliferating neural progenitors, we analyzed expression of Ki67, a proliferation marker, 24 hr after EdU labeling (Figure 2E). We found a significant decrease in the percentage of Ki67 negative cells among EdU⁺ cells in cKO mice, indicating a delay in cell cycle exit (Figure 2F).

To address the cell intrinsic effect of *Mettl14* deletion on cell cycle progression, we performed time-lapse imaging of individual cortical neural progenitor cells (NPCs) cultured from E13.5 mouse cortex. We used a dual reporter system with nuclear localized H2B-mCherry and a GFP-tagged Cdk2 substrate, DNA Helicase B (DHB) (Spencer et al., 2013). Cdk2 becomes active during the G₁-S transition and phosphorylates DHB-GFP, which is then translocated from nucleus to cytoplasm. Therefore, the presence of GFP in the mCherry⁺ nucleus indicates cells in G₁ phase, whereas translocation to the cytoplasm indicates S phase initiation, and continual buildup of cytoplasmic GFP occurs until mitosis (Figure S2A). Quantification of the length between sequential mitoses showed an increase of the total cell cycle length in *Mettl14* cKO NPCs (Figure 2G–H; Movie S1 and S2). Further analysis of different cell cycle phases revealed a specific increase of the S-G₂-M phases in the absence of *Mettl14*, but no difference in the G₁ phase (Figure 2I–J).

To quantify cell cycle characteristics at the population level, we pulsed NPCs with EdU for 30 min and performed flow cytometry analysis 0 or 5 hr later (Figure S2B). We found a significant decrease in the percentage of EdU⁺ cells that divided in *Mettl14* cKO NPCs compared to WT at 5 hr, confirming a delay in cell cycle progression (Figure S2C–D).

Mettl3 regulates embryonic cortical neurogenesis

Consistent with the finding that *Mettl14* is a critical component of the m⁶A methyltransferase complex (Wang et al., 2017), *Mettl14* deletion led to a significant reduction of m⁶A levels in mRNAs from both embryonic mouse cortex in vivo and cultured cortical NPCs (Figure 3A–B). To further assess our model that m⁶A methylation regulates cortical neurogenesis, we compared the phenotype of *Mettl14* cKO to knockdown of *Mettl3*, another critical component of the m⁶A methyltransferase complex (Wang et al., 2017).

We first confirmed effective *Mettl3* knockdown (KD) with Q-PCR and diminished m⁶A content in mRNAs from *Mettl3* KD cells with dot blot analysis (Figure S3A–C and Table S1). We next performed population cell cycle analysis with EdU pulse-chase and flow cytometer quantification (Figure S3D). We found a significant reduction in the percentage of GFP⁺EdU⁺ NPCs that divided upon *Mettl3* KD (Figure 3C–D), similar to the effect of *Mettl14* cKO (Figure S2C–D).

To examine the impact of *Mettl3* KD on RGC behavior in vivo, we electroporated plasmids co-expressing GFP and the shRNA against mouse *Mettl3*, or the control shRNA, in utero at E13.5 and analyzed GFP⁺ cells at E17.5. Newborn neurons normally migrate toward the cortical plate (CP) through the intermediate zone (IZ), whereas self-renewing RGCs remain in the ventricular zone (VZ) and subventricular zone (SVZ) (Taverna et al., 2014). Compared to the control group, GFP⁺ cells with *Mettl3* KD were more abundant in the VZ and SVZ and less abundant in the CP (Figure 3E–F), similar to the result from EdU fate mapping in *Mettl14* cKO mice (Figure 1H). There was also a significant increase in the percentage of GFP⁺Pax6⁺ cells among all GFP⁺ cells with *Mettl3* KD compared to the control group (Figure 3G).

Together, these results indicate that decreasing m⁶A levels by either *Mettl14* cKO or *Mettl3* KD leads to consistent phenotypes of protracted cell cycle progression of cortical NPCs and reduced differentiation of RGCs during mouse embryonic cortical neurogenesis.

m⁶A tags transcripts related to transcription factors, cell cycle, and neurogenesis, and promotes their decay

To gain insight into the molecular mechanism underlying m⁶A regulation of cortical neurogenesis, we performed m⁶A-seq of mouse forebrain at E13.5, a stage enriched for neural stem cells. We identified 4,055 high confidence m⁶A peaks corresponding to 2,059 gene transcripts (Figure S4A and Table S2). Similar to previous findings from cell lines (Meyer and Jaffrey, 2014), our in vivo analysis showed enriched distribution of m⁶A sites near stop codons (Figure S4B). We found no correlation between transcript levels and m⁶A-tagging (Figure S4C). Notably, many transcripts encoding transcription factors were m⁶A-tagged, such as *Pax6*, *Sox1*, *Sox2*, *Emx2*, and *Neurog2/Neurogenin 2* (Figure 4A–B). Gene ontology (GO) and Wikipathways analyses of m⁶A-tagged transcripts revealed enrichment of genes related to cell cycle, stem cell, and neuronal differentiation (Figure 4A–C and Table S3). We observed m⁶A-tagging of a similar group of transcripts in cortical NPCs derived from E13.5 mouse cortex (Figure S4D and Table S1).

To determine the functional consequence of m⁶A-tagging, we explored whether *Mettl14* deletion affects decay of m⁶A-tagged transcripts. Cortical NPCs derived from E13.5 WT and *Mettl14* cKO mice were treated with Actinomycin D to halt *de novo* transcription, and RNA-seq was performed 0 and 5 hr later to obtain the ratio of mRNA levels for each gene in order to measure their stability (Figure S4E). Across the transcriptomes, m⁶A-tagged transcripts exhibited significantly lower ratios compared to non m⁶A-tagged transcripts in the WT NPCs, and this difference was reduced in cKO NPCs (Figure 4D and Table S4). Direct comparison of WT and *Mettl14* cKO NPCs showed that m⁶A-tagged transcripts exhibited a larger increase in their ratio compared to non-tagged transcripts upon *Mettl14* deletion; one m⁶A tag per transcript was sufficient to increase the ratio and there was a minimal additional effect of more tagging sites (Figure 4E). It should be noted that our m⁶A-seq method could not determine whether multiple sites are simultaneously methylated in the same transcript. We confirmed our result with the direct measurement of the half-life of a selected group of transcripts (Figure 4F and S4F; Table S1).

All together, these results support a model that m⁶A methylation of mRNAs related to cell cycle and neurogenesis confers their rapid turnover during the dynamic progress of cortical neurogenesis; a lack of m⁶A-tagging attenuates the decay of these mRNAs, resulting in deficits in temporal specification and cell cycle progression of NPCs.

***Mettl14* deletion uncovers transcriptional pre-patterning for normal cortical neurogenesis**

Among the 2,059 m⁶A-tagged genes in the E13.5 mouse cortex, two major GO terms were generation of neurons and neuronal differentiation (Figure 4C). For example, IPC marker *Tbr2* and *Neurog2*, and neuronal markers *Neurod1* and *Neurod2* (Hevner et al., 2006), were m⁶A-tagged in E13.5 forebrain in vivo (Figure 5A) and in cultured cortical NPCs (Figure S4D). Q-PCR analysis of total mRNA showed increased levels of *Tbr2*, *Neurog2*, *Neurod1*,

and *Neurod2*, but not non-tagged *Rad17*, in *Mett14* cKO compared to WT NPCs (Figure 5B and Table S1). This result raised the possibility that neuronal lineage genes are already expressed in neural stem cells and their levels are actively suppressed post-transcriptionally by m⁶A-dependent decay; alternatively, *Mett14* deletion may transcriptionally upregulate these neuronal genes.

To differentiate between these two possibilities, we quantified the levels of nascent mRNA using the 4-thiouridine (4sU) metabolic labeling approach (Duffy et al., 2015). We found comparable and even lower levels of nascent mRNA of neuronal lineage genes, such as *Tbr2*, *Neurog2*, and *Neurod2*, in *Mett14*^{-/-} NPCs in comparison to WT NPCs (Figure 5B and Table S1). The lower levels of nascent mRNA observed for some neuronal lineage genes in *Mett14* cKO NPCs could be explained by a negative feedback loop at the level of transcription, originating from elevated expression of stem cell genes, such as *Emx2* and *Sox1* (Figure 5B). Similarly, we found comparable levels of pre-mRNA for neuronal lineage genes in *Mett14* cKO compared to WT NPCs (Figure S5A and Table S1), suggesting that the increase in the total mRNA of neuronal lineage genes in *Mett14* cKO NPCs is not due to transcriptional upregulation. Together, these results support that neuronal lineage genes are already expressed in neural stem cells under normal cortical neurogenesis. Consistent with our result, mining the published single-cell RNA-seq dataset (Telley et al., 2016) revealed expression of neuronal lineage genes, such as *Tbr2*, *Neurog2*, *Neurod6* and *Tubb3/Tuj1*, in individual RGCs in the embryonic mouse cortex in vivo (Figure S5B).

We next examined *Tbr2* and *Neurod1* protein levels in RGCs in vivo. Pax6⁺*Tbr2*⁺ cells were localized in the SVZ in WT at E17.5, but extended into the VZ in *Mett14* cKO mice (Figure 5C–D). Pax6⁺*Neurod1*⁺ cells were rare, but detectable just above the SVZ in WT cortices. In contrast, cKO mice exhibited a significantly increased number of Pax6⁺*Neurod1*⁺ cells with a much broader distribution, including in the SVZ and VZ (Figure 5E–F). To specifically examine expression in RGCs, we pulse-labeled juxtaventricular newborn cells by FlashTag (FT) (Telley et al., 2016). We found a significantly increased number of FT⁺Pax6⁺*Tbr2*⁺ and FT⁺Pax6⁺*Neurod1*⁺ cells in *Mett14* cKO cortex compared to those in WT 3 hr after labeling (Figure S5C–D). Given that FT⁺ cells at 3 hr upon labeling are exclusively undifferentiated RGCs (Telley et al., 2016), these results suggest that *Mett14* regulates neuronal lineage gene expression directly in RGCs.

To further assess our model that mRNA decay regulates neuronal lineage gene expression in RGCs, we performed in vivo knockdown experiments for the components of CCR4-NOT complex (*Cnot7* and *Cnot1*), a major cytoplasmic mRNA deadenylase complex responsible for mRNA decay (Du et al., 2016; Schoenberg and Maquat, 2012). Both *Cnot7* KD and *Cnot1* KD led to increased numbers of *Tbr2*⁺Pax6⁺ and *Neurod1*⁺Pax6⁺ cells and location closer to the ventricular surface compared to the control shRNA (Figure S5E–F), phenotypes resembling *Mett14* cKO (Figure 5C–F).

Taken together, our results suggest heightened transcriptional coordination and a previously unappreciated transcriptional pre-patterning mechanism for mammalian cortical neurogenesis, in which late IPC and neuronal genes are already transcribed in cortical neural

stem cells and these transcripts are down regulated post-transcriptionally by m⁶A-dependent decay.

METTL14 regulates cell cycle progression of human cortical NPCs

We next examined whether m⁶A function is conserved in human cortical neurogenesis. Using a previously developed protocol (Yoon et al., 2014), we differentiated human iPSCs into a highly pure population of NESTIN⁺SOX2⁺ NPCs (hNPCs; 96.4 ± 1% among all cells; *n* = 5; Figure S6A). We co-expressed GFP and the validated shRNA against human *METTL14* in these hNPCs (Figure S6B). After 4 days, we labeled cells with EdU for 30 min and performed cell cycle analysis with flow cytometer quantification 14 hr later (Figure S6C). Similar to results from mouse *Mettl14* cKO NPCs (Figure S2C–D), we found a significant decrease in the percentage of GFP⁺EdU⁺ hNPCs that divided with *METTL14* KD, indicating a delayed cell cycle progression (Figure 6A–B).

We recently developed a human iPSC-derived forebrain organoid model, which exhibits transcriptome profiles similar to fetal human cortex during development up to the second trimester (Qian et al., 2016). Around day 47, these forebrain organoids resemble mouse cortical neurogenesis at E13.5 (Figure S6D). We microinjected plasmids co-expressing GFP and the shRNA against human *METTL14*, or the control shRNA, into the lumen of forebrain organoids and performed electroporation to transfect RGCs (Figure S6E). After 7 days, we pulsed organoids with EdU for 1 hr and performed cell cycle analysis of GFP⁺ cells 14 hr later (Figure S6F). We observed a significant decrease in the percentage of GFP⁺EdU⁺ cells that divided with *METTL14* KD (Figure 6C–D). Together, these results indicate that m⁶A mRNA methylation plays a conserved role in regulating cortical NPC cell cycle progression in both mouse and human.

m⁶A-seq of human forebrain brain organoids and fetal brain reveals conserved and unique m⁶A landscape features compared to embryonic mouse forebrain

Finally, we performed m⁶A-seq of day 47 human forebrain organoids. We detected 11,994 high confidence m⁶A peaks associated with 4,702 transcripts (Figure S7A and Table S2). Our previous systematic RNA-seq analyses of human forebrain organoids at different stages revealed that transcriptomes of organoids around day 47 were similar to human fetal cortex at 8–12 post-conception weeks (PCW) (Qian et al., 2016). We further performed m⁶A-seq of PCW11 fetal human brain and identified 10,980 high confidence peaks associated with 5,049 transcripts (Figure S7B and Table S2). m⁶A sites were enriched near transcription start sites and stop codons for both human samples (Figure S7C–D). Furthermore, m⁶A profiles from both samples showed significant overlap (Figure 7B). GO analysis of m⁶A-tagged transcripts shared in both samples showed enrichment of genes related to neurogenesis, neuronal differentiation and development (Figure 7C and Table S5). Many recently identified risk genes for schizophrenia and autistic spectrum disorders have been shown to be dynamically expressed and play critical roles during mammalian embryonic brain development (Ohi et al., 2016; Tebbenkamp et al., 2014). Notably, disease ontology analysis of these m⁶A-tagged genes shared in both human samples showed enrichment related to mental disorders, mental retardation, schizophrenia and bipolar disorder (Figure 7C and Table S5).

We further performed comparison among m⁶A landscapes during mouse and human cortical neurogenesis. About 19.3%, 34.7% and 31.4% of detected transcripts exhibited m⁶A-tagging in E13.5 mouse brain, day 47 human forebrain organoids, and PCW11 human fetal brain, respectively (Figure S7E). Therefore, m⁶A mRNA methylation appears to be more prevalent in human. Among transcripts expressed in all three samples, 856 genes were commonly m⁶A-tagged (Figure 7D). These commonly m⁶A-tagged transcripts are enriched for genes related to neurogenesis and neuronal differentiation (Figure S7F and Table S5). Notably, 1,173 transcripts were expressed in both species, but only m⁶A-tagged in both human samples (Figure 7D). Ontology analysis of these human-specific m⁶A-tagged transcripts showed enrichment of genes related to mental disorders and mental retardation (Figure 7E–F and Table S5). Among genes associated with the 108 loci recently identified for genetic risk of schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics, 2014), 60 genes were m⁶A-tagged in human and 21 genes were uniquely tagged in both human forebrain organoids and fetal brain, but not in mouse E13.5 forebrain. On the other hand, the gene set of m⁶A-tagged transcripts shared between mouse and human is enriched for oncogenic processes (Figure 7E).

DISCUSSION

From flies to mammals, neurogenesis is a highly coordinated process with sequential waves of gene expression (Kohwi and Doe, 2013). Here we revealed a critical role of m⁶A mRNA methylation in this process in the mammalian system in vivo. Our results suggest a model that m⁶A-tagging of transcripts related to neural stem cells, cell cycle, and neuronal differentiation confers their rapid turnover to control the transcriptome composition at different phases of the dynamic cortical neurogenesis process. The observation of RGCs expressing markers thought to be expressed only in late IPCs and post-mitotic neurons in *Mettl14* cKO mice led to the discovery of transcriptional pre-patterning in normal cortical neurogenesis and identifies m⁶A mRNA methylation as a key mechanism to prevent precocious expression of genes of later lineage status at the protein level in stem cells. We also provide the emerging “epitranscriptomic” field with databases of m⁶A mRNA landscapes of mouse and human cortical neurogenesis and identify intriguing human-specific features.

Transcriptional pre-patterning for cortical neurogenesis

The concept of pre-patterning initially came from analysis of chromatin states within multipotent progenitors to regulate the fate choice for liver and pancreas (Xu et al., 2011). Recent genome-wide mapping studies have suggested that epigenetic pre-patterning is important for spatio-temporal regulation of gene expression and may be a widespread phenomenon in cell fate decision (Chen and Dent, 2014). Our study reveals transcriptional pre-patterning in normal cortical neural stem cells in vivo. Consistent with our model, Pax6 has been shown to bind and activate both *Tbr2* and *Neurod1* promoters (Sansom et al., 2009). We showed that pre-patterning transcripts are tagged with m⁶A and subjected to rapid decay, therefore most of them are present in low levels among the bulk mRNA preparation and little protein under normal conditions – a likely reason why such a mechanism has escaped detection in previous studies. While epigenetic mechanisms play a key role in

transcriptional regulation during neurogenesis (Yao et al., 2016), epitranscriptomic regulation as a post-transcriptional mechanism could provide the speed and additional specificity, while maintaining plasticity of gene expression. By working in concert, the epigenetic landscape can permit transcription of certain genes, such as genes defining late lineage states, while the epitranscriptome prevents aberrant protein production. Future studies are needed to investigate whether transcriptional pre-patterning is a general mechanism in fate specification of other stem cells during development.

Heightened transcriptional coordination of mammalian cortical neurogenesis by m⁶A

Our study provides the *in vivo* evidence in the mammalian system to support the emerging notion that m⁶A methylation plays a critical role in developmental fate transition. The precise and predictable developmental schedule of cortical neurogenesis requires rapid, tightly controlled changes in gene expression (Okano and Temple, 2009). Our results suggest that epitranscriptomic m⁶A-tagging, via regulation of mRNA decay, provides a key mechanism for temporal control of dynamic gene expression, which in turn regulates cell cycle progression of cortical neural stem cells in both mouse and human.

There are three major categories of m⁶A-tagged transcripts in the embryonic mouse brain. First, many classic transcription factors involved in neural stem cell maintenance and neurogenesis, such as *Pax6*, *Sox2*, *Emx2*, and *Tbr2*, are m⁶A-tagged and subject to rapid decay. Second, cell cycle-related transcripts, such as *Cdk9*, *Ccnh/Cyclin H*, and *Cdkn1C/p57*, are m⁶A-tagged. Functionally, the loss of m⁶A-tagging leads to prolonged cell cycle progression of cortical NPCs, resulting in delayed generation of different neuronal subtypes, extension of cortical neurogenesis into postnatal stages and deficits in astrocyte generation *in vivo*. Third, transcripts that were generally thought to be expressed only in later IPCs and post-mitotic neurons, such as *Neurod1* and *Neurod2*, are m⁶A-tagged and expressed in neural stem cells. While expression of transcription factors is known to overlap during different stages of mammalian cortical neurogenesis (Hevner et al., 2006), our finding suggests a greater degree of transcriptional coordination than previously thought. On the other hand, expression of detectable neuronal proteins in a significant number of RGCs located in the SVZ in the absence of *Mettl14* highlights the critical role of the epitranscriptomic mechanism in preventing precocious gene expression during the normal process of mammalian cortical neurogenesis.

Conserved and unique features of human m⁶A landscape during cortical neurogenesis

Our study provides databases of m⁶A mRNA landscapes during mouse and human cortical neurogenesis. Consistent with a similar role for m⁶A mRNA methylation in regulating cell cycle progression of cultured human NPCs and mouse NPCs *in vitro* and *in vivo*, the shared m⁶A-tagged transcripts in our mouse and human samples are enriched with genes related to neural stem cells, cell cycle, and neurogenesis. Notably, many genes associated with genetic risk for mental disorders, such as schizophrenia and autistic spectrum disorders, are only m⁶A-tagged in humans, but not in mice, raising the possibility that epitranscriptomic dysregulation may contribute to these human brain disorders. So far, one association study found evidence of ALKBH5 in conferring genetic risk for major depression disorder (Du et

al., 2015), and two studies identified association of FTO mutations with growth retardation and developmental delay (Boissel et al., 2009; Daoud et al., 2016).

In summary, our study identifies a critical and conserved role of an m⁶A epitranscriptomic mechanism in the temporal control of mammalian cortical neurogenesis via promotion of mRNA decay of transcripts related to transcription factors, neural stem cells, cell cycle, and neuronal differentiation. Future studies will address how this epitranscriptomic mechanism interacts with various epigenetic mechanisms to coordinate dynamic transcriptomes during brain development, and how dysregulation of epitranscriptomic mechanisms may contribute to brain disorders.

STAR METHODS

KEY RESOURCES TABLE

The table highlights the genetically modified organisms and strains, cell lines, reagents, software, and source data **essential** to reproduce results presented in the manuscript. Depending on the nature of the study, this may include standard laboratory materials (i.e., food chow for metabolism studies), but the Table is **not** meant to be comprehensive list of all materials and resources used (e.g., essential chemicals such as SDS, sucrose, or standard culture media don't need to be listed in the Table). **Items in the Table must also be reported in the Method Details section within the context of their use.** The number of **primers and RNA sequences** that may be listed in the Table is restricted to no more than ten each. If there are more than ten primers or RNA sequences to report, please provide this information as a supplementary document and reference this file (e.g., See Table S1 for XX) in the Key Resources Table.

Please note that ALL references cited in the Key Resources Table must be included in the References list. Please report the information as follows:

- **REAGENT or RESOURCE:** Provide full descriptive name of the item so that it can be identified and linked with its description in the manuscript (e.g., provide version number for software, host source for antibody, strain name). In the Experimental Models section, please include all models used in the paper and describe each line/strain as: model organism: name used for strain/line in paper: genotype. (i.e., Mouse: OXTR^{fl/fl}: B6.129(SJL)-Oxtr^{tm1.1Wsy/J}). In the Biological Samples section, please list all samples obtained from commercial sources or biological repositories. Please note that software mentioned in the Methods Details or Data and Software Availability section needs to be also included in the table. See the sample Table at the end of this document for examples of how to report reagents.
- **SOURCE:** Report the company, manufacturer, or individual that provided the item or where the item can be obtained (e.g., stock center or repository). For materials distributed by Addgene, please cite the article describing the plasmid and include "Addgene" as part of the identifier. If an item is from another lab, please include the name of the principal investigator and a citation if it has been previously published. If the material is being reported for the first time in the

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 - **A NOTE ABOUT RRIDs:** We highly recommend using RRIDs as the identifier (in particular for antibodies and organisms, but also for software tools and databases). For more details on how to obtain or generate an RRID for existing or newly generated resources, please [visit the RII](#) or [search for RRIDs](#).

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact Hongjun Song (shongjun@mail.med.upenn.edu). There are no restrictions on any data or materials presented in this paper.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals—Exons 7, 8, and 9 of mouse *Mettl14* were targeted by inserting a single loxP site in intron 6 and an FRT-flanked neomycin resistance gene coupled with a loxP site in intron

9, with the consideration that they contain the DPWW active motif (Figure S1B). The targeting construct was electroporated into 129 mESCs, selected for neomycin resistance, screened for homologous recombination by Southern blotting, and selected mESC clones were used to generate chimeric mice by injection into C57BL/6J mouse blastocysts. Chimeric mice were bred to wild type C57BL/6J mice to test for germline transmission of the mutant allele, which was identified by PCR. The PCR-positive lines were crossed with a β -actin promoter-driven Flp recombinase to remove the neomycin resistance gene via FRT site recombination. The neomycin cassette-deleted mice were identified by PCR, and the resultant *Mettl14^{fl/fl}* allele and *Nestin-Cre^{+Tg}* mice (Jackson Laboratory stock: 003771) were used to generate *Nestin-Cre^{+Tg}; Mettl14^{+f}* mice and *Nestin-Cre^{+/+}; Mettl14^{fl/fl}* mice. WT and cKO mice were generated by crossing *Nestin-Cre^{+Tg}; Mettl14^{+f}* males and *Nestin-Cre^{+/+}; Mettl14^{fl/fl}* females.

For in utero electroporation analysis, timed-pregnant CD1 mice (Charles River Laboratory) at E13.5 were used as previously described (Yoon et al., 2014). Timed pregnant mice were euthanized by cervical dislocation, and embryos were euthanized by decapitation before the dissection step. All animal procedures used in this study were performed in accordance with the protocol approved by the Institutional Animal Care and Use Committee of Johns Hopkins University School of Medicine.

Primary mouse NPCs—Mouse NPCs were isolated from *Mettl14* WT and cKO mouse embryonic cortices and cultured in Neurobasal medium (Gibco BRL) containing 20 ng/ml FGF2, 20 ng/ml EGF, 5 mg/ml heparin, 2% B27 (v/v, Gibco BRL), Glutamax (Invitrogen), Penicillin/Streptomycin (Invitrogen) on culture dishes pre-coated with Matrigel matrix (2%, Corning).

Human iPSC cultures and fetal brain sample—The human iPSC line used in the current study (C1) was fully characterized (Wen et al., 2014; Yoon et al., 2014). iPSCs were cultured in stem cell medium, consisting of DMEM:F12 (Invitrogen) supplemented with 20% Knockout Serum Replacer (Gibco), 1X Non-essential Amino Acids (Invitrogen), 1X Penicillin/Streptomycin (Invitrogen), 1X 2-Mercaptoethanol (Millipore), 1X Glutamax (Invitrogen), and 10 ng/ml FGF-2 (Peprotech). Culture medium was changed every day. Human iPSCs were passaged every week onto a new plate pre-seeded with irradiated CF1 mouse embryonic fibroblasts (Charles River Laboratory). Human iPSCs were detached from the plate by treatment of 1 mg/ml Collagenase Type IV (Invitrogen) for 1 hr. iPSC colonies were further dissociated into smaller pieces by manual pipetting. All studies were performed under approved protocols of Johns Hopkins University School of Medicine. Human iPSCs were differentiated into primitive hNPCs according to a previously published protocol (Li et al., 2011). Briefly, iPSCs were passaged onto MEF feeders, and after 3 days, induction medium containing Advanced DMEM:F12 (50%) and Neurobasal medium (50%), CHIR99201 (4 μ M, Cellgentech), SB431542 (3 μ M, Cellgentech), Bovine serum albumin (5 μ g/ml, Sigma), hLIF (10 ng/ml, Millipore), Compound E (0.1 μ M, EMD Millipore), Glutamax (Invitrogen), Pen/Strep, supplemented with N2 and B27 (Invitrogen), was added to the culture. After 6 days of differentiation, hNPCs were dissociated with Accutase

(Invitrogen) and plated, with the aid of a ROCK inhibitor (Y-27632, 3 μ M, Cellagentech), onto culture dishes pre-coated with Matrigel matrix (2%, Corning).

The PCW11 fetal human cortical tissue was used for m⁶A-seq. All procedures used in this study were performed in accordance with the protocol approved by the Institutional Stem Cell Research Oversight Committee of Johns Hopkins University School of Medicine and Lieber Institute for Brain Development.

METHOD DETAILS

DNA constructs—For knockdown experiments for mouse genes, short hairpin RNA sequences (see KEY RESOURCE TABLE) were cloned into the retroviral vector expressing GFP under the control of the EF1 α promoter and a specific shRNA under the control of human U6 promoter (pUEG) as previously described (Ge et al., 2006). For knockdown experiments for human *METTL14*, a short hairpin RNA sequence was cloned into the lentiviral vector expressing GFP under the control of the human ubiquitin C promoter and the specific shRNA under the control of human U6 promoter (cFUGW: Addgene plasmid 14883) as previously described (Yoon et al., 2014). The efficacy of each shRNA was confirmed in mouse B16-F10 cells (ATCC), or hNPCs derived from the C1 iPSC line.

In utero electroporation and FlashTag—In utero electroporation was performed as described previously (Yoon et al., 2014). In brief, timed-pregnant CD1 mice (Charles River Laboratory) at E13.5 or E14.5 were anesthetized and the uterine horns were exposed and approximately 1 to 2 μ l of plasmid DNA, 0.5 μ g/ μ l pCAG-GFP (Addgene plasmid: 11150) and 2.5 μ g/ μ l cFUGW plasmid with the control shRNA, or the shRNA against mouse *Mettl3*, *Cnot1* and *Cnot7*, was injected manually into the lateral ventricles of embryos using a calibrated micropipette. Five pulses (40 V, 50 ms in duration with a 950 ms interval) were delivered across the uterus with two 5-mm electrode paddles (CUY650-5, Nepa Gene) positioned on either side of the head by a square wave electroporator (CUY21SC, Nepa Gene). After electroporation, the uterus was placed back in the abdominal cavity and the wound was sutured. Mouse embryos were analyzed at E17.5. For FlashTag of RGCs, 1 μ l of 10 μ M of a carboxyfluorescein succinimidyl ester (CellTrace CFSE, ThermoFisher) was injected into the lateral ventricle of the E17.5 embryos using a calibrated micropipette. Mouse embryos were collected 3 hr later, fixed with with 4% paraformaldehyde in PBS overnight at 4°C for analysis. All animal procedures were performed in accordance with the protocol approved by the Johns Hopkins Institutional Animal Care and Use Committee.

Immunohistology and confocal imaging—For EdU labeling, timed pregnant mice were injected with EdU (150 mg/kg bodyweight, Invitrogen) at defined time points before euthanasia. For immunostaining of tissue sections, brains were fixed with 4% paraformaldehyde in PBS overnight at 4°C as previously described (Yoon et al., 2014). Samples were cryoprotected in 30% sucrose in PBS, embedded in OCT compound, and sectioned coronally (20 μ m-thickness) on a Leica CM3050S cryostat. Brain sections were blocked and permeabilized with the blocking solution (5% normal donkey serum, 3% Bovine serum albumin, and 0.1% Triton X-100 in PBS) for 1 hr at room temperature, followed by incubation with primary antibodies diluted in the blocking solution at 4°C

overnight. After washing, secondary antibodies diluted in blocking solution were applied to the sections for 1 hr at room temperature. Nuclei were visualized by incubating for 10 min with 0.1 $\mu\text{g/ml}$ 4,6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific) in PBS. For EdU labeling, Click-iT EdU Alexa Fluor 647 Imaging Kit (Thermo Fisher Scientific) was used following the manufacturer's protocol (Qian et al., 2016). Stained sections were mounted with ProLong Gold anti-fade reagents (Thermo Fisher Scientific) and analyzed. All the antibodies used are listed in KEY RESOURCE TABLE.

Mouse and human NPC electroporation—Approximately 1.0×10^6 mouse or human NPCs were resuspended in 100 μL Mouse Neural Stem Cell Nucleofector Solution from the Lonza Nucleofector Kit for Mouse Neural Stem Cells (Lonza, VAPG-1004). Additionally, 10 μg of the appropriate plasmid was added to the cell solution. The solution was then placed in a cuvette provided in the Nucleofector Kit and electroporated using a Lonza Nucleofector 2b device (LONZA) (Ma et al., 2008). Next, the cells were resuspended in NPC media as described above with Rock Inhibitor (Y-27632, 3 μM , Cellgientech) to reduce cell death. Cells were allowed to grow for at least 3 days before analysis.

Human forebrain organoid culture—Protocols for generation of forebrain organoids were detailed previously (Qian et al., 2016; Xu et al., 2016; Yoon et al., 2017). Briefly, human iPSCs were cultured in stem cell medium, consisting of DMEM:F12 (Invitrogen) supplemented with 20% Knockout Serum Replacer (Gibco), 1X Non-essential Amino Acids (Invitrogen), 1X Penicillin/Streptomycin (Invitrogen), 1X 2-Mercaptoethanol (Millipore), 1X Glutamax (Invitrogen), and 10 ng/ml FGF-2 (Peprotech) on irradiated CF1 mouse embryonic fibroblasts (Charles River). On day 1, iPSC colonies were detached by treatment of 1 mg/ml Collagenase Type IV (Invitrogen) for 1 hr and transferred to an Ultra-Low attachment 6-well plate (Corning Costar), containing 3 ml of stem cell medium (without FGF-2), plus 2 μM Dorsomorphine (Sigma) and 2 μM A83-01 (Tocris). On days 5–6, half of the medium was replaced with induction medium consisting of DMEM:F12, 1X N2 Supplement (Invitrogen), 1X Penicillin/Streptomycin, 1X Non-essential Amino Acids, 1X Glutamax, 1 μM CHIR99021 (Cellgientech), and 1 μM SB-431542 (Cellgientech). On day 7, organoids were embedded in Matrigel (Corning) and continued to grow in induction medium for 6 more days. On day 14, embedded organoids were mechanically dissociated from Matrigel and transferred to each well of a 12-well spinning bioreactor (Spin Ω) containing differentiation medium, consisting of DMEM:F12, 1X N2 and B27 Supplements (Invitrogen), 1X Penicillin/Streptomycin, 1X 2-Mercaptoethanol, 1X Non-essential Amino Acids, 2.5 $\mu\text{g/ml}$ Insulin (Sigma).

Forebrain organoid electroporation and analysis—Day 45 forebrain organoids were transferred into PBS solution in a 10 cm petri dish for electroporation. A mixture of 0.5 μl of plasmid DNA and 0.05% Fast green was injected into the lumen of neural tube structures in forebrain organoids using a calibrated micropipette (Yoon et al., 2017). About 3–4 locations on one side of each forebrain organoid were targeted by the injection. The DNA-injected side of the organoid was placed toward the positive electrode in the middle of 5 mm gap of electrode paddles (CUY650-5, Nepa Gene). Five pulses (40 V, 50 ms in duration with a 950 ms interval) were delivered by a square wave electroporator (CUY21SC, Nepa Gene). After

electroporation, organoids were transferred back to the SpinΩ bioreactor for further culturing.

Analysis of cell cycle progression by EdU pulse labeling—Analyses of cell cycle progression of mouse NPCs, hNPCs, and dissociated human forebrain organoids were performed as described previously (Terry and White, 2006; Wang et al., 2014b). In brief, mouse or human NPCs were pulsed by 10 μM EdU (ThermoFisher) for 30 min and washed thoroughly with NPC media. For human forebrain organoids, 10 μM EdU directly applied to culture media and organoids were incubated in the SpinΩ bioreactor for 1 hr to ensure complete penetrance, then washed thoroughly with culture media. After defined time points, cells were dissociated by Accutase, fixed with 4% paraformaldehyde in PBS for 20 min at 4°C, stained with Click-iT EdU Alexa 647 Flow Cytometry Kits (ThermoFisher) for Flow Cytometry following manufacturer's protocol. Cells were stained with Vybrant DyeCycle Violet (ThermoFisher) or 7-AAD (ThermoFisher) for DNA content and applied to flow cytometry using BD LSR II Flow Cytometer (BD Bioscience). EdU⁺ or GFP⁺EdU⁺ cells were gated and DNA content of those cells was analyzed compared to that of whole cell population. Percentages of divided cells among EdU⁺ or GFP⁺EdU⁺ population (G₁ or G₀ phase determined by DNA content) during defined time intervals were quantified from four independent experiments.

Time-lapse live imaging of mouse NPCs—96-well glass bottom microplates (655892, Geiner bio-one) were coated with phenol red-free Matrigel (356237, Corning). After electroporation of mNPCs with 10 μg CDK2-sensor plasmid (pPGK-H2B-mCherry-DHB (aa994-1087)-GFP), cells were plated onto the microplates at a density of 3,000 cells per well and allowed to adhere overnight. Cells were imaged using a Nikon Eclipse Ti fluorescent microscope controlled by Metamorph microscopy automation software. Temperature (37°C), CO₂ (5%), and humidity were held constant throughout experiments. Five blank positions in a well containing Matrigel and media only were used to flat field mNPC images using custom software. ImageJ was used to merge the green and red channels. To quantify the total cell cycle length, time was measured from the first cell division to the next cell division of one or both daughters. To quantify the G₁ phase length, time was measured from one cell division to the time point of significant reduction in the ratio of green/red intensity in the nucleus of the cell. S phase entry was quantitatively defined as the time when the cytoplasmic/nuclear ratio of green/red was approximately 1, as previously described (Spencer et al., 2013). A nuclear marker, H2B-mCherry, was used in the plasmid sensor to accurately segment the cytoplasm and the nucleus. The time point from S phase entry through the second cell division was then quantified as S-G₂-M length.

RNA purification and quantitative RT-PCR analysis—For gene expression analysis, total RNA fraction was isolated from cultured NPC samples with RNeasy Mini Kit (Qiagen), treated with DNaseI and reverse-transcribed into the first-strand cDNA with SuperScript III (Invitrogen). cDNAs were used for SYBR-green based quantitative real-time PCR to measure the expression level of target genes with the T method (ABI). All the primers used for quantitative PCR were listed in Table S1.

Western blot analysis—Forebrains from E17.5 embryos were quickly dissected out and homogenized in RIPA buffer (50 mM Tris pH 7.5, 120 mM NaCl, 1% Triton X-100, 0.5% Sodium Deoxycholate, 0.1% SDS, 5 mM EDTA, Phosphatase Inhibitor Cocktail (Cell Signaling), protease inhibitor cocktail (Sigma). Lysates were incubated for 15 min on ice and centrifuged at 15,000g for 15 min at 4°C. Supernatant was collected and boiled for 5 min in Laemmli sample buffer (Biorad), resolved by SDS PAGE, transferred to PVDF membrane, and immunoblotted. Primary antibodies are listed in KEY RESOURCE TABLE. Quantification of bands was performed using ImageJ software.

m⁶A dot blot assay—mRNA was harvested from homogenized forebrains at embryonic stages E15.5 and E17.5 using Dynabeads mRNA Direct Purification Kit (61011, Ambion). Four biological replicates were pooled for each sample to ensure sufficient concentration of mRNA. Dots were applied to an Amersham Hybond-N⁺ membrane (GE Healthcare) in duplicate as 100 ng mRNA per 1 µl dot. After complete drying, the mRNA was crosslinked to the membrane using a UV Stratalinker 2400 by running the auto-crosslink program twice. The membrane was then washed in PBST three times and blocked with 5% skim milk in PBST for 2 hr. The PBST wash was repeated and the membrane was incubated with primary anti-m⁶A antibody (212B11, Synaptic Systems) at 1:1000 dilution for 2 hr at room temperature. After 3 washes in PBST, the membrane was incubated in HRP-conjugated anti-mouse IgG secondary antibody for 2 hr at room temperature, then washed again 3 times in PBST. Finally, the membrane was visualized using SuperSignal West Dura Extended Duration Substrate (34075, Thermo Scientific). To confirm equal mRNA loading, the membrane was stained with 0.02% methylene blue in 0.3 M sodium acetate (pH 5.2) and quantified m⁶A levels were normalized to amount of mRNA loaded. Four biological samples in technical duplicates for each time point were used.

m⁶A-sequencing—m⁶A profiling was performed as previously described (Dominissini et al., 2012). For m⁶A profiling of mouse developing brain, forebrains from WT E13.5 embryos were dissected. For m⁶A profiling of human organoids, 25 to 30 forebrain organoids at day 47 were used. For m⁶A profiling of PCW11 fetal human brain, cortex from 2 PCW11 fetuses were dissected. The total RNA was extracted using RNeasy Mini Kit (Qiagen). mRNA was isolated using the Dynabeads mRNA Purification Kit (Invitrogen) and mRNA was fragmented via sonication to 100–200 base pairs. m⁶A pull-down was performed using a rabbit polyclonal anti-m⁶A antibody (Synaptic systems), and immunoprecipitation with protein G dynabeads (ThermoFisher). m⁶A-tagged mRNAs were competitively eluted from beads with free N⁶-methyladenosine. cDNA libraries from pulled-down RNA and input RNA were prepared using the NEBNext® Ultra™ DNA Library Prep Kit for Illumina®. The experiment was performed with three technical replicates. For m⁶A profiling of day 47 human forebrain organoids, the same procedure was followed, with the exception that the experiment was performed with two technical replicates because of the amount of samples required.

m⁶A mRNA immunoprecipitation and Q-PCR—Total RNA from NPCs cultured from WT E13.5 mouse forebrain was extracted using RNeasy Mini Kit (Qiagen) and mRNA was isolated using the Dynabeads mRNA Purification Kit (Invitrogen). 1% of input mRNA was

reserved for reverse transcription. Full length m⁶A tagged transcripts were pulled-down using a rabbit polyclonal anti-m⁶A antibody (Synaptic systems) and a mock pull-down was done with normal rabbit IgG (Cell Signaling Technologies). Immunoprecipitation was performed with protein G dynabeads (Thermo Fisher). m⁶A-tagged mRNAs were competitively eluted from beads with free N⁶-methyladenosine. Reverse transcription of input, m⁶A pull-down and mock pull-down mRNA was performed using the SuperScript® III First-Strand Synthesis System for RT-PCR (Thermo Fisher). cDNA was used for SYBR-green based quantitative real-time PCR. Enrichment of m⁶A tagged genes in m⁶A pull-down over input was calculated by comparing relative concentrations using Ct values (2^{-Ct}) and dividing each concentration by the relative concentration of the input. The concentrations of the immunoprecipitated RNA were then divided by the concentration in the input RNA and multiplied by 100, to obtain the percentage of transcripts in the m⁶A immunoprecipitation relative to the input. This value was then normalized to enrichment in the mock (IgG) pull-down, which was also calculated using relative concentrations to determine a percentage of the input. Primers used are listed in Table S1.

Bioinformatic analyses of m⁶A-seq—cDNA libraries from input and m⁶A pull-down were sequenced on the Illumina Nextseq platform, using a 50-cycle single-end run. Pre-processing of reads was performed using the FASTX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), namely adapters were clipped, poor quality reads were filtered out, and identical reads were collapsed. Pre-processed reads from E13.5 mouse forebrains were aligned to the mouse genome (build GRCm38/mm10), and reads from the human organoids and fetal brain to the human genome (build GRCh37/hg19), using Tophat2 (Kim et al., 2013) with default settings. m⁶A-tagged regions were identified using the MACS2 peak calling algorithm (Zhang et al., 2008), with the input library as background. For identifying high confidence m⁶A regions, peaks were intersected in a pairwise fashion among all replicates using the BedTools package (Quinlan and Hall, 2010). Peaks that overlap in at least 50% of their length among 2 or more samples were designated as high confidence m⁶A regions.

For representative coverage plots of m⁶A and input libraries, RNA-seq read alignments in bam format were transformed to bedGraph format and normalized for library size using the genomecov function from the BedTools package (Quinlan and Hall, 2010). Analysis of m⁶A peak enrichment was performed based on 5 non-overlapping transcript segments defined as follows: Transcription start site (TSS) segment [TSS, TSS+200bp], 5' UTR [TSS+201bp, CDS start-1bp], coding region (CDS) [CDS start, CDS stop-101bp], stop codon segment [CDS stop-100bp, CDS stop+100bp], 3' UTR [CDS stop+101bp, TTS]. Each high confidence peak was annotated to one of these regions using the BedTools package and fold enrichment was calculated from the ratio between observed peaks per region and expected number of peaks normalized by average region size. For analysis of correlation between gene expression levels and m⁶A peak fold change, we calculated RPKMs from input RNA seq libraries, using gene counts obtained with the htseq-count function from the HTSeq python package (Anders et al., 2015) that were normalized by library size and gene length defined as the length of its longest transcript. Fold changes for m⁶A peaks were obtained from MACS2 output.

Functional annotation and disease ontology—To assess enrichment of GO terms specific to a biological process, the ToppFunn module of the ToppGene Suite (Chen et al., 2009) was used. A hypergeometric probability mass function with Benjamini Hochberg FDR correction was used to identify significant enrichment for GO terms. Analysis of enrichment for Wikipathways terms was performed using ConsensusPathDB (Herwig, 2016), which calculates enrichment p-values using the Wilcoxon's matched-pairs signed-rank test, and Benjamini Hochberg FDR correction.

Disease association analysis was performed using WebGestalt (Zhang et al., 2005), which uses a hypergeometric method and Benjamini Hochberg FDR correction. Protein interaction network figures were generated using Cytoscape 3.3.0 (Shannon et al., 2003), with the Reactome FI plugin.

RNA degradation assay—cDNA libraries were prepared from cultured NPCs from E13.5 WT and *Mettl14* cKO cortex, at 0 and 5 hr post Actinomycin D treatment, using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina®. The experiment was performed with three replicates per condition. Sequencing was performed on the Illumina Nextseq platform, using a 100-cycle single-end run. Pre-processing of reads was performed using the FASTX toolkit. Gene expression levels were quantified using the RSEM package (Li and Dewey, 2011), which maps reads to the transcriptome using the aligner tool Bowtie2 (Langmead and Salzberg, 2012). Expected counts per gene per sample were combined into a count matrix, and this matrix was used as input for differential expression analysis using the EBSeq package (Leng et al., 2013), which uses empirical Bayesian methods to calculate the posterior probability of a gene being differentially expressed (PPDE). Posterior fold changes per gene between cKO and WT were obtained at time 0 and 5 hr after Actinomycin D treatment. Fold changes at 5 hr were normalized by fold changes at 0 hr (no Actinomycin D treatment) to specifically identify genes that degrade at a different slower rate in the cKO compared to WT, regardless of baseline changes in gene expression between two conditions. Genes with a normalized fold change higher than 2 in cKO over WT at 5 hr were considered as to be differentially degraded (Table S4).

Half-life measurement of m⁶A-tagged transcripts—Mouse NPCs were cultured in standard 6 well culture plates to approximately 80% confluence. Actinomycin D (Sigma) was added at a concentration of 5 μM. Cells were collected at three time points after addition (0, 3 hr, 5 hr) by washing once with PBS, then lysing the cells in Buffer RLT from the RNeasy Kit (Qiagen) with 1% β-Mercaptoethanol. A cell scraper was used to remove all cells from the well plate. Each sample was normalized for cell number by quantifying DNA content using a Quant-IT PicoGreen dsDNA Assay Kit (ThermoFisher) according to manufacturer instructions. Equal amounts of cellular contents, as measured by DNA quantity, were taken from each sample and 1 pg of luciferase control RNA (Promega) was added to each sample before RNA purification. Total RNA was then purified using an RNeasy Kit and reverse transcribed using the SuperScript II First-Strand Synthesis System (Thermo Fisher). Real time PCR was performed on a Step One Plus cycler from Applied Biosystems with Fast SYBR® Green Master Mix. Standard curves were generated by plotting CT values against the known initial concentration of luciferase control RNA, and

then used to derive mRNA concentration of each target gene at each time point. The \ln^{mRNA} concentrations at time point 0, 3 and 5 hr were then used to perform a linear regression as a function of time, and identify the slope of said line as the decay rate (k) (Figure S4F). Half life was calculated with the following formula: $t_{1/2} = \ln 2 / k_{decay}$ (Chen et al., 2008).

Metabolic labeling and purification of nascent RNA—4sU labeling of nascent RNA was performed as previously described (Duffy et al., 2015). Mouse NPCs from E13.5 WT and *Mett114* cKO forebrain were cultured in standard 6 well culture plates to approximately 80% confluence, treated with 500 μ M of 4sU (Carbosynth) for 1 hr, washed with PBS, and harvested with TRIzol reagent (ThermoFisher). Samples were extracted by chloroform twice and precipitated with isopropanol. Biotinylation of 4sU-RNA were carried out in a total volume of 250 μ l, containing 70 μ g total RNA, 10 mM HEPES (pH 7.5), 1 mM EDTA, and 5 μ g MTSEA biotin-XX (Biotium) freshly dissolved in DMF (final concentration of DMF = 20%). Reactions were incubated at RT for 30 min in the dark, and excess biotin reagents were removed by chloroform extraction twice. Purified RNA was dissolved in 50 μ l RNase-free water and denatured at 65°C for 10 min, followed by rapid cooling on ice for 5 min. Biotinylated RNA was separated from non-labeled RNA by incubating with 100 μ l Streptavidin Magnetic Beads (ThermoFisher) for 20 min at RT. Beads were washed twice with high-salt wash buffer (500 μ l each, 100 mM Tris- HCl pH 7.4, 10 mM EDTA, 1 M NaCl, and 0.1% Tween-20). 4sU-RNA was eluted with 100 μ l freshly prepared 100 mM DTT followed by a second elution with an additional 100 μ l 5 min later. RNA was recovered using the MinElute Spin columns (Qiagen) according to the instructions of the manufacturer, and applied for Q-PCR analysis.

Comparison between human and mouse m⁶A-seq datasets—For comparison of m⁶A sequencing data from day 47 human forebrain organoids, PCW11 fetal human cortex, and mouse E13.5 forebrains, we restricted our analysis to expressed genes with a one-to-one ortholog between species. For determining expressed genes, we calculated RPKMs (as stated above) from input libraries, and used a threshold of RPKM > 1.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data in figure panels reflect several independent experiments performed on different days. An estimate of variation within each group of data is indicated using standard error of the mean (SEM). We performed unpaired Student's t-test for assessing the significance of differences between two treatments (See each figure for details).

Analyses of mouse cortical neurogenesis in vivo—For quantitative analysis of electroporated neocortices, only GFP⁺ cells localized within the dorso-lateral cortex were examined. 3 × 3 tiled images were obtained to cover the electroporated region of each coronal section with a 20x or 40x objective by scanning microscope (Zeiss LSM 800) and compared with equivalent sections in littermate counterparts. Quantifications were performed using Imaris software (Bitplane). Specifically, for quantification of cell fate after in utero electroporation, GFP⁺ cells were marked, and GFP⁺Pax6⁺ cells were defined and counted based on the intensity of Pax6 immunofluorescence in GFP⁺ cells measured with the same criteria among different groups using Imaris software. For the distribution of GFP⁺

cells in each layer, the borders between different layers were defined by Pax6 immunofluorescence (VZ/SVZ) and DAPI staining (SVZ/IZ and IZ/CP). For quantification of cell fate in WT and *Mettl14* cKO mice at E17.5, P0 and P5, the regions of the primary somatosensory cortex were identified and the numbers of Pax6⁺, Tbr2⁺, S100β⁺, Ctip2⁺, Satb2⁺ or Tbr1⁺ cells were counted in each vertical column with 100 μm width. For distribution plots, the distances between soma of EdU⁺Pax6⁺, Pax6⁺, Tbr2⁺ or Neurod1⁺ cells and the ventricular surface were calculated. Only EdU⁺Pax6⁺ cells within 200 μm distance from the ventricular surface were measured, and the histogram of cell location in every 20 μm interval from the ventricular surface was plotted as a percentage. For distribution plots of Pax6⁺Tbr2⁺ or Pax6⁺Neurod1⁺ cells, the distances between soma of cells and the ventricular surface were calculated and the numbers of cells per 100 μm² area in every 20 μm interval from the ventricular surface were plotted as density distribution. All quantifications were performed with 4–10 brain sections from at least 4 animals. Data are presented as the mean ± SEM and statistical significance was assessed using unpaired Student's t-test.

DATA AND SOFTWARE AVAILABILITY

The access number for the data for m⁶A-seq reported in this study is NCBI GEO: GSE99017.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- m⁶A depletion leads to prolonged cell cycle progression of cortical neural progenitors
- m⁶A promotes decay of tagged neurogenesis-related transcripts
- Transcriptional pre-patterning for normal cortical neurogenesis

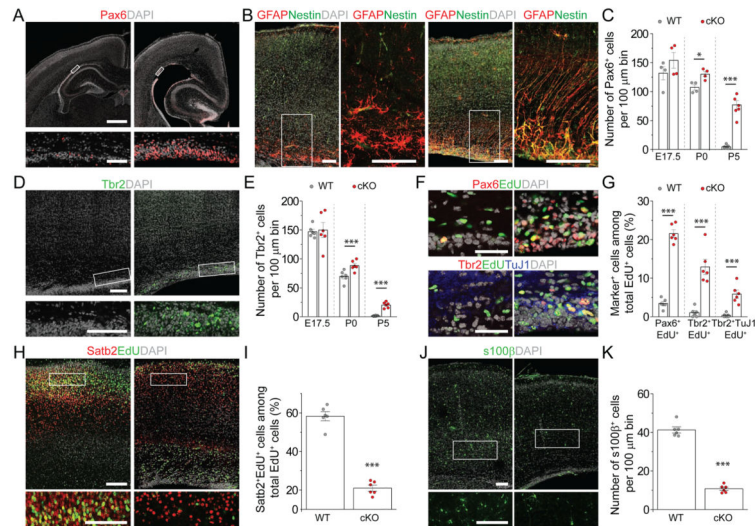


Figure 1. Nervous system *Mettl14* deletion results in residual radial glia cells and ongoing neurogenesis in the postnatal mouse cortex

(A–C) Presence of neurogenic RGCs in P5 *Nestin-Cre;Mettl14^{fl/fl}* cKO cortices. Shown are sample confocal images (A, B) and quantifications (C). Regions in white boxes are shown at a higher magnification. Scale bars: 500 μm (A, top panel), 50 μm (A, bottom panel), 100 μm (B). Values in (C) represent mean ± SEM ($n = 4-7$; ***: $P < 0.001$; *: $P < 0.05$; unpaired Student's t-test).

(D–E) Presence of IPCs in P5 cKO cortices. Shown are sample confocal images (D; scale bars: 100 μm) and quantification (E). Values represent mean ± SEM ($n = 6$; ***: $P < 0.001$; **: $P < 0.01$; unpaired Student's t-test).

(F–G) Ongoing neurogenesis in P5 cKO cortices. P5 pups were injected with EdU and analyzed 48 hr later. Shown in (F) are sample confocal images of the ventricular side of the primary somatosensory cortex. Arrows indicate Pax6⁺EdU⁺ cells (top) and Tbr2⁺TuJ1⁺EdU⁺ cells (bottom). Scale bars: 100 μm. Quantification of EdU⁺ cells with different markers is shown in (G). Values represent mean ± SEM ($n = 6$; ***: $P < 0.001$; unpaired Student's t-test).

(H–K) Reduced production of upper-layer neurons and astrocytes in cKO cortices. Pregnant mice were injected with EdU at E15.5 and analyzed at P5. Shown are sample confocal images (H, J; scale bars: 100 μm) and quantification (I, K). Values represent mean ± SEM ($n = 6$; ***: $P < 0.001$; unpaired Student's t-test).

See also Figure S1

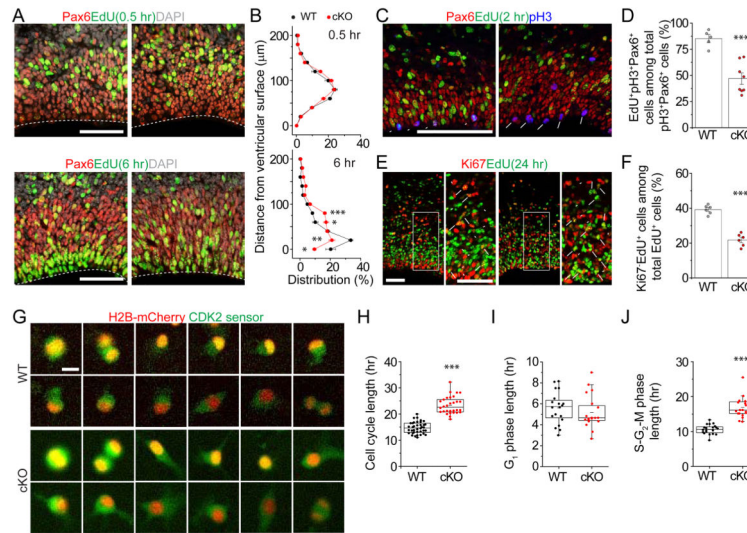


Figure 2. *Mettl14*^{-/-} RGCs and NPCs exhibit prolonged cell cycle progression

(A–B) Abnormal INM of RGCs in *Mettl14* cKO cortices. Pregnant mice were injected with EdU at E17.5 and analyzed 0.5 or 6 hr later. Shown are sample confocal images (A; scale bars: 50 µm) and quantification of the distance from Pax6⁺EdU⁺ nuclei to the ventricular surface (B). Values for the percentages of nuclei in each 20 µm bin represent mean ± SEM ($n = 4$; ***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$; unpaired Student's t-test).

(C–D) Delayed S to M phase transition of RGCs in *Mettl14* cKO mice. Pregnant mice were injected with EdU at E17.5 and analyzed 2 hr later. Shown in (C) are sample confocal images. Arrowheads point to Pax6⁺PH3⁺EdU⁺ cells and arrows point to Pax6⁺PH3⁺EdU⁻ cells. Scale bar: 50 µm. Shown in (D) is the quantification of the percentage of Pax6⁺PH3⁺EdU⁺ cells, representing cells proceeded from S to M phase during the 2 hr chase, among total Pax6⁺PH3⁺ cells. Values represent mean ± SEM ($n = 5$ for WT and $n = 8$ for cKO; ***: $P < 0.001$; unpaired Student's t-test).

(E–F) Delayed cell cycle exit of neural progenitors in *Mettl14* cKO mice. Pregnant mice were injected with EdU at E17.5 and analyzed 24 hr later. Shown in (E) are sample confocal images. Arrowheads point to Ki67⁻EdU⁺ cells and arrows point to Ki67⁺EdU⁻ cells. Scale bar: 50 µm. Shown in (F) is the quantification of the percentage of Ki67⁻EdU⁺ cells, representing cells exited from cell cycle, among total EdU⁺ cells. Values represent mean ± SEM ($n = 6$; ***: $P < 0.001$; unpaired Student's t-test).

(G–J) Time-lapse imaging analysis of mouse NPCs showing prolonged S-G₂-M phase length in the absence of *Mettl14*. WT and cKO mouse NPCs were electroporated with plasmid co-expressing a Cdk2 sensor (green) and the H2B-mCherry nuclear marker (red), cultured for 2 days, and imaged for 48 hr. Shown in (G) are sample time-lapse images with time stamps. Scale bars: 10 µm. Also shown are box plots of quantifications for the total cell cycle length (H; $n = 38$ for WT and $n = 30$ for cKO), G₁ phase length (I; $n = 20$), and S-G₂-M phase length (J; $n = 20$). Each dot represents data from one NPC (***: $P < 0.001$; unpaired Student's t-test).

See also Figure S2.

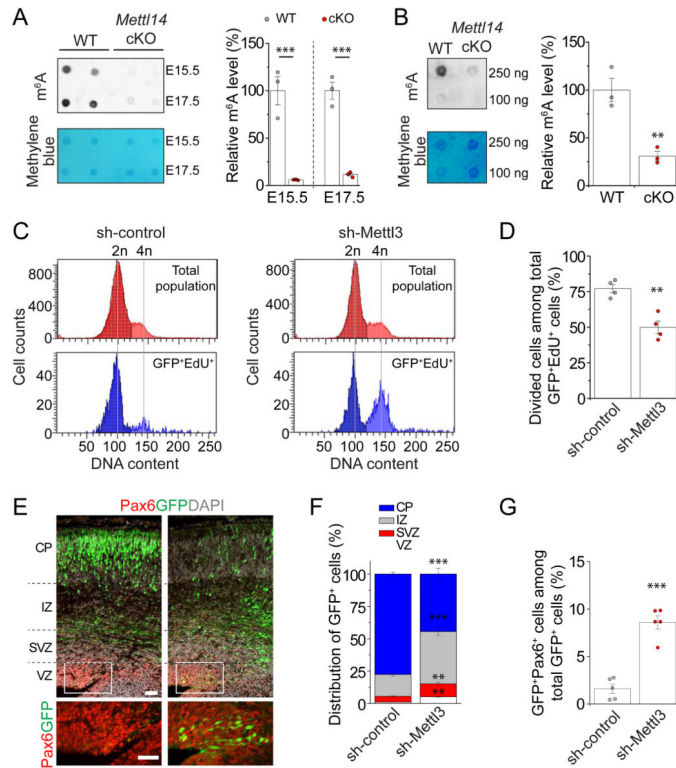


Figure 3. *Mettl3* regulates cell cycle progression of NPCs and maintenance of embryonic cortical RGCs

(A) Depletion of m^6A -tagging on mRNAs purified from E15.5 and E17.5 *Mettl14* cKO mouse forebrain. Shown in the left panels are sample images of m^6A dot blot and methylene blue staining (for loading controls). Data were normalized to the averaged levels of WT samples and quantification is shown in the right panel. Values represent mean \pm SEM ($n = 3$; **: $P < 0.01$; unpaired Student's t-test).

(B) Depletion of m^6A -tagging on mRNAs purified from *Mettl14* cKO NPCs. Values represent mean \pm SEM ($n = 3$; **: $P < 0.01$; unpaired Student's t-test).

(C–D) Flow cytometry analysis of cell cycle status of mouse NPCs. NPCs were electroporated to co-express GFP and the control shRNA, or the shRNA against *Mettl3*. At day 4, NPCs were pulse-labeled with EdU (10 μ M) for 30 min, cultured for 9 hr, followed by EdU and DNA content (DyeCycle Violet) staining and flow cytometry analysis. Shown are sample histograms of DNA content from GFP⁺EdU⁺ cells and the total cell population (as a reference; C) and quantification (D). Values in (D) represent mean \pm SEM ($n = 4$; **: $P < 0.01$; unpaired Student's t-test).

(E–G) Embryonic mouse cortices were electroporated in utero at E13.5 to co-express GFP and shRNA-control, or GFP and shRNA-*Mettl3*, and analyzed at E17.5. Shown in (E) are sample confocal images. Scale bars: 50 μ m. The distribution of GFP⁺ cells in each zone (F) and the percentage of GFP⁺Pax6⁺ cells among total GFP⁺ cells (G) were quantified. VZ: ventricular zone; SVZ: subventricular zone; IZ: intermediate zone; CP: cortical plate. Values represent mean \pm SEM ($n = 4$; ***: $P < 0.001$; **: $P < 0.01$; unpaired Student's t-test). See also Figure S3.

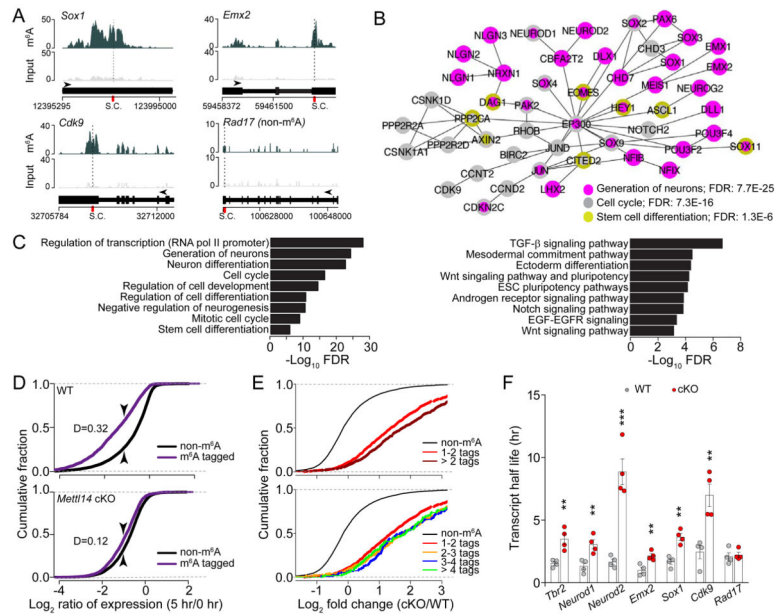


Figure 4. m⁶A tags transcripts related to transcription factors, cell cycle, and neuronal differentiation in the embryonic mouse brain, and promotes their decay
 (A) Coverage plots from m⁶A-seq of E13.5 mouse forebrains showing representative examples of m⁶A-tagged (*Sox1*, *Emx2*, and *Cdk9*) and non m⁶A-tagged (*Rad17*) transcripts. Top and middle panels show read coverages normalized by library sizes from m⁶A pulled-down and input libraries, respectively, and bottom panels show gene structures (arrows point to the direction of transcription; S.C.: stop codon).
 (B–C) GO analysis of m⁶A-tagged genes reveals enrichment for biological process terms related to transcription factors, neurogenesis, cell cycle, and stem cell differentiation. Also shown is Wikipathways gene set enrichment analysis. FDR: false discovery rate.
 (D) Cumulative distribution of Log₂(gene expression ratios) at time 5 hr post ActD over time 0 hr for m⁶A tagged genes (purple line) and non-m⁶A tagged genes (black line) for WT and *Mettl14* cKO NPCs. D = value of Kolmogorov Smirnov test statistic corresponding to maximum difference between methylated and non-methylated distributions.
 (E) Cumulative distribution of log₂(fold change in ratios of gene expression) at 5 hr post ActD treatment over time 0 hr upon *Mettl14* deletion. Top panel shows cumulative distribution for non-targets (black line) and transcripts with 1–1.9 m⁶A sites on average (bright red line), or 2 or more sites on average (dark red line). Bottom panel shows cumulative distribution for non-targets and m⁶A-tagged transcripts with 1–2, 2–3, 3–4, and 4 or more sites on average.
 (F) Summary of half-life of a group of transcripts in WT and *Mettl14* cKO NPCs. Values represent mean ± SEM (*n* = 4; ***: *P* < 0.001; **: *P* < 0.01; unpaired Student’s t-test. See also Figure S4, and Table S2, S3 and S4.

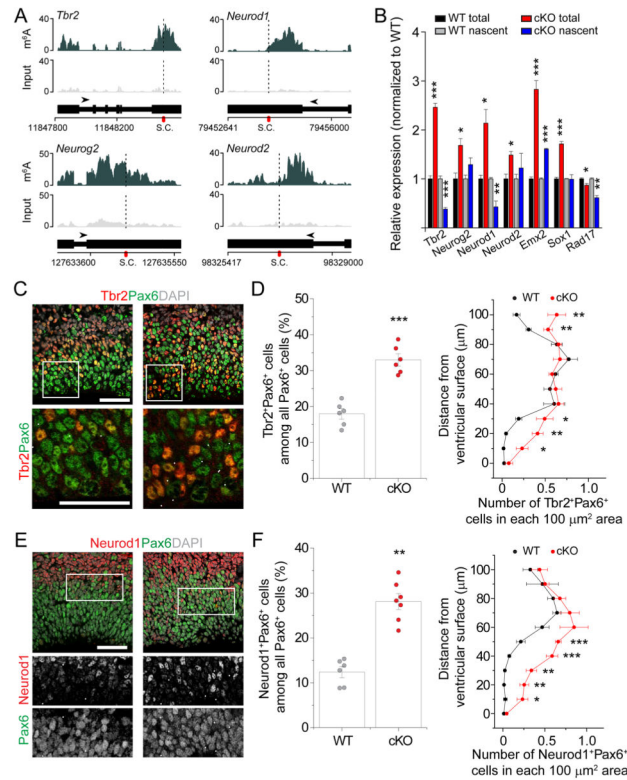


Figure 5. Post-transcriptional regulation of pre-patterning gene levels and protein production by m⁶A signaling in cortical neural stem cells

(A) Coverage plots from m⁶A-seq of E13.5 mouse forebrains showing representative examples of m⁶A-tagged IPC (*Tbr2* and *Neurog2*) and neuronal (*Neurod1* and *Neurod2*) genes.

(B) Q-PCR analysis of total mRNA and 4sU-purified nascent mRNA from WT and *Mett14* cKO NPCs. All Ct values were first normalized to *Gapdh* control (not m⁶A-tagged), which were similar in both WT and cKO NPCs. The ratio (cKO over WT) was calculated for each experiment and values represent mean \pm SEM ($n = 3$ cultures; ***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$; unpaired Student's t-test).

(C–F) Precocious expression of Tbr2 and Neurod1 proteins in RGCs in E17.5 *Mett14* cKO mice in vivo. Shown are sample confocal images (C, E; scale bars: 50 μ m) and quantifications of the percentage of Tbr2⁺Pax6⁺ cells (D), or Neurod1⁺Pax6⁺ cells (F), among total Pax6⁺ cells (left panels, $n = 6$) and the density distribution of Tbr2⁺Pax6⁺ (D), or Neurod1⁺Pax6⁺ cells (F), from the ventricular surface (right panels, $n = 4$). Arrows indicate Tbr2⁺Pax6⁺ (C) or Neurod1⁺Pax6⁺ cells (E). Values in (D, F) represent mean \pm SEM (***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$; unpaired Student's t-test).

See also Figure S5.

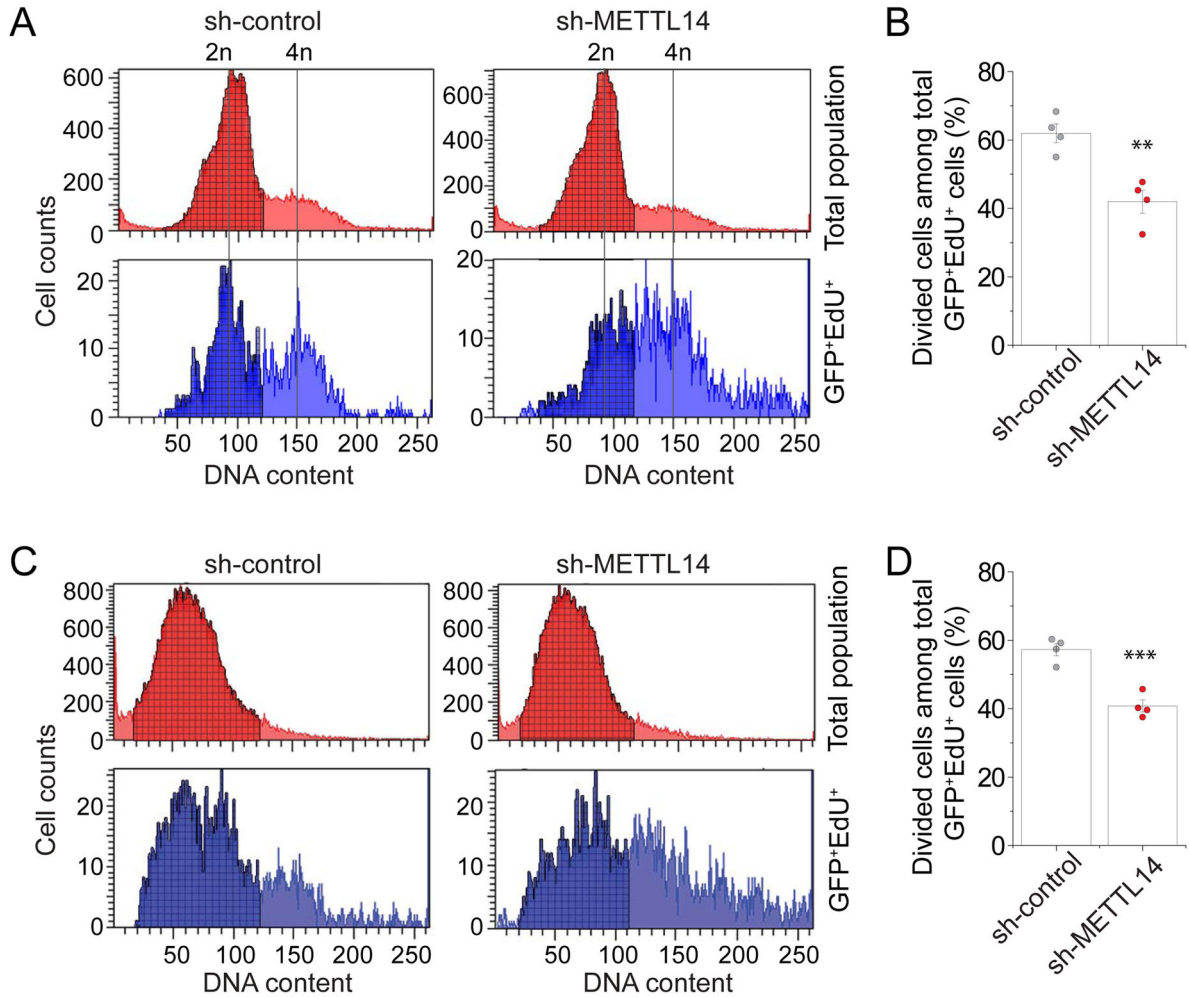


Figure 6. METTL14 regulates cell cycle progression of human NPCs

(A–B) Flow cytometry analysis of cell cycle progression of hNPCs with *METTL14* KD.

Human NPCs were electroporated to co-express GFP and shRNA-control, or shRNA-*METTL14*. After 4 days, hNPCs were pulse-labeled with EdU (10 μ M) for 30 min, incubated for 14 hr, followed by EdU and DNA content (DyeCycle Violet) staining and flow cytometry analysis, similarly as in Figure 3C–D. Values represent mean \pm SEM ($n = 4$; **: $P < 0.01$; unpaired Student's *t*-test).

(C–D) Flow cytometry analysis of cell cycle progression with *METTL14* KD in human forebrain organoids. Day 45 forebrain organoids were electroporated to co-express GFP and shRNA-control, or shRNA-*METTL14*. After 7 days, forebrain organoids were pulse-labeled with EdU (10 μ M) for 1 hr, cultured further for 14 hr, followed by dissociation and analysis similarly as in Figure 3C–D. Values represent mean \pm SEM ($n = 4$; ***: $P < 0.001$; unpaired Student's *t*-test).

See also Figure S6.

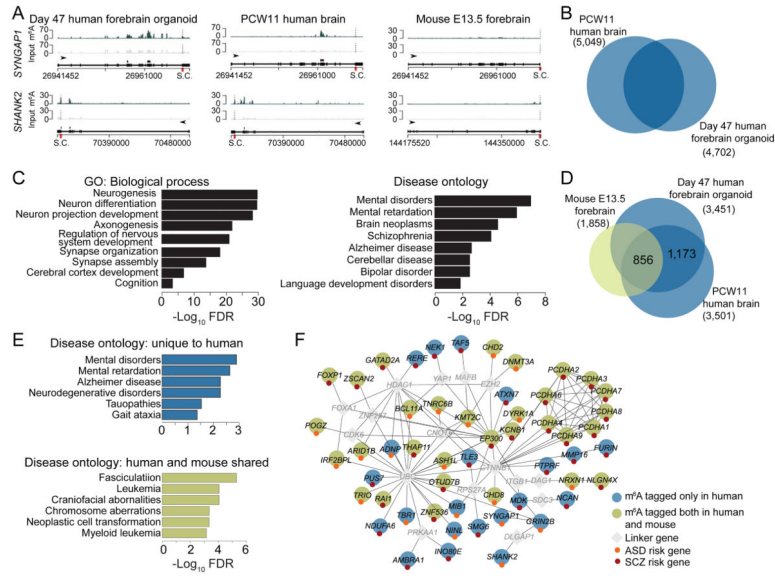


Figure 7. Conserved and unique features of m⁶A mRNA methylation in human forebrain organoids, human fetal brain and embryonic mouse forebrain

(A) Representative plots of two m⁶A-tagged transcripts in day 47 human forebrain organoids and PCW11 human fetal brain, but not in mouse E13.5 forebrain.

(B) Venn diagram showing shared m⁶A-tagged transcripts between day 47 human forebrain organoids and PCW11 fetal human brain.

(C) GO and disease ontology analyses of shared m⁶A-tagged genes in day 47 human forebrain organoids and PCW11 human fetal brain.

(D) Venn diagram showing shared and unique m⁶A-tagged transcripts among mouse E13.5 forebrain, day 47 human forebrain cortex, and PCW11 fetal human brain. Only ortholog genes expressed in all three samples were used for analysis.

(E–F) Disease ontology analysis of transcripts uniquely m⁶A-tagged in human shows enrichment for neurodevelopmental diseases, whereas disease ontology analysis of commonly m⁶A-tagged transcripts showed enrichment for oncogenic processes.

See also Figure S7, and Table S2 and S5.

KEY RESOURCE TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-Pax6	BioLegend	PRB-278P RRID: AB_2313780
Mouse anti-Pax6	BD Bioscience	561664 RRID: AB_10895587
Rabbit anti-Tbr2	Abcam	ab23345 RRID: AB_778267
Chicken anti-GFP	Aveslab	GFP-1020 RRID: AB_10000240
Rabbit anti-Ki67	Thermo Fisher	RM-9106 RRID: AB_2335745
Goat anti-Sox2	Santa Cruz	sc-17320 RRID: AB_2286684
Chicken anti-Nestin	Aveslab	NES RRID: AB_2314882
Rabbit anti-m ⁶ A	Synaptic Systems	202 003 RRID: AB_2279214
Mouse anti-m ⁶ A	Synaptic Systems	202 111 RRID: AB_2619891
Rabbit anti-Actin	Cytoskeleton	AAN01 RRID: AB_10708070
Rabbit anti-GFAP	Dako	Z0334 RRID: AB_10013382
Mouse anti- β III Tubulin (TuJ1)	Sigma-Aldrich	T3952 RRID: AB_1841226
Rabbit anti-S100 β	Abcam	ab52642 RRID: AB_882426
Mouse anti-SATB2	Abcam	ab92446 RRID: AB_10563678
Rabbit anti-Tbr1	Abcam	ab31940 RRID: AB_2200219
Rat anti-CTIP2	Abcam	ab18465 RRID: AB_2064130
Rabbit anti-Phospho-Histone H3	Cell Signaling	9701S RRID: AB_331534
Rabbit anti-Mettl14	ABclonal	AB8530
Mouse anti-Neurod1	Abcam	ab60704 RRID: AB_943491
Goat anti-rabbit IgG-HRP	Santa Cruz	sc-2004 RRID: AB_631746
Goat anti-mouse IgG-HRP	Santa Cruz	sc-2005 RRID: AB_631736
Chemicals, Peptides, and Recombinant Proteins		
Actinomycin D	Sigma-Aldrich	A1410
DAPI	Thermo Fisher Scientific	D1306 RRID: AB_2629482
StemPro Accutase Cell Dissociation Reagent	Thermo Fisher Scientific	A1110501

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Matrigel Matrix	Corning	354277
7-AAD	Thermo Fisher Scientific	A1310
Vybrant DyeCycle Violet	Thermo Fisher Scientific	V35003
Y-27632	Cellagen Technology	C9127-2s
EdU	Thermo Fisher Scientific	A10044
Phosphatase Inhibitor Cocktail	Cell Signaling	5870
Protease Inhibitor Cocktail	Sigma	P8340
4x Laemmli Sample Buffer	Bio-Rad	1610747
Methylene blue	Sigma-Aldrich	M9140
A83-01	Stemcell Technologies	72022
Dorsomorphin	Stemcell Technologies	72102
SB431542	Stemcell Technologies	72232
CHIR99021	Stemcell Technologies	72052
Dulbecco's Phosphate-Buffered Saline (DPBS)	Corning	21-031
Dulbecco's Modification of Eagle's Medium (DMEM)	Corning	10-013
DMEM/F-12, HEPES	Gibco	11330-032
Neurobasal® Medium	Gibco	21103049
KnockOut™ Serum Replacement	Gibco	10828028
GlutaMAX™ Supplement	Gibco	35050061
MEM Non-Essential Amino Acids Solution	Gibco	11140050
Penicillin-Streptomycin (10,000 U/mL)	Gibco	15140122
2-Mercaptoethanol	Gibco	21985023
N-2 Supplement	Gibco	17502048
B-27® Supplement	Gibco	17504044
Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix	Corning	354230
Insulin solution	Sigma-Aldrich	I0516
Fetal Bovine Serum (FBS)	Corning	35-010
0.1% Gelatin in Water	Stemcell Technologies	7903
Advanced DMEM/F-12	Gibco	12634010
Human recombinant LIF	Stemcell Technologies	78055
Compound E	Stemcell Technologies	73952
Costar® 6 Well Clear Flat Bottom Ultra Low Attachment plate	Sigma-Aldrich	CLS3471
Recombinant Human FGF-basic	Peptotech	100-18B
SUPERase In RNase Inhibitor	Thermo Fisher Scientific	AM2694
TURBO DNase (2 U/μL)	Thermo Fisher Scientific	AM2238
N6-Methyladenosine monophosphate sodium salt	Sigma-Aldrich	M2780
Agencourt AMPure XP	Beckman	A63880
Dynabeads® Protein G for Immunoprecipitation	Thermo Fisher Scientific	10003D
CellTrace CFSE	Thermo Fisher Scientific	C34554

REAGENT or RESOURCE	SOURCE	IDENTIFIER
luciferase control RNA	Promega	L4561
TRIzol Reagent	Thermo Fisher Scientific	15596026
4-thiouridine	Carbosynth	T4509
MTSEA biotin- XX	Biotium	90066
Dimethylformamide (DMF)	Sigma-Aldrich	D4551
Pierce Streptavidin Magnetic Bead	Thermo Fisher Scientific	88816
Critical Commercial Assays		
Click-iT EdU Assay Kits for Flow Cytometry	Thermo Fisher Scientific	C10635
Click-iT EdU Alexa Fluor 647 Imaging Kit	Thermo Fisher Scientific	C10340
Dynabeads mRNA Purification Kit	Thermo Fisher Scientific	61006
SuperScript III First-Strand Synthesis System	Thermo Fisher Scientific	18080051
Fast SYBR Green Master Mix	Thermo Fisher Scientific	4385610
NEBNext Ultra RNA Library Prep Kit for Illumina	New England Biolabs	E7530
Dynabeads mRNA DIRECT Purification Kit	Invitrogen	61011
RNeasy Mini Kit	Qiagen	74106
Quant-IT PicoGreen dsDNA Assay Kit	Thermo Fisher Scientific	P7589
RNeasy MinElute Kit	Qiagen	74204
Deposited Data		
Raw and analyzed data	This Paper	GSE99017
Experimental Models: Cell Lines		
B16-F10	ATCC	CRL-6475 RRID: CVCL_0159
C12 (iPSC from normal human foreskin fibroblasts)	Wen et al., 2014	N/A
Experimental Models: Organisms/Strains		
Mouse: B6.Cg-Tg(Nes-cre)1Kln/J	Jackson Laboratory	003771 RRID: IMSR_JAX:003771
Mouse: <i>Mettl14</i> ^{flxed;flxed}	From Dr. Chuan He	N/A
Mouse: CrI:CD1(ICR)	Charles River Laboratory	RRID: IMSR_CRL:22
Mouse: CrI:CF188	Charles River Laboratory	RRID:IMSR_CRL:23
Oligonucleotides		
shRNA control sequence for mouse and human: UUCUCCGAACGUGUCACGU	Qiagen	SI03650318
shRNA targeting sequence: mouse <i>Mettl3</i> : GCACACUGAUGAATCUUUAGG	Wang et al., 2014	N/A
shRNA targeting sequence: human <i>Mettl14</i> : CCUGAAAUUGCAAUUAUAGAA	Wang et al., 2014	N/A
shRNA targeting sequence: mouse <i>Cnot1</i> : GUGGACAAUUUAACCAAGA	Du et al., 2016	N/A
shRNA targeting sequence: mouse <i>Cnot7</i> : AACAAGUCUACAUUACACCGC	Du et al., 2016	N/A
Primers for Q-PCR analysis	See Table S1	N/A
Recombinant DNA		
pUEG	Ge et al., 2006	N/A
cFUGW	Lois et al. 2002	Addgene plasmid 14883

REAGENT or RESOURCE	SOURCE	IDENTIFIER
pCAG-GFP	Matsuda and Cepko, 2004	Addgene plasmid: 11150
pPGK-H2B-mCherry-2A-DHB(aa994-1087)-GFP	From Dr. Sergi Regot (Spencer et al., 2013)	N/A
Software and Algorithms		
Image J	NIH	https://imagej.nih.gov/ij/
Imaris	Bitplane	http://www.bitplane.com/imaris/imas
MATLAB	MathWorks	https://www.mathworks.com/products/matlab.html
Bowtie2	Langmead and Salzberg, 2012	http://bowtie-bio.sourceforge.net/bowtie2/index.shtml
Tophat2	Kim et al., 2013	https://ccb.jhu.edu/software/tophat/index.shtml
Fastx Toolkit	http://hannonlab.cshl.edu/fastx_toolkit/	http://hannonlab.cshl.edu/fastx_toolkit/download.html
MACS2	Zhang et al. 2008	https://github.com/taoliu/MACS/wiki/Install-macs2
BedTools	Quimlan and Hall, 2010	Bedtools.readthedocs.io/en/latest/content/installation.html
Metagene R Package	Beauparlant et al., 2014	http://bioconductor.org/packages/release/bioc/html/metagene.html
HTSeq	Anders et al., 2014	http://www-huber.embl.de/HTSeq/doc/install.html
DESeq2	Love et al., 2014	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
RSEM	Li and Dewey, 2011	https://github.com/deweylab/RSEM
EBSeq	Leng et al., 2013	https://github.com/lengning/EBSeq
Toppgene	Chen et al., 2009	https://toppgene.cchmc.org
ConsensusPathDB	Herwig, 2016	http://cpdb.molgen.mpg.de
Webgestalt	Zhang et al., 2005	http://www.webgestalt.org/option.php
Cytoscape	Shannon et al., 2003	http://www.cytoscape.org/download.php
Single-cell transcriptomic atlas of the developing neocortex	Telley et al., 2016	http://genebrowser.unige.ch/science2016/
Other		
SuperSignal West Dura Extended Duration Substrate	Thermo Fisher Scientific	34075
Nucleofector Kits for Mouse Neural Stem Cells	Lonza	VAPG-1004
Nucleofector 2b Device	Lonza	AAB-1001
Square wave electroporator	Nepa Gene	CUY21SC
Tweezers with platinum disk electrode	Nepa Gene	CUY650-5
Immun-Blot PVDF Membrane	Bio-Rad	1620177
Amersham Hybond-N+ membrane	GE Healthcare	RPN119B
Stratagene Stratalinker 2400 UV Crosslinker	Agilent Genomics	53274-1
12-well Spinning Bioreactor	Qian et al. 2016	N/A