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The m⁶A reader YTHDF2 regulates mRNA transport and axon outgrowth in developing neurons

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Abstract

Proper nervous system development is critical for brain function, and deficits in neural development are implicated in many brain disorders. Neurons are distinctly polarized cells where mRNA can be transported to distal structures like axons and dendrites. Recent discoveries of widespread mRNA chemical modifications raise the question of their post-transcriptional regulatory role in brain development and function. N⁶-methyladenosine (m⁶A), installed by the METTL3/METTL14 methyltransferase complex, is the most prevalent internal mRNA modification, influencing stability, translation, splicing, and localization. However, the impact of m⁶A modification on RNA transport in developing neurons is not well understood.

In this study, we find that the ablation of *Mettl14* in postmitotic neurons leads to impaired axonal projection during corticogenesis. RNA-seq and single-molecule in situ hybridization reveal mislocalization of mRNAs in neurites of neurons with m⁶A loss-of-function. Furthermore, m⁶A-SAC-seq to identify a single nucleotide resolution m⁶A maps in the perinatal brain uncovers m⁶A-tagged transcripts associated with synapse organization, mRNA processing, and axonogenesis. We also identify YTHDF2 as the reader protein responsible for mRNA transport in callosal projection axons. YTHDF2 interacts with motor proteins, translational regulators, and microtubules to facilitate distal transport of m⁶A-tagged mRNA. Our data suggest that FMRP may serve as a context-guiding interactor that reshapes the YTHDF2 complex by recruiting specific cofactors and motor proteins, thereby promoting transport rather than degradation of m⁶A-tagged transcripts. Together, these findings provide insight into the epitranscriptomic mechanisms governing axon projection and guidance during mammalian cortical neurogenesis.

Introduction

Neurodevelopment is a precisely orchestrated process wherein neural stem cells sequentially give rise to various types of neurons and glia, which then migrate and integrate into distinct regions of the nervous system. Precise regulation of gene expression is crucial for many steps of developmental processes, where mRNA modifications, particularly N⁶-methyladenosine (m⁶A) modification, play a pivotal role in post-transcriptional regulation. m⁶A modification, the most prevalent internal mRNA modification in eukaryotic organisms, is involved in various aspects of neurodevelopment^{1,2}. For example, the absence of m⁶A induces the accumulation of m⁶A-tagged mRNAs related to temporal and cell-type specific transcription factors, leading to delayed transitions in developmental competency³. Additionally, spatiotemporal progression of neurogenesis is significantly impaired in knockout mice lacking YTHDF2, an m⁶A reader^{4,5}. These results suggest that dynamic mRNA m⁶A modification adds an additional layer to gene expression regulation during neurodevelopment, with dysregulation contributing to various neurological problems^{6,7}.

Neurons are highly polarized cells with extensive neuronal processes including dendrites and axons. Recent studies with advanced sequencing technology and high throughput mRNA imaging have revealed a myriad of different mRNA molecules in neuronal processes, suggesting that RNA localization is a prevalent method to sort out proteins to distinct subcellular compartments of neurons⁸⁻¹⁰. In neurons, long-distance transport of mRNAs to distal subcellular

compartments allows dynamic local protein translation upon external stimuli, which is essential for activity-dependent neuronal wiring during neurodevelopment and synaptic plasticity in the adult brain. Many studies have also shown that local translation of specific mRNA subsets at the axon terminal is required for axonal growth during development^{11,12} as well as in mature brains¹⁰. The general principles for targeting an mRNA to a specific subcellular location are still elusive, but several molecular mechanisms suggest that cis-elements of mRNA serve as critical signals for trafficking to dendrites and axons. For example, regulatory elements depending on primary sequence or secondary structure in 3'UTRs of mRNA are essential for active transport by facilitating the anchoring of RNA and associated RNA-binding proteins to cytoskeletons¹³. It has also been suggested that m⁶A modification may serve as a cis-regulatory element for mRNA distal trafficking in neurons. Notably, a recent study reported that m⁶A-modified mRNAs are restricted to the soma due to m⁶A-mediated enhanced degradation (passive regulation)¹⁴. In contrast, other studies have proposed that m⁶A modification in the 3'UTR facilitates the distal translocation of these mRNAs (active regulation)^{15,16}. Therefore, further mechanistic investigations are necessary to understand m⁶A-mediated regulation of mRNA transport in light of these contradictory observations in diverse physiological contexts. In addition, the precise m⁶A-mediated mechanism for recruiting RNA binding proteins (RBPs) and motor proteins to regulate mRNA trafficking processes, remains complex and not fully elucidated.

In this study, we elucidate the critical role of m⁶A modification in axon projection and neurite development during corticogenesis. Employing a single nucleotide resolution m⁶A profiling using m⁶A-selective allyl chemical labeling and sequencing (m⁶A-SAC-seq), we unveil the quantity and stoichiometry of m⁶A modification in the developing brain *in vivo*. Investigation

into the stability and axonal localization of m⁶A-tagged mRNAs suggests that m⁶A modification in 3'UTR, along with its reader YTHDF2, facilitates the distal translocation of target mRNAs in neurons. Through analysis of YTHDF2 interactome in the perinatal brain, we identified the YTHDF2-transport complex, which includes motor proteins and FMRP, as functionally essential for transporting target mRNAs bearing m⁶A tagging.

Results

***Mettl14* ablation leads to impaired axon projection and reduced growth cone advance**

A previous study using *Nestin-Cre; Mettl14^{f/f}* (*M14* cKO) mice demonstrated the critical role of m⁶A methylation in mammalian cortical neurogenesis³. While gene expression regulation by m⁶A mRNA modification in neural progenitor cells has been extensively studied, the *in vivo* functional roles of m⁶A in differentiating newborn neurons have remained unexplored. To investigate this, we performed *in utero* electroporation of plasmid vectors expressing Cre recombinase (hCRE-IRES-GFP-RV)¹⁷ and RFP at E14.5 into the somatosensory cortex and monitored differentiating neurons at P5 (Fig. 1a). Electroporated neurons were mostly SATB2⁺ upper layer neurons and migrated into layer 2/3 at P5 both in control (*Mettl14^{+/f}*) and *M14* cKO (*Mettl14^{f/f}*), suggesting that METTL14 depletion did not affect neuronal subtype specification and neuronal migration in this experimental setting (Supplementary Fig. 1a-c). Callosal axons originating from *Cre*-electroporated projection neurons traversed the corpus callosum (CC) to reach the contralateral hemisphere, forming axonal arbors. However, axon projections of *M14* cKO neurons exhibited reduced axon projections in cortical layers and arborization on the

contralateral side compared to the control group at P5 (Fig. 1a-c). Additionally, to mitigate potential secondary effects resulting from m⁶A depletion in neural progenitor cells (NPCs), we conducted *in utero* electroporation at E14.5 using a neuron-specific Cre driver plasmid under the control of the Doublecortin (*Dcx*) promoter, driving the expression of both Cre recombinase and EGFP (*Dcx*-CRE-IRES-GFP)¹⁸, and analyzed at P5. The results are consistent with our findings using hCRE-IRES-GFP-RV, showing reduced axon projection and disrupted arborization during the corticogenesis upon m⁶A depletion (Supplementary Fig.1d-f).. Furthermore, we found that axon projections and arborization were more dramatically reduced contralaterally after the *Mettl14* depletion in postmitotic neurons compared to the control at P14 (Fig. 1d-e).

To further examine the effects of m⁶A depletion on postmitotic differentiating neurons, we cultured primary cortical neurons after acute *Mettl14* deletion. Following complete cell adhesion and the initiation of polarization (day *in vitro* 1, DIV1), we infected the neurons with AAV-mCherry-IRES-Cre viruses and analyzed at DIV6, a time point when neurons typically display well-defined axon-dendrite polarity¹⁹. We confirmed a similar population of neuronal subpopulation between control and cKO primary neurons (Supplementary Fig. 1g-h), despite efficient depletion of *Mettl14* and m⁶A by AAV-mCherry-IRES-Cre virus infection in the primary culture (Supplementary Fig. 1i-j). We observed a significant reduction in the longest neurite length of *M14* cKO neurons and an increase in axon collateral branching compared with control neurons at DIV6 (Fig. 1f-h), suggesting that the neurite development is impaired after m⁶A depletion. Last, we also examined the dynamics of growth cones by live imaging, which revealed that *Mettl14* deletion significantly reduced the velocity of growth cone advance and increased transient protrusion formation compared to the control at DIV6 (Fig. 1i-k, Supplementary Movie 1,2). Collectively, these findings indicate that m⁶A RNA modification

plays an essential role in axon projection, neurite development, and branching, as well as normal growth cone dynamics during neuronal differentiation.

A single nucleotide resolution, quantitative m⁶A mapping reveals transcripts related to axon development with high m⁶A stoichiometry

To investigate the landscape of the m⁶A epitranscriptome in the brain at single nucleotide resolution, we used m⁶A-SAC-seq on the P0 mouse forebrain (Supplementary Fig. 2a)²⁰. To identify METTL14-dependent m⁶A sites, we filtered out peaks according to the previous protocol²¹, using m⁶A-SAC-seq data from WT forebrain and *M14* cKO forebrain with 2 biological replicates for each (Supplementary Fig. 2b). We identified 10,304 m⁶A sites in 3,830 genes, which showed enrichment at the DRACH motif and a stop codon-proximal 3'UTR distribution, consistent with previous reports (Fig. 2a-b, Supplementary Data 1). We also confirmed that our m⁶A-modified transcripts highly overlapped with previously reported m⁶A-modified transcripts in a neuronal context¹⁶, suggesting the consistency of m⁶A targets across different methods (Supplementary Fig. 2c). The m⁶A-modified transcripts showed strong enrichment for genes associated with synapse organization, axonogenesis, positive regulation of cell projection organization, and regulation of synapse, implying that m⁶A-modified RNAs play a key role in neuronal differentiation during early postnatal cortical development (Fig. 2c).

We further investigated the m⁶A-SAC-seq result, including characteristics of stoichiometries and the position of m⁶A sites. Interestingly, the average m⁶A stoichiometry in 3'UTR is higher than in the CDS and the 5'UTR (Fig. 2d). This higher methylation stoichiometry in 3'UTR is a unique characteristic of the brain epitranscriptome compared to previously reported m⁶A-SAC-seq results in HeLa and HEK293 cells (Supplementary Fig. 2d)²⁰.

We also performed Gene Ontology (GO) enrichment analysis²² with genes categorized by m⁶A position or stoichiometry. Although intronic and long non-coding methylated transcripts showed enrichment for genes associated with RNA processing, other positions, including CDS, 5'UTR, and 3'UTR, did not show differential enrichment (Supplementary Fig. 2e). Furthermore, average methylation stoichiometry among transcripts also did not display differential enrichment between different transcripts set (Supplementary Fig. 2f). However, Gene Set Enrichment Analysis (GSEA) revealed that high m⁶A stoichiometry was significantly and positively related to regulation of synapse assembly, nervous system development, and axon development (Fig. 2e, Supplementary Fig. 2g).

Next, we performed an RNA stability assay to determine the effects of m⁶A methylation in a neuronal context (Supplementary Data 2). As previously described³, cultured primary cortical neurons from WT and *M14* cKO mice were treated with Actinomycin D, and RNA-seq was performed at 0 and 5 hours to estimate the RNA stability of each transcript. Across the transcriptome, m⁶A-modified transcripts showed increased relative stability in the *M14* cKO condition compared to WT (Supplementary Fig. 3a), and the number of m⁶A sites affected the stability (Supplementary Fig. 3b). We utilized m⁶A position data and found that depletion of m⁶A in the 3'UTR stabilized transcripts more than depletions in the CDS and 5'UTR (Fig. 2f). Moreover, the stability of transcripts with m⁶A only in the CDS changed slightly less, while the stability of transcripts with m⁶A only in the 3'UTR or with m⁶A in both regions increased more significantly, suggesting that m⁶A in the 3'UTR has a more pronounced effect on mRNA stability (Supplementary Fig. 3c). Interestingly, based on m⁶A stoichiometry data, we found that transcripts with high m⁶A stoichiometry were less stabilized than transcripts with low m⁶A stoichiometry in the *M14* cKO neurons (Fig. 2g). This tendency became more evident when

comparing the top 10% of m⁶A stoichiometry transcripts to the bottom 10% (Supplementary Fig. 3d). Altogether, these results support that m⁶A-tagged transcripts are directly related to neurodevelopmental processes and axon development in the postnatal brain, which are dysregulated in the *M14* cKO neurons by increased mRNA stability. Of note, the results of m⁶A-SAC-seq and mRNA stability assay imply that high m⁶A stoichiometry transcripts related to axon development (Fig. 2h, Supplementary Fig. 3e) are not solely regulated at the level of RNA stability as previously described¹⁴. This suggests that m⁶A may exert additional functions beyond promoting mRNA degradation, potentially influencing mRNA localization, translation efficiency, or RNP complex stabilization in neurons.

***Mettl14* loss-of-function reduced a subset of transcripts in neurites in differentiating neurons**

To examine the functional contribution of m⁶A modification in developing axons, we conducted RNA-seq using microdissected tissue from the axon-enriched CC of cortical projection neurons (layer 2/3) at P4 (referred to as CC-seq), a time-point at which the maximum number of axons are distally projected in the callosal region and remain in the white matter before entering the cortical plates (Supplementary Fig. 4a)²³. The enrichment of previously reported axonally localized mRNAs, such as *Abce1*, *Gtpbp4*, and *Npm1*,²⁴ was confirmed by qPCR (Supplementary Fig. 4b). As a result, we identified 1,864 decreased transcripts and 1,761 increased transcripts in the CC of *M14* cKO compared to that of WT (Fig. 3a, Supplementary Data 3).

To further investigate the relationship between RNA stability and localization, we compared our axon-enriched CC-seq results with the RNA stability assay in WT and *M14* cKO

primary neurons. We categorized the mRNAs in each quadrant (Q1, Q2, Q3, Q4; Fig. 3b) based on changes in stability and axonal localization upon *Mettl14* ablation. For example, Q1 represents mRNAs that are more stabilized, but their axonal localization is also increased in the *M14* cKO condition. Q2 represents mRNAs that are more stabilized, and their axonal localization is significantly reduced in the *M14* cKO condition. mRNAs in Q1 and Q2 groups (stability difference > 0.5) are both related to axonogenesis and synaptic development (Supplementary Fig. 4c-d). However, while GO analysis of the Q1 group showed similar GO terms regardless of m⁶A tagging as revealed by m⁶A-SAC-seq, the Q2 group displayed distinct GO terms depending on the presence of m⁶A tagging, including axonogenesis and growth cone (Fig. 3c). These findings suggest that m⁶A modification may not only regulate stability but also the axonal localization of mRNAs related to axonogenesis and growth cone.

To examine whether m⁶A-modified transcripts fail to localize to neurites under the *M14* cKO condition, we selected target transcripts by comparing our CC-seq, m⁶A-SAC-seq, and RNA stability assay dataset to previous datasets of axonally localized RNA (Fig. 3d)²⁵. For the control experiments, we selected *Gap43* and *Camk2b* mRNAs, which have m⁶A peaks but whose mRNA abundance in axons was not significantly changed by *Mettl14* ablation in CC-seq (Fig. 3a). To investigate whether m⁶A tagging plays a role in the localization of our target transcripts during neuronal differentiation, we performed single-molecule *in situ* hybridization (smFISH) to visualize endogenous *Gap43*, *Camk2b*, *Ncam1*, *Cntn1*, *Ank2*, and *Actb* mRNAs (Fig. 2h and Supplementary Fig. 3e) in control and *M14* cKO conditions (Fig. 3e). We measured the distance from the soma and the relative density of smFISH signals in neurites, and found that *Ncam1*, *Cntn1*, *Ank2*, and *Actb* mRNAs were reduced in neurites in *M14* cKO compared to the control at DIV6. However, levels of *Gap43* and *Camk2b* mRNAs remained unchanged (Fig. 3e-g,

Supplementary Fig. 5c-d). Consequently, the axonal protein levels of NCAM1 and ANK2 were significantly reduced at growth cones, indicating that m⁶A-mediated mRNA transport is essential for proper axonal expression of these target proteins (Supplementary Fig. 5a-b).

To directly visualize RNA transport through neurites, we employed the MS2 system²⁶. We used HA-tdMCP-GFP and BFP-actin-3'UTR-24×MS2 reporters containing a conserved m⁶A site in the 3'UTR (at the 1217th position, located 5nt downstream of the stop codon) (Fig. 3h). RNA dynamics were monitored in control and *Mettl14* cKO neurons using a spinning-disk microscope. Interestingly, anterograde RNA movement was significantly reduced under *M14* cKO conditions (Fig. 3i-j, Supplementary Movie 3,4). Moreover, the total motile time of RNA was also reduced under *M14* cKO conditions (Fig. 3k). To examine the direct role of m⁶A modification in RNA transport, we generated a mutant reporter, BFP-actin-3'UTRΔm⁶A-24×MS2, by introducing an A→G substitution at position 1217 to prevent m⁶A modification. RNA granule movement was then compared between the wild-type and Δm⁶A reporters in mouse primary neurons. As a result, anterograde RNA movement was markedly reduced in the Δm⁶A reporter (Fig. 3l-m, Supplementary Movie 5,6). Also, the total motile time of RNA was reduced in the Δm⁶A reporter (Fig. 3n). These findings demonstrate that a single m⁶A modification within the 3'UTR is critical for efficient anterograde transport of *Actb* mRNAs in developing neurons.

We also conducted live imaging to examine the transport of lysosomes through neurites in *M14* cKO and control groups at DIV6. We did not observe a significant alteration in lysosome transport, indicating that the general transport machinery was unaffected by m⁶A depletion (Supplementary Fig. 5e-h, Supplementary Movie 7,8).

Taken together, although m⁶A tagging generally restricts the stability of target mRNAs, m⁶A modification also has an additional function to positively promote neurite localization of mRNAs related to axonogenesis and growth cone regulation, which is essential for normal axonogenesis.

YTHDF2 is an essential reader that regulates the transport of m⁶A-tagged mRNA during axon development

The fates of m⁶A-tagged mRNAs largely depend on the functions of reader protein ²⁷. The YTHDF reader protein family, which is the most well-studied, interacts with m⁶A through their conserved YTH domain, with all three members (YTHDF1, 2, and 3) regulating the translation or decay of target mRNAs in the cytosol ²⁸. In non-neuronal cells, it has been shown that YTHDF reader proteins regulate subcellular mRNA localization, including P-bodies and stress granules ²⁹. To investigate which reader protein directly engages with m⁶A-tagged transcripts in axon projection, we evaluated the expression patterns of YTHDF1, YTHDF2, and YTHDF3 during corticogenesis. Although YTHDF2 is abundantly expressed in the embryonic cortex, the expression levels of YTHDF1 and YTHDF3 are very low according to the single-cell transcriptome atlas (Supplementary Fig. 6a) ³⁰. Moreover, *Ythdf1* or *Ythdf3* knockout mice did not exhibit obvious developmental abnormalities ⁴. In contrast, *Ythdf2* null knockout mice exhibited embryonic lethality and impaired cortical development ⁵. Furthermore, *YTHDF1* and *YTHDF3* knockdown did not alter the dynamics of growth cones and protrusion formation in cultured primary cortical neurons (Supplementary Fig. 6b-e). Therefore, we generated *Ythdf2* conditional knockout mice by deleting *Ythdf2* in the developing mouse nervous system using the *Nestin-Cre; Ythdf2^{fl/fl}* cKO model (*DF2* cKO) (Supplementary Fig. 6f). YTHDF2 depletion was

confirmed by Western blot analysis of protein lysate from *DF2* cKO and control mice at P0 (Supplementary Fig. 6g). *DF2* cKO mice exhibited decreased body weight and sub-viability, with approximately 30% failing to survive beyond P30 (Supplementary Fig. 6h-i). Additionally, we observed a significant decrease in forelimb grip strength in *DF2* cKO mice compared to controls at P30 (Supplementary Fig. 6j), indicating that YTHDF2 is crucial for normal postnatal development of the nervous system. However, we found that the cytoarchitecture of cortical layer-specific neurons appeared grossly normal compared to control mice (Supplementary Fig. 6k).

To track the axonal projection of *DF2* cKO neurons in detail, we performed *in utero* electroporation of plasmid vectors expressing Cre recombinase with GFP at E14.5 into the somatosensory cortex and monitored differentiating neurons at P5 (Fig. 4a). Electroporated neurons were primarily SATB2⁺ upper-layer neurons and migrated into layers 2/3 at P5 in both cKO (*Ythdf2^{f/f}*) and control (*Ythdf2^{f/+}*) mice. We observed that the axonogenesis of cortical projection neurons was significantly reduced in the white matter and cortical layer entry on the contralateral side (Fig. 4b-c).

Moreover, to validate the influence of *Ythdf2* loss-of-function in postmitotic neurons, we infected primary neurons from *Ythdf2^{f/f}* or *Ythdf2^{+/-}* mice with AAV-mCherry-IRES-Cre viruses and analyzed them at DIV6, respectively. We performed live imaging experiments to investigate growth cone mobility and dynamics, and found that *Ythdf2* deletion notably reduced growth cone dynamics and increased transient protrusion formation compared to the control at DIV6 (Fig. 4d-f, Supplementary Fig. 7a, Supplementary Movie 9,10). These results suggest that YTHDF2 is

crucial for axon projection, neurite development and branching, and growth cone dynamics during corticogenesis ³¹.

It is well known that YTHDF2 specifically interacts with the m⁶A-tagged mRNA and leads to its degradation. YTHDF2-depleted zebrafish embryos have an extended lifetime of the YTHDF2-targeted mRNAs ³². Therefore, we examined the RNA stability of m⁶A-tagged RNAs in primary cortical neurons ³. Unexpectedly, m⁶A-modified transcripts showed only a slight stability change in the *DF2* cKO condition (Fig. 4g, Supplementary Data 2). Altogether, we hypothesized that YTHDF2 does not simply degrade RNAs in neuronal context and may protect and promote neurite localization of selected mRNA.

We used smFISH to visualize the localization of *Gap43*, *Ncam1*, *Cntn1*, *Ank2*, and *Actb* mRNAs in both control and *DF2* cKO conditions (Fig. 4i, Supplementary Fig. 7b). In the *DF2* cKO condition, *Ncam1*, *Cntn1*, and *Ank2* mRNAs were found to be reduced in neurites compared to the control, while *Gap43* mRNA remained statistically unchanged (Fig. 4j-k, Supplementary Fig. 7c). Additionally, we observe *Ythdf1* or *Ythdf3* knockdown does not affect RNA localization (Supplementary Fig. 7d, e). These findings indicate that the loss of YTHDF2 function has a negative impact on selected mRNA localization in neurites, consistent with previous results observed in m⁶A depletion conditions (Fig. 3f-g).

Next, we used HA-tdMCP-GFP and BFP-actin-3'UTR-24×MS2 reporters described above to visualize the RNA transport (Fig. 3h). The dynamics of reporter RNA granules were monitored in control and *DF2* cKO neurons using a spinning-disk microscope. As a result, anterograde RNA movement was significantly reduced under *DF2* cKO conditions (Fig. 4l-n, Supplementary Movie 11,12). Moreover, the total motile time of RNA granules was also reduced

under *DF2* cKO conditions (Fig. 4n), consistent with previous results in the m⁶A depletion condition and the m⁶A mutant reporter (Fig. 3k, n).

Collectively, the *DF2* cKO model shows significant neurodevelopmental defects at the *in vivo* and *in vitro* levels. m⁶A-modified mRNAs exhibit transcript-specific changes in stability in the absence of YTHDF2, and YTHDF2 promotes the anterograde localization of specific target mRNAs to neurites. Therefore, YTHDF2 is a key reader protein that selectively protects m⁶A-modified transcripts and localizes them to neurites.

YTHDF2 is associated with RNA trafficking machinery in the developing brain

To identify the m⁶A-dependent protein-interacting complex of YTHDF2 in the developing brain, we conducted co-immunoprecipitation with YTHDF2 in the P0 forebrain, comparing WT and *M14* cKO mice, followed by mass spectrometry analysis (Fig. 5a). Upon depletion of METTL14, 281 proteins showed reduced interaction, while 109 proteins exhibited increased interaction (Fig. 5b, Supplementary Data 4). Gene ontology-based cluster comparison analysis and gene-concept network analysis revealed that m⁶A-dependent protein interactors are involved in cytoskeleton constitution, cytoskeleton binding, and mRNA 3'UTR binding. Notably, KIF5C, a kinesin protein specifically expressed in the nervous system³³, was identified as one of the interacting proteins. Additionally, microtubule components, including TUBB2B, TUBB3, and MAP2, were found to be associated with YTHDF2 in an m⁶A-dependent manner. In contrast, proteins that interact with YTHDF2 independently of m⁶A are primarily associated with mRNA binding or transcription corepressor binding (Fig. 5c-d). To validate our LC-MS/MS results, we performed co-immunoprecipitation with YTHDF2, and examined the pull-down affinity of associated proteins, TUBB3, KIF5C, and FMRP (Fig. 5e-g). We performed Co-IP with anti-YTHDF2

antibody in P0 forebrain lysate of *Mettl14* WT and cKO to validate the interaction with TUBB3 (Fig. 5e). We found that TUBB3 interacts with YTHDF2 with the presence of m⁶A specifically. Also, we performed Co-IP with anti-GFP antibody to pulldown GFP fused with YTHDF2 after co-transfection with KIF5C-myc and FMRP-HA in Neuro2A cells and HEK293T cells respectively (Fig 5.f-g). These experiments showed that both KIF5C and FMRP are interacting with YTHDF2 clearly. With these results, we validated that our LC-MS/MS data (Fig. 5.a-d) is well representing the interactome of YTHDF2 with m⁶A.

To further investigate the association between YTHDF2 and microtubules, we conducted proximity ligation assay (PLA) experiments between YTHDF2 and TUBB3, a neuron-specific tubulin, in primary cortical neurons from WT and *M14* cKO mice. The results revealed a significant reduction in PLA signals in the axons of *M14* cKO neurons compared to those in the axons of WT neurons. This observation suggests that m⁶A modification is crucial for the interaction between YTHDF2 and microtubules in the axons (Fig. 5h-j). Notably, we did not detect an interaction between YTHDF1 and microtubules in Tau-positive axons, in contrast to YTHDF2 (Supplementary Fig. 8a–b). We next hypothesize this interaction is mediated by neuron-specific motor proteins such as KIF5C. To address this, we performed YTHDF2-TUBB3 PLA after the knockdown of *Kif5c* in primary cortical neurons. As a result, PLA signals in the neurites were significantly reduced in the *Kif5c* knockdown condition compared to the control condition (Fig. 5k-m, Supplementary Fig. 8g-h). Next, we also performed YTHDF2-KIF5C PLA in WT and *M14* cKO neurons, observed reduced interactions between YTHDF2 and KIF5C in axons (Fig. 5n-o). Again, we did not detect a robust interaction between YTHDF1 and KIF5C in neurites (Supplementary Fig. 8c). These results suggest that the neuron-specific motor protein

KIF5C connects YTHDF2 with microtubules to promote mRNA transport in a m⁶A-dependent manner.

Taken together, these findings suggest that YTHDF2 interacts with RNA trafficking machinery and microtubules in an m⁶A-dependent manner to facilitate mRNA transport to the neurites.

FMRP is the context-guiding interactor for YTHDF2 for axonal RNA transport in neurons

Next, we hypothesized that YTHDF2 associates with other RBPs and selectively localizes mRNA to the neurites depending on target RNA characteristics, including m⁶A and 3'UTR sequence. When we examined m⁶A stoichiometry across different RBP target mRNAs, FMRP target mRNAs exhibited significantly higher m⁶A stoichiometry than non-target mRNAs (Supplementary Fig. 9a). To further evaluate our model, we performed motif enrichment analysis on sequences proximal to m⁶A sites within the 3'UTRs of the Q2 and Q1 groups (Fig. 3b)³¹. As a result, we found FMRP binding motifs were enriched in Q2 3'UTR sequences, particularly near m⁶A sites, compared with Q1 3'UTR sequences (Fig. 6a, Supplementary Fig. 9b).

FMRP, an RNA-binding protein and translation repressor, is also known to regulate RNA transport^{25,34,35}. It has been reported that YTHDF proteins interact with FMRP to translationally suppress target mRNAs crucial for proper synaptic function and axonal growth^{6,31,36}. As FMRP has been identified as an m⁶A-dependent protein interactor of YTHDF2 (Fig. 5b), we validated the interaction between YTHDF2 and FMRP in primary cortical neurons using PLA. Previously,

YTHDF2 was shown to interact with the mRNA deadenylase complex, including CNOT1, to promote mRNA degradation³⁷. Intriguingly, PLA signals for YTHDF2-FMRP were observed in both the soma and neurites, whereas signals for YTHDF2-CNOT1 were exclusively detected in the perinuclear soma (Fig. 6b-c). In addition, knockdown of *Fmr1* significantly increased the interaction between YTHDF2 and CNOT1 (Fig. 6d-e). These results indicate the presence of two distinct YTHDF2-containing complexes: an RNA transport complex with FMRP and an RNA degradation complex with CNOT1, with FMRP competitively inhibiting the association of m⁶A-tagged mRNA/YTHDF2 with the RNA degradation machinery.

To evaluate the functional significance of FMRP in the association of YTHDF2 with microtubules, we conducted a PLA experiment between YTHDF2 and TUBB3 in control shRNA (*shNS*) and *shFmr1*-treated mouse primary neurons. The results revealed a significant decrease in YTHDF2-TUBB3 PLA signals in the axons of *Fmr1* knockdown neurons compared to those in control neurons (Fig. 6f-g, Supplementary Fig. 9d-e), suggesting that FMRP facilitates the microtubule association of YTHDF2. Conversely, FMRP-TUBB3 PLA signals were markedly reduced in the axons of *Ythdf2* knockout neurons compared to those in control neurons (Fig. 6h-i, Supplementary Fig. 9f-g). In addition, the YTHDF2-KIF5C PLA signal was reduced in *shFmr1*-treated neurons, indicating that FMRP facilitates the association of YTHDF2 with motor proteins in axons (Fig. 6m-n). Collectively, these findings suggest a cooperative interaction between YTHDF2 and FMRP that links m⁶A-tagged mRNAs to microtubules and motor proteins within neurites.

Next, smFISH was employed to investigate the mRNA localization of *Ncam1*, *Cntn1*, *Ank2*, and *Actb* in primary cortical neurons derived from both WT and *Fmr1* knockout mice. Indeed, these experiments revealed a notable reduction in the neurite localization of m⁶A-tagged

Ncam1 mRNA and *Ank2* mRNA in *Fmr1* knockout neurons compared to control neurons (Fig. 6j-k). We note that we do not exclude the possibility that FMRP partially regulates the localization of these mRNAs in a YTHDF2-independent manner, as FMRP is well known to participate in multiple RNA regulatory processes in the neuron^{6,25,31,34–36}.

In conclusion, our findings indicate that FMRP cooperates with YTHDF2 and plays a crucial role in facilitating the efficient transport of m⁶A-tagged mRNAs to neurites.

Discussion

RNA transport in neurons is a fundamental process critical for dynamically regulating gene expression within different subcellular compartments. Unlike most other cells, neurons often exhibit extensive cellular architecture with long processes, such as axons and dendrites, which can span considerable distances. Various RNA-binding proteins and molecular motors, such as kinesins and dyneins, facilitate the transport of RNA granules along the microtubule within neurites. This transport mechanism delivers mRNAs to specific subcellular locations where protein synthesis is required³⁸. In this study, we revealed that m⁶A mRNA modification is essential for proper axon projection and neurite development of cortical projection neurons. A single nucleotide resolution m⁶A mapping and microdissected axon RNA-seq identified m⁶A-tagged mRNAs related to cytoskeletal regulation. The m⁶A reader YTHDF2 is critical for promoting the translocation of these m⁶A-tagged mRNAs to neurites. Mechanistically, the YTHDF2-FMRP transport complex, distinct from the YTHDF2-CNOT1 degradation complex, interacts with motor proteins to facilitate the trafficking of m⁶A-tagged mRNAs along microtubules in developing neurons. These findings suggest that YTHDF2-mediated switching

between m⁶A effector complexes regulates the stability and localization of target mRNAs (Supplementary Fig. 10).

In general, m⁶A-tagged mRNAs are subjected to faster degradation by YTHDF proteins^{28,39}. Recently, it has been reported that highly m⁶A methylated mRNAs are more unstable and less neurite-localized compared to low m⁶A methylated mRNAs in neurons¹⁴, suggesting that m⁶A acts as a determinant to restrict distal mRNA localization (passive regulation of transport on non-m⁶A mRNAs). In contrast, other reports suggest that m⁶A methylation promotes the localization of a subset of neuronal mRNAs to dendrites and axons (active regulation of transport on m⁶A mRNAs)^{16,40}. In our study, a group of mRNAs displayed both increased stability and axonal localization upon the depletion of m⁶A in *M14* cKO (Fig. 3b, Q1). However, other group of mRNAs showed reduced axonal localization despite their increased stability, suggesting the presence of additional active regulation of transport on these target mRNAs (Fig. 3b, Q2). Surprisingly, high m⁶A stoichiometric mRNAs displayed less alteration of stability compared with low m⁶A mRNAs in *M14* cKO. Furthermore, *DF2* cKO neurons displayed reduced neurite mRNA localization without significant alterations in mRNA stability, implying that YTHDF2 has additional roles beyond passively regulating mRNA stability for mRNA localization (Fig. 4g-h). Indeed, YTHDF2 interacts both with the mRNA degradation complex, including CNOT1, and with the mRNA transport complex, including FMRP and kinesins, but in distinct subcellular compartments (Fig. 5b, 5f-g, and Fig. 6b-c). Furthermore, FMRP inhibits the interaction of YTHDF2 with the RNA degradation machinery (Fig. 6d-e). These results suggest that m⁶A-tagged mRNAs are selectively recognized either by the degradation or the transport complex, with FMRP playing a pivotal role in regulating the degradation and transport of m⁶A-tagged mRNAs. Specifically, FMRP protects a subset of m⁶A-tagged mRNAs from degradation by

inhibiting the interaction between YTHDF2 and the RNA degradation machinery, while simultaneously promoting their transport by enhancing the association of YTHDF2 with microtubules and motor proteins (Fig. 6f-g and Fig. 6m-n). Indeed, known FMRP target mRNAs have higher m⁶A stoichiometry compared to FMRP non-target mRNAs (Supplementary Fig. 9a), and high m⁶A stoichiometric mRNAs are less affected in their stability compared to low m⁶A stoichiometric mRNAs upon Mettl14 depletion (Fig. 2g). Consistently, *Drosophila* YTHDF directly interacts with FMRP to cooperatively inhibit the translation of mRNAs involved in axonal growth regulation ⁴¹.

Whereas the previous report showed mossy fiber overgrowth in hippocampal neurons lacking *Ythdf2* ⁴², we observed impaired axonal growth in layer 2/3 cortico-cortical projection neurons. This discrepancy may arise from differences in axonal projection length, as long-range projection neurons require extensive mRNA transport to support growth cone navigation, whereas short mossy fibers may not depend on long-distance mRNA trafficking. Consistently, in *M14* cKO and *DF2* cKO cortical neurons, we observed increased proximal axon collaterals and transient protrusions, but reduced longest-neurite length and slower growth cone navigation (Fig. 1 and Fig. 4). These findings suggest that YTHDF2 plays a predominant role in coordinating mRNA transport and translational repression in long-range projection neurons, thereby promoting efficient growth cone advance while limiting proximal branching. In contrast, YTHDF2 may function more prominently in RNA degradation pathways in short-range projection neurons, where its loss could increase axonal growth by elevating mRNA abundance or protein synthesis in proximal neurites. Together, these observations indicate that YTHDF2 exerts cell-type-specific control over axonal development, warranting further investigation into the molecular mechanisms underlying such diversity.

The three YTHDF paralogs—YTHDF1, YTHDF2, and YTHDF3—share highly conserved protein sequences and have been reported to exhibit functional redundancy in several cellular contexts^{43–45}. However, emerging evidence suggests that differences in their low-complexity domains and post-translational modifications confer distinct physiological functions^{46–48}. Consistent with this, our data indicate that YTHDF proteins are not functionally redundant in regulating mRNA transport during early neuronal differentiation. Instead, YTHDF2 appears to play a selective and direct role in promoting mRNA localization in neurites. For example, although all three YTHDF proteins are expressed at comparable levels in the postnatal brain (data not shown), *DF2* cKO mice exhibit subviability, neurological deficits, and impaired axon projection (Fig. 4 and Supplementary Fig. 6), suggesting that YTHDF1 and YTHDF3 do not compensate for the loss of YTHDF2 *in vivo*. In addition, we find that YTHDF2, but not YTHDF1, associates with neurite microtubules and motor proteins in an m⁶A-dependent manner, suggesting that the subcellular compartment of YTHDF2 function differs from that of YTHDF1 (Supplementary Fig. 8). Consistent with this, loss of *Ythdf2*—but not *Ythdf1* or *Ythdf3*—disrupts neurite localization of target mRNAs and impairs growth cone dynamics (Supplementary Fig. 6 and Supplementary Fig. 7). A recent study demonstrating that YTHDF1 regulates APC mRNA translation in soma and indirectly modulates axonal mRNA transport via APC-associated ribonucleoprotein complexes⁷. In contrast, our data support a model in which YTHDF2 directly engages the cytoskeletal transport machinery to mediate the localization of m⁶A-tagged mRNAs. Notably, neither YTHDF1 nor APC was identified in the YTHDF2 interactome in our *in vivo* co-immunoprecipitation and mass spectrometry analyses (Fig. 5 and Supplementary Data 4), suggesting YTHDF2-transport complex mediates the transport of a distinct set of mRNAs via a separate transport protein complex from the APC-dependent transport machinery. Together,

these findings support a model in which YTHDF family proteins function through mechanistically distinct, non-redundant pathways, with YTHDF1 proposed to regulate APC translation in the soma, whereas YTHDF2 directly controls mRNA transport in neurites.

The m⁶A-modified RNAs and YTHDF reader proteins have recently been suggested as key stickers in liquid-liquid phase separation (LLPS), generating RNA granules that play a crucial role in neuronal RNA transport^{29,49–51}. Our study shows that the localization of YTHDF2 along the neurite significantly decreases under m⁶A depletion conditions (Fig. 5h-j), suggesting that YTHDF2 and mRNA may have a lower tendency to be recruited into neuronal RNA granules without m⁶A. Moreover, the depletion of m⁶A and the subsequent failure to recruit YTHDF2 may alter the physical properties of neuronal transport granules, hindering efficient granule transport, proper localization, and translation, which requires further investigation in the future. Our research suggests that m⁶A modification not only regulates gene expression levels through stability control, but also by modulating subcellular localization in neurites during cortical development. Additionally, we identified the transport machinery involving YTHDF2, motor proteins, and the RNA-binding protein FMRP as key players in m⁶A-mediated mRNA transport. These findings provide insights into how m⁶A reader proteins differentially interpret m⁶A tags through reader-complex switching mechanisms, revealing novel epitranscriptomic mechanisms that govern nervous system development and function.

Methods

Mouse models

Mice with a conditionally removable *Mettl14* allele^{3,52} were crossed with a Nestin promoter-driven Cre recombinase transgenic line (B6.Cg-Tg(Nes-cre)1Kln/J; Jackson Laboratory stock: 003771) to conditionally delete *Mettl14* in the nervous system. The mice with a conditionally removable *Ythdf2* allele⁵³ were received as a gift from Dr. Chuan He and Dr. Xiaoxi Zhuang at the University of Chicago. *Fmr1* allele (B6.129P2-Fmr1^{tm1Cgr}/J; Jackson Laboratory stock:003025) were purchased from Jackson Laboratory. For embryo stage counting, plugs were checked in the morning, considered as E0.5, and transferred to the surgery room on the day of surgery. For *in utero* and *in vitro* electroporation studies, timed pregnant C57BL/6J (wild type) mice were commercially obtained from KRIBB (Taconic), South Korea. Mice were housed in standard conditions on a 12-hour light/dark cycle in a temperature- and humidity-controlled barrier facility and allowed ad libitum access to standard chow and water. The primers used for genotyping are listed in Supplementary Table 1. All procedures were in accordance with the guidelines of and approved by the Institutional Animal Care and Use Committee at KAIST.

Plasmid information

To knockout *in vivo* and *in vitro*, hCRE-IRES-GFP-RV¹⁸ (From Dr. William Paul at the National Institute of Health), Dcx-CRE-IRES-GFP¹⁷ (From Dr. Ulrich Mueller at Johns Hopkins University), CAG-RFP (From Dr. Hongjun Song at the University of Pennsylvania), and pAAV-mCherry-IRES-Cre (Addgene, #55632) were used. To knock down the YTHDF1, YTHDF3, KIF5C, and FMRP, the sh sequence was cloned into the pUEG vector. Further, sh*Fmr1* sequence

was cloned into the pUETdTomato vector (From Dr. Hongjun Song at the University of Pennsylvania)⁵⁴. For the Co-IP, pT7-EGFP-HsYTHDF2 plasmid (Addgene, #148310) and each of pFRT-HA-FLAG-hFMRP (Addgene, #48690) and pRK5-myc-KIF5C (Addgene, #127618) were used. For the RNA live imaging, we used phage-ubc-nls-ha-tdMCP-gfp (Addgene #30649), and pcDNA-puro-TagBFP- β -ACTIN-3'UTR-24xMS2 (Addgene #205161). For the point-mutant reporter (BFP-Actb-3'UTR Δ m⁶A-MS2x24), we used Site-directed Mutagenesis kit (Enzymomics, EZ004S) according to the manufacturer's instructions. In brief, the reporter plasmid was amplified with a pair of primers substituting the conserved m⁶A site with a cytosine residue. Next, PCR product was ligated and transformed into *E. coli* for the selection and amplification. All detailed sequence information is written in Supplementary Table 2.

Mammalian cell culture and Transfection

HEK293T and Neuro2A cell lines were maintained with DMEM high glucose (Welgene, LM 001-05) supplemented with 10% fetal bovine serum (Gibco, #12483020) and 1% penicillin-streptomycin (Welgene, LS 202-02) and subcultured every 3-4 days. All the plasmids were transfected with polyethylenimine MAX (PEI; Polysciences, 24765-1) at DNA (μ g) to PEI (μ l) ratio of 1:3. Cells were harvested 48 hours after transfection for RNA or protein to validate knockdown efficiency or perform immunoprecipitation. The primer sequences are listed in Supplementary Table 1.

Primary culture of mouse cortical neurons

Before processing cortical neuron cultures, glass coverslips (12 mm) were coated with Poly-D-lysine (Gibco, 0.1 mg/ml) for 2-4 hours. Subsequently, laminin (Merck) was added, and the coverslips were incubated for an additional 2 hours. Mice cortices were isolated from embryos at the P0 stage, using HBSS buffer (Wiegand). The meninges were removed from the tissues, which were then washed twice in HBSS buffer. The cortices were triturated and digested in accutase with DNase I for 30 minutes at 37°C. Neurobasal media (Gibco) supplemented with B-27 (Gibco), GlutaMAX (Gibco), penicillin-streptomycin (10,000 U/mL, Gibco), and gentamycin (0.01mg/mL, Sigma) were added. The mixture was centrifuge at 400g, and the pellet was resuspended in media, filtered through a 70 µm cell strainer (SPL Life Sciences), and the cells were counted and plated on the prepared coverslips. The media was initially changed after 8 hours and subsequently every three days. For virus infection, AAV-mCherry-IRES-Cre virus was added to the new culture media, and the plate was returned to the incubator. For further experiments, we fixed the infected neurons with 4% paraformaldehyde (PFA, GeneAll) at DIV6.

***In utero* electroporation**

In utero electroporation experiments were done following the method previously described³. In brief, pregnant mice at E14.5 were anesthetized with isoflurane for laparotomy and the uterine horns were exposed to manipulate embryos. A 1 µl solution of endotoxin-free plasmid DNA with fast green (20X) at a final concentration of 1.5 µg/µl was injected into the lateral ventricle of the embryos using a calibrated microcapillary in a Pneumatic PicoPump setup (WPI).

Electroporation was performed using a Super Electroporator Nepa21 Type II (Nepa Gene), which delivered five transfer pulses at 43V, with a pulse length of 50 msec, an interval of 450 msec, and a decay rate of 40. The electrodes were positioned over the embryo's head atop the

uterus. After electroporation, the uterus was repositioned in the abdominal cavity and sutured with W581 Mersilk (Ethicon), and povidone-iodine was applied to prevent infection. At P5, the pups were perfused and incubated overnight with 4% PFA. The following day, the 4% PFA was replaced by 15% sucrose in PBS and 30% sucrose until the tissues were completely submerged. Cryosectioning was then performed on coronal sections with a thickness of 30 μm . All procedures involving the handling of mice and pups were conducted following the protocol approved by the Institutional Animal Care and Use Committee at KAIST.

***in vitro* electroporation in primary cortical neurons**

We have isolated the primary cortical neurons as described in the primary culture of mouse cortical neurons section. After counting the cells, we washed twice with Opti-MEM to remove serum and collected the fixed number of cells (1.0×10^6) in 100 μl of Opti-MEM and mixed with 10 μg of plasmid DNA. Cells were transferred in EC-002S (green) cuvette (2mm gap) to proceed with electroporation. Culture plates were ready and placed in an incubator with the neurobasal media with various compositions as described in the primary culture of mouse cortical neurons section. Electroporation was performed with poring pulse voltage 275.0V, pulse length 0.5 msec, pulse interval 50.0 msec, number of pulses 2, and decay rate 10% together with transfer pulse voltage 20.0V, pulse length 50.0 msec, pulse interval 50.0, no. of pulses 5 with decay rate 40%. Culture media was immediately added to the cuvette, and the cells were collected and transferred to culture plates. They were then cultured using the standard method, with the media being changed every three days. We observe expression of the reporter protein in polarized cortical neurons after 2 days.

Immunohistochemistry and confocal imaging

The pups were perfused, and their brains were immediately isolated for overnight fixation with 4% paraformaldehyde (PFA). The next day, the PFA was replaced by 15% sucrose in DPBS (Welgene), followed by 30% sucrose until the tissue completely sunk in the solution. The brain was then embedded in an OCT mold and stored at -80°C until needed. Coronal brain sections with a thickness of 30µm were obtained using a cryostat from Leica (CM1860). Antigen retrieval was performed with sodium citrate buffer (pH 6.0) before blocking. The fixed slide for immunocytochemistry was blocked with a blocking buffer for 1 hour at RT as described in publication ³. The primary antibody in the blocking buffer was then added and left overnight at 4°C. After two washes with PBST, the secondary antibody was added for 1 hour at RT. Subsequently, the nuclei were stained with Hoechst buffer for 5 minutes, washed twice with PBST, and finally, mounting media was applied to fix the coverslips. The slides were then stored in the dark at 4°C. Images were captured with a confocal microscope (Nikon TI2) using 20X, 40X, and 60X objective lenses. All the images from the same experimental condition were captured with the same microscope settings.

Live imaging

Cortical neurons were cultured on a confocal glass-bottom dish (SPL 101350) until DIV6. For the axon growth live imaging, we conducted confocal imaging for 3 hours with 5-minute intervals. For the lysosome live imaging, we introduced the cathepsin D-binding natural product pepstatin A-based SiR Lysosome tracker (Spirochrome) in *M14* cKO with control for 1 hour with verapamil, according to the manufacturer's protocol. Confocal imaging was then conducted for 30 minutes with 10-second intervals.

For the RNA live imaging, we co-electroporated phage-ubc-nls-ha-tdMCP-gfp, with pcDNA-puro-TagBFP- β -ACTIN-3'UTR-24xMS2 reporters which retains the endogenous 3'UTR m⁶A motif (the 1217th position, 5nt after the STOP codon) to cortical neurons on a confocal glass-bottom dish and culture to the DIV5~6. Live images were acquired with a Nikon Eclipse Ti inverted microscope equipped with a CSU-10 spinning disk (Yokogawa, Tokyo, Japan), an EMCCD camera, and a 100 $\times$ oil immersion objective (NA 1.49) for 5minutes with 500ms intervals.

RNA-seq library preparation and sequencing

For the corpus callosum (CC) RNA-seq, the mouse P4 brain was isolated and frozen in OCT, and 60 μ m thickness sections were cut using a cryostat. Axon projections from the superficial neurons (layer 2/3) across the CC were manually microdissected and collected in TRIzol (Invitrogen) to store at -80°C until use. Total RNA was isolated from each sample, and further RNA cleanup was performed with the RNAeasy Mini kit (Qiagen). After a total RNA quality check was analyzed from a bioanalyzer (Agilent), a Dyna beads mRNA DIRECT purification kit (Invitrogen) was employed to isolate mRNA from the samples. NEBNext UltraTM RNA Library Prep Kit for Illumina (New England Biolabs) was employed, and the instructed protocol was followed to generate the library. In brief, mRNA was fragmented and reverse-transcribed to generate cDNA. Synthesized double-strand cDNA was purified by Agencourt AMPure XP beads (Thermo Fisher Scientific) and ligated with an adapter. Index primers and universal primers were used to amplify the library after adaptor ligation. Further, AMPure beads were used to purify the cDNA library. Finally, cDNA library quality and concentration were assessed on an Agilent Bioanalyzer.

Quantitative RT-PCR analysis

For gene expression level quantification, real-time PCR was performed. TRIzol reagent (Ambion) was used to isolate total RNA and purified with a QIAGEN RNA purification kit (DNaseI was included). Purified mRNA was reverse-transcribed into the first strand of cDNA with (TAKARA), and further SYBR-green-based qRT-PCR was performed to quantify with qPCR machine (CFX Opus 96, Bio-Rad). All the primers are listed in Supplementary Table 1.

RNA stability assay

Mouse cortical neurons were cultured in an approximately equal number of cells after counting in standard 6-well plates until DIV7. Actinomycin D (Sigma) was added at 5uM concentration and neurons were collected at two time points (0 and 5 hrs) after washing with DPBS, cells were collected in TRIzol by scraping each well. Total RNA was isolated, and the NEBNext Ultra RNA Library Prep Kit was used to generate the library. As described the protocol in RNA-seq library preparation section. Linear regression was performed for time and decay rates were determined.

smFISH

For mouse cortical neuron culture, smFISH was carried out with the RNAscope Fluorescent Detection Multiplex Kit (Advanced Cell Diagnostics Inc.) according to the manufacturer's instructions as previously described with minor changes⁵⁵. Briefly, primary cortical neurons were cultured on a 12mm coverslip until DIV6, then washed with DEPC-PBS and fixed in 4% PFA for 10 minutes at RT. The neurons were serially dehydrated with ethanol in DEPC-PBS (50%, 70%, and 100%), pretreated with protease III for 15 minutes after washing with DEPC-

PBS, and then treated with customized, previously designed probes for 2 hours at 40°C. All solutions, including the RNAscope wash buffer, were used from the provided kit. Following two washes of 2 minutes each at RT with the wash buffer, a fluorescent amplification assay was performed by hybridizing with Amp 1-FL for 30 minutes at 40°C. After washing, the samples were hybridized with Amp 2-FL for 15 minutes at 40°C. The next step involved hybridization with Amp 3-FL for 30 minutes at 40°C, followed by the addition of Amp 4-FL using Alt-A or -B for 15 minutes at 40°C. Finally, Hoechst solution was added for 3 minutes after washing, and mounting media was applied to the slides to fix the coverslips for storage in the dark at 4°C. If required, immunocytochemistry was performed after the smFISH. Confocal imaging was performed to acquire images from more than 5 ROIs from three independent experiments for each probe. Quantification was then conducted to proceed with statistical analysis.

Proximity ligation assay

The NaveniFlex MR PLA kit was employed to perform a proximity ligation assay (PLA) between the following protein pairs: YTHDF2-TUBB3, YTHDF2-FMRP, YTHDF2-CNOT1, FMRP-TUBB3, GFP-KIF5C, and negative controls with IgG. Cortical neurons were prepared on 12mm coverslips and fixed with 4% PFA. After three washes with TBS-T, Naveni blocking buffer was added to the coverslips and incubated at 37°C for 1 hour. Primary antibodies were introduced at the specified dilution and incubated overnight at 4°C. After three washes with TBS-T, NaveniBody was diluted in the provided diluent and added to cover the sample area, followed by incubation for 1 hour at 37°C. After three washes with TBS-T, reaction A mix was added to the samples and incubated for 1 hour at 37°C in a preheated humidity chamber. After three washes with TBS-T, reaction B mix was added to the samples and incubated for 30 minutes

at 37°C in a preheated humidity chamber. Following two washes with TBS-T, reaction C mix was added to the samples and incubated for 90 minutes at 37°C in a preheated humidity chamber. After a TBS wash, nuclear staining was performed with Hoechst buffer. After two washes with TBS, mounting media was applied to the slides to fix the coverslips for storage in the dark at 4°C. If required, immunocytochemistry was performed after the PLA.

Co-immunoprecipitation (Co-IP)

For Co-IP with endogenous protein lysates, the forebrain was dissected from P0 C57BL/6 mouse brains and dissociated using dissociation buffer (0.1% Triton-X 100, 0.5% protease inhibitor cocktail in DPBS) with a tissue homogenizer. The tissue was sonicated under the following conditions: ON 30 sec, OFF 30 sec, for 15 cycles at medium strength using a Bioruptor (Diagenode). To obtain the protein lysate, the sonicated tissue was centrifuged at 15,000g for 15 minutes at 4°C, after which the supernatant was collected, and the pellet discarded.

For Co-IP from cells with overexpression of target proteins, pT7-EGFP-HsYTHDF2 plasmid and each of pFRT-HA-FLAG-hFMRP and pRK5-myc-KIF5C plasmid were transfected into HEK293T and Neuro2A cells using PEI. Cell pellets were lysed with lysis buffer (150mM NaCl, 50mM Tris-HCL pH 7.4, 1mM EDTA, and 1% Triton-X-100) containing a protease inhibitor cocktail (200:1, Millipore, 535140) and sonicated. The supernatant was collected after centrifugation at 15,000g for 20 min at 4 °C.

For antibody binding, 5 µg of antibody was incubated with a total of 30 µL of Dynabeads Protein A (Invitrogen) and Dynabeads Protein G (Invitrogen) in a 1:1 mixture at 4°C for 3 hours with rotation. Meanwhile, 1 mg of protein lysate was pre-cleared with 10 µL of Dynabeads Protein A and Dynabeads Protein G at 4°C for 1 hour with rotation. The pre-cleared lysate was then added

to the Dynabeads-conjugated antibody and incubated overnight at 4°C with rotation. After incubation, the protein-bound Dynabeads were washed six times with dissociation buffer for forebrain derived samples, and washing buffer (150mM NaCl, 50mM Tris-HCl pH 7.4, 1mM EDTA, and 0.1% Triton-X-100) supplemented with protease inhibitor cocktail (200:1) for cell derived samples. To elute the protein from the beads, it was heated for 5 min at 95 °C within 5X SDS-PAGE loading buffer (LPS solution) and subjected to western blot.

Western blot

20µg of proteins per sample were loaded onto a 12% acrylamide gel (LumiBio) and run at 90V to 120V in 1X Tris-Glycine SDS buffer (LPS solution). After the gel run, proteins were transferred to a PVDF membrane at 300mA for 90 minutes in 1X Tris-Glycine buffer (LPS solution) with 20% methanol. The membrane was then blocked with blocking buffer (5% BSA and 0.1% Triton X-100 in 1X TBS buffer) for 1 hour at room temperature (RT). The primary antibody was applied at a 1:500 dilution in blocking buffer and incubated overnight at 4°C. The membrane was washed three times with wash buffer (0.1% Triton X-100 in 1X TBS buffer) for 10 minutes each. The secondary antibody was applied at a 1:2000 dilution in 1:5 diluted blocking buffer and incubated for 90 minutes at RT. The HRP signal was detected using SuperSignal™ West Dura Extended Duration Substrate (Thermo Fisher Scientific), and imaging was performed with a Chemi-Doc (Bio-Rad).

Peptide preparation and LC-MS/MS analysis

Co-IP products with internal protein from forebrain conducted followed the protocol described above were used for LC-MS/MS. For final extraction, 50 µL of 2X SDS-PAGE loading buffer

(LPS) was added, and the mixture was incubated at 95°C for 10 minutes. The interacting proteins were loaded onto a 12% acrylamide gel (LumiBio) and run at 60V. To extract total protein for proteomic analysis, the gel was excised and washed three times with HPLC-grade water (Fisher) and 30% acetonitrile (Fisher). After incubation with 10 mM dithiothreitol (DTT, Sigma-Aldrich) at 56 °C for 20 minutes, 100% ACN was added to the gel and then discarded. Next, 100 mM iodoacetamide (IAA, Sigma-Aldrich) was added to the gel and incubated at RT in the dark for 20 minutes. 100% ACN was added again and discarded. The gel was dried using a lyophilizer. A 1:50 ratio of trypsin/Lys-C mixture (Promega) solution was added to the dried gel and incubated overnight at 37 °C. The supernatant containing the extracted peptides was collected in a new E-tube. After drying, the peptides were applied to a C18 spin column (Thermo Fisher Scientific), and the eluents were dried using a lyophilizer.

For LC-MS/MS analysis, the extracted peptides from in-gel digestion were analyzed using a Q-Exactive™ Plus hybrid quadrupole orbitrap mass spectrometer (Thermo Fisher Scientific) interfaced with an EASY-Spray source. Chromatographic separation of the injected peptides was performed on an UltiMate™ 3000 RSLCnano System (Thermo Fisher Scientific) equipped with an Acclaim PepMap™ 100 (75 µm × 2 cm, 3 µm, NanoViper, Thermo Fisher Scientific) as the loading column and an EASY-Spray column PepMap™ RSLC C18 (75 µm × 50 cm, 2 µm, Thermo Fisher Scientific) as the separation column. The extracted peptides, dissolved in HPLC-grade water containing 0.1% formic acid, were loaded via an RS autosampler and separated using a linear gradient of acetonitrile (ACN)/water containing 0.1% formic acid at a flow rate of 300 nL/min. The LC eluent was electro-sprayed from the analytical column, and a voltage of 2.0 kV was applied via the liquid junction of the nanospray source. The peptide

mixtures were separated using a 5% to 40% ACN gradient over 40 minutes. The total chromatographic run time was 135 min for each sample.

The analysis method involved a full mass spectrometry (MS) scan over a range of 350–2000 m/z, followed by data-dependent MS/MS (MS²) on the ten most intense ions from the full MS scan. The mass spectrometer was operated in data-dependent acquisition mode and calibrated using the proposed calibration solution according to the manufacturer's instructions. Full MS scans were acquired at a resolution of 70,000, followed by HCD MS/MS scans at a resolution of 17,500. The AGC target was set to 1×10^6 for MS² scans, with maximum injection times of 100 ms, respectively. Precursor ions were isolated using a 2.0 m/z isolation window and fragmented using a normalized collision energy (NCE) of 27. The MS² scan range was automatically determined based on the precursor m/z, with a fixed first mass of m/z 50. For the database search, tandem mass spectra were processed using Proteome Discoverer software version 2.4 SP1 (Thermo Fisher Scientific). Spectral data were searched against the mouse Uniprot database (release version 2022_05). The analysis workflow included four nodes: Spectrum Files (data input), Spectrum Selector (spectrum and feature retrieval), Sequest HT (sequence database search), and Percolator (peptide spectral match (PSM) validation and FDR analysis). All identified proteins had an FDR $\leq 1\%$, calculated at the peptide level and validated based on the q-value. Search parameters allowed for tryptic specificity of up to two missed-cleavages, with carbamidomethylation of cysteine as a static modification and oxidation of methionine as a dynamic modification. The mass search parameters for +1, +2, and +3 ions included mass tolerances of 20 ppm for precursor ions and 0.5 Da for fragment ions.

Each LC-MS/MS sample was analyzed as a single replicate. Protein enrichment was calculated by comparing normalized PSM counts between anti-YTHDF2 immunoprecipitation samples and IgG controls. Proteins exhibiting an absolute log₂ fold change greater than 0.5 relative to IgG controls were defined as differentially associated proteins. Gene ontology-based compare cluster analysis and gene-concept network analysis were conducted with the R package clusterProfiler (v 4.6.2) ²². Statistical analyses were performed as indicated in the corresponding figure legends.

m⁶A-SAC-seq analysis

P0 forebrains from *Nestin-Cre; Mettl14^{f/+}* pups and *Nestin-Cre; Mettl14^{f/f}* pups were dissected. Total RNA was extracted using TriZol (Invitrogen). mRNA was purified using the Dynabeads mRNA Purification Kit (Invitrogen). The poly(A) tail of each mRNA sample was removed with oligo(dT) and RNase H (NEB), then fragmented with 10x zinc fragmentation buffer, followed by treatment with PNK enzyme (NEB). Samples were cleaned up with silane beads, and a 2% calibration spike-in mix was added to the reaction, which was then treated with T4 RNA ligase 2, truncated KQ (NEB), and a 3'-adaptor, followed by overnight incubation. The excessive RNA adaptor was digested by adding 5' Deadenylase (NEB) and RecJf (NEB). Then, Dynabeads C1 (Thermo Fisher Scientific) resuspended in 2x binding/wash buffer was added to ligation mix to purify the sample with washing the beads in 0.1% PBST (v/v) and 1× binding/wash buffer and twice in Tris-HCl. Beads were resuspended in RNase-free H₂O and then, one-third of sample was separated as RNA-seq input and stored at -80 °C. With the tube in magnetic rack, the half of water was removed. The rest of resuspended beads were linearized and added with 10× buffer, SUPERase In RNase Inhibitor (Thermo Fisher Scientific), allylic-SAM, and MjDim1 enzyme for

m⁶A labeling. The supernatant was removed, and the beads were resuspended in water. Labeling reaction was repeated once more. Beads were washed sequentially with 0.1% PBST, 1× binding/wash buffer, and Tris–HCl. Iodine(I₂) was added and kept in the dark at RT for 1 h, then Na₂S₂SO₃ was added to quench I₂. Beads were washed sequentially with 0.1% PBST, 1× binding/wash buffer, and Tris–HCl. To both samples of SAC-seq and RNA-seq, RT primer and dNTP was added. Then, AMV reverse transcriptase reaction buffer (NEB), RNaseOUT, Actinomycin D, and HIV RT enzyme (Worthington Biochemical) were added for reverse transcription. After RT reaction, RNase-free H₂O and 10% SDS were added. Resuspended samples were cleaned with Silane beads washed with buffer RLT, then extracted with RNase-free H₂O. For cDNA adaptor ligation, cDNA adaptor was added and linearized. 10× T4 RNA ligase reaction buffer, ATP, 50% PEG8000, DMSO, and Co(NH₃)₆Cl₃ were added, then T4 RNA ligase 1 was added followed by overnight incubation. Ligated samples were cleaned with silane beads washed with buffer RLT, followed by extraction with RNase-free H₂O. For library construction, NEBNext Ultra II Q5 Master Mix and NEBNext adaptors were used. Amplified libraries were purified with Ampure XP beads. The purified libraries were sent to Macrogen for next-generation deep sequencing with Illumina HiSeq X in paired-end 2 × 100 bp read length. The adapters of all raw m⁶A-SAC-seq data fastq file is trimmed with Cutadapt (version 3.4). Filtered fastq files were aligned to the mouse genome (mm10) with STAR (version 2.7.10b). To detect the mutation sites on the transcriptome, the mapping results were filtered by masking flags to eliminate unmapped, secondary, low-quality, or duplicate reads for downstream analysis. Then, the bam file was splitted into forward and reverse reads. The mutations of each site are detected with customized tool, cpup (version 0.1.0, <https://github.com/y9c/cpup>) Sites with more than one mutation reads in any library are reported in the output file. Detected sites are filtered

with their mutation ratio between mutation occurred samples and untreated samples. Following criteria is applied to identify appropriate site; Average mutation ratio of the untreated input libraries should be less than 2.5% or the maximum number of mutations is 1, average mutation ratio of the treated libraries should be more than 5%, There should be 3 or more mutations in the treated libraries, The minimum sequencing depth of all the libraries should be more than 20. To annotate the gene or transcript name, relative position to transcript and motif sequence at RNA level, python package variant (<https://github.com/y9c/cpup>) were used for final m⁶A sites. SAC gene body plot was generated through R package Guitar (v2.14.0). Integrative genomics viewer (IGV, version 2.15.2) was used to visualize m⁶A-SAC-seq results.

Image analysis

For the acquisition and quantification of images, we utilized the open-source online tool Fiji⁵⁶. To ensure linearity and consistency across samples, minimum and maximum threshold signals were determined. Quantification was performed to obtain binary values from pixel intensity. Callosal axon projection values were normalized by RFP-positive soma (grey zone) and binned by axon projection before and after midline. Contralateral fluorescence intensity of the layer 1~6 values were normalized to relative RFP-positive soma at the ipsilateral position with the S1 region z-stacked image¹². For tracking *in vitro* primary neuron samples, the Fiji plugin NeuronJ was used⁵⁷. The neurite length was measured as the distance between a clearly identifiable neurite tip and the center of the nucleus along axonal tracks labeled with Tau. The collateral branching was measured as the number of branching points in a neuron and normalized by total neurite length. The protrusion transiently disappeared within 15 min were counted during neuron live imaging. Quantification of smFISH single molecules and PLA analysis were conducted using NeuronJ by measuring the distance between a single RNA molecule and the center of the nucleus along axon

tracks labeled with fluorescent proteins or Tau. The normalized density of smFISH and PLA was quantified by calculating the ratio of neurite signal per total signal and normalizing it with the control ratio. For representative images in figures, maximum intensity projections of Z-stacked images were processed using Fiji. All analyses were performed on uncropped images. For RNA live images, RNA molecules observed > 60 frames were selected for consistency. Anterograde and retrograde movement were counted to calculate the fraction of transport and motile time. Movements longer than 2 μm were categorized into the antero or retro group, and those shorter than 2 μm were categorized as the interrupted group⁵⁸.

RNA-seq data analysis

The RNA-seq data from CCseq and RNA stability assay were aligned to the mm10 mouse genome, computed expression level with mm10 mouse annotation from illumina iGenomes database. All procedure was done by the RSEM (v1.3.3) and STAR (v2.7.10b) with default parameters. Downstream analysis was done with the DESeq2 (1.38.3) package in R. Transcripts with normalized count below 10 were removed from downstream analysis. Differential expression was done by “DESeq” function with default parameters. On CCseq, low expressing transcripts with base mean below 70 were removed from downstream analysis. Significantly localized transcripts were identified by $\text{Log}_2\text{foldchange} > |0.5|$, and p-adjust value < 0.05 . On stability assay, relative stability difference was estimated by $[5\text{hr } \text{Log}_2\text{foldchange} - 0\text{hr } \text{Log}_2\text{foldchange}]$ between each experimental condition and significantly stability altered transcripts identified by $\text{dLFC} > |1.0|$. Selected gene sets were used gene ontology (GO) and gene set enrichment analysis (GSEA) with R package ClusterProfiler (v 4.6.2). Selected gene set 3'UTR sequence were analyzed by AME (v5.5.5).

Statistical analysis

All quantified data were analyzed using either Microsoft Excel or R(4.2.2). Data distributions were assumed to be normal, although this was not formally tested. Sample sizes were not pre-determined based on statistical methods but were consistent with those reported in previous publications^{3,27}. For comparisons of genotype or knockdown samples, an unpaired two-tailed Student's t-test was used. For comparisons of two distribution sets, the Kolmogorov–Smirnov test was employed. No data were excluded from the analysis. All visualized plots were generated using the R package ggplot2(3.4.4) or individual tools described on each method parts. All image analyses were replicated in at least three independent samples, and all experiments showing representative figures were replicated with similar results.

Data availability

Sequencing data used in the manuscript have been deposited in the Gene Expression Omnibus under accession codes GSE277229, GSE277230, and GSE277231. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD080605. Source data are provided with this paper. Due to the large size of the raw imaging datasets, these data are available from the corresponding authors upon request.

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Author Contributions

K.-J.Y. conceived the project. K.-J.Y., B.K., A.K., H.H., and M.P. designed the experiments. H.H., E.C., H.L., and J.H.Y. performed immunoprecipitation–mass spectrometry analysis. S.-M.L. performed the m⁶A-SAC-seq experiments, and C.-W.L. prepared materials for these experiments. H.H. and B.K. performed sequencing experiments and bioinformatic analyses. A.K. performed in utero electroporation experiments and histological analyses. B.K., H.H., and M.P. performed RNA live-imaging experiments and analyses. M.P. performed immunoprecipitation experiments and biochemical analyses. S.L., H.-J.J., and W.-I.K. performed behavioral analyses. B.K. performed smFISH, PLA, and most of the other experiments. S.H.B., K.-H.L., J.-H.M., J.Y., and C.-W.P. performed additional experiments and analyzed data. J.-S.W., J.H.Y., Y.K.K., and K.-J.Y. supervised the research. B.K., A.K., H.H., M.P., E.C., and K.-J.Y. wrote the manuscript, and all authors reviewed, edited, and approved the final manuscript.

Competing Interests

The authors declare no competing interests.

Figure Legends

Fig. 1. *Mettl14* deletion during corticogenesis leads to reduced axon projection, shorter neurite length, increased axon collaterals, and slower growth cone dynamics. (a-c) Corpus callosal axon projection in control and *Mettl14*^{fl/fl} P5 brains after electroporation of hCRE-IRES-GFP-RV and pCAG-RFP plasmids at E14.5. **(a)** The upper panel shows ipsilateral to contralateral axon projections crossing the white matter and corpus callosum area in coronal sections. The lower panels (i, ii) are selected to magnify the corpus callosum and projected axon

terminals (scale bars: 1 mm, i, ii; scale bars: 200 μ m). **(b)** The frequency distribution plot represents RFP intensity during axon projections from the ipsilateral to the contralateral position. (confidence level=0.99) **(c)** The integrated density of the RFP signal per soma was quantified. **(d-e)** Corpus callosal axon projection in control and *Mettl14*^{f/f} P14 brains after electroporation of Dcx-CRE-IRES-GFP and pCAG-RFP plasmids. **(d)** The upper panels show representative images of ipsilateral to contralateral axon projections. The lower panels display z-projected images with higher magnification of axonal arborization in the S1 region. (scale bars: 1 mm). The plot represents the quantitative intensity of RFP in total contralateral branching, projections, and arborization normalized to the RFP⁺ soma intensity of upper-layer neurons. **(e)** Quantitative analysis of contralateral branching distribution along the radial axis of the cortical wall. (confidence level=0.99) **(f-k)** *M14* cKO primary neurons exhibited abnormal neurite growth at DIV6. Shown in **(f)** are representative confocal images and skeletal images (scale bars: 100 μ m). Neurite length and collateral branching frequency were quantified in **(g)** and **(h)**. **(i)** Live imaging of growth cones in control and *M14* cKO neurons over 3 hours at DIV6. Red arrowheads indicate the locations of growth cones (scale bar: 20 μ m). Shown in **(j)** is the quantification of total growth length divided by tracking time and **(k)** is the quantification of the transient protrusion. Significance was evaluated using two-tailed unpaired Student's t-tests (c, d, g, h, and j). The significance of RFP intensity across the midline was evaluated using two-way ANOVA (b). * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$. Statistical details and sample sizes are provided in the Supplementary Data 5.

Fig. 2. A single nucleotide resolution, quantitative m⁶A mapping revealed transcripts related to axon development with high m⁶A stoichiometries

(a) Metagene profile of the identified m⁶A sites aligned on mRNA, showing enrichment in the 3'UTR, as previously reported. **(b)** Motif enrichment of the identified m⁶A sites. **(c)** GO analysis for the identified m⁶A-modified genes. **(d)** The m⁶A stoichiometry in different RNA regions, including the 3'UTR, CDS, 5'UTR, intronic, and intergenic regions. The boxplot represents differences compared to the CDS. **(e)** Gene Set Enrichment Analysis (GSEA) for the position in the m⁶A fraction ranked genes. **(f-g)** Cumulative curves and violin plots showing the distribution of stability differences between control and *M14* cKO neurons. **(f)** Genes were classified by m⁶A site, and differences were compared for each group. **(g)** Genes were classified by m⁶A fraction and divided into two groups, and differences were compared between groups. **(h)** IGV plots for the m⁶A peaks and stoichiometry in representative genes *Ank2*, *Actb*, *Ncam1*, and *Gap43*. Significance was evaluated using two-tailed unpaired Student's t-tests (d, f, and g). The significance of two gene sets was evaluated using the two-side Kolmogorov-Smirnov test (f, g), and hypergeometric distribution for GO analysis (c). ns, not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Statistical details and sample sizes are provided in the Supplementary Data 5.

Fig. 3. m⁶A modification is crucial for the neurite translocation of cytoskeletal mRNAs in differentiating neurons. **(a)** The volcano plot shows transcripts that are depleted and enriched in the corpus callosum of P4 *M14* cKO mice. Red indicates decreased m⁶A-modified transcripts, blue indicates increased m⁶A-modified transcripts and targets of interest are highlighted. **(b)** Relationship between RNA stability and changes in CC transcript abundance in WT and *M14* cKO conditions. Red indicates m⁶A-modified transcripts, and targets of interest are highlighted. **(c)** Gene Ontology analysis of Q1 and Q2 transcripts depends on m⁶A modification. **(d)** The schematic diagram for the selection of target transcripts. **(e-g)** *M14* cKO primary cultured

neurons show depleted RNA localization. Shown in (e) are representative smFISH signals (green) representing endogenous *Gap43*, *Camk2b*, *Ncam1*, *Cntn1*, *Ank2*, and *Actb* (scale bars: 20 μ m). The distance from the nucleus is quantified in (f) and the relative density of smFISH puncta in neurites compared to the total is quantified in (g). **(h)** The schematic diagram of the RNA live imaging MS2 system. Created in BioRender. Yoon, K. (2026) <https://BioRender.com/icye89e> **(i-k)** *M14* cKO primary cultured neurons show depleted anterograde RNA localization. Shown in (i) anterograde (blue arrowheads) and interrupted (grey arrowheads) RNA reporter are indicated (scale bars: 10 μ m). The anterograde fraction of RNA reporter motility was quantified in (j) and the total motile time of RNA granules was compared with each condition in (k). **(l-n)**. The m⁶A point-mutated reporter granules showed depleted anterograde RNA localization. Shown in (l) anterograde (blue arrowheads) and interrupted (grey arrowheads) RNA reporter granules are indicated (scale bars: 10 μ m). The anterograde fraction of RNA reporter motility was quantified in (m) and the total motile time of RNA granules was compared with each condition in (n). Significance was evaluated using two-tailed unpaired Student's t-tests (f, g, j, and m). The significance of GO analysis was evaluated using hypergeometric distribution (c). The significance of two RNA motility time sets was evaluated using the one-side Kolmogorov-Smirnov test (k, n). ns, not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Statistical details and sample sizes are provided in in the Supplementary Data 5.

Fig. 4. *Ythdf2* deletion reduces axonal projection and neurite length and impairs growth cone dynamics in *DF2* cKO. (a-c) Corpus callosal axon projections in control and *YTHDF2*^{ff} cKO P5 brains, examined by *in utero* electroporation. **(a)** The upper panel shows ipsilateral to

contralateral axon projections and corpus callosum area in coronal sections. The lower panels (i, ii) are selected to magnify the corpus callosum and projected axon terminals (scale bars: 1 mm, i, ii; scale bars: 200 μ m). **(b)** The frequency distribution plot represents GFP intensity during axon projections. (confidence level=0.99). **(c)** The integrated density of the GFP signal of axons per soma was quantified. **(d)** Neurite projections within 3 hours at DIV6 (scale bars: 20 μ m). Shown in **(e)** is the quantification of total growth length divided by tracking time, and **(f)** is the quantification of the transient protrusion. **(g)** Cumulative curves and violin plots showing the distribution of stability differences between control and *DF2* cKO neurons. Genes were classified by m⁶A modification, and differences were compared within each group. **(h-j)** *DF2* cKO primary cultured neuron showed reduced neurite RNA localization. Shown in **(h)** are representative smFISH signals (Green) representing endogenous *Gap43*, *Ncam1*, *Cntn1*, *Ank2*, *Actb* (Scale bars: 20 μ m). The distance from the nucleus was quantified in **(i)**, and the relative density of smFISH puncta in neurites compared to the total is quantified in **(j)**. **(k-m)** *DF2* cKO primary cultured neurons show depleted anterograde RNA localization. Shown in **(k)** anterograde (blue arrowheads) and interrupted (grey arrowheads) RNA reporter granules are indicated (scale bars: 10 μ m). The anterograde fraction of RNA reporter motility was quantified in **(l)**, and the total motile time of RNA granules was compared with each condition in **(m)**. The significance was evaluated using two-tailed unpaired Student's t-tests (c, e, f, g, i, j, and l). The significance of GFP intensity across the midline was evaluated using two-way ANOVA (b). The significance of RNA motility time sets was evaluated using the one-side Kolmogorov-Smirnov test (m). ns, not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Statistical details and sample sizes are provided in in the Supplementary Data 5.

Fig. 5. YTHDF2 interacts with the transport machinery and neurite microtubules in an

m⁶A-dependent manner. (a) Schematic diagram for YTHDF2 co-IP LC-MS/MS. Created in

BioRender. Yoon, K. (2026) <https://BioRender.com/h97k678> **(b)** MA plot visualizing the

differentially interacting proteins with normalized PSM values. Red circles highlight proteins

involved in the RNA localization process (GO:0006403). **(c)** Gene Ontology analysis of the

proteins captured with YTHDF2 co-IP LC-MS/MS. m⁶A-dependent proteins are those captured

at higher levels in WT than *M14* cKO (Log₂ FC > 0.5), while m⁶A-independent proteins are

captured in both WT and *M14* cKO. **(d)** Network analysis of proteins identified from YTHDF2

co-IP LC-MS/MS. Blue circles indicate proteins with higher enrichment and red circles indicate

proteins with lower enrichment in WT compared to *M14* cKO (|Log₂ FC| > 0.5). **(e–g)** Western

blot validation of YTHDF2 interactors identified by LC–MS/MS. Shown in (e) is the interaction

between TUBB3 and YTHDF2, validated by co-immunoprecipitation using an anti-YTHDF2

antibody in P0 forebrain tissue from *Mettl14* WT and cKO mice. Interactions of YTHDF2-GFP

with KIF5C-myc in Neuro2A cells (f) and with HA-FMRP in HEK293T cells (g) validated by

co-IP using an anti-GFP antibody. **(h–j)** YTHDF2 localization to the neurite decreased in the

M14 cKO primary neurons at DIV6. Shown in (h) are representative PLA signals (Green)

showing endogenous YTHDF2-TUBB3 association (Scale bar: 20μm). The distance from the

nucleus (i) and the relative density (j) is quantified. **(k–m)** YTHDF2 localization to neurites

decreased in primary neurons upon *Kif5c* knockdown. Shown in (k) are representative PLA

signals indicating endogenous YTHDF2-TUBB3 association in control and *Kif5c* knockdown

conditions (scale bar: 20μm). The distance from the nucleus (l) and the relative density (m) was

quantified. **(n–o)** YTHDF2-KIF5C localization to neurites decreased in *M14* cKO primary

neurons. Shown in (n) are representative PLA signals showing YTHDF2+GFP-KIF5C association (Scale bar: 20 μ m). The relative density is quantified in (o).

Significance was evaluated using two-tailed unpaired Student's t-tests (i, j, l, m, and o). The significance of GO analysis was evaluated using hypergeometric distribution (c). ns, not significant, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Statistical details and sample sizes are provided in the Supplementary Data 5.

Fig. 6. FMRP interacts with YTHDF2 to facilitate transport of m⁶A-tagged mRNAs to neurites in developing neurons.

(a) Motif enrichment analysis of the Q2 3'UTR m⁶A proximal sequence (± 50 bp). **(b-c)** YTHDF2 is associated with functional cofactors in different subcellular locations. Shown in (b) are representative PLA signals (green), representing endogenous YTHDF2-CNOT1 and YTHDF2-FMRP associations (Scale bar: 20 μ m). The yellow arrowheads indicate PLA signals in the neurites. The distribution of PLA signals in neurites is quantified in (c). **(d-e)** YTHDF2-CNOT1 associations increased in *Fmr1* KD mouse primary neurons. Shown in (d) are representative PLA signals indicating endogenous YTHDF2-CNOT1 associations in control and *Fmr1* KD mouse primary neurons (Scale bar: 20 μ m). The number of PLA signals per neuron was quantified in (e). **(f-g)** YTHDF2 localization to axons decreased in *Fmr1* KD mouse primary neurons. Shown in (f) are representative PLA signals and Tau staining indicating endogenous YTHDF2-TUBB3 associations in control and *Fmr1* KD mouse primary neurons (Scale bar: 20 μ m). The relative density on tau is quantified in (g). **(h-i)** FMRP localization to axons decreased in *DF2* cKO mouse primary neurons. Shown in (h) are representative PLA signals and

Tau indicating endogenous FMRP-TUBB3 associations in control and *DF2* cKO mouse primary neurons (Scale bar: 20 μ m). The relative density on Tau is quantified in (i). **(j-l)** RNA localization to neurites decreased in *Fmr1* KO mouse primary neurons. Shown in (j) are representative smFISH signals (green) representing endogenous *Ncam1*, *Cntn1*, *Ank2*, and *Actb* in control and *Fmr1* KO conditions (Scale bar: 20 μ m). The distance from the nucleus was quantified in (k), and the relative density of smFISH puncta in neurites compared to the total is quantified in (l). **(m-n)** YTHDF2-KIF5C complex localization to neurites decreased in *Fmr1* KD mouse primary neurons. Shown in (m) are representative PLA signals showing YTHDF2+GFP-KIF5C association (Scale bar: 20 μ m). The relative density is quantified in (n).

Significance was evaluated using two-tailed unpaired Student's t-tests (e, g, i, k, l, and n). ns, not significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Statistical details and sample sizes are provided in the Supplementary Data 5.











