The RNA-binding protein FMRP facilitates the nuclear export of N6-methyladenosine–containing mRNAs

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N6-Methyladenosine (m6A) is the most abundant post-transcriptional mRNA modification in eukaryotes and exerts many of its effects on gene expression through reader proteins that bind specifically to m6A-containing transcripts. Fragile X mental retardation protein (FMRP), an RNA-binding protein, has previously been shown to affect the translation of target mRNAs and trafficking of m6A-modified transcripts. Loss of function of FMRP causes fragile X syndrome, the most common form of inherited intellectual disability in humans. Using HEK293T cells, siRNA-mediated gene knockdown, cytoplasmic and nuclear fractions, RNA-Seq, and LC-MS/MS analyses, we demonstrate here that FMRP binds directly to a collection of m6A sites on mRNAs. FMRP depletion increased mRNA m6A levels in the nucleus. Moreover, the abundance of FMRP targets in the cytoplasm relative to the nucleus was decreased in Fmr1-KO mice, an effect also observed in highly methylated genes. We conclude that FMRP may affect the nuclear export of m6A-modified RNA targets.

Fragile X syndrome (FXS) is the most commonly occurring form of inherited intellectual disability in humans, affecting ~1 in 4,000 males and 1 in 8,000 females (1). FXS is characterized by cognitive impairment, autistic behaviors, and seizures and results from loss of function of the fragile X mental retardation protein (FMRP) (2). FMRP is an mRNA-binding protein that represses the translation of its target transcripts by stalling ribosome translocation (3–5). FMRP also plays roles in mRNA localization, trafficking FMRP-associated mRNA granules in neurons from the soma to neurites (6). Although FMRP is predominantly localized in the cytoplasm, it contains nuclear localization and nuclear export signals, which allow FMRP to enter the nucleus (7). As localization of FMRP within nuclear pores has been observed, it has been suggested that FMRP shuttles between the cytoplasm and nucleus (8). Whether it plays any role in the export of mRNA targets, as well as for which mRNA targets it may facilitate nuclear export, are unknown.

N6-Methyladenosine (m6A), the most abundant chemical modification of mRNA in eukaryotes, performs critical functions in gene expression regulation, impacting processes such as translation, decay, splicing, and nuclear export (9–11). Many of these functions are carried out through “reader” proteins that bind preferentially to mRNAs containing m6A. We recently found that FMRP is an m6A reader protein that regulates the translation of m6A-containing transcripts (12). As reader proteins may play multiple roles (13), we wondered whether the shuttling activity of FMRP also affects m6A-modified transcripts. In this study, we show that FMRP facilitates the nuclear export of transcripts containing m6A.

Results

FMRP binds directly to a collection of m6A sites on mRNAs

To determine whether FMRP binds directly to m6A on mRNA rather than indirectly to unmethylated regions of m6A-containing transcripts, we combined approaches of photoactivatable ribonucleoside–enhanced cross-linking and immunoprecipitation (PAR-CLIP) and anti-m6A immunoprecipitation (m6A-IP) in HEK293T cells. PAR-CLIP identified ACU and GGAC as the top two motifs of FMRP direct binding sites (Fig. 1A and Table S1), agreeing with previous studies (5). These two motifs both contain a part of the consensus motif of m6A, (G/A/U)(G=A)Am6A(U>A>C) (9); therefore, we wondered whether FMRP–binding sites can be m6A-modified when the two motifs overlap. To test this hypothesis, we performed m6A-IP on RNA fragments (~20 nucleotides) bound by FMRP in the PAR-CLIP experiment. Indeed, this assay produced RNA libraries that could be subjected to sequencing, demonstrating that regions of RNA
directly bound by FMRP contain m^6^A. The top consensus motif of RNA isolated by m^6^A-IP following FMRP PAR-CLIP matched the full length of the m^6^A motif (Fig. 1B). In contrast, the FMRP I304N mutant, which has decreased RNA-binding capacity (12, 14), did not show significant RNA bound in the PAR-CLIP m^6^A-IP fraction (Table S1). Peak distribution analyses of FMRP PAR-CLIP peaks and PAR-CLIP-m^6^A-IP peaks showed that m^6^A-modified binding sites of FMRP tend to cluster in the CDS and 3’UTR regions of transcripts (Fig. S1).

We also performed m^6^A profiling (m^6^A-Seq) in HEK293T cells and the mouse cortical tissue (Tables S2 and S3). FMRP PAR-CLIP targets contained more m^6^A sites than nontargets in HEK293T cells (Fig. 1C). FMRP targets in the brain have been reported using FMRP polysome CLIP-Seq in C57BL/6 mouse cortices (4). Consistently, FMRP targets in the brain contained more m^6^A than nontargets (Fig. 1D). Thus, FMRP targets are preferentially m^6^A modified and FMRP directly targets a collection of m^6^A sites on mRNAs.

**FMRP mediates cytoplasmic-nuclear distribution of m^6^A-modified transcripts in HEK293T cells and the mouse cortex**

To explore whether FMRP affects metabolism of methylated mRNAs, we depleted FMRP from HEK293T cells by siRNA knockdown and quantified mRNA m^6^A levels from total, cytoplasmic, and nuclear lysate from siCtrl versus siFMR1 HEK293T cells (n = 2, biological replicates). Error bars, mean ± S.D., p < 0.0001 (Mann–Whitney U test). The numbers of genes for each group are shown in parentheses (C and D). Box plot elements are as follows: center line, median; box limits, upper and lower quartiles; whiskers, Tukey.

Next, we tested the role of FMRP in nuclear export of m^6^A-modified target transcripts; however, we cannot exclude the possibility that the increases in m^6^A levels are due in part to an effect of FMRP on mRNA stability. Next, we tested the role of FMRP in nuclear export in vivo using mouse tissues. We performed RNA-Seq on cytoplasmic and nuclear fractions of cortices of postnatal day 11 (P11) WT and Fmr1 knockout (KO) mice (Table S5). Effective cytoplasmic-nuclear fractionation was confirmed by Western blotting (Fig. S2D). In Fmr1-KO mice, there was a decrease in the abundance of FMRP targets (4) containing m^6^A in the cytoplasm relative to the nucleus (Fig. 2C), but no difference in overall cytoplasmic or overall nuclear abundance (Fig. S3A). We observed no difference in the abundance of FMRP nontargets in the cytoplasm relative to the nucleus (Fig. 2C), nor overall (Fig. S3A). Interestingly, we also saw that there was a smaller but noticeable difference in the abundance of methylated FMRP nontargets in the cytoplasm relative to the nucleus (Fig. 2, C and D). Thus, FMRP may affect nuclear export indirectly or target more transcripts than have been previously determined (4). The difference in the abundance of FMRP nontargets in the cytoplasm relative to the nucleus (Fig. 2C, compare with S3A) may be due to limitations in the RNA-Seq analysis, such as poorer sequencing coverage or lower signal-to-noise ratio in the cytoplasmic fraction.
dance of transcripts in the cytoplasm relative to the nucleus was especially significant for transcripts that were heavily methylated (containing three or more m6A peaks) (Fig. 2D), although no difference in overall cytoplasmic or nuclear abundance of transcripts was observed (Fig. S3B).

Interestingly, a separate analysis of the top 40 targets of FMRP associated with phenotypes (16) showed that these targets demonstrated a decrease in cytoplasmic level relative to nuclear level (Fig. 3A), a finding that we also largely confirmed by RT-qPCR (Fig. 3B). Overall, these results suggest that FMRP facilitates the nuclear export of its m6A-modified targets.

Finally, to explore the subcellular localization of m6A-modified targets of FMRP in a dynamic state rather than at a steady state, we metabolically labeled nascent RNA transcripts in HEK293T cells with 5-ethynyl uridine (EU) for 1 h. EU-labeled nascent transcripts were further modified with a biotin tag and captured by streptavidin pulldown. The cytoplasmic and nuclear distribution of nascent copies was quantified using RT-qPCR. We tested targets of FMRP that were bound directly to an m6A site on the transcript, as demonstrated by our findings in the PAR-CLIP-m6A-IP (Fig. 1). Depletion of FMRP caused the accumulation of several m6A-modified FMRP targets in the nucleus, but not of transcripts that are FMRP nontargets (Fig. S4A). These data support our findings that FMRP facilitates the nuclear export of RNA transcripts, but the results could also be interpreted as an effect of FMRP in repressing the transcription of its targets. To exclude this possibility, we performed the same experiment using nascent RNA from the whole-cell rather than nuclear or cytoplasmic fractions. Depletion of FMRP did not increase the transcription of m6A-modified targets of FMRP (Fig. S4B). Our data suggest that depletion of FMRP attenuates nuclear export of its target transcripts.

**Discussion**

FMRP is an mRNA-binding protein with established roles in affecting translation and trafficking of its targets (4–8). Our studies here suggest a potential additional role: the nuclear export of m6A-containing transcripts (Fig. S5). By performing m6A-Seq directly on RNA fragments purified from FMRP PAR-CLIP assays, we confirmed the presence of m6A methylation at a collection of FMRP-binding sites in HEK293T cells (Fig. 1B). FMRP depletion in HEK293T cells resulted in accumulation of nuclear mRNA m6A level (Fig. 2A) and nuclear retention of nascent transcripts for some of its targets (Fig. S4), without a significant global effect on target abundance (Fig. S2C) or stability of m6A-modified transcripts (Fig. 2B). In the cortex of P11 Fmr1-KO mice, the distribution of FMRP targets and m6A-modified transcripts in the nuclei and cytoplasm is perturbed (Fig. 2, C and D), including those associated with phenotypes of the KO mice (Fig. 3).

**Potential mechanisms of FMRP-mediated mRNA nuclear export**

FMRP may mediate nuclear export of m6A-modified transcripts through different mechanisms. The first is through nuclear RNA export factors, such as NXF2. FMRP depletion in HEK293T cells resulted in accumulation of nuclear mRNA m6A level (Fig. 2A) and nuclear retention of nascent transcripts for some of its targets (Fig. S4), without a significant global effect on target abundance (Fig. S2C) or stability of m6A-modified transcripts (Fig. 2B). In the cortex of P11 Fmr1-KO mice, the distribution of FMRP targets and m6A-modified transcripts in the nuclei and cytoplasm is perturbed (Fig. 2, C and D), including those associated with phenotypes of the KO mice (Fig. 3).
ously shown to interact with mRNA nuclear export factor NXF2 in mouse male germ cells and hippocampal neurons (17). The interaction between NXF2 and FMRP may be important for the export of m6A-modified transcripts from the nucleus. The second mechanism is through a selective mRNA export pathway mediated by CRM1 (18). During preparation of this manuscript, Edens et al. (19) reported that FMRP interacts with CRM1 as an adaptor protein to modulate nuclear export of FMRP targets, a process necessary for proper neural progenitor cell differentiation. Further studies are needed to profile interactions between FMRP and nuclear export machineries, and how the specificity of these interactions is achieved is also not yet known.

Aside from being exported, mRNA in the nucleus also undergoes nuclear decay. The nuclear accumulation of mRNA m6A methylation and FMRP targets in FMRP-deficient cells (Fig. 2) may also be due to impaired nuclear decay. However, this is likely not a main contributor, because a similar effect was observed for nascent transcripts marked by 1-h metabolic labeling (Fig. S4), where a decay effect is less dominant. In fact, an equally (if not more) possible scenario would be that a faster decay takes place as a result of abnormal nuclear accumulation of transcripts (20) in the absence of FMRP, counteracting the m6A mRNA accumulation. This may explain why a larger effect was observed in metabolic labeling assays than in steady-state RNA-Seq (Fig. S4 versus Fig. 2 (C and D)).

m6A methylation and mRNA nuclear export

In the mouse cortex, FMRP depletion appears to impact nuclear export of m6A-modified transcripts in general, especially for the highly m6A-modified transcripts (Fig. 2D). One possible reason is that the list of FMRP targets in the mouse cortex generated by polysome CLIP-Seq (4) may only be a subset of all the transcripts bound by FMRP due to the defined cellular compartment and the detection limit of the CLIP assay. Another possibility is that FMRP may affect nuclear export of an m6A-modified transcript without directly binding to it, especially for those transcripts with multiple m6A methylation sites, as suggested in Fig. 2C. FMRP may interact with other m6A-binding proteins to facilitate export of RNA as a complex. It has been recently reported that highly m6A-methylated transcripts, together with YTHDF proteins, undergo liquid-liquid phase separation in the cytosol, especially during stress response (21). A similar situation may occur during the nuclear export step for those highly m6A-modified transcripts, where FMRP may play a role.

Because FMRP is a reader of m6A, its potential role associated with methylated transcripts may be interesting. For example, it is known that FMRP inhibits the translation of its targets in neurons (3–5). It is possible that FMRP directly impacts translation by binding m6A. Another possibility is that FMRP affects translation by interacting or competing with YTHDF proteins, which affect translation and are also known to interact or compete with FMRP to affect mRNA stability (15, 22–24). We show here yet another potential effect of FMRP’s role as a reader of m6A: FMRP could affect nuclear mRNA export through recognizing m6A modified mRNAs. This work adds to the expanding body of evidence that m6A reader proteins may play multiple roles. All of these proposed functions could operate concurrently and may be dependent on the functions of protein partners as well as cellular context. Given that different m6A reader proteins appear to have similar roles, future studies dissecting each potential pathway using mouse models are required to fully understand how these proteins target different transcripts and how they may complement or hinder the function of similar reader proteins.

Importantly, our studies here are limited to the function of FMRP in affecting the export of methylated transcripts. It will be important to determine whether the other roles of FMRP, including ribosome stalling and mRNA localization within the cytoplasm, are carried out with respect to the methylation status of transcripts. These studies would shed light not only on the function of FMRP, but on the comprehensive function of m6A in a physiological setting.

Experimental procedures

PAR-CLIP-m6A-Seq

PAR-CLIP-m6A-Seq was performed as described previously (25), with modifications. To begin, PAR-CLIP was performed starting with 600 million HEK293T cells stably expressing FLAG-HA-FMRP. FMRP-RNA complex was SDS-PAGE–purified with size selection from 130 to 200 kDa, and RNA fragments were extracted via ethanol precipitation after proteinase K digestion of the gel slices. The purified RNA pellet was dissolved in 12 μl of RNase-free water, of which 3 μl was set aside as input.

The remaining 9 μl of the purified RNA was then subjected to m6A-Seq, which was performed as described previously (26) with modifications. Briefly, the RNA was mixed with 2.5 mg of affinity-purified anti-m6A polyclonal antibody (Synaptic Systems) in IP buffer (150 mM NaCl, 0.1% (v/v) Nonidet P-40, and 10 mM Tris-HCl, pH 7.4) and incubated for 2 h at 4 °C. The antibody-RNA complex was isolated by incubation with protein A beads (Invitrogen) at 4 °C for 2 h. The beads were washed three times and eluted competitively with an m6A monophosphate solution. RNA in the eluate was isolated using RNA Clean and Concentrator (Zymo Research). Library construction was performed by NEBNext small RNA sample preparation kit (New England Biolabs).

Animals

All animal experiments conducted within this study have been approved by the Institutional Animal Care and Use Committee of Northwestern University (protocol #ISO0006359) and performed in accordance with federal regulations governing the use of animals in laboratory research. The Fmr1 KO mice (Jackson Laboratory #003025 or Fmr1-KO1) were obtained from Dr. Anis Contractor’s laboratory. This mouse line was created and initially characterized by the Dutch-Belgian Fragile X Consortium (27) and backcrossed onto the C57BL/6 background and distributed by Dr. David Nelson’s laboratory at Baylor College of Medicine (Houston, TX).

Mammalian cell culture and siRNA knockdown

The HEK293T cell line used in this study was purchased from ATCC and grown in Dulbecco’s modified Eagle’s medium.
formed as described previously (22, 26). Briefly, after removing mm10 RefSeq and UCSC hg38 RefSeq. Data analyses were performed with enrichment score (Fig. S1).

To call m6A peaks, the longest isoform of each human gene was scanned using a 100-nt sliding window with 10-nt steps. To reduce bias from potentially inaccurate gene structure annotation and the arbitrary usage of the longest isoform, windows with read counts less than one-twentieth of the top window in both m6A IP and input sample were excluded. For each gene, the read count in each window was normalized by the median count of all windows of that gene. A negative binomial model was used to identify the differential windows between IP and input samples by using the edgeR package (29). The window was called positive if false discovery rate was $<1\%$ and log$_{2}$(enrichment score) was $\geq 1$. Overlapping positive windows were merged. The following four numbers were calculated to obtain the enrichment score of each peak (or window): read count of the IP sample in the current peak/window ($a$), median read count of the IP sample in all 100-nt windows on the current mRNA ($b$), read count of the input sample in the current peak/window ($c$), and median read count of the input sample in all 100-nt windows on the current mRNA ($d$). The enrichment score of each window was calculated as $(a \times d)/(b \times c)$. Peaks with enrichment score $\geq 2$ were considered as m6A peaks. In samples from mouse cortices, peaks with enrichment $\geq 2$ in both cytoplasmic and nuclear fractions were considered as m6A peaks.

For PAR-CLIP, we analyzed data using PARalyzer with default settings (30). For PAR-CLIP-m6A-Seq, two algorithms were utilized to generate identify m6A peaks: 1) PARalyzer (30), where the PAR-CLIP-m6A-IP sample was analyzed as a PAR-CLIP sample using PARalyzer with default settings, assuming efficient m6A immunoprecipitation and base conversion; 2) MACS (31), where the PAR-CLIP sample and the PAR-CLIP-m6A-IP sample were treated as an “input” and an “IP” and m6A sites were called based on enrichment score calculated by MACS. Finally, the intersection of m6A sites generated by the two methods was kept as high-confidence PAR-CLIP-m6A sites (Fig. S1).

For integrative analyses, 1) only transcripts with significant expression (RPKM $> 1$ in all samples of a batch of RNA-Seq) were kept for further analyses, and 2) nonparametric Mann–Whitney $U$ test (Wilcoxon rank-sum test, two-sided) was applied to RNA-Seq data analysis, as reported previously (22).

**LC-MS/MS**

LC-MS/MS was performed as reported previously (24). Briefly, about 50–100 ng of mRNA were digested by nuclease P1 (1 unit, Wako) in 20 μl of buffer containing 20 mM NH$_4$OAc (pH 5.3) at 42 °C for 2 h, followed by the addition of 1 μl of FastAP (Thermo Fisher Scientific #EF0651) with 2.5 μl of 10× FastAP buffer, incubated at 37 °C for 4 h. After an additional incubation at 37 °C for 2 h, the sample was diluted to 50 μl and filtered (0.22-μm pore size, 4-mm diameter; Millipore), and 5

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**Cytoplasmic and nuclear fractionation**

The nuclear and cytoplasmic fractions of P11 Fmr1-/-KO and WT mouse cortices were isolated using the NE-PER kit (Thermo Fisher Scientific #78835) following the manufacturer’s instructions.

The nuclear and cytoplasmic fractions of HEK293T cells were isolated using the following procedure. Three million HEK293T cells were lysed on ice for 5 min using 60 μl of Nonidet P-40 lysis buffer (0.075% Nonidet P-40, 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1:100 complete EDTA-free protease inhibitor (Roche Applied Science), 1:100 SUPERaseIn inhibitor (Thermo Fisher Scientific AM2694). The cell lysate was layered over 150 μl of chilled sucrose cushion (24% RNase-free sucrose in Nonidet P-40 lysis buffer) and centrifuged at 4 °C for 10 min at 15,000 × g. The supernatant was collected as the cytoplasmic fraction. The pellet was rinsed four times with PBS and collected as the nuclear fraction.

**RNA isolation and RNA-Seq**

RNA was extracted from the total, cytoplasmic, and/or nuclear fractions using TRizol. mRNA was further purified using either one or two rounds of the mRNA DIRECT kit (Thermo Fisher Scientific #61012) and one round of the RiboMinus Eukaryotic kit version 2 (Thermo Fisher Scientific #A15015) or two rounds of the mRNA RNA-Seq library preparation was performed with TruSeq stranded mRNA sample preparation kit (Illumina).

**m6A-Seq**

m6A-Seq was performed as described previously (26). Briefly, 100 μg of total RNA was extracted from P11 WT mouse cortices (cytoplasmic and nuclear fractions), as well as HEK293T cells, using TRizol following the manufacturer’s protocol. Poly(A) mRNA was enriched using the Dynabeads mRNA DIRECT Kit (Ambion) following the manufacturer’s protocols. mRNA was sonicated to ~100 nt, mixed with 2.5 μg of affinity-purified anti-m6A polyclonal antibody (Synaptic Systems) in IP buffer (150 mM NaCl, 0.1% Nonidet P-40, and 10 mM Tris-HCl, pH 7.4), and incubated for 2 h at 4 °C. The antibody–RNA complex was isolated by incubation with protein A beads (Invitrogen) at 4 °C for 2 h. The beads were washed three times and eluted competitively with an m6A monophosphate solution. RNA in the eluate was isolated using RNA Clean and Concentrator (Zymo Research) and used for library preparation with the TruSeq stranded mRNA sample preparation kit (Illumina).

**Data analysis**

Gene structure annotations were downloaded from UCSC mm10 RefSeq and UCSC hg38 RefSeq. Data analyses were performed as described previously (22, 26). Briefly, after removing adapters, sequencing reads were aligned to the reference genome (mm10) using TopHat (version 2.0.14) (28). For m6A-Seq, the longest isoform was used if multiple isoforms were detected. Aligned reads were extended to 100 nt (average fragment size) and converted from genome-based coordinates to isoform-based coordinates to eliminate interference from introns in peak calling.

To call m6A peaks, the longest isoform of each human gene was scanned using a 100-nt sliding window with 10-nt steps. To reduce bias from potentially inaccurate gene structure annotation and the arbitrary usage of the longest isoform, windows with read counts less than one-twentieth of the top window in both m6A IP and input sample were excluded. For each gene, the read count in each window was normalized by the median count of all windows of that gene. A negative binomial model was used to identify the differential windows between IP and input samples by using the edgeR package (29). The window was called positive if false discovery rate was $<1\%$ and log$_{2}$(enrichment score) was $\geq 1$. Overlapping positive windows were merged. The following four numbers were calculated to obtain the enrichment score of each peak (or window): read count of the IP sample in the current peak/window ($a$), median read count of the IP sample in all 100-nt windows on the current mRNA ($b$), read count of the input sample in the current peak/window ($c$), and median read count of the input sample in all 100-nt windows on the current mRNA ($d$). The enrichment score of each window was calculated as $(a \times d)/(b \times c)$. Peaks with enrichment score $\geq 2$ were considered as m6A peaks. In samples from mouse cortices, peaks with enrichment $\geq 2$ in both cytoplasmic and nuclear fractions were considered as m6A peaks.

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For integrative analyses, 1) only transcripts with significant expression (RPKM $> 1$ in all samples of a batch of RNA-Seq) were kept for further analyses, and 2) nonparametric Mann–Whitney $U$ test (Wilcoxon rank-sum test, two-sided) was applied to RNA-Seq data analysis, as reported previously (22).
μl of the solution was subjected to LC-MS/MS. Nucleosides were separated by reverse-phase ultraperformance LC on a C18 column with on-line MS detection using an Agilent 6140 QQQ triple-quadrupole LC mass spectrometer in positive electrospray ionization mode. The nucleosides were quantified by using retention time and the nucleoside-to-base ion mass transitions of 284–152 (G), 282.1–150.1 (m6A), 268–136 (A), 245–113.1 (U), and 244–112 (C). Quantification was performed compared with the standard curve obtained from pure nucleoside standards running with the same batch of samples. The m6A level was calculated as the ratio of m6A to A based on the calibrated concentrations (32).

**EU labeling of nascent RNA**

We followed the manual of the Click-it™ Nascent RNA Capture Kit (C10365, Thermo Fisher Scientific). Briefly, 6-cm plates of HEK293T cells were treated with either siCtrl or siFMR1–10 for 48 h before being labeled with 200 μM EU in culture medium for 1 h. Cells were then collected by scraping, with one-eighth saved in TRIzol as the whole-cell fraction and the remaining seven-eighths subjected to cytoplasmic nuclear fractionation as described above. Total RNAs from the whole cell, the cytoplasmic fraction, and the nuclear fraction were purified using TRIzol and RNA precipitation. Afterward, the click reaction was performed for each sample with 3.0 μg of total RNAs and azide-biotin (final concentration 0.5 mm) in a 50-μl volume. The RNAs after biotinylation were purified again by ethanol precipitation.

The biotinylated RNAs were then captured by Dynabeads® MyOne™ Streptavidin T1 magnetic beads. 250 ng of RNA from the last step and 20 μl of T1 beads were used per IP, and cDNAs were synthesized on beads in a 40-μl volume. The cDNAs were synthesized on beads in a 40-μl volume. The cDNAs were used for cDNA quantification of the following transcripts with primer sequences listed:

- CAAA (forward) and CCTCAACAGCG (forward) and ACCACCCTGTTGCTGTAG-CAAT (reverse).
- CAAA (forward) and CCTCAACAGCG (forward) and ACCACCCTGTTGCTGTAG-CAAT (reverse).
- CAAA (forward) and CCTCAACAGCG (forward) and ACCACCCTGTTGCTGTAG-CAAT (reverse).
- CAAA (forward) and CCTCAACAGCG (forward) and ACCACCCTGTTGCTGTAG-CAAT (reverse).
- CAAA (forward) and CCTCAACAGCG (forward) and ACCACCCTGTTGCTGTAG-CAAT (reverse).
- CAAA (forward) and CCTCAACAGCG (forward) and ACCACCCTGTTGCTGTAG-CAAT (reverse).
- CAAA (forward) and CCTCAACAGCG (forward) and ACCACCCTGTTGCTGTAG-CAAT (reverse).
- CAAA (forward) and CCTCAACAGCG (forward) and ACCACCCTGTTGCTGTAG-CAAT (reverse).
- CAAA (forward) and CCTCAACAGCG (forward) and ACCACCCTGTTGCTGTAG-CAAT (reverse).

**Actinomycin D treatment**

Actinomycin D was added to the culture medium to a final concentration of 5 μg/ml for 3 or 6 h before cells were collected in TRIzol. Total RNAs were extracted by RT-qPCR or LC-MS/MS. LC-MS/MS quantification of m6A after transcription inhibition was fitted to a model of exponential decay, using the one-phase decay nonlinear fitting model from the software GraphPad Prism 7 (GraphPad Software, La Jolla, CA).

\[
y = \left( y_0 - \text{plateau} \right) \times \exp(-k \times t) + \text{plateau} \quad \text{(Eq. 1)}
\]

where \(y\) is m6A/A(%), \(t\) is time after transcription inhibition (hours), and \(k\) is decay rate constant. The half-lifetime is calculated as \(\ln 2/k\).

**Antibodies**

The antibodies used in this study are listed as follows in the format of name (catalogue; supplier; application/amount used).
- rabbit anti-FMRP (ab17722; Abcam; Western blotting/1:1,000);
- goat anti-GAPDH horseradish peroxidase (A00192-100; GenScript; Western blotting/1:5,000); rabbit anti-Lamin A/C (ab83306; Abcam; Western blotting/1:10,000).

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