Supplementary information, Data S1

Materials and methods

Protein purification in *E.coli*

The human YTHDF3 gene was subcloned into pGEX-5X-2 expression vector with GST-tag. Then recombinant GST-YTHDF3 proteins were induced to express in *E.coli* strain BL21 (DE3) and purified by FPLC using Bio-Scale Mini Profinity GST cartridge (Bio-rad) according to the manufacturer's instructions.

Protein purification in mammalian cells

293T cells were transiently transfected with Flag-tagged RPL/RPS plasmids (vigene), after 48 h, cells were lysed with lysis buffer (50 mM Tris-HCl, pH 7.4, 500 mM NaCl, 1% NP-40) and then sonicated (10% output, 10s on, 20s off) for 1 min. Cell debris was removed by centrifugation and the crude lysates were incubated with Flag beads for 4 h at 4 ℃. After 5 times washing with lysis buffer, the beads-bound proteins were eluted with 1 mg/ml 3×Flag peptide for 1 h at 4 ℃.

GST pull down

Flag-RPL3, Flag-RPLP0, Flag-RPS2, Flag-RPS3 and Flag-RPS15 were pre-cleared with glutathione sepharose (GE Healthcare) in NETN buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl pH 7.4, 0.5% NP-40). The pre-cleared Flag-tagged proteins were mixed with GST or GST-YTHDF3 protein and equilibrated glutathione sepharose overnight at 4 ℃ separately. After washing the beads five times with NETN buffer, proteins bound to the beads were analyzed by 10% SDS-PAGE followed by immunoblotting with HRP-conjugated anti-Flag and anti-GST antibodies (Abcam).

Nascent protein synthesis

Cells were plated on coverslips in 6-well plates and transfected at 40% confluence with 100 nM 5'-CY3 labeled siRNAs targeting human YTHDF3 or negative controls using RFect siRNA Transfection reagent (BIO-TRAN) and then incubated for 8 h. After 12 h recovery, cells were transfected again with the respective siRNAs. 12 h later, cells were

cultured in methionine-free complete media for 1 h, and then labelled with AHA (Invitrogen) in methionine-free complete media for 4 h. The cells were subsequently fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.25% Triton X-100 in phosphate buffered saline (PBS) for 15 min on ice. Cells were blocked for 15 min at room temperature in PBS containing 3% BSA. Detection of AHA-labelled protein samples were performed by using the Click-iT® Cell Reaction Buffer Kit and Click-iT® Metabolic Labeling Reagents (Invitrogen) according to the manufacturer's instructions. The coverslips were mounted with DAPI-containing mounting medium (Vector Laboratories) and sealed with nail polish. Fluorescent images were acquired by Leica TCS SP8 confocal microscope.

For the rescue assay, HeLa cells grown on coverslips were first transfected with 100 nM siRNA targeting YTHDF3. After 24 h, the cells were then co-transfected with siRNAs, Myc-EV, Myc-tagged insensitive wild-type and m^6A binding defective YTHDF3 plasmids as indicated by Lipofectamine 2000 (Invitrogen). 24 h later, immunofluorescence staining was performed as described above.

Plasmid construction and antibodies

Total RNA was extracted from 293T cells using TRI Reagent (Sigma) and cDNA synthesized by Reverse Transcription PCR using RevertAidTM First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, K1622). The human YTHDF1 gene open reading frame encoding the full-length protein was amplified and subcloned into S-protein/FLAG/SBP (streptavidin-bindign protein) triple-tagged vector with the following primers:

for (Sal I): 5'-ACGCGTCGACACATGTCGGCCACCAGCGTGGAC-3',

rev (BamH I): 5'-CGCGGATCCTCATTGTTTGTTTCGACTCTGCCG-3'.

The human YTHDF3 gene open reading frame encoding the full-length protein was subcloned into S-protein/FLAG/SBP (streptavidin-bindign protein) triple-tagged vector and pCMV-Myc (Sigma) vector with the following primers, respectively:

SFB-for (Sal I): 5'- ACGCGTCGACACATGTTCTATCTTGATTTGACTCTG-3',

SFB-rev (BamH I): 5'- CGCGGATCCTTATTGTTTGTTTCTATTTCTCTCC-3',

Myc-for (EcoR I): 5’- CGGAATTCGGATGTTCTATCTTGATTTGACTCTG-3’, Myc-rev (Not I): 5’- ATAGTTTAGCGGCCGCTTATTGTTTGTTTCTATTTCTCTCC -3’.

pCMV-Myc-YTHDF3-m^{6}A binding defective (Myc-DM: W438A and W492A), pCMV-Myc-YTHDF3-insensitive (Myc-WT-Ins) and pCMV-Myc-YTHDF3-m^{6}A binding defective-Insensitive (Myc-DM-Ins) plasmids were generated with point mutations by QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene). Primers for pCMV-Myc-YTHDF3-DM are as follows:

for(W438A):TTATTACCATGCTCAGTACTACACGCGATAGAGTATTTAATGGA ACGATG,

rev(W438A):CATCGTTCCATTAAATACTCTATCGCGTGTAGTACTGAGCATGG TAATAA.

for (W492A): CTTCCACTTATCCTGAGACGCGACACCAGCATACGCATTA,

rev (W492A): TAATGCGTATGCTGGTGTCGCGTCTCAGGATAAGTGGAAG.

Primers for pCMV-Myc-YTHDF3-WT-Ins and pCMV-Myc-YTHDF3-DM-Ins are as follows:

1,GGACAAGTGGATCTCAGGGACAAT(A)C(G)A(C)ACACAAAGTTCTGCTTA TAGT.

2, CAGCAGTGGTATGACTAGC(T)ATT(A)GCA(G)ACCAATAGTGTGCCCCCA.

3,TTGATTGGAATCTGAAGAATGGACGT(C)GTG(A)TTT(C)ATAATTAAAAGC TACTCTGAGGATG.

The following antibodies were used: rabbit-anti-LRPAP1 (ABclonal), rabbit-anti-EEF1G (ABclonal), rabbit-anti-EIF4E (ABclonal), rabbit-anti-EIF2S2 (ABclonal), rabbit-anti-ADAR1 (Abcam), rabbit-anti-RPS15 (Abcam), rabbit-anti-RPS3 (Abcam) rabbit-anti-RPLP0 (ABclonal), rabbit-anti-RPL3 (Abcam), rabbit-anti-RPS15 (Abcam), rabbit-anti-YTHDF3 (Abcam), rabbit-anti-YTHDF1 (proteintech).

Plasmid transfection and RNA interference

10 μg plasmids and 30 μl polyethylenimine (Polysciences) were mixed in serum and antibiotic-free DMEM. After 15 min, the mixture was introduced into the cells cultured

in 10 cm dish. After 4 h, medium was replaced with fresh medium and cells were harvested after 48 h incubation for immunoprecipitation.

siRNA duplexes (GenePharma, China) were transfected into cells at a final concentration of 100 nM by using Lipofectamine RNAiMAX™ (Invitrogen) according to the manufacturer's instructions. The siRNA information is listed as follows:

YTHDF1 #1: 5'- GCUCCAUUAAGUACUCCAU -3',

YTHDF1 #2: 5'- CCUCCACCCAUAAAGCAUA -3',

YTHDF1 #3: 5'- GGAUACAGUUCAUGACAAU -3',

YTHDF3 #1: 5'- GGACGUGUGUUUAUAAUUA -3',

YTHDF3 #2: 5'- GACUAGCAUUGCAACCAAU -3',

YTHDF3 #3: 5'- GGACAAUCAACACAAAGU-3',

Scrambled siRNA (siCTRL): 5'-UUCUCCGACGUGUCACGU-3'.

For immunofluorescence experiments the above siRNAs labeled with Cy3 as indicated were synthesized by GenePharma, China.

Tandem affinity purification and Mass spectrometry

293T cells were transiently transfected with triply-tagged YTHDF1 plasmid (Flag-SFB-YTHDF1) or triply-tagged YTHDF3 plasmid (Flag-SFB-YTHDF3). After 40 h incubation, the cells were harvested and lysed with NETN buffer on ice for 30 min. Cell debris was removed by centrifugation and the crude lysates were incubated with streptavidin-Sepharose beads (Amersham Biosciences) for 1 h at 4 ℃. The bead-bound proteins were eluted twice with 1 mg/ml biotin (Sigma) for 1 h at 4 ℃. The eluates were further incubated with S-protein-agarose (Novagen) for 1 h at 4 ℃ and washed 3 times with NETN buffer. The proteins bound to S-protein-agarose beads were subjected to SDS-PAGE and visualized by Coomassie Blue staining. The identities of eluted proteins were revealed by mass spectrometry analysis (peptideatlas access number PASS00934). The Q Exactive mass spectrometry data (Thermo Fisher Scientific) were searched against SwissProt human database using 15 ppm peptide mass tolerance and 20 mmu fragment mass tolerance.

RNA Isolation, cDNA Synthesis and Semi-quantitative PCR

Total RNA was extracted from HeLa cells using TRIzol reagent (Invitrogen) following the manufacturer's instruction. cDNA was synthesized using RevertAidTM First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, K1622) with Oligo-dT primers following manufacturer's recommendations. PCR reactions were carried out on a VeritiTM 96-Well Thermal Cycler (Applied Biosystems, 9902) in 20 μl reaction volume. All PCR reactions were performed in three independent biological repeats. The following primers were synthesized (GenePharma, China) and used in the study:

YTHDF1-forward: CTCAGCATGGGGGACAAGTG,

YTHDF1-reverse: GAGGAGCTGACGTCCCCAAT,

YTHDF3-forward: ACAAGTGGATCTCAGGGAC,

YTHDF3-reverse: GCTGAGGCTGCTGGATTAT,

LRPAP1-forward: TTTCCGCCTACCACAACCTC,

LRPAP1-reverse: TCTTCTGCTCCGGGTCAAAC,

EEF1G-forward: GAGGACACACTCTCTGTGGC,

EEF1G-reverse: ATCCAGTTTCCGCCATGTGT,

EIF4E-forward: CCCCTACAGAACAGATGGGC,

EIF4E-reverse: GGTCACTTCGTCTCTGCTGT,

ADAR1-forward: TGACAGAGTGCCAGCTGAAG,

ADAR1-reverse: AGAAGCCATGGAGTTGGTCG,

EIF2S2-forward: ACCAGAGGATGACCTTGACA,

EIF2S2-reverse: CCACTTGTACCCAATTCAGCC,

ACTIN-forward: AGCCATGTACGTAGCCATCC,

ACTIN-reverse: CTCTCAGCTGTGGTGGTGAA.

PAR-CLIP Assay

PAR-CLIP-seq and PAR-CLIP-biotin chemiluminescent nucleic acid detection were performed as follows. In brief, human HeLa cells with specific gene knockdown and plasmids overexpression were cultured in medium supplemented with 200 μM 4-thio-uridine (4SU) (Sigma, T4509) for 14 h, and then irradiated once with 400 mJ/cm2 at

365 nm using the UV crosslinker BLX-E365 (Vilber) to induce crosslinking. Cells were collected and lysed in 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM EDTA, 0.5% (v/v) NP-40, 1 mM NaF, 1 × Protease Inhibitor Cocktail (Sigma, P8340), 0.04 U/ml RNasin (Promega, N251B), and rotated at 4 ℃ for 30 min. Cell debris was removed by centrifugation and the soluble fractions (3-4 mg/ml) were digested by 1 U/μl RNase T1 at 22 ℃ in a water bath for 15 min and cooled on ice for 5 min. Then the soluble fractions were incubated with anti-Flag M2 magnetic beads (Sigma, M8823) or PierceTM anti-Myc magnetic beads (Thermo, 88843) for about 2 h at 4 ℃. The immunoprecipitates were then washed three times with IP wash buffer (50 mM Tris-HCl pH 7.5, 300 mM NaCl, 0.05% (v/v) NP-40, 1 × Protease Inhibitor Cocktail (Sigma, P8340), 0.04 U/ml RNasin (Promega, N251B)). The above obtained samples were digested with 10 U/μl RNase T1 again at 22°C in a water bath for 15 min, cooled on ice for 5 min. Beads were washed three times in high salt wash buffer (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.05% (v/v) NP-40, 1 × Protease inhibitor cocktail (Sigma, P8340), 0.04 U/ml RNasin (Promega, N251B)) with 5 min rotation. Protein-RNA-beads mixture were re-suspended in 100 μl dephosphorylation buffer (50 mM Tris-HCl pH 7.9, 100 mM NaCl, 10 mM $MgCl_2$) and incubated with 0.5 U/μl calf intestinal alkaline phosphatase (CIAP, NEB, M0290L) for 10 min at 37 ℃. The beads were then washed twice with phosphatase wash buffer (50 mM Tris-HCl pH 7.5, 20 mM EGTA, 0.5% (v/v) Triton X-100).

For PAR-CLIP-biotin chemiluminescent nucleic acid detection, protein-RNA complex was labeled with biotin by RNA 3' End Biotinylation kit (Thermo, 20160) following the manufacturer's instructions. Then the beads were washed three times with IP wash buffer (50 mM Tris-HCl pH 7.5, 300 mM NaCl, 0.05% (v/v) NP-40, 1 × Protease Inhibitor Cocktail (Sigma, P8340ein complex, and then transferred to PVDF membrane, detected by chemiluminescent nucleic acid detection module (Thermo, 89880) following the manufacturer's instructions. One fourth of the sample was separated by SDS-PAGE to detect protein immunoprecipitation complex, and then transferred to PVDF membrane, detected by chemiluminescent nucleic acid detection module (Thermo, 89880) following the manufacturer's instructions. One tenth and one fourth

of the samples were separated by SDS-PAGE to detect protein immunoprecipitation efficiency and m^6A level respectively.

For PAR-CLIP-seq, protein-RNA complex was washed twice with PNK buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$), incubated with 1 mM ATP and 1 U/μl T4 PNK (NEB, M0201L) in one original bead volume for 1 h at 37 ℃ followed by three times washing with PNK buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$). Then the protein-RNA mixture were re-suspended in 30 μl 4 × SDS loading buffer (Invitrogen, NP0007) and 90 μl 1 × SDS loading buffer (Invitrogen, NP0007), boiled at 95 ℃ for 10 min. Then the sample was separated by SDS-PAGE, transferred to PVDF membrane. The membrane were cut into small piece and digested by 4 μg/μl proteinase K (Roche, 03115828001) in 200 μl PK buffer (100 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM EDTA) for 20 min at 37°C following 200 μl PK-urea buffer (100 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM EDTA, 7 M urea) incubation for 20 min at 37 ℃. RNA were followed by three times washing and precipitated in ethanol with the help of glycogen (Thermo, R0551). RNAs in ethanol were sent for the small RNA library construction and deep sequencing.

PAR-CLIP data analysis

The PAR-CLIP-seq reads of the YTHDF1 and YTHDF3 proteins were stripped of the adaptor sequence with the Cutadapt software (version 1.2.1) [1]. Reads that were less than 18 nt in length or contained an ambiguous nucleotide were discarded by Trimmomatic (version 0.30) [2]. The remained reads were aligned to the human genome (version hg19), with up to two mismatches allowed, by the Bowtie algorithm [3]. Mapped locations were only reported for those with the minimum number of observed mismatches for each read. Clusters were identified by PARalyzer (v1.1) with the default parameters [4]. Each cluster was annotated based on the Ensembl (release 72) gene annotation information by applying BEDTools' intersectBed (version 2.16.2) [5]. The clusters overlapped with discovered m^6A peaks in previous study [6] were kept for downstream analysis. The PAR-CLIP-seq data have been uploaded to GSA database (http://gsa.big.ac.cn/) and can be accessed via accession number PRJCA000273.

Motif identification among clusters

The motifs in clusters were identified by HOMER (v4.7) [7]. All clusters mapping to exon regions were used as the target sequences, and a set of background clusters was generated with the BEDTools' shuffleBed program by randomly shuffling the regions to the same size as the clusters throughout the gene regions.

mRNA translation efficiency

Human ribosome profiling and mRNA input data were downloaded from GEO database with access number GSE63591 [8]. The translation efficiency were estimated by ribosome-bound fragments and mRNA input fragments as follows.

$$Translation\ Efficiency = \frac{Fragments_{ribosome\ bound}}{Fragments_{mRNA\ input}}$$

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