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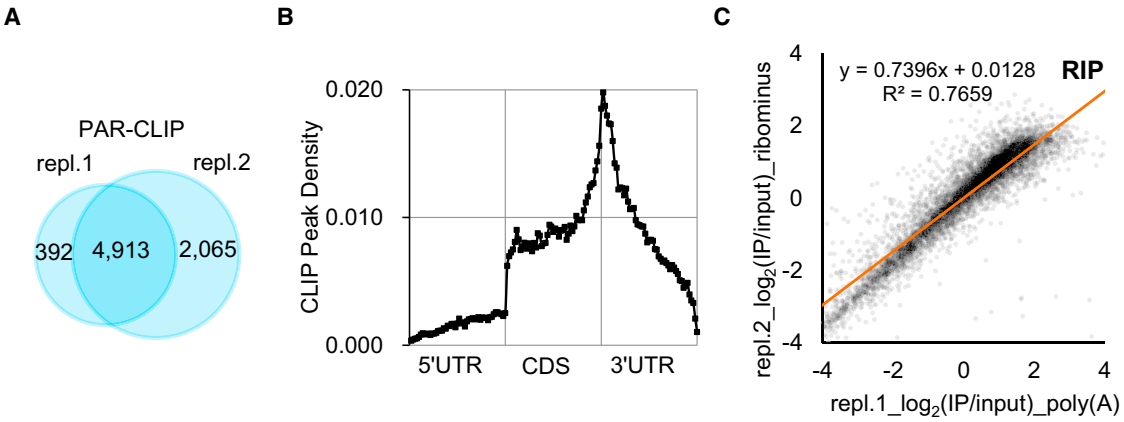

Figure S1. Identification of YTHDF1 RNA Targets by PAR-CLIP and RIP, Related to Figure 1

(A) Overlap of two biological replicates for the PAR-CLIP data of YTHDF1. Numbers are total genes identified in each sample.

(B) Sub-transcript distribution of the YTHDF1-binding sites.

(C) Correlation of enrichment fold as $\log_2$(IP/input) between two technical RIP replicates. In repl.1 the input mRNA was purified by poly(A) selection, whereas in repl.2 the input RNA was processed by rRNA removal.

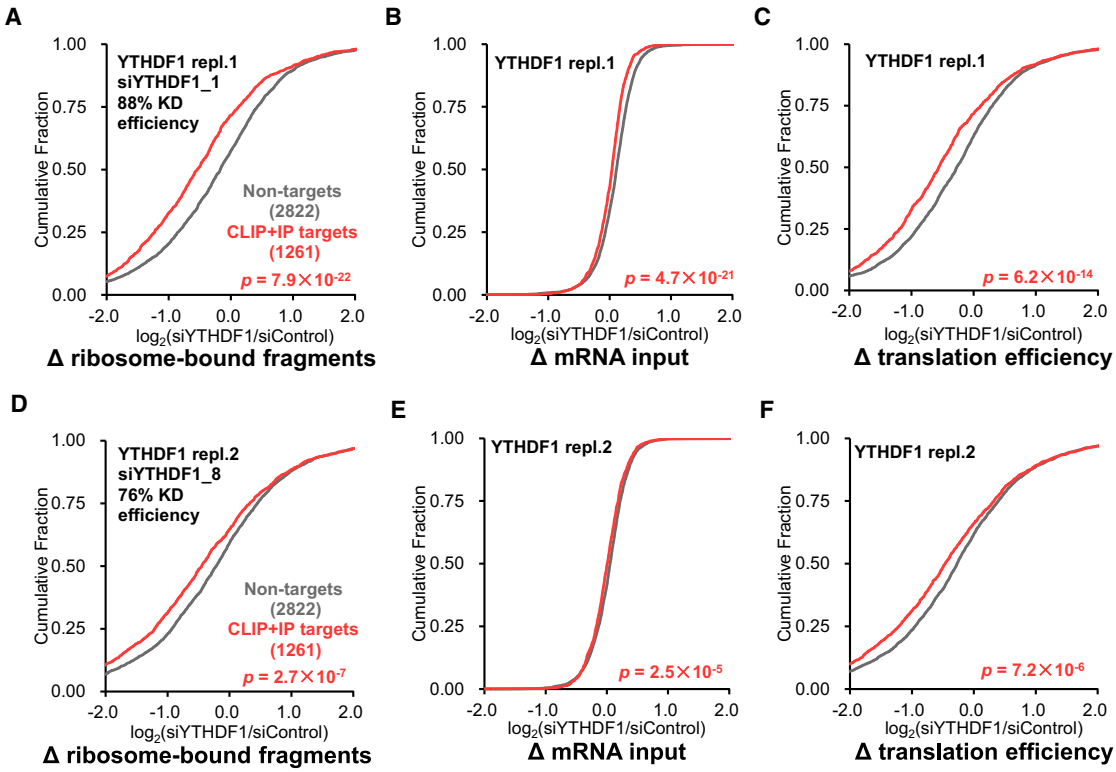

Figure S2. Comparisons of Biological Replicates of Ribosome Profiling Results, Related to Figure 2

The *log*$_2$-fold changes for cumulative distribution of ribosome-bound fragments (A and D), mRNA input (B and E), and translation efficiency (C and F, ratio of ribosome-bound fragments and mRNA input) between siYTHDF1 (YTHDF1 knockdown) and siControl (knockdown control) for non-targets (gray) and common targets of PAR-CLIP and RIP (red). Biological repl.1 (A–C) and biological repl.2 (D–F) were prepared using different siYTHDF1 siRNA sequences. The extent of translation reduction caused by YTHDF1 knockdown correlates with the YTHDF1 knockdown efficiency. p values were calculated using two-sided Mann-Whitney test.

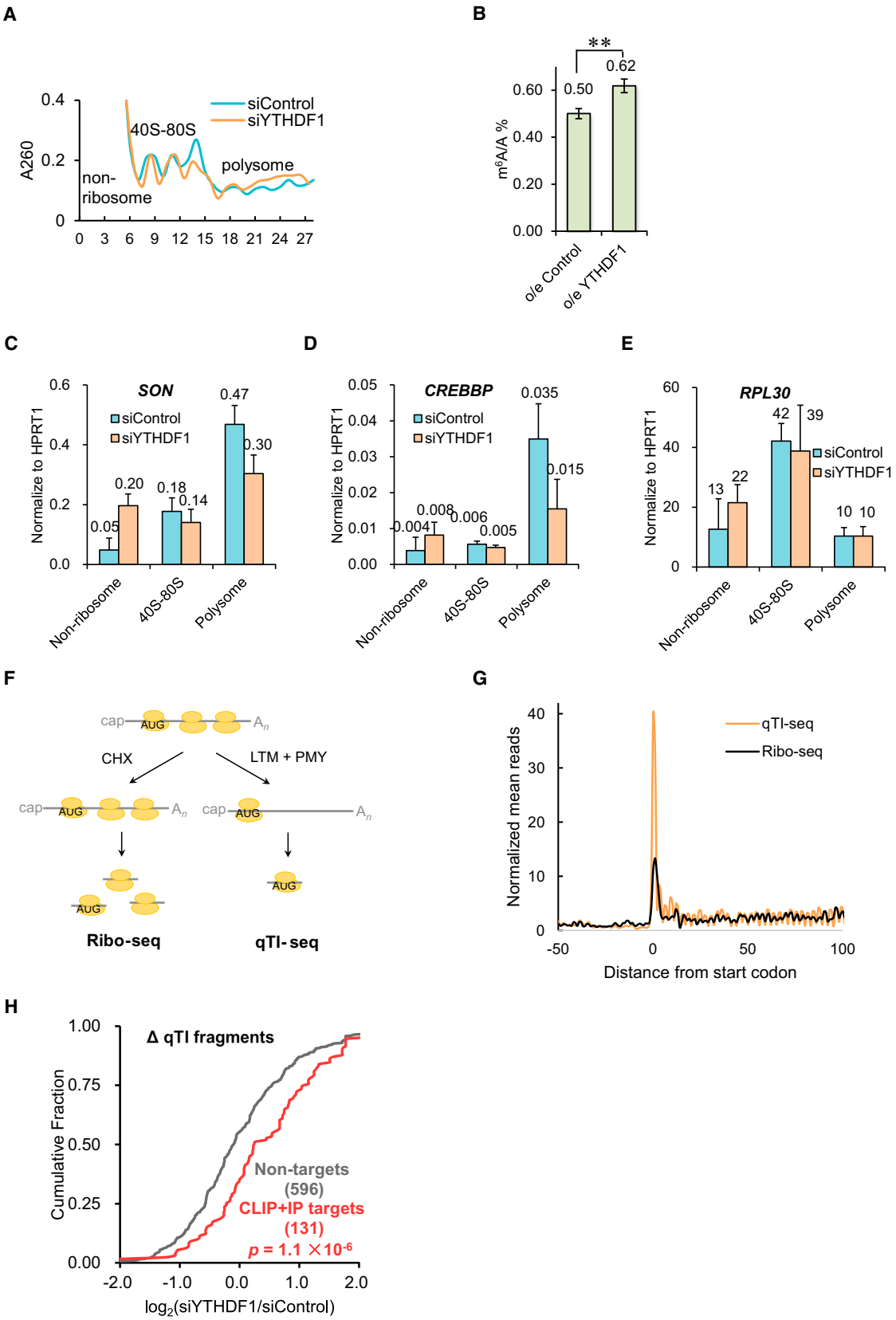

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Figure S3. YTHDF1 Alters the Translation Dynamics of m^{6}A-Modified mRNA, Related to Figure 3

(A) Polysome profiles of YTHDF1 knockdown and control samples.

(B) Quantification of the m^{6}A/A ratio of poly(A)-selected total mRNA by LC-MS/MS for the YTHDF1 overexpression sample compared to control after 24 hr. p values were determined using two-sided Student's t test for paired samples. Error bars represent mean ± SD, p = 0.0015, n = 6 (three biological replicates × two technical replicates).

(C–E) Subcellular re-distribution of representative RNAs under YTHDF1 knockdown. The *SON* (C) and *CREBBP* (D) mRNA levels increased in the non-ribosome mRNP portion but decreased in the polysome portion under siYTHDF1 compared to siControl. *RPL30* mRNA (E) is not a target of YTHDF1, and did not show a decrease in the polysome portion upon YTHDF1 knockdown. Error bars represent mean ± SD, n = 4 (two biological replicates × two technical replicates).

(F) Scheme of ribosome profiling (Ribo-seq) and quantitative translation initiation sequencing (qTI-seq). CHX, cycloheximide; LTX, lactimidomycin; PMY, puromycin.

(G) Meta-gene analysis of Ribo-seq and qTI-seq. Normalized ribosome-protected fragment (RPF) reads were averaged across the entire transcriptome and aligned at the annotated start codon.

(H) Cumulative distribution of the log_2-fold changes of qTI-seq reads between the YTHDF1 knockdown and knockdown control samples for non-targets (gray) and common targets based on PAR-CLIP and RIP (red).

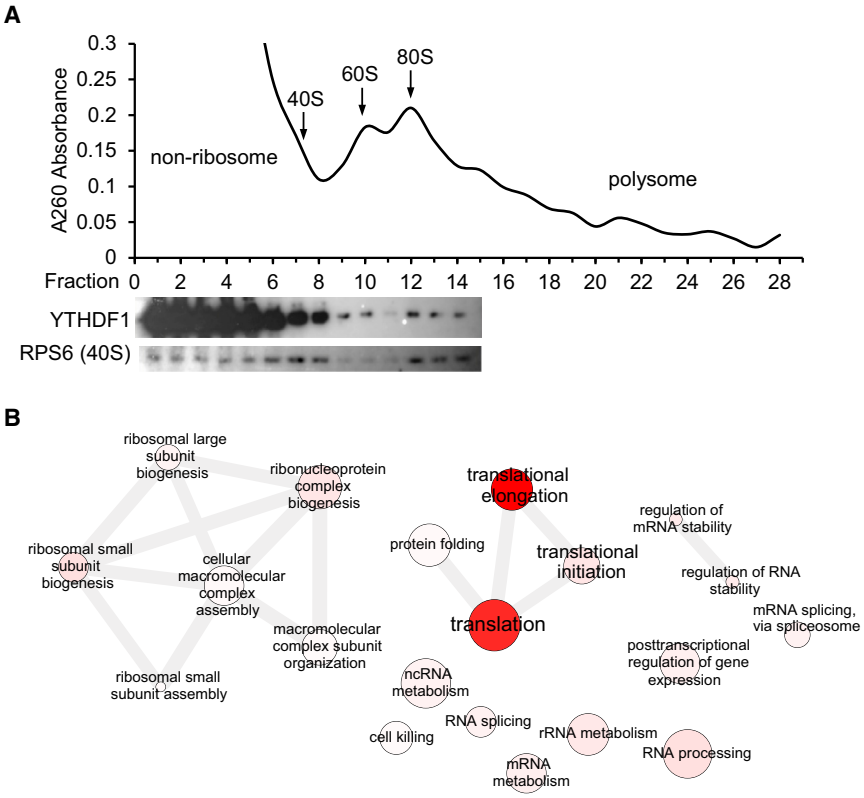

Figure S4. YTHDF1 Associates with Translation Machinery, Related to Figure 5

(A) Western blotting of Flag-tagged YTHDF1 on each fraction of 10%–50% sucrose gradient showing that YTHDF1 associates with 40S and 80S ribosome. The fractions were grouped to non-ribosomal mRNPs, 40S–80S, and polysome. RPS6 is a protein subunit of 40S ribosome. The YTHDF1 HeLa stable line was treated with 0.5% formaldehyde for 7 min before harvesting cells.

(B) Gene ontology analysis of the YTHDF1 protein interactome obtained by co-immunoprecipitation and mass spectrometry identification.

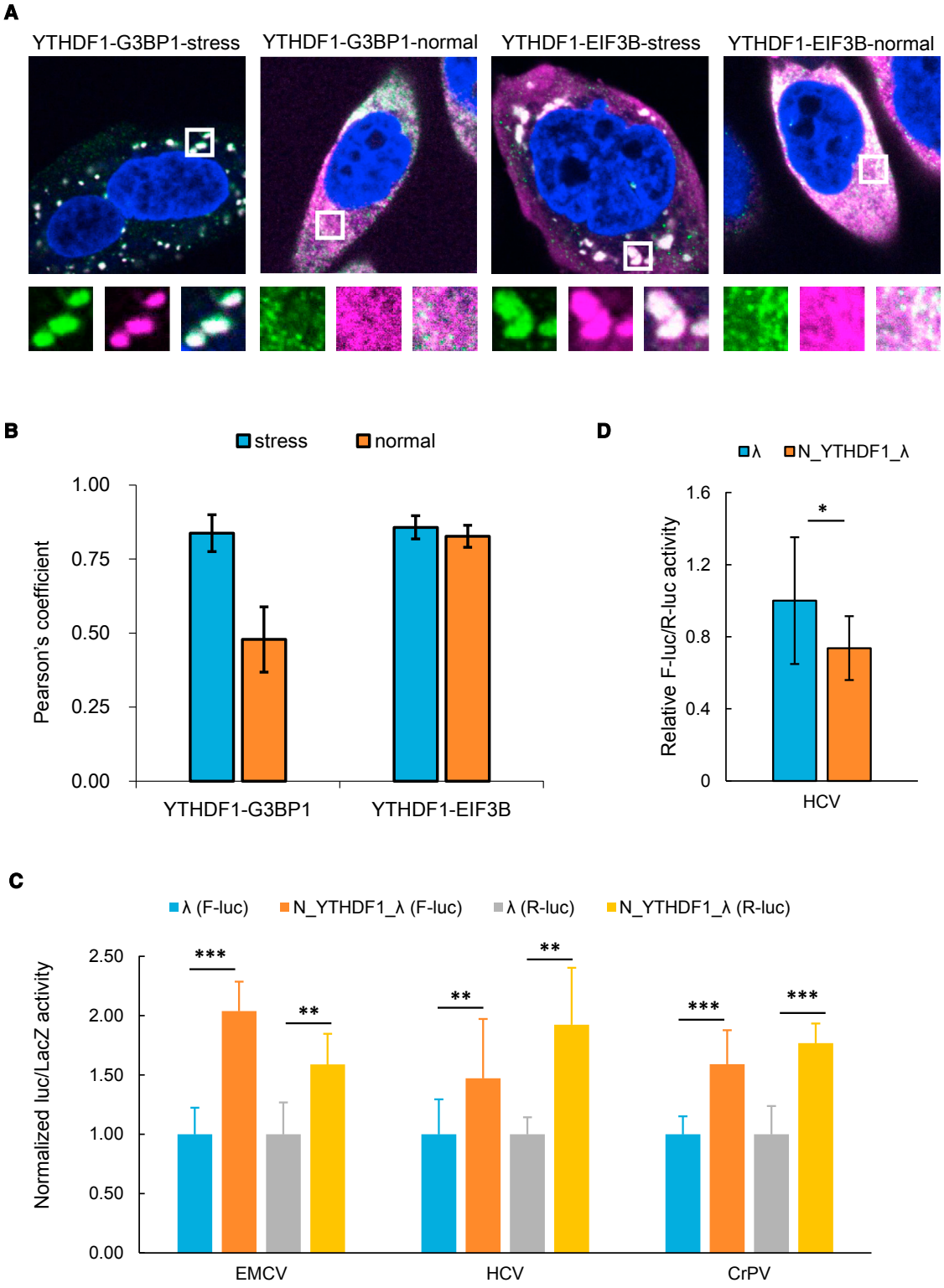

Figure S5. Interaction of YTHDF1 with Protein Markers, Related to Figure 5

(A) Fluorescence immunostaining of Flag-tagged YTHDF1 (green, anti-Flag, Alexa 488) and protein markers (G3BP1 for stress granules and EIF3B for translation machinery; magenta of Alexa 647 is the color for the marker, green + magenta = white for the co-localization spot). The scale of the magnified region (while frame) is 1.7 μm × 1.7 μm.

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(B) Co-localization between YTHDF1 and different protein markers were characterized by the Pearson's coefficient, for each pair, n = 4. The Pearson's coefficient is used to quantify the degree of colocalization between two fluorophores used to label the corresponding proteins. YTHDF1 co-localizes better with stress granules after arsenite treatment (1 mM, 1 hr) compared to the normal growth condition. It showed high co-localization with translation machinery under both normal and stress conditions, with the latter slightly better.

(C) Tethering assay showing the result of both cap-dependent translation (R-luc) and IRES-dependent translation (F-luc) normalized with LacZ activity as transfection control. The effects of N_YTHDF1_λ tethering on cap-dependent translation are comparable for all three groups. EMCV-dependent translation showed significantly more increase while the HCV and CrPV data revealed slightly less increase compared to the cap-dependent translation. Error bars, mean ± SD, p (EMCV-F-luc) = 2.5×10^{-4}, *P* (EMCV-R-luc) = 7.4×10^{-3}, *P* (HCV-F-luc) = 1.5×10^{-3}, *P* (HCV-R-luc) = 3.0×10^{-3}, *P* (CrPV-F-luc) = 7.4×10^{-4}, *P* (CrPV-R-luc) = 3.7×10^{-4}, two-sided Student's t test for paired samples, n = 8 (biological replicates).

(D) N_YTHDF1_λ tethering did not show statistically significant effect on the HCV IRES reporter. Error bars, mean ± SD, p (HCV) = 0.037, two-sided Student's t test for paired samples, n = 8 (biological replicates).

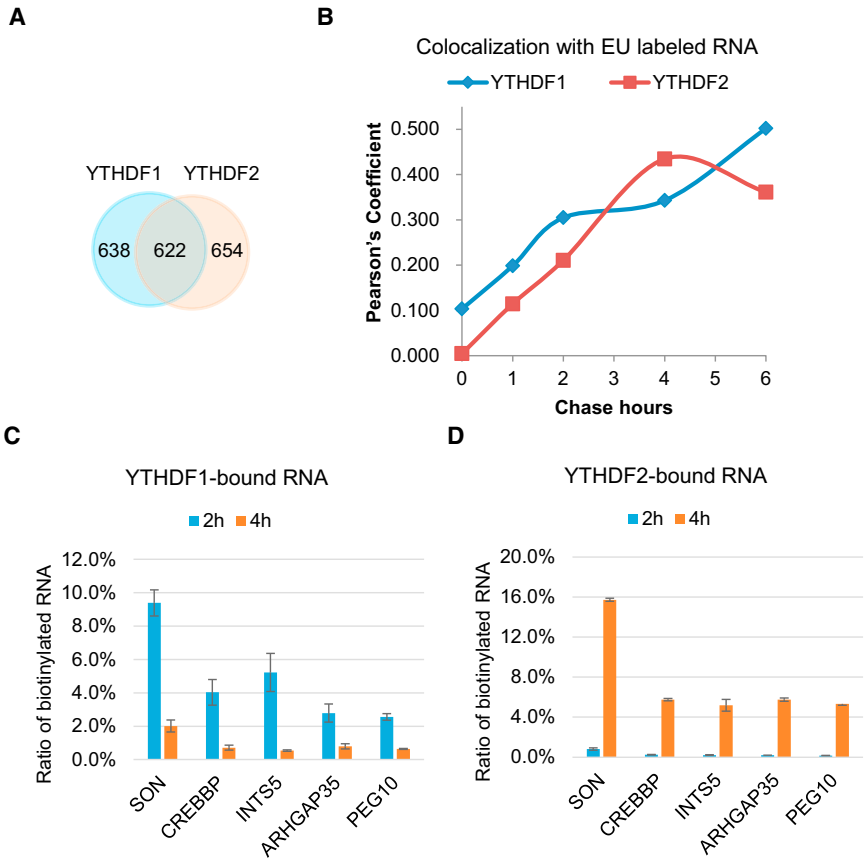

Figure S6. Temporal Order of YTHDF1 and YTHDF2 Binding to Their Common RNA Targets, Related to Figure 6

(A) Overlap of YTHDF1 and YTHDF2 mRNA Targets. Fifty percent of YTHDF1 targets are also YTHDF2 targets.

(B) Nascently transcribed RNAs were labeled by ethynyl-uridine (EU) for 1 hr and the association of YTHDF1 or YTHDF2 with the nascent RNA was characterized by co-localization analysis at indicated time points. The Pearson's coefficient is used to quantify the degree of colocalization between the two fluorophores that were used to label the YTH protein and nascent RNA.

(C and D) After one hour pulse of EU labeling, RNAs that associate with YTHDF1 or YTHDF2 were isolated at 2 or 4 hr post-labeling. YTHDF1- or YTHDF2-bound RNAs (input) were further biotinylated via Click reaction. Nascent (biotinylated) RNAs were captured by Streptavidin-conjugated magnetic beads. For the five YTHDF1 and YTHDF2 common target genes, the amounts of their nascent RNAs in the YTHDF-bound portions were determined by qPCR, and the ratios of their captured amounts to input were calculated and shown in the figure. The results show that at 2 hr post labeling YTHDF1 binds nascent RNAs at a higher percentage than YTHDF2 (C), whereas at 4 hr post-labeling YTHDF2 binds more nascent RNAs than YTHDF1 (D). Therefore, these transcripts encounter with YTHDF1 earlier than YTHDF2. Error bars represent mean ± SD, n = 2 (two technical replicates).

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Supplemental Information

N^6-methyladenosine Modulates Messenger RNA Translation Efficiency

Xiao Wang, Boxuan Simen Zhao, Ian A. Roundtree, Zhike Lu, Dali Han, Honghui Ma, Xiaocheng Weng, Kai Chen, Hailing Shi, and Chuan He

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmid construction and protein expression

Flag-tagged YTHDF1 was cloned from commercial cDNA clones (Open Biosystems) into vector pcDNA 3.0 (EcoRI, XhoI; forward primer, CGTACGAATTCATGGATTACAAGGACGACGATGACAAGATGTCGGCCACCAGCG; reverse primer, CCATACTCGAGTCATTGTTTGTTTCGACTCTGCC. Plasmids with high purity for mammalian cell transfection were prepared with a Maxiprep kit (Qiagen).

λ peptide sequence (MDAQTRRRERRAEKQAQWKAAN) was fused to the C-terminus of N-YTHDF1 by subcloning N-YTHDF1 into pcDNA 3.0 with forward primer containing Flag-tag sequence and reverse primer containing λ peptide sequence (pcDNA-Flag-Y1Nλ, BamHI, XhoI; forward primer, CGTACGGATCCATGGATTACAAGGACGACGATGACAAGATGTCGGCCACCAGCG; reverse primer, CTATGGCTCGAGTCAGTTTGCAGCTTTCCATTGAGCTTGTTTCTCAGCGCGACGCTCACGTC GTCGTGTTTGTGCGTCCATACCGACGCTGGGGGCAGAATT). The construction of λ peptide control that contains an N-terminal Flag tag and GGS spacer (pcDNA-Flag-λ), the N_YTHDF2_λ construct and the tether reporter pmirGlo-Ptight-5BoxB were reported previously (Wang et al., 2014).
IRES-containing tethering reporter plasmids were constructed by inserting the 5BoxB sequence into the 3'UTR of the original IRES reporter plasmids (pRF-HCV, pRF-EMCV, pRF-CrPV. Gifts from Prof. Anne Willis) (Hertz et al., 2013). 5BoxB insert was amplified using the following primer with single restriction site FseI (forward primer, CGATACGGCCGGCCTTCCCTAAGTCCAACTACCAAAC; reverse primer, CTATGGGGCCGGCCATAATATCCTCGATAGGGCCC).

Mammalian cell culture, siRNA knockdown and plasmid transfection

Human HeLa cell line used in this study was purchased from ATCC (CCL-2) and grown in DMEM (Gibco, 11965) media supplemented with 10% FBS and 1% 100 × Pen/Strep (Gibco). HeLa Tet-off cell line was purchased from Clontech and grown in DMEM (Gibco) media supplemented with 10% FBS (Tet system approved, Clontech), 1% 100 × Pen/Strep (Gibco) and 200 μg ml^{-1} G418 (Clontech). AllStars negative control siRNA from Qiagen (1027281) was used as control siRNA in knockdown experiments. *YTHDF1* siRNAs were ordered from Qiagen. (Hs_YTHDF1_1 with target sequence CCGCGTCTAGTTGTTCATGAA; Hs_YTHDF1_8 with target sequence CAGGCTGGAGAATAACGACAA). *YTHDF2* siRNA was reported previously (Wang et al., 2014). METTL3 siRNA was reported previously (Liu et al., 2014). Transfection was achieved by using Lipofectamine RNAiMAX (Invitrogen) for siRNA, and Lipofectamine LTX Plus (Invitrogen) for transfection of one or multiple types of plasmids (tethering assay) following the manufacturer's protocols.

RNA isolation

mRNA isolation for LC-MS/MS: total RNA was isolated from wild-type or transiently transfected cells with TRIzol reagent (Invitrogen). mRNA was extracted using FastTrack® MAG mRNA Isolation Kits (Invitrogen) or Ultracapture mRNA Isolation Kit (Wisegene) followed by further removal of contaminated rRNA using RiboMinus Eukaryote Kit v2 (Ambion). mRNA concentration was measured by NanoDrop. Total RNA isolation for RT–PCR: following the instruction of RNeasy kit (Qiagen) with DNase I digestion step. Ethanol precipitation: to the RNA solution being purified or concentrated, 1/10

volume of 3 M NaOAc, pH 5.5, 1 μl glycogen (10 mg ml^{-1}) and 2.7 volume of 100% ethanol were added, stored at –80°C for 1 h to overnight, and then centrifuged at 15,000*g* for 15 min. After removing the supernatant, the pellet was washed twice by using 1 ml 75% ethanol, and dissolved in the appropriate amount of RNase-free water as indicated.

PAR-CLIP

We followed the previously reported protocol (Wang et al., 2014). Two biological replicates were conducted.

RIP-seq

The procedure was reported previously (Wang et al., 2014). Input mRNAs were prepared by either Poly(A) selection (replicate 1, FastTrack MAG Micro mRNA isolation kit, Invitrogen) or rRNA removal (replicate 2, RiboMinus Eukaryote Kit v2, Ambion). Input mRNA and IP with 150-200 ng RNA of each sample were used to generate the library using TruSeq stranded mRNA sample preparation kit (Illumina).

Ribosome profiling

Ribosome profiling was conducted using ARTseq Ribosome Profiling Kit (Mammalian, Epicentre). Two biological replicates with the use of different YTHDF1 siRNA sequences (siYTHDF1_1 and siYTHDF1_8) were studied to avoid potential off-target effects of specific siRNA. The manufacturer's procedure was followed exactly until library construction. For library construction, the end structures of the RNA fragments for ribosome profiling and mRNA input were repaired using T4 PNK: (1) 3′ de-phosphorylation: RNA (20 μl) was mixed with 2.5 μl PNK buffer and 1 μl T4 PNK, and kept at 37°C for 1 h; (2) 5′-phosphorylation: to the reaction mixture, 1 μl 10 mM ATP and 1 μl extra T4 PNK were added, and the mixture was kept at 37°C for 30 min. The RNA was purified by RNA clean and concentrator (Zymo), and finally dissolved in 10 μl water. The library was constructed by NEBNext small RNA sample preparation kit for high-throughput sequencing (NEB). The sequencing data obtained from ribosome profiling were denoted as ribosome-bound fragments and that from RNA input as mRNA input. Translation efficiency was defined as the ratio of ribosome-protected fragments and mRNA input, which reflected the relative occupancy of 80S ribosome per mRNA species (Ingolia et al., 2009).

RNA-seq for mRNA lifetime measurements

Two 10-cm plates of HeLa cells were transfected with YTHDF1 siRNA or control siRNA at 30% confluency. After 6 h, each 10-cm plate was re-seeded into three 6-cm plates, and each plate was controlled to afford the same amount of cells. After 48 h, actinomycin D was added to 5 μg ml^{-1} at 6 h, 3 h, and 0 h before trypsinization collection. The total RNA was purified by RNeasy kit (Qiagen). Before construction of the library with Tru-seq mRNA sample preparation kit (Illumina), ERCC RNA spike-in control (Ambion) was added to each sample (0.1 μl per sample) proportional to total RNA.

Quantitative translation initiation sequencing (qTI-seq)

We followed the previously reported protocol (Gao et al., 2015). Briefly, two 15-cm plates of cells were harvested in 5 ml cold PBS and collected by centrifugation. 400 μL lysis buffer containing lactimidomycin (LTM; 5 μM) was added to the cell pellets. Cells were then incubated on ice for 10 min. After centrifugation at 4 °C and 12,000g for 15 min, the supernatant was saved for puromycin treatment (25 μM, room temperature 15 min). Puromycin-treated samples were then subjected to the same

procedure as regular ribosome profiling. Libraries were constructed similarly and the data analysis was carried out according to the protocol provided (Gao et al., 2015).

Sequencing data analysis

General pre-processing of reads: All samples were sequenced by illumine Hiseq2000 with single end 100 or 50-bp read length. For libraries that generated from small RNA (PAR-CLIP and ribosome profiling), the adapters was trimmed by using FASTX-Toolkit (Pearson et al., 1997). The deep sequencing data were mapped to Human genome version hg19 by Tophat version 2.0 (Trapnell et al., 2009) without any gaps and allowed for at most two mismatches. RIP and Ribosome profiling were analyzed by DESeq (Anders and Huber, 2010) to generate RPKM (reads per kilobase, per million reads). mRNA lifetime data were analyzed by Cuffdiff version 2.0 (Trapnell et al., 2010) to calculate RPKM.

Data analysis for each experiment: (1) for RIP, enrichment fold was calculated as $\log_2$(IP/input); (2) PAR-CLIP data were analyzed by PARalyzerv1.1 (Corcoran et al., 2011) with default settings. Binding motif was analyzed by HOMER (v4.7) (Heinz et al., 2010); (3) for ribosome profiling, only genes with RPKM >1 were used for analysis and the change fold was calculated as $\log_2$(siYTHDF1/siControl); (4) for qTI-seq, mapped reads that fall within a 51bp window centering at annotated translation initiation sites (TIS) were fed to Cuffdiff 2.0 to calculate differential expression of TIS occupancy between siYTHDF1 and siControl samples, and Figure S3H were plotted with the average of two biological replicates using different siYTHDF1 sequences; (5) for mRNA lifetime profiling: RKPM were converted to attomole by linear-fitting of the RNA spike-in.

The degradation rate of RNA *k* was estimated by

$$\ln\left(\frac{A_t}{A_0}\right) = -kt$$

where *t* is transcription inhibition time (h), A_t and A_0 represent mRNA quantity (attomole) at time *t* and time 0. Two *k* values were calculated: time 3 h versus time 0 h, and time 6 h versus time 0 h. The final lifetime was calculated by using the average of k_{3h} and k_{6h}.

$$t_{\frac{1}{2}} = \frac{2\ln 2}{k_{3h} + k_{6h}}$$

Integrative data analysis and statistics: PAR-CLIP targets were defined as reproducible gene targets among two biological replicates (4,913). RIP targets (1,747) were genes with $\log_2$(IP/input) > 1. The overlap of PAR-CLIP and RIP targets were defined as CLIP+IP targets (1,261). And non-targets (2,822) should meet the conditions of: (1) complementary set of PAR-CLIP targets; (2) RIP enrichment fold <0. For the comparison of PAR-CLIP and m^6A peaks, at least 1 bp overlap was applied as the criteria of overlap peaks. Two biological replicates were conducted for ribosome profiling and mRNA lifetime profiling, respectively. Genes with sufficient expression level (RPKM >1) were subjected to further analysis. The change fold that used in the main text is the average of the two $\log_2$(siYTHDF1/siControl) values. Nonparametric Mann–Whitney *U*-test (Wilcoxon rank-sum test, two sided, significance level = 0.05) was applied in ribosome profiling data analysis as previous reported (Bazzini et al., 2012). Gene Ontology (GO) term analyses were performed by DAVID (Huang et al., 2009). Top 30 terms were then selected for visualizations by the interactive graph function of REVIGO (Supek et al., 2011).

Data accession: all the raw data and processed files have been deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) are accessible under GSE63591. The analysis of Figure 1C utilized data from GSE46705. Figure 6B utilized data from GSE49339.

LC-MS/MS

100-200 ng of mRNA was digested by nuclease P1 (2 U) in 25 μl of buffer containing 10 mM of NH_4OAc (pH=5.3) at 42°C for 2 h, followed by the addition of NH_4HCO_3 (1 M, 3 μl, freshly made) and alkaline phosphatase (0.5 U). 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 μl of the solution was injected into LC-MS/MS. Nucleosides were separated by reverse phase ultra-performance liquid chromatography on a C18 column with on-line mass spectrometry detection using an Agilent 6410 QQQ triple-quadrupole LC mass spectrometer in positive electrospray ionization mode. The nucleosides were quantified by using the nucleoside to base ion mass transitions of 282 to 150 (m^6A), and 268 to 136 (A). Quantification was performed in comparison with the standard curve obtained from pure nucleoside standards running on the same batch of samples. The ratio of m^6A to A was calculated based on the calibrated concentrations (Jia et al., 2011).

RT–PCR

Real-time PCR (RT–PCR) was performed to assess the relative abundance of mRNA. All RNA templates used for RT–PCR were pre-treated with on column DNase I in the purification step. When the examined gene has more than one isoform, only region shared by all isoforms were selected for primer design to evaluate the overall expression of that gene. RT–PCR was performed by using Platinum one-step kit (Invitrogen) with 200–400 ng total RNA template or 10–20 ng mRNA template. *HPRT1* was used as an internal control because: (1) *HPRT1* mRNA does not have m^6A peak based on the m^6A profiling data; (2) *HPRT1* mRNA was not bound by YTHDF1 or YTHDF2 from the PAR-CLIP and RIP sequencing data; (3) *HPRT1* showed relatively invariant expression upon YTHDF1 or YTHDF2 knockdown based on the RNA-seq data; (4) *HPRT1* is a house-keeping gene. The primer sequences of *SON*, *CREBBP*, *RPL30*, *HPRT1*, F-luc and Rluc have been reported previously (Wang et al., 2014).
ARHGAP35: TTAGCAGGGGCATGAATAGG; GATCTCGCTGAGGTCTCCAC.
INTS5: CAGCTCCTGGGACTGGTAAG; ACTTGCCCACCAAACAGTTC.
PEG10: GGCAGTGCATTCACATTGAG; GAGGCACAGGTTCAGCTTTC.

Polysome profiling

Four 15-cm plates of HeLa cells were prepared for each sample (siControl, siYTHDF1 or YTHDF1 stable expression line). Before collection, cycloheximide (CHX) was added to the media at 100 μg ml^{-1} for 2 min. The media was removed, and the cells were collected by cell lifter with 5 ml cold PBS with CHX (100 μg ml^{-1}). The cell suspension was spun at 400*g* for 2 min and the cell pellet was washed once by 5 ml PBS-CHX per plate. 1 ml lysis buffer (10 mM Tris, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 100 μg ml^{-1} CHX, 0.5% Triton-X-100, freshly add 1:100 protease inhibitor, 40 U ml^{-1} SUPERasin) was added to suspend the cells and then kept on ice for 15 min with occasional pipetting and rotating. After centrifugation at 15,000*g* for 15 min, the supernatant (~1.2 ml) was collected and absorbance tested at 260 nm (150–200 $A_{260\,nm}$ ml^{-1}).

A 10/50% w/v sucrose gradient was prepared in a lysis buffer without Triton-X-100. Supernatant from previous step was loaded onto the sucrose gradient and centrifuged at 4°C for 4 h at 27,500 r.p.m. (Beckman, rotor SW28). The sample was then fractioned and analyzed by Gradient Station (BioCamp)

equipped with ECONO UV monitor (BioRad) and fraction collector (FC203B, Gilson). The fractions resulting from sucrose gradient were used for western blotting or RNA extraction. For western blotting, 10 μl of the sucrose fraction were loaded to each well. For RNA analysis, the fractions were then categorized into three main sub-types: non-ribosome portion, 40S–80S and polysome based on western results. They were pooled to isolate total RNA by TRIzol reagent for RT–PCR and mRNA for LC-MS/MS test of the m^6A/A ratio.

Tandem affinity purification of YTHDF1 protein interactome and mass spectrometry identification

We followed the previously reported protocol (Shi et al., 2003). Briefly, stable expression HeLa cell line with double-tagged YTHDF1 (N terminal Flag and HA in tandem) was created by puromycin selection. The control cell line with expression of only tandem Flag and HA peptides was created similarly. Thirty 15 cm plates of YTHDF1 or control HeLa stable line cells were collected by cell lifter and suspended in ice-cold PBS (5 mL per plate). The cell pellets were pooled and washed once with 30 mL cold PBS. 45 mL hypotonic buffer (10 mM Tris, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$) was added into the tube. The pellet was loosened by inverting the tube and swollen after 15 min incubation on ice. The cells were collected at 3000 rpm for 10 min at 4 ^oC. The pellet volume roughly doubled and supernatant was carefully removed by pipet. Equal volume of hypotonic buffer with protease inhibitor and SUPERasin was added. The mixture was homogenizd by Douncing pestle 25 to 30 times and then was spin down at 3500 rpm at 4 ^oC for 15 min. The resulted pellet (nuclear part) was discarded and the supernatant was pre-S100 (cytoplasmic part). The supernatant was transferred into a new tube with 10X buffer (300 mM Tris, pH 7.4, 1.4M KCl, 30 mM $MgCl_2$) at 10% volume of total supernatant volume added and well mixed. The mixture was cleared by ultra-centrifuge at 27,500 rpm for 60 min at 4 ^oC. The resulted supernatant (S100) was dialyzed in 1L BC-100 buffer overnight (20 mM Tris, pH 7.4, 100 mM KCl, 20% glycerol, 0.5 mM DTT). The dialysate was cleared at 14,000 rpm for 20 min at 4^oC and the supernatant was transferred to a new tube. 250 μL anti-Flag magnetic beads (Sigma) were washed by washing buffer (50 mM Tris, pH 7.9, 100 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.1% NP-40, 10% glycerol, protease inhibitor and SUPERasin) three time, combined with the cleared cytoplasmic extract and incubated at 4^oC for 4 h with rotation. The beads were then washed by washing buffer for four times, followed by incubation with 300 μL elution solution containing 3 × Flag peptide (0.5 mg/ml in washing buffer, Sigma) at 4°C for 1 h. During this time, 50 μL anti-HA magnetic beads (Pierce) were washed three times with washing buffer. The eluted samples was incubated with anti-HA beads for 3 h at 4^oC followed by four washes with washing buffer (without SUPERasin). The final protein complex was eluted by 200 μL elution buffer (0.5 mg/mL HA peptide in washing buffer, Sigma), purified by TCA precipitation and trypsin digested. The protein mass spectroscopy was performed by Institutes of Biomedical Sciences at Fudan University, Shanghai.

Protein co-immunoprecipitation and western blot

Stable line cells expressing Flag-HA tagged YTHDF1 or Flag-HA peptide were collected by cell lifter (three 15-cm plates for each), and pelleted by centrifuge at 400 *g* for 5 min. The cell pellet was re-suspended with 2 volumes of lysis buffer (150 mM KCl, 10 mM HEPES pH 7.6, 2 mM EDTA, 0.5% NP-40, 0.5 mM DTT, 1:100 protease inhibitor cocktail, 400 U mL^{-1} RNase inhibitor), and incubated on ice for 10 min. To remove the cell debris, the lysate solution was centrifuged at 15,000 *g* for 15 min at 4°C. The lysate was then split into two portions. One of them was subjected to RNase digestion by adding 100 U RNase A and 50 U RNase T1 into the lysate solution and incubating for 20 min at room

temperature. The resulting supernatant was further cleared by passing through a 0.45-μm membrane syringe filter. While 50 μl of cell lysate was saved as Input, the rest was incubated with the anti-Flag M2 magnetic beads (Sigma) in ice-cold NT2 buffer (200 mM NaCl, 50 mM HEPES pH 7.6, 2 mM EDTA, 0.05% NP-40, 0.5 mM DTT, 200 U mL^{-1} RNase inhibitor) for 4 h at 4°C. Afterwards, the beads was subject to extensive wash with 8 × 1 ml portions of ice-cold NT2 buffer, followed by incubation with the elution solution containing 3 × Flag peptide (0.5 mg ml^{-1} in NT2 buffer, Sigma) at 4°C for 2 h. The eluted samples, saved as IP, were analyzed by western blotting. For IP samples, each lane was loaded with 2 μg IP portion; and the input lane were loaded with 10 μg input portion which corresponded to ~1% of overall input.

Fluorescence microscopy

Fluorescent immunostaining: the protocol of (Kedersha and Anderson, 2007) was followed. Cells were grown in an 8-well chamber (Lab-Tek). After treatment indicated in each experiment, cells were washed once in PBS and then fixed in 4% paraformaldehyde in PBS (Alfa Aesar) at room temperature for 15 min. The fixing solution was removed, and ice-cold methanol was immediately added to each chamber and incubated for 10 min at room temperature. Cells were rinsed once in PBS and incubated with blocking solution (10% FBS with PBST) for 1 h at room temperature under rotation. After that, the blocking solution was replaced with primary antibody (diluted by fold indicated in Antibodies section in blocking solution) and incubated overnight at 4°C. After being washed 4 times with PBST (300 μl, 5–10 min for each wash), secondary antibody (1:300 dilution in PBST) was added to the mixture and incubated at room temperature for 1 h. After washing 4 times with PBST (300 μl, 5–10 min for each wash), Slowfade antifade reagent with DAPI (Invitrogen) was added to the slides for direct imaging.

Image capture and analysis: images were captured by Leica TCS SP2 AOBS Laser Scanning Confocal microscope, analyzed by ImageJ. The colocalization was quantified by JAcoP (ImageJ plug-in) and the Pearson coefficients were gained under Costes' automatic threshold (Bolte and Cordelières, 2006).

Tethering assay

Basic setting: 50 ng reporter plasmid (pmirGlo-pTight-5BoxB) and 450 ng effecter plasmid (λ, N-YTHDF1-λ, N-YTHDF2-λ, or both N-YTHDF1-λ and N-YTHDF2-λ in pcDNA3.0) were used to transfect HeLa cells in each well of six-well plate at 60~80% confluency under doxycycline (100 ng/mL) inhibition. After 6 h, transfection mixture was replaced with fresh media containing doxycycline. After 18 h, each well was trypsin-digested, extensively washed with PBS and re-seeded into 96-well plate (1:20) and 12-well plate (1:2) without doxycycline. 8 hours after reseeding and induction, cells in 96-well plate were assayed by Dual-Glo Luciferase Assay Systems (Promega). Firefly luciferase (F-luc) activity was normalized by *Renilla* luciferase (R-luc) to evaluate translation of the reporter. Samples in 12-well plate were processed to extract total RNA (DNase I digested), followed by RT–PCR quantification. The amount of F-luc mRNA was also normalized by that of R-luc mRNA.

For pulse-induction: 2 h after reseeding, 500 ng/mL doxycycline was added into the wells, ending the 2-hour pulse induction of transcription. Then the translation dynamics was monitored by assaying cells each hour from reseeding (set as $t = 0$) to 8 h after reseeding (except 7 h).

For stress-recovery: 2 h after reseeding, 500 ng/mL doxycycline was added into the wells, ending the 2-hour pulse induction of transcription. At the same time, 1 mM sodium arsenite was also added into the wells, and removed at 3 h, completing the 1-hour stress treatment. The translation dynamics was monitored by assaying cells each hour from reseeding (set as $t = 0$) to 8 h after reseeding (except 7 h).

For translation efficiency evaluation: Protein production was calculated by normalized luciferase signal (F-luc/R-luc) after 8 h post reseeding and induction. mRNA abundance was quantified by qRT-PCR with normalization (F-luc/R-luc) after 8 h post reseeding and induction.

For IRES-containing tethering reporter assay: 5 ng reporter plasmid (pRF-HCV-5BoxB, pRF-EMCV-5BoxB, pRF-CrPV-5BoxB), 45 ng effecter plasmid (λ and N-YTHDF1-λ in pcDNA3.0), and 5 ng control plasmid (pJ7-LacZ) were used to transfect ~ 1.5×10^5 HeLa cells in each well of a 96-well plate. After 24 h half of the cells were assayed by Dual-Glo Luciferase Assay Systems (Promega) to measure F-luc and R-luc activity. The other half of the cells were assayed by Galacto-Light Plus System (Applied Biosystems) to measure the LacZ activity, which was then used to normalize both F-luc and R-luc signals. Finally, F-luc activity was normalized by R-luc to evaluate IRES-dependent expression compared to cap-dependent expression.

Pulse-chase experiment by metabolic labeling of nascent mRNA

Pulse-chase experiment was designed to investigate the sequential binding of YTHDF1 and YTHDF2. Two metabolites, 4-thiouridine (4SU) and 5-ethinyluridine (EU), were each used in the following experiments. They mimic the natural form of uridine and can be incorporated into the newly transcribed nascent mRNA. By following these nascent mRNA and studying the proteins they interact with in temporal order, we could reveal the binding sequence of YTHDF1 and YTHDF2 during the lifetime of an mRNA.

LC-MS/MS test with 4-thiouridine (4SU) labeling: one 15-cm plate of stable line HeLa cells expressing Flag-HA tagged YTHDF1 or Flag-HA tagged YTHDF2 was cultured with 0.5 mM 4SU containing media for 4 h. Then 4SU was removed and media were replaced with fresh ones. Cells were collected after chasing for 2 h or 4 h. Immunopreciptitation of YTH-bound fraction was performed using anti-Flag magnetic beads. RNA bound by YTHDF1 or YTHDF2 was extracted from IP fraction and mRNA was purified accordingly. LC-MS/MS was carried out as previously described. The amount of 4SU in each mRNA portion indicates the extent of association between each YTH protein and nascent mRNA.

Colocalization and qPCR test with 5-ethinyluridine (EU) labeling: one 15-cm plate of stable line HeLa cells expressing Flag-HA tagged YTHDF1 or Flag-HA tagged YTHDF2 was cultured with 0.1 mM EU containing media for 1 h. Then EU was removed and media were replaced with fresh ones. For colocalization study, cells were fixed after chasing for 2 h, 4 h, 6 h or 8h. Then cells were treated with Click-iT RNA Alexa Fluor 594 Imaging Kit (Life Technologies) and imaged accordingly. Colocalization analysis was carried out as described previously (Bolte and Cordelières, 2006; Wang et al., 2014). The Pearson's coefficient is used to quantify the degree of colocalization between two fluorophores. More colocalization indicates more association between the YTH protein and nascent mRNA. For qPCR test, cells were collected after chasing for 2 h or 4 h. Total RNA was isolated and subjected to biotinylation by Click reaction using Click-iT Nascent RNA Capture Kit (Life Technologies) following manufacturer's protocol. The cDNA of both input RNA and captured nascent RNA were synthesized and subjected to qPCR test using qPCR primers for common targets of YTHDF1 and YTHDF2.

Antibodies

The antibodies used in this study were listed below in the format of name (application; catalogue; supplier; dilution fold): Rabbit anti-YTHDF1 (Western; ab99080; Abcam; 1,000). Mouse anti-Flag HRP conjugate (Western; A5892; Sigma; 5000). Goat anti-GAPDH HRP conjugate (Western; A00192;

GeneScript; 15,000). Rat anti-Flag (IF; 637304; Biolegend; 300). Mouse anti-RPS6 (Western; #2317; Cell signaling; 1,000). Rabbit anti-G3BP1 (Western; A302-033A; Bethyl; 2,000). Mouse anti-G3BP1 (IF; sc-365338; Santa Cruz; 200). Goat anti-EIF3B (IF; sc-16377; Santa Cruz Biotech; 100) (Western; 500). Rabbit anti-EIF3A (Western; A302-002A; Bethyl; 2,000). Donkey anti-rat Alexa 488 (IF; A21208; Molecular Probes; 300). Goat anti-rabbit Alexa 647 (IF; A21446; Molecular Probes; 300). Goat anti-mouse Alexa 647 (IF; A21236; Molecular Probes; 300). Donkey anti-goat Alexa 647 (IF; A21447; Molecular Probes; 300).

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