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YTHDF2 recognition of N^1-methyladenosine (m^{1}A)-modified RNA is associated with transcript destabilization

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Abstract

Epitranscriptomic modifications play an important role in RNA function and can impact gene expression. Here, we apply a chemical proteomics approach to investigate readers of N^1-methyladenosine (m^{1}A), a poorly characterized modification on mammalian mRNA. We find that YTHDF proteins, known m^{6}A readers, recognize m^{1}A-modified sequences in a methylation-specific manner. We characterize binding of recombinant YTHDF1/2 proteins to m^{1}A-modified oligonucleotides to demonstrate that these interactions can exhibit comparable affinity to m^{6}A-recognition events, and occur in diverse sequence contexts. Further, we demonstrate YTHDF2 interacts specifically with endogenously modified m^{1}A transcripts. Finally, we deplete cellular YTHDF2 to show that the abundance of m^{1}A-modified transcripts is increased in its absence. Similarly, increasing m^{1}A levels through depletion of ALKBH3, an m^{1}A eraser protein, destabilizes known m^{1}A-containing RNAs. Our results shed light on the function of m^{1}A on mRNA, and provide a mechanistic framework to further evaluate the role of m^{1}A in biological processes.

INTRODUCTION

Chemical modifications on macromolecules play an essential role in biological processes. Post-transcriptional modifications of cellular RNA are widespread throughout biology[1] and represent an evolutionarily conserved strategy to modulate the properties of this central molecule of life. Investigation into the function of modifications occurring on structured RNAs has revealed how these marks can regulate base pairing, nucleic acid folding, and interactions with associated proteins[2–3]. In contrast, there still exist major gaps in our understanding of mRNA modification chemistry and the mechanisms by which these modifications affect biological processes.

Transcriptome-wide mapping of N^6-methyladenosine (m^{6}A), an abundant modification on eukaryotic mRNA,[4–5] has led to the proposal of RNA epigenetics or epitranscriptomics[6–7]. N^6-methyladenosine perturbs RNA structure[8] and functions as an epitope for the recruitment of modification-specific reader proteins[9–10], resulting in the modulation of RNA stability[11],

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ASSOCIATED CONTENT

Supporting Information. The Supporting Information including supplemental figures, experimental methods, oligonucleotide characterization, mass spectrometry proteomics data, and RNA sequencing is available free of charge on the ACS Publications website.

protein translation[12], splicing[13], and nuclear export[14]. Recent studies have demonstrated a role for the m^6A pathway in diverse biology occurring in normal[15–17] and disease[18–19] contexts. The effect of m^6A on mRNA behavior suggests that other modifications[20] may function similarly; however, currently we lack insights into the roles of many modifications.

One such modification is N^1-methyladenosine (m^1A), which has been found on mRNA through transcriptomic and mass spectrometric studies[21–22]. Currently, there exists substantial controversy over the sites and frequency of m^1A modification on mRNA[23–24]. While initial estimates based on m^1A immunoprecipitation and sequencing suggested that thousands of m^1A sites existed[22], recent single-nucleotide mapping approaches have arrived at more conservative numbers ranging from ~15–500[25–26]. The reported single-nucleotide strategies combine immunoprecipitation with m^1A-induced mutations or truncations during reverse transcription (RT) to localize modifications at high resolution. Since the propensity of specific RT enzymes to stall or misincorporate opposite an m^1A site is not fully understood, the conditions and criteria applied to map m^1A are likely responsible for the large discrepancy between studies. In addition, factors such as sequencing depth and RNA preparation could also explain the disparity in called m^1A sites. While our manuscript was in review, a new single-nucleotide approach for m^1A mapping relying upon an engineered RT enzyme was used to identify 215 m^1A sites on mRNA[27]. This number of sites is within the range of earlier estimates, however, only a fraction of them overlap with prior studies suggesting that further work will be needed in order to generate a definitive m^1A map.

Beyond mapping, elucidating m^1A-associated proteins is critical towards understanding its regulation and function. Thus far, the demethylases ALKBH1 and ALKBH3 have been characterized as m^1A erasers[28–29] and TRMT6/61a was shown to install a subset of m^1A mRNA sites[25–26]. Investigation of m^1A readers, however, has lagged behind. Recently, Yang and co-workers used affinity proteomics with an m^1A-modified oligonucleotide to identify YTH-domain proteins (specifically, YTHDF1–3 and YTHDC1) as m^1A readers[30]. This is an intriguing finding given that YTH-domain proteins are well-established m^6A readers. Although the reported m^1A-specific interactions were low affinity (K_d ~ 10–100 μM), this study provides insight into how protein-m^1A recognition could regulate mRNA behavior and warrants further investigation. Recently, we developed an RNA chemical proteomics approach to profile modification-specific readers and anti-readers[10]. Our approach relies upon photocrosslinking with synthetic, diazirine-containing oligonucleotides and enables stringent identification of direct interaction partners. Here, we apply our method to identify m^1A-specific binding proteins. We find that YTHDF proteins bind specifically to m^1A-modified sequences. Interestingly, the related YTH-domain protein YTHDC1 shows no m^1A-specific RNA binding. We further investigate the interactions of recombinant YTHDF1/2 using biophysical assays in order to demonstrate that these binding events are high affinity and occur in diverse sequence contexts. Finally, we show that YTHDF2 binds to endogenous m^1A-modified transcripts and provide evidence that m^1A destabilizes RNA transcripts, thereby demonstrating a role for this mark in post-transcriptional gene regulation.

RESULTS

Chemoproteomic probes for identifying m^{1}A reader proteins

To discover m^{1}A readers, we used our previously reported RNA chemical proteomics method[10]. First, we designed synthetic RNA probes containing m^{1}A, a photo-activatable diazirine-modified uridine residue (5-DzU), and biotin as an affinity handle for protein enrichment (Figure 1a). We embedded m^{1}A within a purine-rich consensus motif (GAGGA*G) generated from a subset of m^{1}A sites enriched upon knockdown of the m^{1}A eraser ALKBH3[21]. This motif is also similar to one recently identified using an evolved reverse transcriptase enzyme for m^{1}A mapping[27]. To avoid possible Dimroth rearrangement of m^{1}A to m^{6}A[31], we synthesized m^{1}A-containing probe **1** as well as the unmethylated control probe **2** (Figure 1a, Table S1) using ultra-mild phosphoramidite chemistry.

Next, we applied our probes to identify m^{1}A readers in HeLa cells. As described previously[10], HeLa cell lysate was incubated with either probe **1** or **2** and exposed to 365 nm UV light (Figure 1b). Cross-linked RNA-protein complexes were next enriched with streptavidin, eluted with mild RNase treatment, and analyzed by LC-MS/MS to identify peptides and quantify abundance by spectral counting. Three independent replicates were performed, and a volcano plot was generated to represent the data (Figure 1c).

Analysis of our proteomics data revealed a number of proteins exhibiting significant ($p < 0.05$) and probe-specific photocrosslinking behavior. Among these hits, we found proteins from the YTH-domain family[32], which are well-established m^{6}A reader proteins. In particular, YTHDF1 and YTHDF2 exhibited 27–32-fold preference for the m^{1}A probe over the unmethylated probe (Figure 1c). The identification of these proteins in both our study and by Wang and co-workers[30], using different m^{1}A-containing sequences and different enrichment approaches speaks to the generality of m^{1}A-recognition by YTHDF proteins. In contrast to the earlier report[30], YTHDC1, another YTH-domain protein that binds to m^{6}A-modified sequences, was not identified in our data indicating that m^{1}A recognition is likely unique to the YTHDF clade of YTH-domain proteins. In addition, we also found adenylate kinase (AK1), enriched 5-fold against the m^{1}A probe. While AK1 is primarily an enzyme involved in nucleotide metabolism, it has been reported to associate with RNA *in vitro*[33]. We did not observe additional m^{1}A specific proteins that met our cutoff (p-value <0.05 and >4-fold enrichment). On the left side of the volcano plot, we identified several putative anti-readers, proteins that bind specifically to unmodified sequences. These included ZNF335 and KIAA1522, enriched 5-fold and 10-fold by the unmethylated probe, respectively (Figure 1c). Our data did not reveal any interactions between unmethylated sequences and Ku70/80 or ILF2/3 as reported previously[30], which may reflect that these interactions are sequence specific or indirect. Due to the strong connection between YTHDF proteins and mRNA regulation, we chose to focus on these proteins for further study.

YTHDF1/2 bind preferentially to m^{1}A-modified transcripts

To further investigate our findings, we biochemically characterized the interaction between m^{1}A-containing RNA and YTHDF1/2 proteins. For *in vitro* binding assays, we synthesized a new set of RNA oligonucleotides, **3** and **4** (Table S1), with identical sequence to probes **1**

and **2** except lacking the 5-DzU photocrosslinker and containing 3' fluorescein instead of biotin. We also generated recombinant YTH domains from YTHDF1 and YTHDF2, which have been shown to interact specifically with m^6A-modified RNA oligonucleotides[3435]. Next, we performed electrophoretic mobility shift assay (EMSA) to measure binding of each protein to the m^1A-containing and corresponding unmethylated RNA oligonucleotides. We observed tight-binding of YTHDF1 and YTHDF2 to the m^1A-modified oligonucleotide (Figure 2a–2d, K_d = 0.13 +/− 0.047 μM for YTHDF1:probe **3**; K_d = 0.39 +/− 0.030 μM for YTHDF2:probe **3**). These values are similar to those reported for YTHDF1/2 binding to m^6A-modified sequences [34–35]. In contrast, both proteins interacted more weakly with the corresponding unmethylated oligonucleotides, as characterized by a ~5-fold higher K_d for the YTHDF1:probe **4** complex and a >3.5-fold higher K_d for YTHDF2:probe **4** binding (saturation binding was not reached at the highest protein concentration tested) (Figure 2a–2d). Further, we confirmed the binding preference of YTHDF1 and YTHDF2 for the m^1A-containing sequence by microscale thermophoresis (MST) assay, which showed similar trends to those measured by EMSA (Figure S1 and Figure 2a–2d).

We next investigated the generality of m^1A recognition by YTHDF1/2. We synthesized fluorescein-labeled oligonucleotides **5** and **6** (Table S1), which contain m^1A/A within an entirely different sequence context (UUUUA*AA) from the A/G-rich motif interrogated above. This motif was also identified as a cellular substrate of ALKBH3[21]. We measured the affinity of YTHDF1 and YTHDF2 for probes **5** and **6** by EMSA and observed tight binding for the m^1A-containing probe **5** (Figure 2e–2h, K_d = 0.15 +/− 0.044 μM for YTHDF1:probe **5**; Kd = 0.35 +/− 0.048 μM for YTHDF2:probe **5**), similar to the measured affinity of YTHDF1/2 for the A/G-rich probe **3** sequence. For both YTH-domain proteins, we observed a >5-fold reduction in affinity for the interaction with the corresponding unmethylated oligo **6** (Fig. 2e–2h) with binding failing to reach saturation with YTHDF2 (Figure 2h). Taken together, our results demonstrate that YTHDF1 and YTHDF2, canonical m^6A-reader proteins, can also bind to diverse m^1A-modified sequences in a methylation-specific fashion.

YTHDC1 does not recognize m^1A-modified sequences

Interestingly, YTHDC1, another YTH-domain containing m^6A reader[36], was not enriched by m^1A in our proteomics data. To explore this, we measured the affinity of recombinant YTHDC1 for oligos **3**/**4** and **5**/**6** using EMSA. In both sequence contexts, we observed a slight preference for YTHDC1 binding to the unmethylated sequence, although the affinities were modest and binding curves did not reach saturation (Figure 3a–3d). In contrast, YTHDC1 exhibited high-affinity binding to probes **7** and **8**, analogous sequences containing a single m^6A residue (Figure 3a–3d and Table S1, K_d = 0.068 +/− 0.033 μM for YTHDC1:probe **7**; K_d = 0.12 +/− 0.010 μM for YTHDC1:probe **8**), with 27–60-fold lower K_d than what was measured for binding to the corresponding m^1A-modified sequences. As a related comparison, we also measured the affinity of YTHDF1/2 for the m^6A-modified probes **7** and **8**. While binding to these sequences was tighter than to the analogous m^1A sequences (probes **3** and **5**) (Figure S2), the difference in affinity was much less drastic than for YTHDC1. Moreover, as these sequences do not represent canonical m^6A-modified motifs, they may not exist in this form in the cell. The large difference in binding of YTHDC1 to the m^6A- and m^1A-modified sequences also strongly suggests that our

synthetic m^1A-containing oligonucleotides have not undergone Dimroth rearrangement, a base-catalyzed chemical transformation that would convert m^1A to m^6A [31]. We further confirmed that the extent of Dimroth rearrangement in our m^1A probes was minimal using LC-MS/MS (Figure S3) Altogether, our binding assay results demonstrate that among YTH-domain protein, m^1A recognition is specific to YTHDF1/2.

YTHDF2 promotes the degradation of m^1A-containing transcripts in cells

After characterizing binding between YTHDF2 and m^1A- RNA *in vitro*, we next investigated the biological effect of YTHDF2-m^1A-binding in human cells. YTHDF2 has been shown to promote the destabilization of m^6A-modified RNA by direct binding of these transcripts through its C-terminal YTH-domain and recruitment to processing bodies (sites of mRNA decay) via its P/Q/N-rich N-terminal domain[11]. Moreover, the simple recruitment of YTHDF2 to mRNA transcripts (independent of m^6A modification) is sufficient to facilitate their degradation[37]. Therefore, we hypothesized that m^1A-directed YTHDF2 recruitment could also lead to degradation of m^1A-modified transcripts (Figure 4a and 4b). To test this, we first needed to establish that YTHDF2 binds to endogenous m^1A-modified RNAs. Towards this end, we generated a stable cell line containing 3xFLAG-tagged YTHDF2 (Fig. S7) and performed RNA immunoprecipitation coupled with LC-MS/MS (RIP-LC-MS/MS). In brief, YTHDF2-RNA complexes were immunoprecipitated from cells and RNA nucleosides were nalyzed using QQQ-LC-MS. We quantified the levels of m^1A/A and found a ratio of 0.19%, well above reported values for total polyadenylated RNA[21] (Figure 4c and Figure S5). As a control, we performed the same experiment with G3BP1, an abundant RNA binding protein with no known affinity for m^1A and found the m^1A/A levels to be ~7-fold lower (Figure 4c), consistent with a preference of YTHDF2 for binding to m^1A-modified transcripts.

We next investigated regulation of annotated m^1A-modified transcripts by YTDHF2. For this purpose, we employed several published datasets including transcriptome-wide abundance data upon YTHDF2 knockdown[11] and the three reported single-nucleotide m^1A maps[25–27]. Among m^1A-modified transcripts found in all 3 maps, only *PRUNE* and *BRD2* were identified in the YTHDF2 RNAseq dataset and both transcripts increase in abundance upon YTHDF2 knockdown (Figure S8). Analysis of m^1A-modified transcripts shared between the Zhou *et al.*[27] and Li *et al.*[26] maps for which YTHDF2 knockdown data exists identified 8 transcripts (*ARHGDIA, ACTB, ILF3, PRPF8, HNRNPU, BRD2, PRUNE,* and *MAVS*) – of these 7 were upregulated upon YTHDF2 knockdown (Figure S8). Further, we performed analysis of candidate m^1A transcripts upon YTHDF2 siRNA knockdown using RT-qPCR. We focused on transcripts containing m^1A and lacking m^6A (Figure 4b) to avoid the possibility that the observed effects would be due to the presence of m^6A, as well as those that were annotated YTHDF2 substrates based upon PAR-CLIP analysis[11]. We picked three transcripts (CHPF2, ARHGAP35, and DCAF8) to characterize by RT-qPCR. Consistent with the previous study[11], upon siRNA knockdown of YTHDF2 (Figure S4), we observed a significant 1.5–2-fold increase (relative to cells transfected with a scrambled siRNA oligo) in transcript abundance for CHPF2 and DCAF8, indicating a role of YTHDF2 in their destabilization (Figure 4d).

Finally, we took advantage of the known role of ALKBH3 as an m^1A eraser enzyme[21] to probe the effect of m^1A on transcript stability. We depleted ALKBH3 (which should concomitantly increase m^1A levels) in HeLa cells using siRNA-mediated knockdown (Figure S6) and analyzed RNA transcript abundance compared to a scrambled siRNA control. We interpreted our results in the context of the Zhou *et al.*[27], Li *et al.*[26], and Safra *et al.*[25] m^1A maps. For the first two maps, which identify between 200–500 sites on mRNA, we found a statistically significant decrease in transcript abundance upon ALKBH3 knockdown (Figure 4e), suggesting that m^1A destabilizes RNA transcripts, likely through a YTHDF2-dependent mechanism. We did not observe an overall decrease in abundance for the handful of transcripts from the Safra *et al.* study[25], perhaps because the m^1A levels on these transcripts do not significantly increase upon ALKBH3 knockdown.

DISCUSSION

In this manuscript, we use chemical proteomics to profile the m^1A interactome in HeLa cells. Our results demonstrate that YTHDF proteins recognize m^1A-modified RNA in diverse sequence contexts. Further, we show that m^1A-modified transcripts in cells exhibit lower stability, likely through YTHDF2-mediated mRNA decay, as has been demonstrated for m^6A-modified mRNAs[11].

YTH-domain proteins are established readers of m^6A-modified mRNA transcripts[32], but their interactions with other methylated bases remain largely unexplored. Wang and co-workers[30] recently reported direct binding between YTH-domain proteins including YTHDF1, YTHDF2, and YTHDC1 and an m^1A-modified RNA oligonucleotide. Our study supports and expands upon their findings with regards to YTHDF1/2, however, we were unable to observe m^1A-specific binding by YTHDC1. The observed discrepancy may be due to sequence context effects or specific assay conditions. Notably, Wang and co-workers found that YTHDC1 binds considerably less tightly to their m^1A-modified oligonucleotide than YTHDF1/2, which is in line with our observations. How YTHDF proteins can bind to both m^6A and m^1A residues is still unclear. In YTHDF2, the m^6A methyl group is accommodated in a hydrophobic cavity lined with aromatic Trp residues [34], therefore we speculate that the positively charged m^1A may engage in cation-π interactions with these amino acid side chains. While YTHDC1 also possesses a similar aromatic cage [36], studies examining the substrate specificity of YTHDC1 in comparison to YTHDF1/2 have found that YTHDC1 has a stronger preference for specific nucleotides surrounding the m^6A site [38–39], which may preclude the recognition of an alternate substrate conformation required for accommodation of the m^1A residue. Interestingly, we also find substantial affinity between YTHDF1/2 and unmethylated sequences, suggesting that YTHDF1/2 may have a broad role in modulating mRNA translation and metabolism in both a methylation dependent and independent fashion. The ability of YTH-domain proteins to bind diversely modified transcripts will likely depend on the relative concentrations of these molecules in cells, as well as additional mechanisms governing RNA and protein localization. Further, while our biochemical studies provide insight into preferences for different marks, they likely do not fully reflect the native interactions, which may exhibit additional selectivity for adenosine methylation. Additional work will be necessary to elucidate the principles of substrate recognition by these proteins in their native context.

We provide evidence indicating that m^1A destabilizes mRNA transcripts. Combined with our biochemical analysis of YTHDF2-m^1A binding, this suggests that YTHDF2 recognizes and facilitates the degradation of m^1A-modified RNA, likely in a manner similar to its role in m^6A RNA turnover. We cannot exclude the possibility that other mechanisms exist for the clearance of m^1A-modified transcripts, however these mechanisms remain as yet unknown.

Mapping m^1A sites on eukaryotic mRNA has presented a considerable challenge to the field[24]. While there is still a lack of clarity on the exact number and frequency of m^1A modification sites in mammalian cells, we are encouraged that among the transcripts found by multiple studies, several exhibit YTHDF2-dependent abundance. Just as well, our biochemical finding that YTDHF proteins can recognize diverse m^1A-modified sequence motifs suggests that this is a general phenomenon. While more robust approaches to m^1A sequencing will be required in order to definitively characterize the m^1A epitranscriptome, our work demonstrates the functional potential of m^1A on mRNA through the recruitment of YTHDF reader proteins, providing support for the regulatory role of this modification in mRNA biology.

METHODS

General Experimental Procedures.

A complete description of methods is given in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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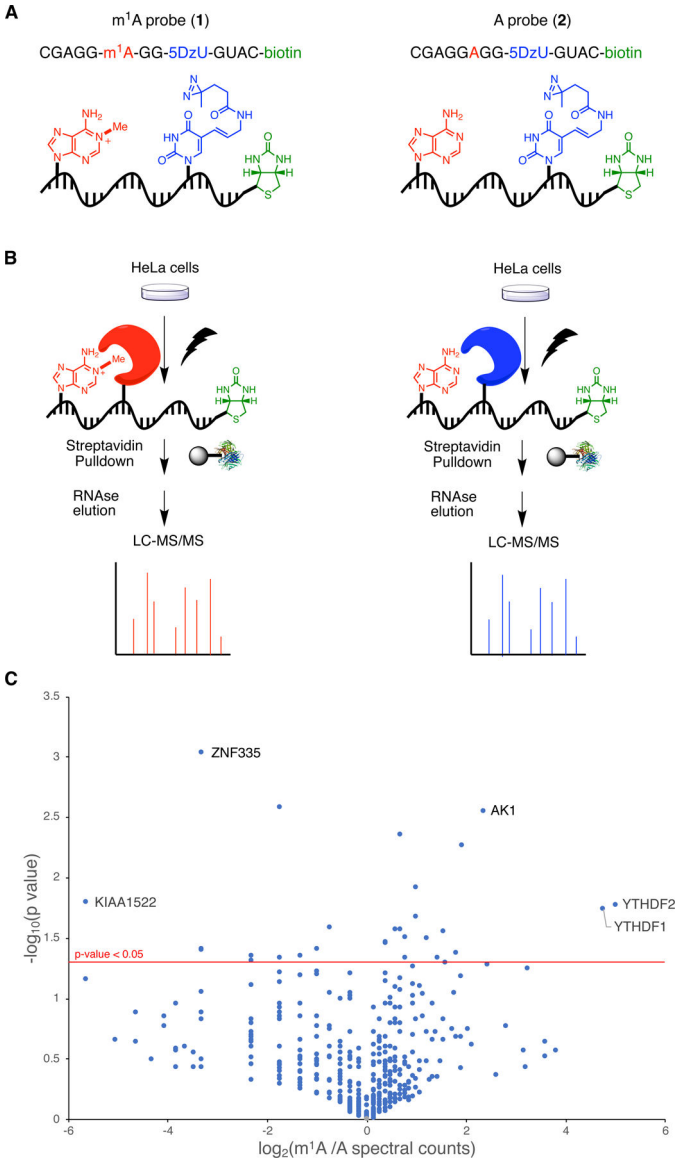

Figure 1.

Chemoproteomic profiling of the m^{1}A interactome. a) Photocrosslinking RNA probes used in this work. b) Schematic for comparative proteomics workflow. c) Volcano plot of protein enrichment from m^{1}A proteomics experiments.

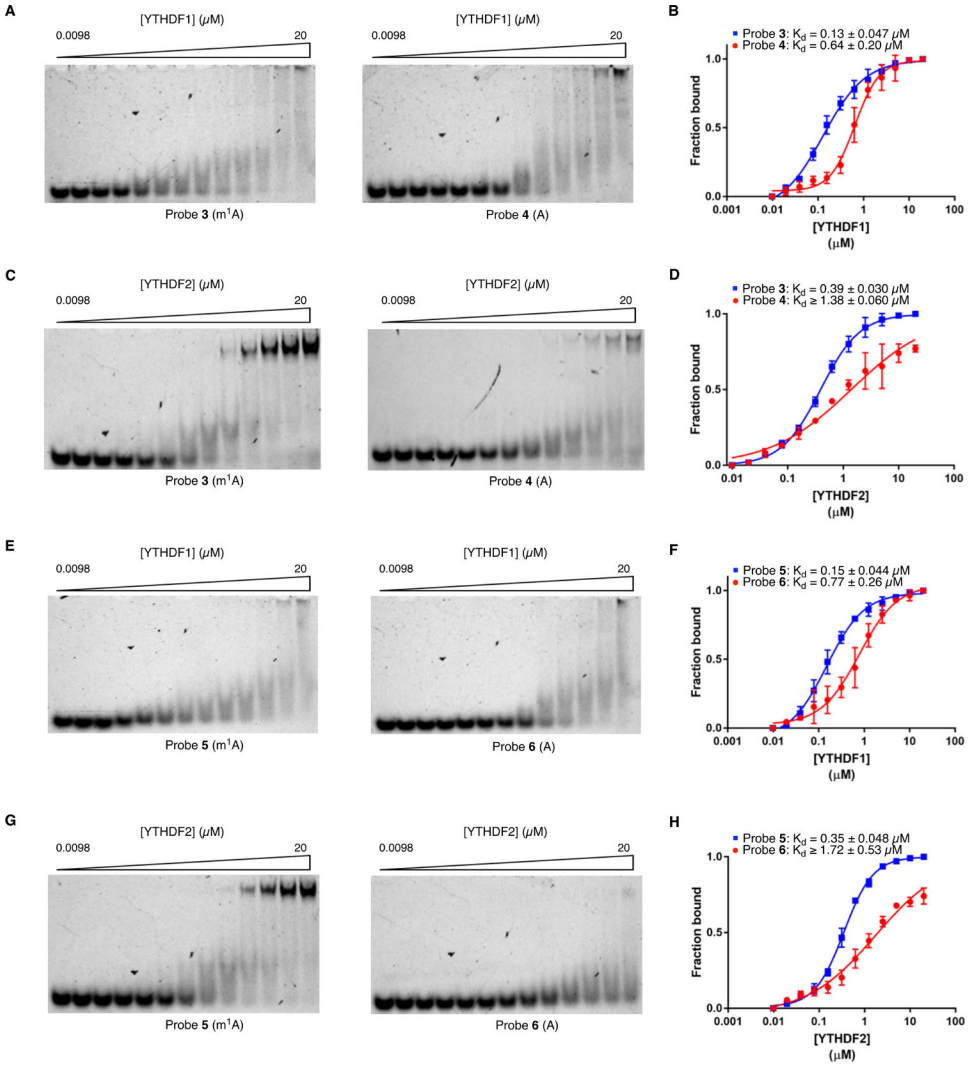

Figure 2.

Characterization of YTHDF1/2-m^{1}A binding. **a)** EMSA for YTHDF1 and probe **3**/**4**. **b)** Quantification of YTHDF1-probe **3**/**4** binding. **c)** EMSA for YTHDF2 and probes **3** /**4**. **d)** Quantification of YTHDF2-probe **3**/**4** binding. **e)** EMSA images YTHDF1 and probes **5**/**6**. **f)** Quantification of YTHDF1-probe **5**/**6** binding. **g)** EMSA for YTHDF2 and probes **5**/**6**. **h)** Quantification of YTHDF2-probe **5**/**6** binding.

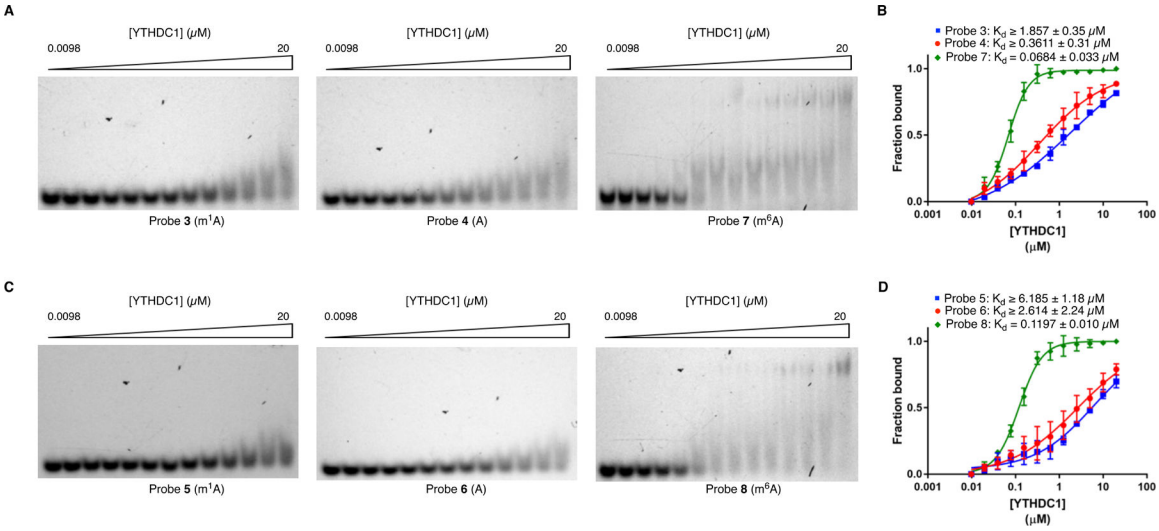

Figure 3.

Characterization of YTHDC1-m^{1}A/m^{6}A binding. **a)** EMSA for YTHDC1 and probes **3**, **4**, or **7**. **b)** Quantification of YTHDC1-probe **3**/**4**/**7** binding. **c)** EMSA for YTHDC1 and probes **5**, **6**, or **8**. **d)** Quantification of YTHDC1-probe **5**/**6**/**8** binding.

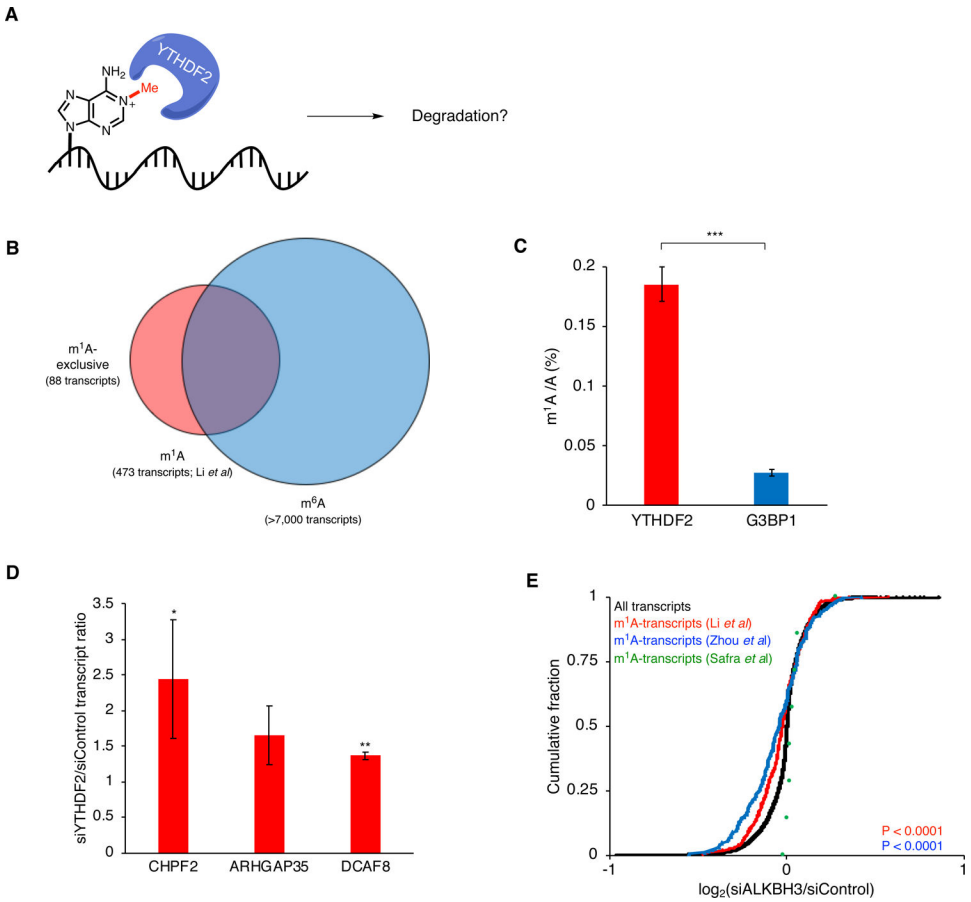

Figure 4.

YTHDF2-m^{1}A regulation in living cells. **a)** Model for YTHDF2-m^{1}A-mediated regulation of transcript stability. **b)** Venn diagram to classify transcripts as m^{1}A-exclusive, m^{6}A-exclusive, and both m^{1}A- and m^{6}A-modified. **c)** LC-MS/MS quantification of m^{1}A levels in RNA enriched by immunoprecipitation of 3xFLAG-tagged YTHDF2 or G3BP1. **d)** Relative abundance of exclusive m^{1}A-modified transcripts upon YTHDF2 knockdown. **e)** Cumulative distribution of transcript abundance upon ALKBH3 knockdown (*, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$).