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Chemoproteomic Approaches to Studying RNA Modification-Associated Proteins

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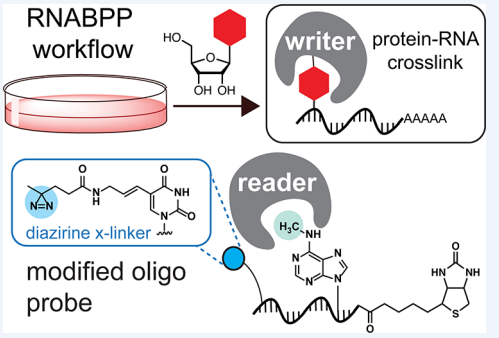
CONSPECTUS: The function of cellular RNA is modulated by a host of post-transcriptional chemical modifications installed by dedicated RNA-modifying enzymes. RNA modifications are widespread in biology, occurring in all kingdoms of life and in all classes of RNA molecules. They regulate RNA structure, folding, and protein−RNA interactions, and have important roles in fundamental gene expression processes involving mRNA, tRNA, rRNA, and other types of RNA species. Our understanding of RNA modifications has advanced considerably; however, there are still many outstanding questions regarding the distribution of modifications across all RNA transcripts and their biological function. One of the major challenges in the study of RNA modifications is the lack of sequencing methods for the transcriptome-wide mapping of different RNA-modification structures. Furthermore, we lack general strategies to characterize RNA-modifying enzymes and RNA-modification reader proteins. Therefore, there is a need for new approaches to enable integrated studies of RNA-modification chemistry and biology.



In this Account, we describe our development and application of chemoproteomic strategies for the study of RNA-modification-associated proteins. We present two orthogonal methods based on nucleoside and oligonucleotide chemical probes: 1) RNA-mediated activity-based protein profiling (RNABPP), a metabolic labeling strategy based on reactive modified nucleoside probes to profile RNA-modifying enzymes in cells and 2) photo-cross-linkable diazirine-containing synthetic oligonucleotide probes for identifying RNA-modification reader proteins.

We use RNABPP with C5-modified cytidine and uridine nucleosides to capture diverse RNA-pyrimidine-modifying enzymes including methyltransferases, dihydrouridine synthases, and RNA dioxygenase enzymes. Metabolic labeling facilitates the mechanism-based cross-linking of RNA-modifying enzymes with their native RNA substrates in cells. Covalent RNA−protein complexes are then isolated by denaturing oligo(dT) pulldown, and cross-linked proteins are identified by quantitative proteomics. Once suitable modified nucleosides have been identified as mechanism-based proteomic probes, they can be further deployed in transcriptome-wide sequencing experiments to profile the substrates of RNA-modifying enzymes at nucleotide resolution. Using 5-fluorouridine-mediated RNA−protein cross-linking and sequencing, we analyzed the substrates of human dihydrouridine synthase DUS3L. 5-Ethynylcytidine-mediated cross-linking enabled the investigation of ALKBH1 substrates. We also characterized the functions of these RNA-modifying enzymes in human cells by using genetic knockouts and protein translation reporters.

We profiled RNA readers for N^6-methyladenosine (m^6A) and N^1-methyladenosine (m^1A) using a comparative proteomic workflow based on diazirine-containing modified oligonucleotide probes. Our approach enables quantitative proteome-wide analysis of the preference of RNA-binding proteins for modified nucleotides across a range of affinities. Interestingly, we found that YTH-domain proteins YTHDF1/2 can bind to both m^6A and m^1A to mediate transcript destabilization. Furthermore, m^6A also inhibits stress granule proteins from binding to RNA.

Taken together, we demonstrate the application of chemical probing strategies, together with proteomic and transcriptomic workflows, to reveal new insights into the biological roles of RNA modifications and their associated proteins.

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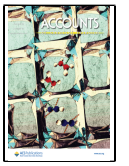

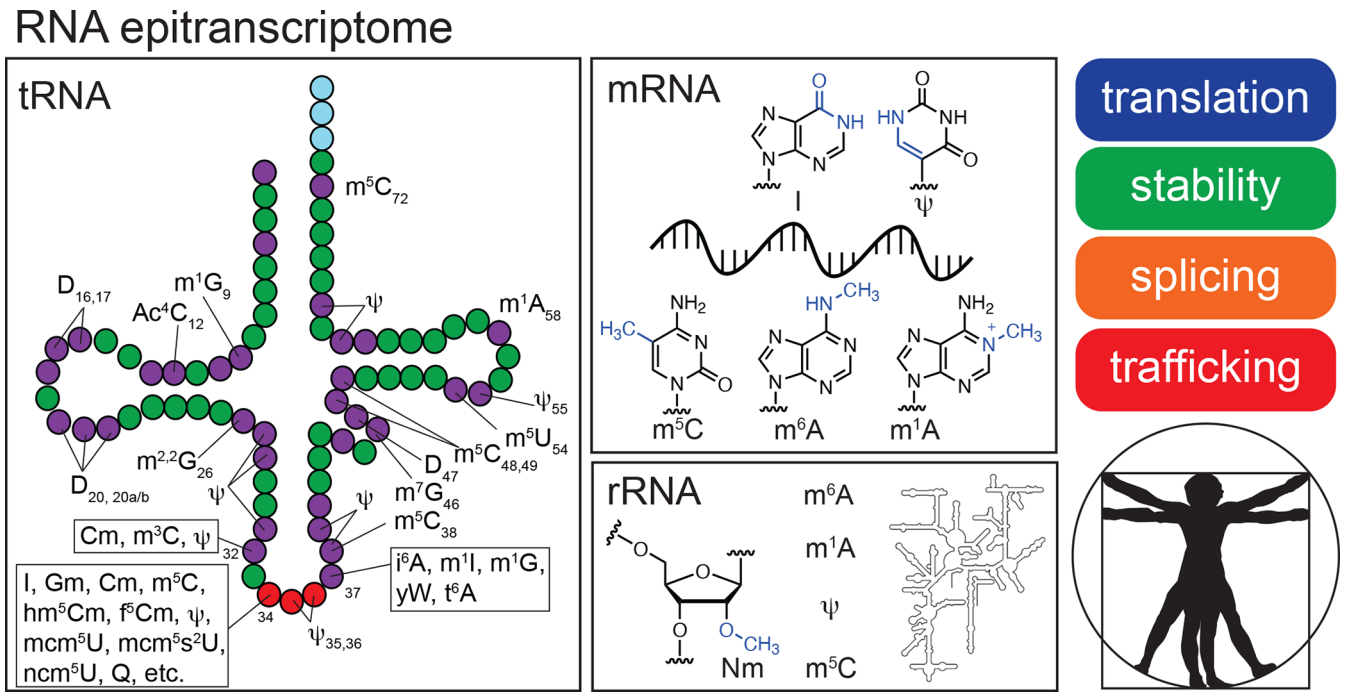

Figure 1. Major RNA modifications on tRNA, rRNA, and mRNA.

using metabolic labeling with 5-fluoropyrimidine nucleosides for RNA-modifying enzyme discovery and the first characterization of human DUS3L.

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1. INTRODUCTION

RNA modifications play an important role in regulating RNA function in biology. Over 150 different post-transcriptional modifications of RNA have been described,[5] and modifications are found in all kingdoms of life and across all classes of RNA (Figure 1). RNA modifications have roles in a myriad of processes including mRNA splicing, export, translation, and metabolism.[6] Modifications on noncoding RNAs, such as tRNA and rRNA, impact RNA structure, assembly, and processing and affect protein translation efficiency and fidelity.[7] Less abundant noncoding RNA species including miRNAs and lncRNAs are also post-transcriptionally modified. Whereas the study of RNA modifications began in the 1950s with the discovery of pseudouridine,[8] the "fifth nucleotide", there are still major gaps in our understanding of the transcriptome-wide distribution of RNA modifications and their role in RNA biochemistry and biological processes.

The most heavily modified RNA species are tRNAs, containing on average ∼13 modified nucleotides out of a total of 76[7] (Figure 1). tRNAs function as adaptor molecules in protein synthesis, and modifications can dynamically impact protein translation through the regulation of tRNA structure, stability, and folding as well as codon−anticodon base pairing and the recognition of tRNA by aminoacyl-tRNA synthetase enzymes. Although tRNA modifications have been better studied than modifications on other types of RNA, most investigation has been performed in model organisms such as *E. coli* and yeast, and the homologous mammalian tRNA modifications and tRNA-modifying enzymes are still relatively poorly characterized. Importantly, the dysregulation of tRNA modifications has been implicated in a host of human diseases known as tRNA modopathies.[9] mRNAs also contain multiple different types of modifications,[6] and more recent studies have illuminated the importance of modifications such as N^6-methyladenosine (m^6A), pseudouridine (ψ), and inosine (I), among others, in mRNA biology.

The study of RNA modifications is enabled by technologies for mapping modification sites and characterizing RNA-modification-associated proteins including RNA-modifying enzymes ("writers" and "erasers") responsible for regulating modification levels and RNA-binding proteins that recognize modified RNAs ("readers") and modulate their behavior. As many RNA modifications do not perturb Watson−Crick base pairing and are therefore undetectable to common nucleic acid sequencing platforms, modification-specific sequencing methods,[10] including antibody enrichment or chemoenzymatic

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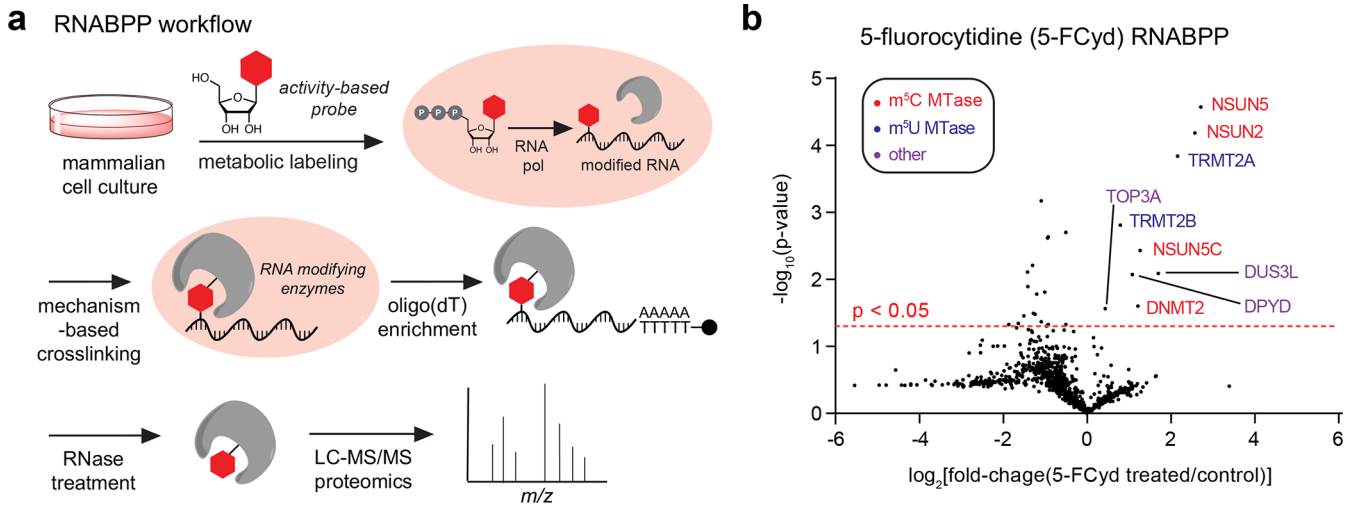

Figure 2. Activity-based profiling of RNA-modifying enzymes using RNABPP. (a) An activity-based cross-linker is fed to cells and incorporated into cellular RNA though metabolic labeling. RNA-modifying enzymes are cross-linked with their endogenous substrates. mRNA-specific enzymes are isolated with oligo(dT) enrichment, released by RNase digestion, and identified by LC-MS/MS proteomics. (b) Volcano plot showing distinct classes of RNA-modifying enzymes discovered in the 5-FCyd-RNABPP experiment. Adapted from ref 1 with permission from Springer Nature, copyright 2021.

approaches, combined with nucleic acid mass spectrometry are necessary to decipher modifications. Furthermore, matching modifications and RNA-modifying enzymes and identifying RNA modification readers are critical to uncovering the function of RNA modifications in biology. In this Account, we describe our efforts to develop chemical probes that can be applied in proteomic workflows to broadly profile RNA-modifying enzymes and RNA modification reader proteins in an unbiased manner. Together with these chemoproteomic approaches, we also develop modification-specific transcriptome-wide sequencing methods and explore the biological consequences of depleting RNA-modifying enzymes and RNA modification readers.

2. ACTIVITY-BASED PROFILING OF RNA PYRIMIDINE-MODIFYING ENZYMES

2.1. RNA-Modifying Enzyme Discovery and ABPP

The study of RNA modifications and the corresponding RNA-modifying enzymes that regulate these modifications go hand-in-hand. Historically, the identification of RNA modifications and RNA-modifying enzymes has been disjointed, as many modified nucleotide structures were elucidated decades before[11] the first characterization of the relevant writer enzymes. As a result, functional studies on the biological role of RNA modifications have lagged far behind the characterization of modified nucleotide structures. Furthermore, whereas most of the RNA-modifying enzymes in single-celled model organisms such as *E. coli* and yeast have now been identified,[12] there are still major gaps in the characterization of RNA writer/eraser enzymes in higher eukaryotes. Current approaches for identifying and characterizing enzymes rely upon low-throughput screening or assignment based on homology to known RNA-modifying enzymes.[13−15]

Activity-based protein profiling (ABPP) has emerged as a versatile strategy for the proteome-wide study and discovery of enzymes in their native context as well as for target ID and small-molecule screening.[16,17] ABPP probes typically contain multiple elements including a recognition motif for the target enzyme family, a reactive mechanism-based "warhead" that forms a covalent bond with the enzyme, and a reporter group such as a fluorophore or affinity handle for detection and/or enrichment. The design of ABPP probes provides mechanistic insight into enzyme activity, which facilitates the downstream characterization of captured enzymes. Over the last ∼20 years, the ABPP strategy has undergone significant evolution and adaptation and has been successfully applied to a diverse array of enzyme classes. In proteome-wide analyses using Cys-reactive ABPP probes[18−20] or photoreactive ABPP probes based on *S*-adenosyl homocysteine (SAH),[21] known and putative RNA-modifying enzymes including methyltransferases, pseudouridine synthases, dihydrouridine synthases, and adenosine deaminases have been identified. However, dedicated mechanism-based ABPP probes to selectively capture RNA-modifying enzymes have not been reported. This is likely due to the diversity of RNA modification structures (which require comparably diverse enzyme chemistries) as well as the challenge of developing small molecules that can mimic protein−nucleic acid molecular recognition events. Leveraging ABPP technology to study RNA-modifying enzymes should provide new insights into this important class of proteins.

2.2. RNA-Mediated Activity-Based Protein Profiling (RNABPP)

We developed RNA-mediated activity-based protein profiling (RNABPP) as a strategy to profile RNA-modifying enzymes and their substrates in cells or whole organisms[1] (Figure 2a). Rather than employing a small-molecule ABPP probe, we envisioned that mechanism-based reactive groups could be incorporated into cellular RNA through metabolic labeling with modified nucleosides. In this manner, the cell would synthesize its own activity-based probe by metabolizing the modified nucleoside to the corresponding modified nucleotide triphosphate (NTP) and incorporating the modified NTP into native transcripts during transcription. Next, covalent mRNA-enzyme complexes could be isolated from cells by oligo(dT) enrichment[22,23] (performed under denaturing conditions), followed by the identification of proteins by mass spectrometry-based proteomics. In a complementary workflow, the same activity-based nucleoside probe could be used for cross-linking and immunoprecipitation

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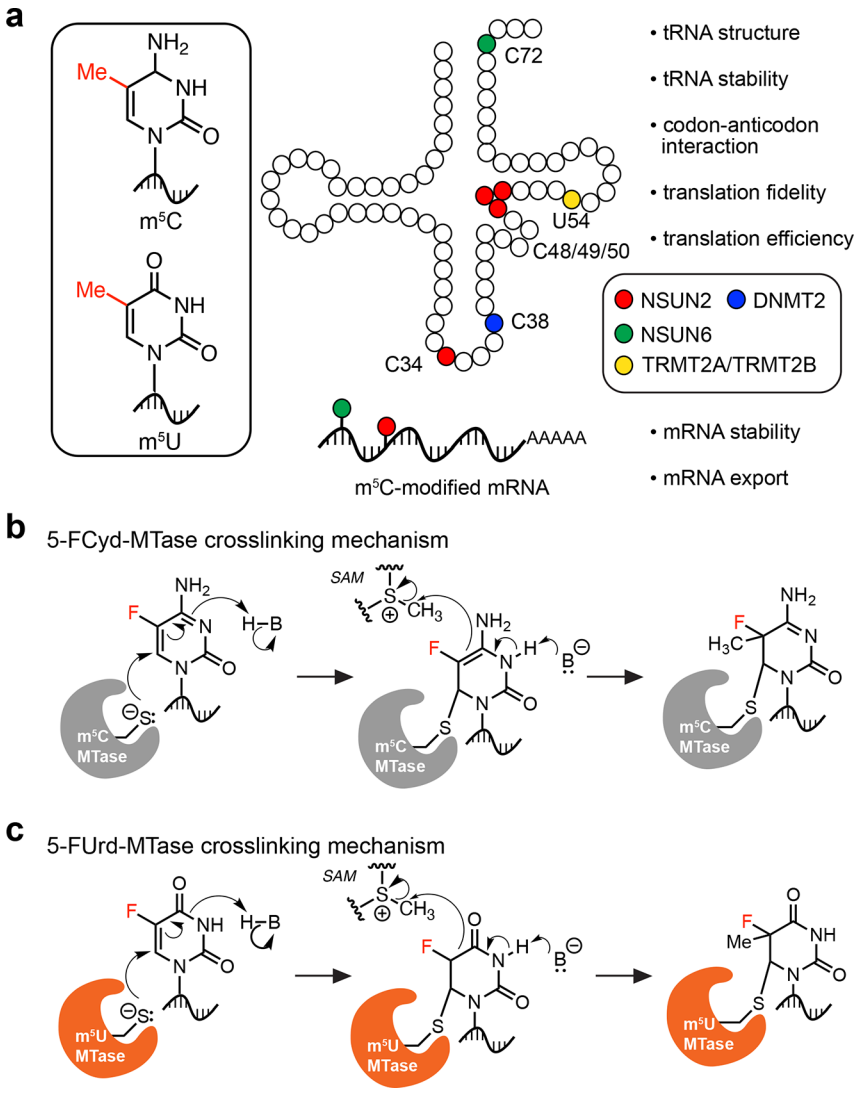

Figure 3. RNA m^5C and m^5U modifications and proposed cross-linking mechanisms for m^5C/m^5U methyltransferases. (a) Known m^5C and m^5U sites in tRNA and their biological functions. (b) and (c) Proposed cross-linking mechanisms of m^5C and m^5U MTases with 5-FCyd- and 5-FUrd-modified RNA, respectively.

(CLIP)[24] analysis combined with high-throughput RNA sequencing to identify enzyme substrate sites. Whereas RNABPP is constrained by the requirement for the metabolic incorporation of the modified nucleoside, many organisms have functional nucleotide salvage pathways, and strategies exist for bypassing some of the more selective metabolic activation steps.[25] Furthermore, metabolic incorporation of modified probes into cellular RNA transcripts is advantageous in that it allows the interrogation of enzyme activity on the native substrates.

In our first application of RNABPP, we chose 5-fluorocytidine (5-FCyd) as a metabolically incorporated mechanism-based probe to study RNA m^5C methyltransferases.[1] Nucleoside LC-QQQ-MS demonstrated efficient RNA labeling with 5-FCyd in a mammalian cell culture. In addition to the incorporation of 5-fluorocytosine, the treatment also resulted in the generation of 5-fluorouracil in RNA, which is likely due to the conversion of 5-FCyd to 5-fluorouridine (5-FUrd) by cytidine deaminase activity in cells. This allowed the dual capture of both cytidine- and uridine-modifying enzymes in one experiment, resulting in profiling data for three distinct classes of RNA-modifying enzymes (Figure 2b): 5-methylcytidine (m^5C) methyltransferases (MTases), 5-methyluridine (m^5U) MTases, and dihydrouridine synthases (DUS).

2.3. Profiling RNA m^5C and m^5U Methyltransferases with 5-Fluoropyrimidine RNABPP

5-Methylcytidine (m^5C) has been identified in various types of RNAs, including rRNA, tRNA, mRNA, and other noncoding RNAs[26] (Figure 3a). The installation of m^5C on tRNAs has been the best studied. In this context, m^5C functions to promote tRNA stability[27] and regulate the production of tRNA-derived fragments (tRFs).[28] In contrast, whereas m^5C has been reported on mRNA,[29,30] its distribution, biological function, and the identity of key mRNA m^5C writer enzymes are less well understood. All known mammalian m^5C writers, which includes NOL1/NOP2/SUN (NSUN) family proteins and DNMT2, use a highly conserved reaction mechanism involving the attack of a catalytic Cys residue on the C6 position of the substrate cytosine, followed by methylation at C5 and enzyme release through β-elimination[26] (Figure 3b). In analogous enzymes, the presence of a fluorine atom at C5 is known to inhibit enzyme release,[31] effectively capturing the enzyme in action. We

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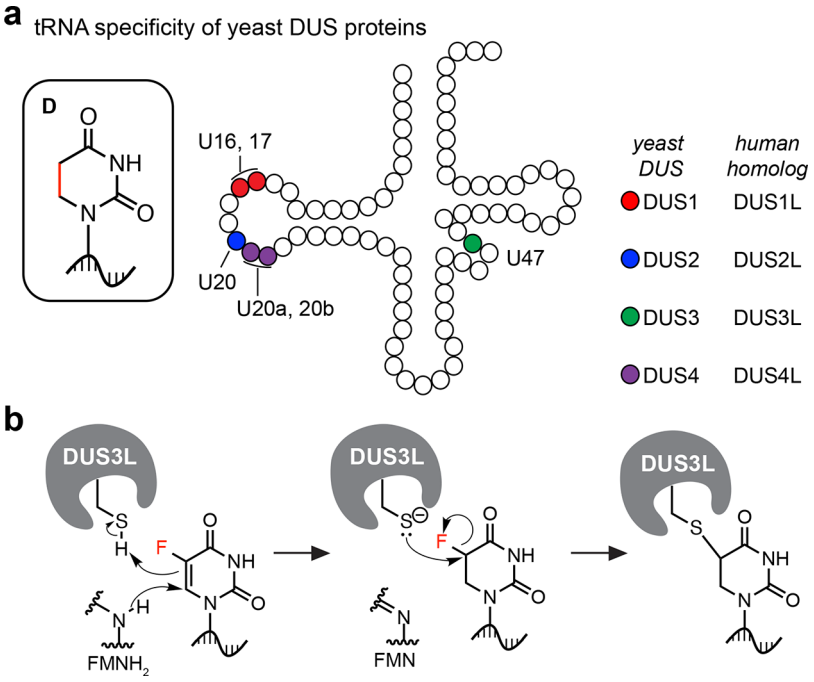

Figure 4. D modification and proposed cross-linking mechanism for DUS enzymes. (a) Identified D sites in yeast tRNA with their corresponding modifying enzymes. (b) Proposed cross-linking mechanism of DUS3L with 5-FUrd-RNA.

therefore reasoned that metabolic labeling with 5-fluorocytidine (5-FCyd) would induce mechanism-based cross-links between m^5C MTases and their RNA substrates. Indeed, our 5-FCyd RNABPP experiment identified NSUN2, NSUN5, and DNMT2 (Figure 2b), and we further demonstrated that NSUN2 is the major m^5C mRNA MTase, consistent with bisulfite sequencing maps.[29]

5-FUrd can react with m^5U MTases as its catalytic mechanism mirrors that of m^5C MTases[32] (Figure 3c). TRMT2A and TRMT2B are mammalian homologues of TrmA/Trm2,[33] which install the highly conserved m^5U54 on tRNA. Concurrent with our studies, TRMT2A/B were shown to install m^5U54 on tRNA in mammals.[34,35] Both enzymes were recovered in our proteomics experiment, suggesting their involvement in catalyzing m^5U formation on human mRNA. We used nucleoside LC-QQQ-MS to measure m^5U on poly(A)-enriched RNA and show partial reduction upon TRMT2A depletion, which is in line with a recent report.[36] However, the lack of dedicated sequencing methods and the low abundance of mRNA m^5U make the reliable assignment of modification sites challenging.

2.4. Identification of a Mechanism-Based Cross-Link between DUS3L and 5-FUrd RNA

To our surprise, DUS3L, a human homologue of yeast DUS3,[37] was also recovered in our 5-FCyd RNABPP study[1] (Figure 2b). D is a widely conserved and abundant tRNA modification found across various domains of life (Figure 4a), but relatively little is known about its function and we lack methods to map D sites transcriptome-wide. Based on biochemical[38] and structural[39] studies with homologous DUS enzymes, we propose that DUS3L cross-links with 5-FUrd using the following mechanism: flavin-dependent reduction of the C5═C6 double bond to generate 5-fluorodihydrouracil concomitant with deprotonation of the catalytic Cys residue, followed by an SN_2-type attack of the Cys thiolate on the C5 position with fluoride serving as the leaving group (Figure 4b). In line with this proposal, mutant DUS3L C396A does not cross-link with 5-FUrd-RNA.[1] We also measured reduced D levels in total RNA, small RNA, and poly(A)-enriched RNA upon DUS3L KO, confirming that this enzyme is a bona fide mammalian DUS.

2.5. Transcriptome-Wide Characterization of DUS3L Substrates

To comprehensively investigate the substrates of DUS3L, we conducted 5-FUrd-mediated DUS3L CLIP sequencing (5-FUrd-iCLIP) (Figure 5a).[1] In this strategy, metabolic labeling with 5-FUrd mediates DUS3L-RNA cross-linking in cells. Next, complexes are immunoprecipitated and digested, and RNA is prepared for Illumina sequencing. The residual peptide-RNA adduct at the cross-linking sites impedes transcription, resulting in the generation of reverse transcription termination at these sites ("RT stops"), which can be used to map DUS3L-mediated D sites at near-nucleotide resolution.

A majority of DUS3L-cross-linked RNAs were tRNA (Figure 5b). In addition, rRNA, mRNA, and long noncoding RNAs were also identified. RT stop analysis revealed prominent tRNA peaks around U47 in the tRNA variable loop (Figure 5c), and motif enrichment analysis of DUS3L peaks using MEME revealed the D consensus sequence "AAGGTTG", matching sequences commonly found in the tRNA variable loop (Figure 5d). These findings indicate that tRNA U47 is the major substrate for DUS3L, consistent with observations for yeast DUS3.[40] However, our sequencing results reveal the presence of D47 in a significantly larger repertoire of tRNAs in humans.

Regarding the presence of D on mRNA, our CLIP results identified mRNA peaks, and two recent studies using $NaBH_4$-based sequencing detected mRNA D sites in both yeast and human mRNA.[41,42] Nonetheless, due to the scarcity of D on mRNA as measured by nucleoside LC-QQQ-MS and the potential for false positives in RT-stop-based RNA modification sequencing methods, orthogonal strategies and further validation will be needed to robustly map D on mRNA.

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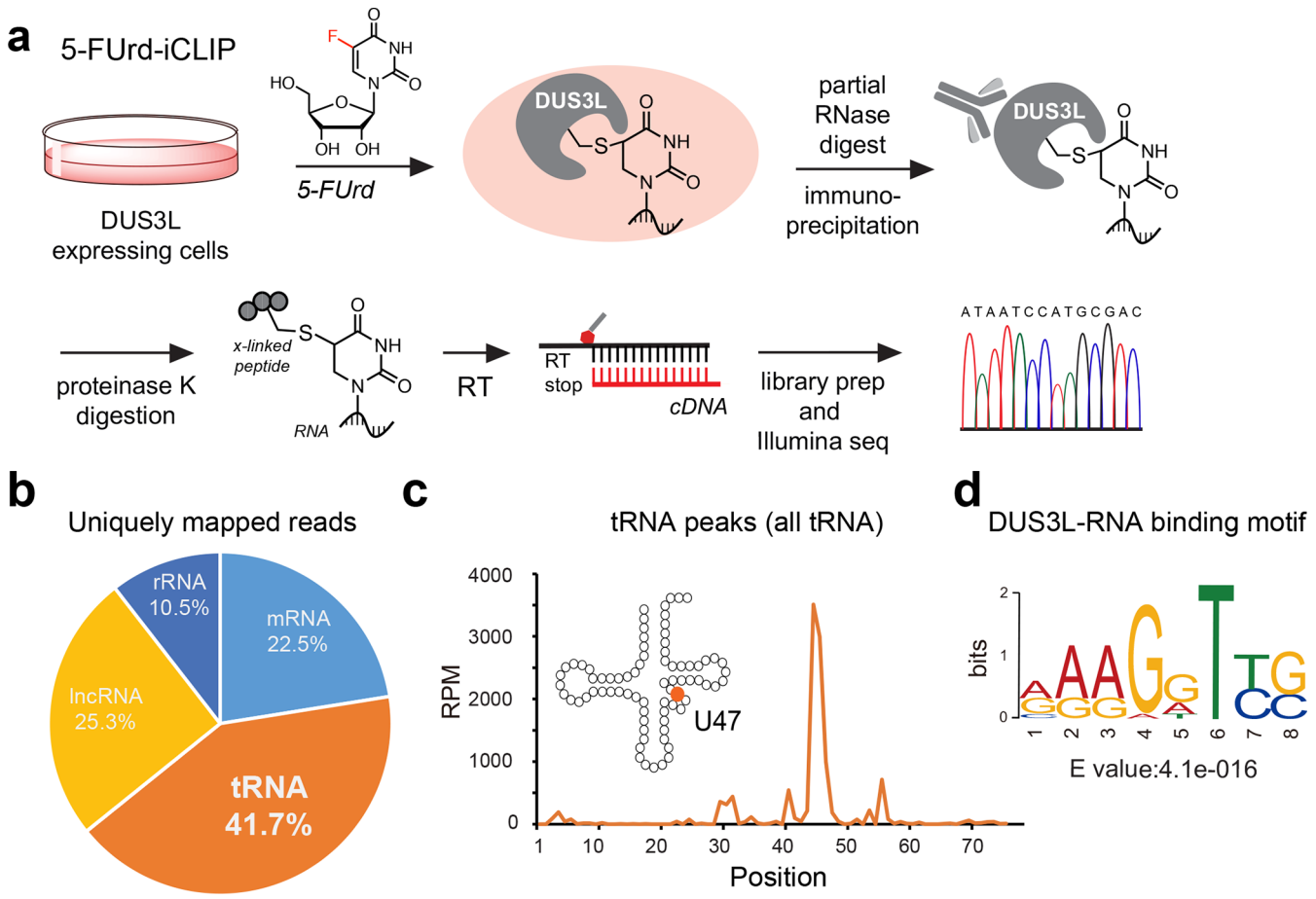

Figure 5. Mapping DUS3L substrates transcriptome-wide with 5-FUrd-iCLIP. (a) Scheme for 5-FUrd-iCLIP. DUS3L-RNA complexes are cross-linked in cells through 5-FUrd metabolic labeling and recovered by immunoprecipitation. The cross-link site impedes RT progression, resulting in RT stop signatures. (b) Pie chart showing the distribution of DUS3L-RNA cross-links. (c) Coverage of cross-link sites mapping to tRNA from the DUS3L 5-FUrd-iCLIP experiment. (d) Consensus motif of DUS3L-dependent D modification in human tRNAs from MEME analysis. Adapted from ref 1 with permission from Springer Nature, copyright 2021.

2.6. Biological Roles of D and DUS3L

Despite their widespread abundance and evolutionary conservation, the biological function of D and DUS enzymes has remained enigmatic. Yeast and *E. coli* DUS KO strains do not exhibit significant growth defects.[37,43,44] The reduction of the C5═C6 double bond modulates the nucleotide conformation and base-stacking interactions,[45] which have been associated with increased conformational flexibility and a preference for loop over base-paired structures. Interestingly, cold-loving psychrophilic bacteria[46] have higher levels of D in RNA, and D is elevated in cancer cells.[47,48] We generated DUS3L KO cells and found that the cell viability and bulk protein translation rate were impaired.[1] These phenotypes are most likely related to the absence of D on tRNA but could also potentially stem from the mRNA sites. Finet et al.[41] reported that D modification on tubulin mRNA in fission yeast plays an important role in repressing tubulin protein expression during meiosis. Moving forward, an additional investigation of the effect of D on tRNA and mRNA biology and the biological roles of the four mammalian DUS enzymes will be needed to advance our understanding of this fundamental RNA modification.

2.7. Outlook

The RNABPP strategy takes advantage of the nucleotide salvage pathway to enable the selective capture of RNA-modifying enzymes. As cross-linking occurs in living cells, enzymes form covalent linkages with their endogenous substrates. Covalent enzyme-RNA complexes can then be analyzed by using either proteomics or RNA sequencing. With a careful choice of nucleoside chemistry, RNABPP can be a powerful approach to interrogating diverse classes of RNA-modifying enzymes. In our work, we have shown that 5-fluoropyrimidines are versatile metabolically incorporated mechanism-based probes, but any modified nucleoside compatible with nucleotide salvage pathways can be employed. Furthermore, because cross-linking does not require additional treatment such as UV irradiation, this method holds promise for application in more complex biological systems, including tissues and animal models.

3. PROFILING RNA M^5C OXIDATION

3.1. RNA 5-Hydroxymethylcytidine and 5-Formylcytidine

The discovery of 5-hydroxymethylcytosine on DNA and the TET enzymes responsible for its installation in 2009[49,50] was a watershed moment in mammalian epigenetics as this modification and its biosynthesis from 5-methylcytosine hinted at a long-searched-for molecular mechanism for DNA demethylation and corresponding epigenetic reprogramming. The flurry of activity that has followed has included the identification of 5-hydroxymethylcytidine (hm^5C) on RNA[51,52] and studies of TET enzymes as bifunctional RNA/DNA modifying enzymes;[53] however, the characterization and

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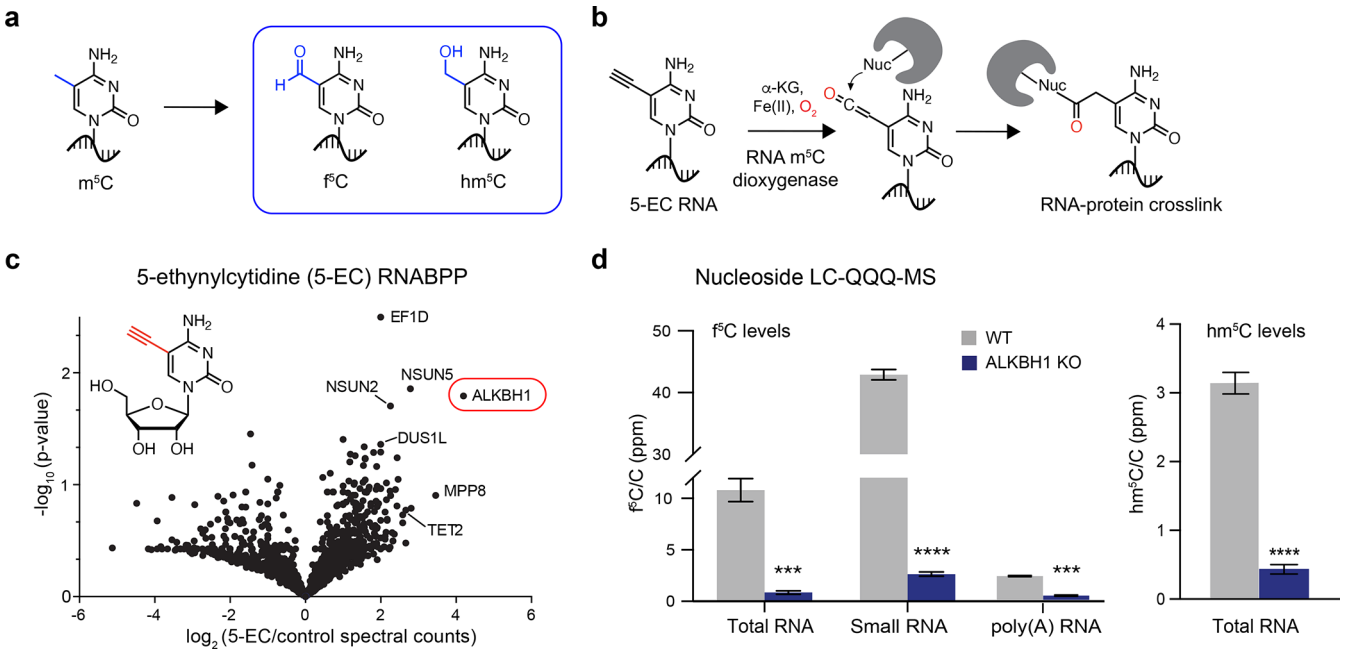

Figure 6. Profiling RNA m^{5}C oxidizing enzymes. (a) Structures of 5-formylcytidine (f^{5}C) and 5-hydroxymethylcytidine (hm^{5}C). (b) Mechanism-based cross-linking of m^{5}C oxidizing enzymes using 5-ethynylcytidine (5-EC)-modified RNA. (c) 5-EC RNABPP proteomics data set. (d) Nucleoside LC-QQQ-MS analysis of f^{5}C and hm^{5}C levels in RNA extracted from WT HEK293T or genetically matched ALKBH1 KO cells. Adapted with permission from ref 2, copyright (2022) the authors, some rights reserved; exclusive licensee Springer Nature. Distributed under a Creative Commons Attribution License 4.0 (CC BY).

mapping of hm^{5}C sites on RNA have presented a considerable challenge. In addition to hm^{5}C, the presence of 5-formylcytidine (f^{5}C) at the wobble base of Mt-tRNA-Met has been known since the 1990s.[54] The biosynthesis of f^{5}C on Mt-tRNA-Met was elucidated by several groups[55−57] and requires the installation of 5-methylcytidine (m^{5}C) by NSUN3, followed by oxidation to f^{5}C by the Fe(II), a-KG-dependent dioxygenase ALKBH1. Suzuki and co-workers also showed that ALKBH1 can install 2′-*O*-methyl-5-hydroxymethylcytidine and 2′-*O*-methyl-5-formylcytidine at the wobble base of tRNA-Leu-CAA,[58] but limited data exists regarding these modifications.

3.2. 5-EC RNABPP

To further investigate the oxidation products of m^{5}C on RNA and characterize the relevant writer enzymes, we applied RNABPP with 5-ethynylcytidine (5-EC)[2] (Figure 6a,b). Deoxyoligonucleotides containing 5-ethynylcytosine were previously shown to cross-link with TET enzymes;[59] therefore, we reasoned that the corresponding ribonucleoside could be used as a metabolic activity-based probe for RNA m^{5}C oxidizing enzymes. The oxidation of 5-EC is thought to produce a highly reactive ketene intermediate which can cross-link with proximal nucleophlic residues in the enzyme active site. Indeed, we were able to show that 5-EC can be incorporated metabolically into RNA and can form mechanism-based cross-links with TET enzymes in cells. Interestingly, while proteomic data from the 5-EC RNABPP experiment did identify TET2 (consistent with previous studies showing that TET2 among the mammalian TET enzymes is most likely to modify RNA), the major hit was ALKBH1 (Figure 6c). We further showed using LC-QQQ-MS that ALKBH1 is the major f^{5}C- and hm^{5}C-generating enzyme in HEK293T cells (Figure 6d) and that these modifications depend on m^{5}C methyltransferases NSUN2 and NSUN3.

3.3. Mapping ALKBH1 Substrates

To map ALKBH1 modification sites, we employed three orthogonal modification sequencing strategies.[2] Analogous to our RNABPP study with 5-fluoropyrimidines, we performed ALKBH1 iCLIP using 5-EC metabolic labeling to induce RNA-protein cross-links (Figure 7a). In this assay, ALKBH1 cross-links primarily localized to cytosolic tRNAs, and we found that the most abundant cross-link site occurred at the wobble base of tRNA-Leu-CAA (consistent with the prior identification of hm^5*Cm*/f^5*Cm* at this site) (Figure 7b,c). We also found cross-links on other tRNAs, including Mt-tRNA-Met, although 5-EC may induce artificial cross-linking with RNAs that would not normally contain suitable m^{5}C sites for ALKBH1 modification. We next focused on characterizing f^{5}C sites using two different sequencing chemistries—reduction of f^{5}C to D using pyridine borane[60,61] (Figure 7d) and oxime formation with aldehyde reactive probe (ARP).[62] Unfortunately, chemical sequencing methods for hm^{5}C have not been well developed, which precluded further study of this modification. Using pyridine borane conversion chemistry and enrichment of f^{5}C-containing RNAs with ARP, we found that the wobble bases of Mt-tRNA-Met and tRNA-Leu-CAA are the major f^{5}C sites in the human transcriptome (Figure 7e,f), and we quantified the modification stoichiometry at both sites. We were able to find minor modification sites; however, these will need further characterization. We further showed that the depletion of ALKBH1 resulted in translation defects on Leu codons, implicating ALKBH1-dependent wobble base modifications in decoding. Taken together, our work provides a new activity-based profiling approach for studying RNA m^{5}C oxidation and reveals the distribution of hm^{5}C and f^{5}C modification sites across the transcriptome. Moving forward, focused studies of ALKBH1-dependent sites on tRNA-Leu-CAA should be prioritized, as this appears to be the major cytosolic ALKBH1 substrate and f^{5}C

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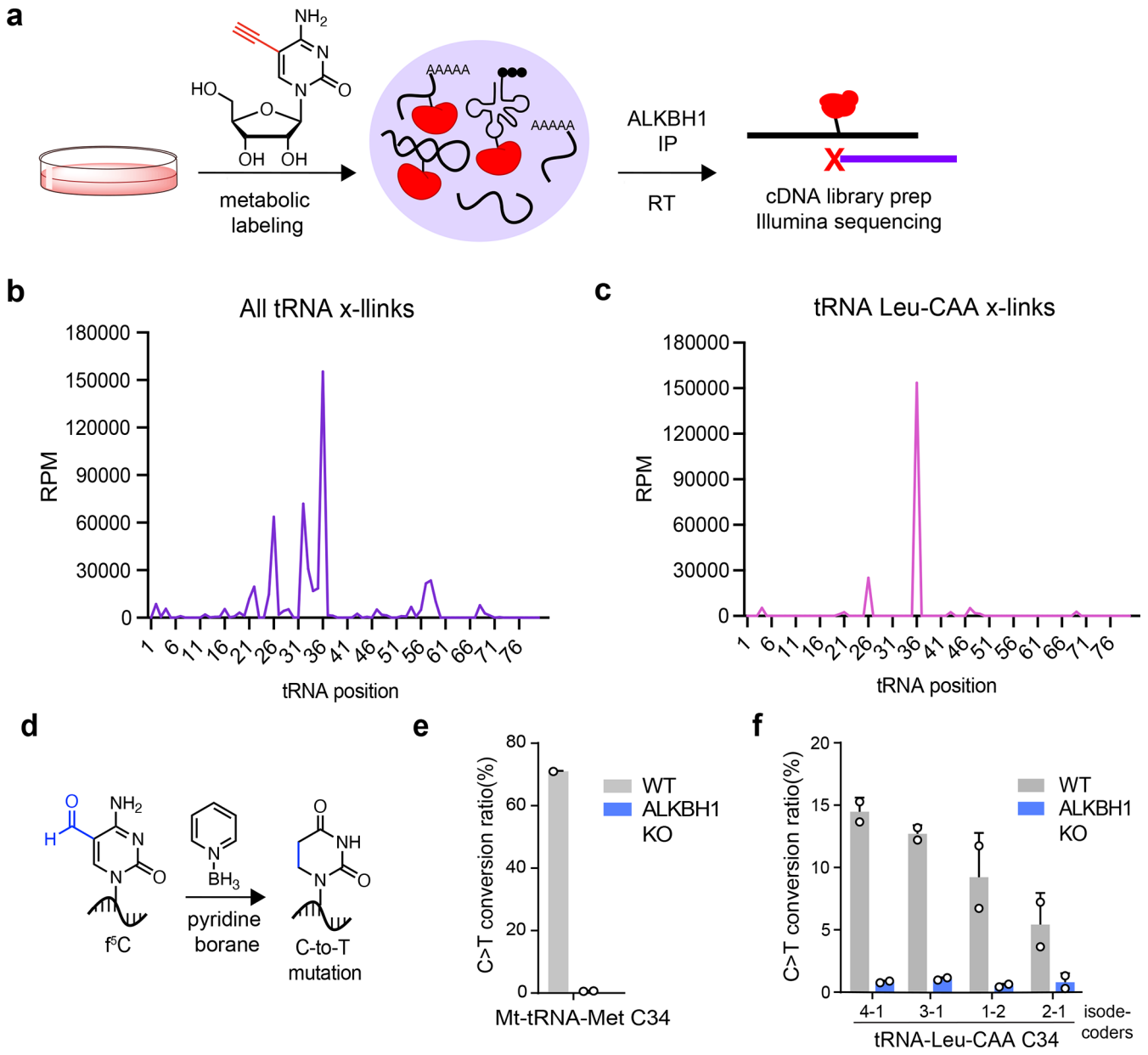

Figure 7. Mapping ALKBH1-dependent hm^{5}C/f^{5}C sites. (a) 5-EC-iCLIP workflow. (b) Cross-link sites from all tRNAs detected using 5-EC-iCLIP for ALKBH1. (c) Cross-link sites mapping to tRNA-Leu-CAA detected by ALKBH1 5-EC-iCLIP analysis. (d) Pyridine borane-based sequencing of f^{5}C sites on RNA. (e) Targeted pyridine borane-based sequencing of f^{5}C34 on Mt-tRNA-Met isolated from WT or ALKBH1 KO cells. (f) Targeted pyridine-borane-based sequencing of f^{5}C34 on tRNA-Leu-CAA isolated from WT or ALKBH1 KO cells. Adapted with permission from ref 2, copyright (2022) the authors, some rights reserved; exclusive licensee Springer Nature. Distributed under a Creative Commons Attribution License 4.0 (CC BY).

modification site. Further studies of hm^{5}C will likely require the development of more specific chemical tools to profile the distribution of this modification.

4. CHEMICAL PROBES TO IDENTIFY RNA MODIFICATION READER PROTEINS

4.1. Reading the RNA Modification Code

RNA modifications can affect mRNA behavior through the recruitment of modification-specific RNA-binding proteins (RBPs), commonly referred to as "reader" proteins.[63] These proteins bind to modified RNAs in a manner that depends on the presence of the modified nucleotide, often within a unique sequence context. RBPs play an important role in RNA function including the regulation of splicing, localization, stability, and translation; therefore, RBPs that "read" modifications serve to specifically modulate the activity of modified transcripts. For some modifications, such as m^{6}A and m^{5}C, their recognition by protein readers is thought to be one of the major mechanisms underlying their role in RNA regulation. In addition, modifications on mRNA can also impact the behavior of macromolecular RNP complexes including the ribosome and the splicesome; however, the direct nature of these molecular recognition events is harder to define.

Identifying and characterizing RNA modification readers is challenging since mRNA modifications are typically substoichiometric, and conventional proteomic and transcriptomic workflows to study RNA−protein interactions do not

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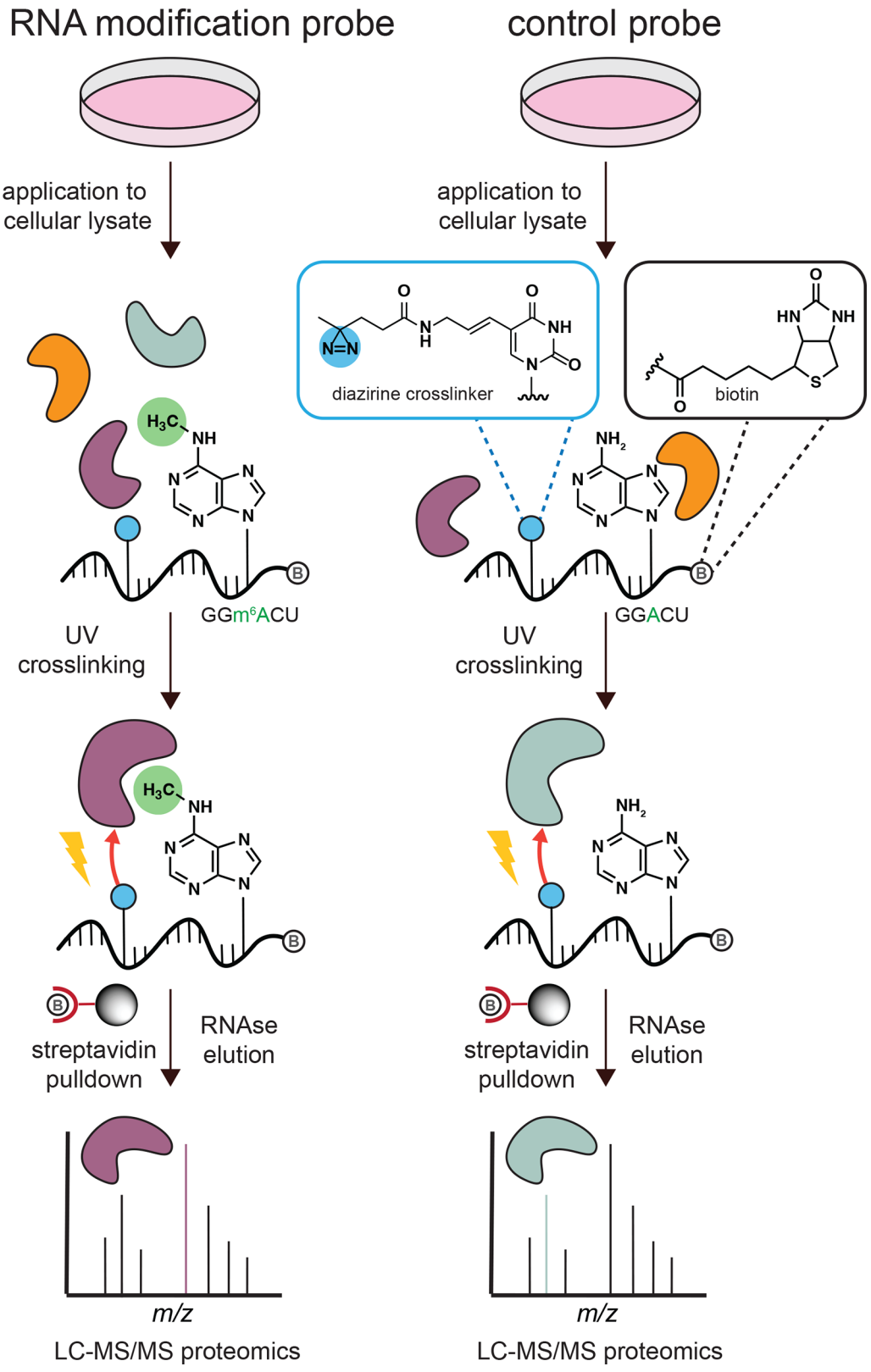

Figure 8. Chemoproteomic workflow for identifying RNA modification-specific reader proteins using synthetic diazirine-containing oligonucleotides. Oligonucleotide probes for N^6-methyladenosine (m^6A) are shown.

specifically target or enrich for modification-dependent interactions. Standard methods require chemical synthesis of a modified oligonucleotide bait for affinity purification−mass spectrometry (AP−MS); however, AP−MS methods do not perform well with low affinity reader-modification interactions and cannot distinguish direct vs indirect binders. To streamline the workflow for characterizing modification reader proteins, we developed a comparative chemoproteomic pipeline (Figure 8)

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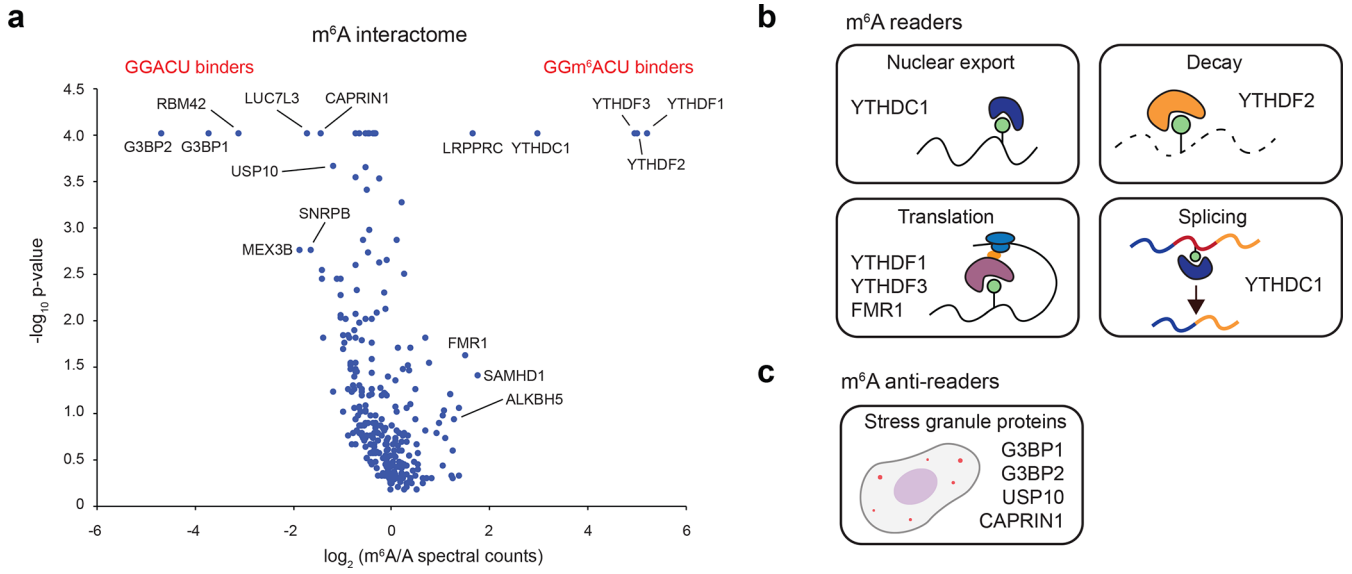

Figure 9. Proteomic profiling of the RNA m^{6}A interactome. (a) Volcano plot of protein enrichment (m^{6}A/A spectral counts) and *p* values from three independent biological replicates using GG(m^{6}A/A)CU-based probes. (b) Reported m^{6}A reader functions from identified proteins. (c) Reported m^{6}A antireader functions from identified proteins. Adapted with permission from ref 3, copyright (2017) American Chemical Society.

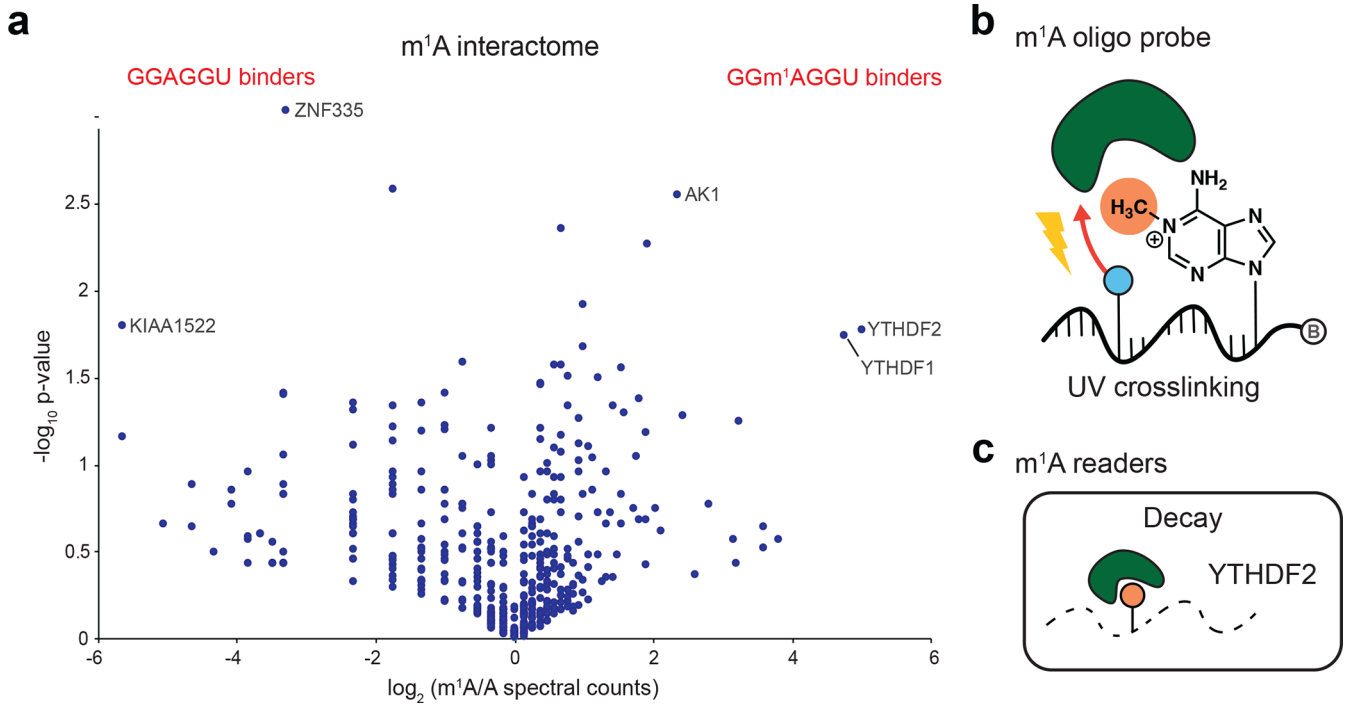

Figure 10. Proteomic profiling of the RNA m^{1}A interactome. (a) Volcano plot of protein enrichment (m^{1}A/A spectral counts) and *p* values from three independent biological replicates. (b) Oligonucleotide probe used for the comparative proteomic workflow. (c) Function of YTHDF2-m^{1}A interaction. Adapted with permission from ref 4, copyright (2020) American Chemical Society.

based upon photo-cross-linking with synthetic oligonucleotides,[3] affinity pulldown, and quantitative proteomic analysis, as described below.

4.2. Chemoproteomics with Diazirine-Modified m^{6}A Oligoprobes

We envisioned that the application of photoaffinity labeling (PAL) to a synthetic oligonucleotide containing the RNA modification of interest would enable efficient capture of RNA modification readers in the cellular lysate. We started with N^6-methyladenosine (m^{6}A), since readers had been described previously[64] and this modification exhibits a major consensus sequence motif (i.e., GGm^{6}ACU).[65,66] Whereas UV photo-cross-linking is used widely to stabilize native nucleic acid–protein interactions, the cross-linking efficiency is low. We therefore incorporated a diazirine functional group into a synthetic oligonucleotide using postsynthesis acylation of a 5-aminoallyluridine-modified oligo with an NHS-alkyldiazirine (Figure 8). The choice of this strategy, as opposed to the incorporation of the diazirine at the 5′ or 3′ end of the sequence, allowed placement of the photo-cross-linker two nucleotides

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distance from the modified m^{6}A nucleotide, maximizing the likelihood of capturing direct m^{6}A binders. Oligonucleotide probes also contained a biotin residue at the 3′ end of the sequence. We validated our probe design by measuring cross-linking between a diazirine-modified m^{6}A oligo and purified YTH domain from YTHDF2 or YTHDC1, known m^{6}A-specific binding proteins, and were able to demonstrate m^{6}A-dependent binding and cross-linking.[3] Additionally, our probe compared favorably against analogous oligonucleotides containing 4-thiouridine (4-SU) or 5-iodouridine (5-IU) in place of the diazirine-modified uridine nucleotide.

Having validated our probe design, we set up a comparative chemoproteomic experiment in HeLa cell lysate using diazirine-modified oligonucleotides, one containing a single m^{6}A nucleotide and the other identical except with an unmodified adenosine at the same position. Lysate was photo-cross-linked with either 1 μM probe, followed by streptavidin pulldown under denaturing conditions, mild RNase elution, and label-free proteomic analysis. Our comparative study using m^{6}A and A oligoprobes identified 4/5 of all mammalian YTH-domain proteins (only YTHDC2 was absent from our data) as well as the RBPs LRPPRC and FMR1 as weaker but still m^{6}A-specific binders (Figure 9a,b). Unexpectedly, we also found proteins that photo-cross-linked more strongly to the A oligo than to its m^{6}A-modified analog. The most prominent among these hits was G3BP1/2, as well as CAPRIN1 and USP10 (Figure 9c). We termed these proteins "antireaders" as they appear to be repelled by the m^{6}A modification. Interestingly, the vast majority of RBPs in our data set bound equally well to both methylated and unmethylated probes. A concurrent study from Vermeulen and co-workers[67] using SILAC-based proteomics and oligonucleotides containing tandem repeats of GG(m^{6}A/A)CU motifs arrived at similar findings. More recent reports have identified additional m^{6}A reader proteins, such as IGF2BP1-3[68] and Prrc2a.[69] The absence of these proteins from our data set (and from data in ref 67) is likely due to the requirement for RNA sequence elements beyond the minimal GGm^{6}ACU motif used in our probes.

In a separate study, we used our chemoproteomic method to survey m^{6}A binding proteins in *Drosophila*.[70] The *Drosophila* genome encodes two YTH-domain proteins, namely, Ythdc1 and Ythdf. For this study, we determined that Ythdf binds poorly to the mammalian GGm^{6}ACU sequence; therefore, an oligo probe based on AAm^{6}ACU (which more accurately reflects the native sequence context of m^{6}A in *Drosophila*) was used. Our proteomics survey confirmed that Ythdc1 and Ythdf are the major m^{6}A binding proteins in *Drosophila*. Follow-up studies characterized the role of Ythdf in translational regulation and demonstrated its involvement in learning and memory.

4.3. Chemoproteomics with N^1-Methyladenosine (m^{1}A)-Modified Oligonucleotide Probes

We applied our chemoproteomic platform to study RBPs that recognize m^{1}A-modified RNA and identified YTH-domain proteins YTHDF1 and YTHDF2 as m^{1}A readers[4] (Figure 10a,b). A similar finding was reported by Dai et al.[71] We confirmed our proteomic finding through biochemical characterization by EMSA and MST assay, showing that whereas YTH-domain proteins bind most tightly to m^{6}A-modified RNAs, they bind to m^{1}A over unmethylated sequences. This behavior was exhibited over multiple sequence contexts, indicating that m^{1}A recognition is a general property of YTHDF1/2. Interestingly, YTHDC1 was not found in our proteomic data set and did not bind specifically to m^{1}A-modified RNA over unmethylated sequences in biochemical assays.

To understand the role of the YTHDF2-m^{1}A interaction, we performed siRNA knockdown of YTHDF2 and measured the expression of known m^{1}A-modified sequences.[4] We excluded transcripts containing m^{1}A and m^{6}A since this would confound our analysis. Our findings demonstrate that YTHDF2 destabilizes m^{1}A-modified RNA, similar to its role in m^{6}A biology (Figure 10c). We also perturbed the levels of ALKBH3, a reported m^{1}A demethylase, and performed an RNA-seq analysis. Knockdown of ALKBH3, which should increase m^{1}A levels, also resulted in transcript destabilization. Taken together, our results indicate that m^{1}A destabilizes mRNA transcripts. Similar findings regarding the role of m^{1}A in transcript stability have been recently reported.[72]

5. CONCLUSIONS AND OUTLOOK

In this Account, we have described the development and application of chemoproteomic methods to study writer and reader proteins of RNA modifications. These strategies are based on chemical probes and proteomic workflows that allow the profiling of RNA-modifying enzymes and RNA readers in native proteomes, paving the way for further investigation of the biological role of RNA modifications and their associated protein regulators and effectors.

Our diazirine-oligonucleotide method can be applied to discover readers of synthetically accessible epitranscriptomic RNA modifications. We used this method to identify m^{6}A and m^{1}A readers, but for many other mRNA modifications, there are no known reader proteins. Our method provides a powerful tool to illuminate one possible mechanism by which these modifications can modulate mRNA behavior. Diazirine-based photo-cross-linking can also be employed to study interactions of other small RNA species (in a modification-dependent or -independent manner) that can be generated synthetically, including tRNAs, miRNAs, and therapeutic oligonucleotides.

Our RNABPP strategy represents an important advance in RNA-modifying enzyme analysis methods since it provides an unbiased reactivity-based method to discover and characterize RNA modification writers and their substrates in their native context. We applied this method in cultured human cells with C5-modified pyrimidine nucleosides; however, it can be adapted to any biological system that will tolerate metabolic labeling with modified nucleosides and can be employed with other nucleoside chemistries that are compatible with cellular metabolism. We envision that RNABPP will be a powerful tool in the arsenal of RNA chemists and biologists to discover and characterize new RNA-modifying enzymes. Furthermore, reactivity-based nucleoside probes for RNA writer enzymes can be incorporated into oligonucleotides and used for *in vitro* biochemical and structural studies of enzyme-RNA complexes as well as RNA-modifying enzyme inhibitors.

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†W.D. and N.J.Y. contributed equally. CRediT: **Wei Dai** writing-original draft; **Nathan J Yu** writing-original draft; **Ralph E. Kleiner** writing-original draft.

Notes

The authors declare no competing financial interest.

Biographies

Wei ("Crystal") Dai was born in 1994 in Hubei province, China. She obtained her bachelor's degree at the University of Hong Kong, with a major in chemistry and a minor in French. She also participated in an overseas' exchange program at the University of Edinburgh in the U.K. for a year during her undergraduate study. She recently completed her Ph.D. study at Princeton University under the supervision of Ralph Kleiner, where her research focus was the exploration of RNA-modifying enzymes and the development of advanced sequencing methods for dihydrouridine.

Nathan J. Yu was born in 1995 in Pittsburgh, Pennsylvania (USA). He received his B.S. degree in chemistry from Penn State University, where he performed research with Prof. Ross Hardison and Prof. Bratoljub Milosavljevic. He is currently working towards his Ph.D. at Princeton University under Prof. Ralph Kleiner. Nathan's research focuses on developing methods to profile RNA-modifying enzymes and investigating biological consequences of post-transcriptional modifications.

Ralph E. Kleiner was born in 1983 in Syracuse, New York (USA). He received his A.B. from Princeton University, where he performed research with Michael Hecht, and his Ph.D. from Harvard University under the supervision of David Liu. He pursued postdoctoral studies with Tarun Kapoor at The Rockefeller University. In 2016, he assumed his current position as assistant professor in the Department of Chemistry at Princeton University, where he is also an affiliated faculty member with the Department of Molecular Biology and the Princeton Bioengineering Initiative. His research group develops and applies chemical biology approaches to study nucleic acid function in biological systems, with a focus on the regulation of RNA through post-transcriptional modifications and interactions with RNA-binding proteins.

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