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Account

Chemical Approaches To Investigate Post-transcriptional RNA Regulation

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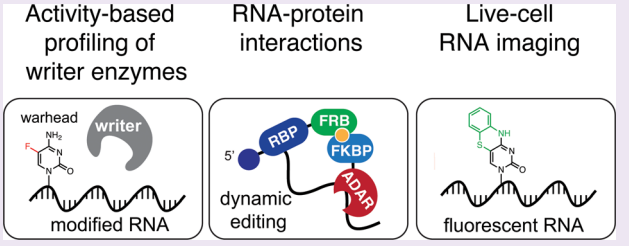
ABSTRACT: RNA plays a central role in biological processes, and its activity is regulated by a host of diverse chemical and biochemical mechanisms including post-transcriptional modification and interactions with RNA-binding proteins. Here, we describe our efforts to illuminate RNA biology through the application of chemical tools, focusing on post-transcriptional regulatory mechanisms. We describe the development of an activity-based protein profiling approach for discovery and characterization of RNA-modifying enzymes. Next, we highlight novel approaches for RNA imaging based upon metabolic labeling with modified nucleosides and engineering of the nucleotide salvage pathway. Finally, we discuss profiling RNA−protein interactions using small molecule-dependent RNA editing and synthetic photo-cross-linkable oligonucleotide probes. Our work provides enabling technologies for deciphering the complexity of RNA and its diverse functions in biology.



1. INTRODUCTION

RNA serves as a central hub regulating the flow of biological information. Whereas the importance of transcriptional programs in biological systems has been appreciated since the pioneering work of Jacob and Monod,[1] the mechanisms regulating RNA *after* it is synthesized, or what is known as post-transcriptional regulation,[2] are not as well understood. Mechanisms regulating RNA post-transcriptionally are of critical importance in biology, as these processes can control the localization, dosage, translatability, and sequence content of an RNA message. Additionally, cells synthesize a variety of noncoding RNAs that control transcription, translation, innate immunity, and genome integrity, among other fundamental processes. How does RNA serve so many diverse roles in biology? The myriad of distinct RNA transcripts, structures, and macromolecular RNA-based assemblies is aided by a large collection of post-transcriptional or epitranscriptomic[3] chemical modifications (Figure 1) installed by RNA-modifying enzymes. These modifications provide chemical and functional diversity beyond the four canonical ribonucleotide building blocks and can be "written" and "erased" by enzymes in response to cellular states or environmental cues. RNA also works in concert with cellular proteins in the form of ribonucleoprotein complexes (RNPs). In humans, it is estimated that >1500 proteins interact with RNA.[4] These proteins are known as RNA-binding proteins (RBPs) and control all aspects of the RNA lifecycle from synthesis to degradation. RNA modification pathways and RBP−RNA interactions are emerging therapeutic targets in a variety of human diseases,[5,6] as they can regulate the expression of disease relevant genes, but there are still many gaps in our

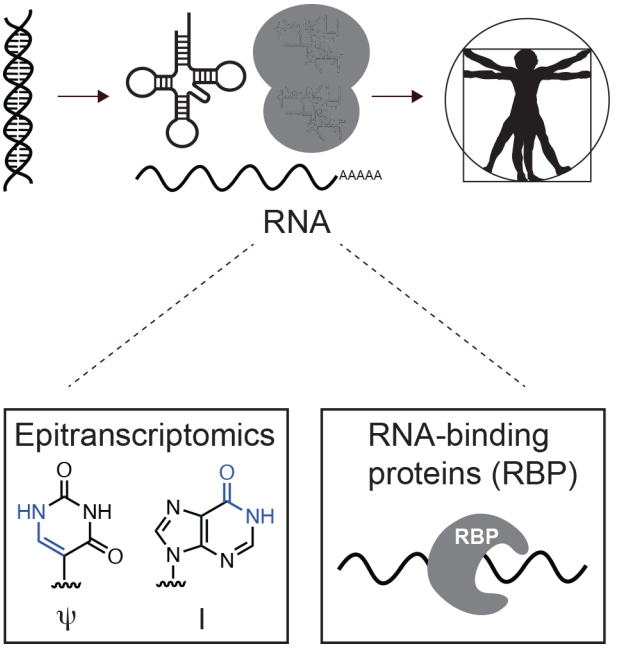


Figure 1. Mechanisms of post-transcriptional RNA regulation.

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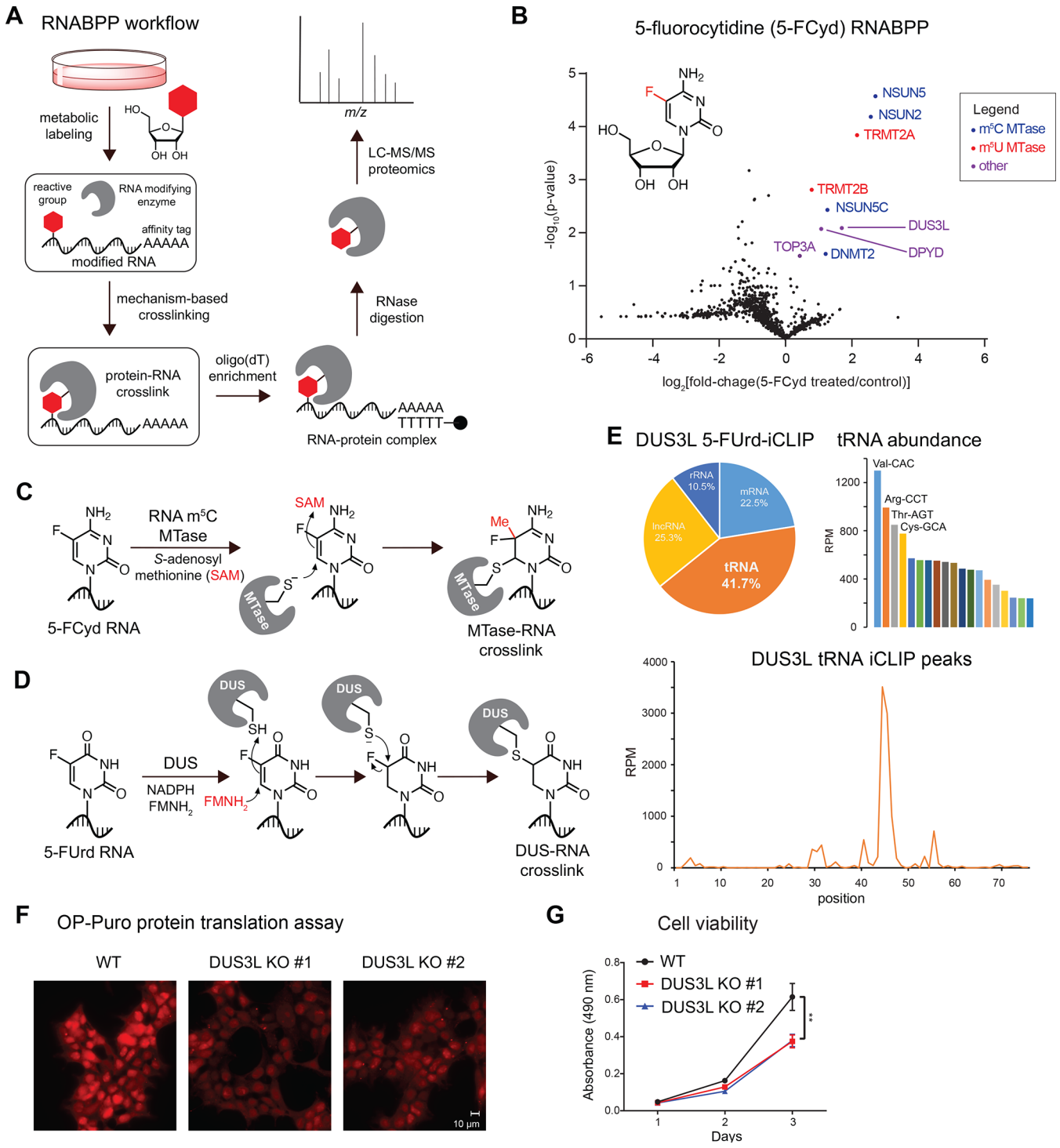

Figure 2. Investigating RNA-modifying enzymes using RNA-mediated activity-based protein profiling (RNABPP). (A) RNABPP schematic. (B) Proteomic profiling of 5-FCyd-reactive proteins in HEK293T cells using RNABPP. (C) Covalent inhibition of RNA m^{5}C methyltransferase by 5-FCyd. (D) Covalent inhibition of dihydrouridine synthases (DUS) by 5-FUrd. (E) Distribution of uniquely mapped reads from DUS3L 5-FUrd-iCLIP experiment. (F) Bulk protein synthesis analysis of DUS3L KO cells using *O*-propargyl puromycin (OP-Puro). (G) Cell viability of DUS3L KO cells. Data in B, E, F, and G were previously published in Dai et al.[17] and are adapted with permission from Springer Nature.

understanding of these molecular phenomena and their role in RNA biology and cellular processes.

Methods for studying RNA biology have undergone a quantum leap with the development of systems level 'omics tools for studying the transcriptome and proteome. In particular, high-throughput sequencing/next generation sequencing (NGS)[7] enables quantification of every RNA expressed in a cell and can characterize transcript isoforms generated through alternative splicing. Further, NGS has been adapted together with a variety of biochemical enrichment and cDNA library preparation methods to allow studies of post-transcriptional processes at the transcriptome-wide level, including RNA−RBP interactions and ribosomal translation. RBP binding sites can be profiled transcriptome-wide using UV photo-cross-linking and

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immunoprecipitation coupled with high-throughput sequencing (i.e., CLIP-seq/HITS-CLIP and related methods).[8] The nuances of protein translation can be measured across the transcriptome by isolating and sequencing ribosome-protected RNA fragments or "footprints", a method known as ribosome profiling or Ribo-Seq.[9]

Whereas NGS serves as a powerful cellular microscope that can be focused on a multitude of RNA-centric processes fundamental to gene expression regulation, there are certain areas in RNA biology that remain dark under its illumination. One such area is the study of epitranscriptomic RNA modifications. A number of the most abundant RNA modifications (e.g., N^6-methyladenosine, pseudouridine, 5-methylcytidine, etc.) are undetectable in standard RNA sequencing experiments because they do not change Watson−Crick pairing properties of the base. Mapping these modifications requires customized sequencing pipelines relying upon modification-specific antibodies or chemoenzymatic transformations,[10] typically together with genetic knockout of appropriate writer enzyme(s) to confirm that the modification signature results from a bonafide modification. Together with modification mapping, investigation of RNA modification-associated proteins including writer/eraser[11] enzymes and reader proteins[12] is critical for functional studies, but methods for identifying and characterizing these proteins are limited. Another area that is difficult to address with canonical NGS analysis is the study of cellular RNA dynamics. Sequencing provides a bulk "snapshot" of all RNA transcripts across a sample of cells; therefore, we lack spatial or temporal information regarding RNAs of interest. RNAs are highly dynamic, constantly undergoing synthesis/turnover and trafficking to different regions of the cell; therefore, such information is critical to understand the regulation of cellular RNA. Finally, even methods where NGS is used successfully, such as CLIP analysis of RNA-binding protein substrates, can benefit from innovation to make these approaches more streamlined and less technically challenging, increasing their accessibility and utility.

In this Account, we describe our efforts to illuminate RNA biology by developing and applying chemistry-inspired approaches to study RNA modifications, RNA-binding proteins, and RNA dynamics. Our studies have integrated nucleic acid chemistry and chemical biology with transcriptomic, proteomic, and cellular imaging modalities to develop enabling methods for understanding the regulation of RNA and its role in fundamental cellular processes.

2. ACTIVITY-BASED PROFILING OF RNA-MODIFYING ENZYMES

Motivation. The properties of RNA are regulated by a large variety of post-transcriptional modifications that affect RNA structure, base pairing, chemical stability, and RNA−protein binding.[3] These modifications enable cellular RNAs to fulfill diverse roles in biology and can be dysregulated in disease states.[13] The characterization and functional interrogation of RNA modifications has been ongoing since the discovery of pseudouridine in the 1950s[14] and has revealed insights into the role of modifications in protein translation, mRNA metabolism, splicing, innate immune recognition, and many other fundamental biological processes; nevertheless, many questions remain regarding the biological functions of RNA modifications, the enzymes regulating modification levels, and the effect of modifications on RNA biochemistry in the cell. One of the major challenges in the study of RNA modifications is the lack of general and unified methods for investigating the >100 structurally distinct chemical modifications known in biology,[15] which has necessitated the development of individualized tools and workflows for each RNA modification of interest. Therefore, strategies that can allow simultaneous investigation of multiple RNA modification chemistries in one biological sample/system should prove highly enabling for deciphering epitranscriptomic complexity and regulation.

Studies of RNA modifications are closely linked with characterization of the relevant RNA-modifying writer enzyme(s) responsible for their installation. Whereas not all RNA modifications are installed by enzymes, and can occur due to nonenzymatic processes, the existence of a dedicated writer enzyme for a modification implies biological intentionality and suggests functional importance, particularly when the enzyme and modification are evolutionarily conserved. Further, knowledge of the relevant writer enzymes for a modification provides a handle for evaluating its biological role, because well-established methods for protein knockdown in cells and organisms can be exploited to deplete modification levels and study function. Historically, characterization of modified ribonucleotides largely preceded identification of the associated writers by several decades, and studies of RNA-modifying enzymes are still lacking, particularly in higher eukaryotes. Current approaches for assigning RNA-modifying enzyme substrates[16] largely rely upon low throughput screening strategies coupling protein knockdown with RNA mass spectrometry or modification sequencing, or simply assign substrates based upon homology to known enzymes in lower organisms such as *E. coli* or yeast. Few general strategies for uncovering RNA-modifying enzymes and establishing relevant substrates have been described.

RNABPP with 5-Fluoropyrimidines. Our lab has pioneered a reactivity-based approach to RNA-modifying enzyme discovery and characterization. Our method, RNA-mediated activity-based protein profiling (RNABPP)[17] (Figure 2A), is inspired by activity-based protein profiling (ABPP),[18] which utilizes reactive small molecule probes to selectively label protein families and amino acids as a function of substrate recognition and reactivity. ABPP has been applied to diverse enzyme classes to uncover novel protein activity and identify ligandable sites but has not been specifically used to profile RNA-modifying enzymes. We envisioned that metabolic labeling of cellular RNA with reactive, modified nucleosides would enable the installation of mechanism-based covalent probes directly into the native substrates. In this manner, cells would synthesize their own activity-based probe by metabolic activation of the nucleoside to the nucleotide triphosphate, followed by transcriptome-wide incorporation into RNAs during transcription. Resulting covalent enzyme−mRNA complexes could then be enriched from cells using oligo-dT pulldown[19,20] and reactive proteins identified using mass spectrometry-based proteomics.

In our first implementation of the RNABPP method,[17] we utilized metabolic labeling with 5-fluorocytidine (5-FCyd) to profile 5-methylcytidine (m^5C) methyltransferase enzymes acting upon mRNA (Figure 2B). RNA m^5C is an abundant epitranscriptomic mark on tRNA,[21] but its prevalence on mRNA has been controversial. It is installed primarily by NSUN/DNMT enzymes that utilize a thymidylate synthase (TS)-like mechanism (Figure 2C) that should be amenable to covalent trapping with 5-FCyd. In our study, we found that metabolically incorporated 5-FCyd could form efficient covalent adducts with multiple m^5C methyltransferase enzymes, including NSUN2,

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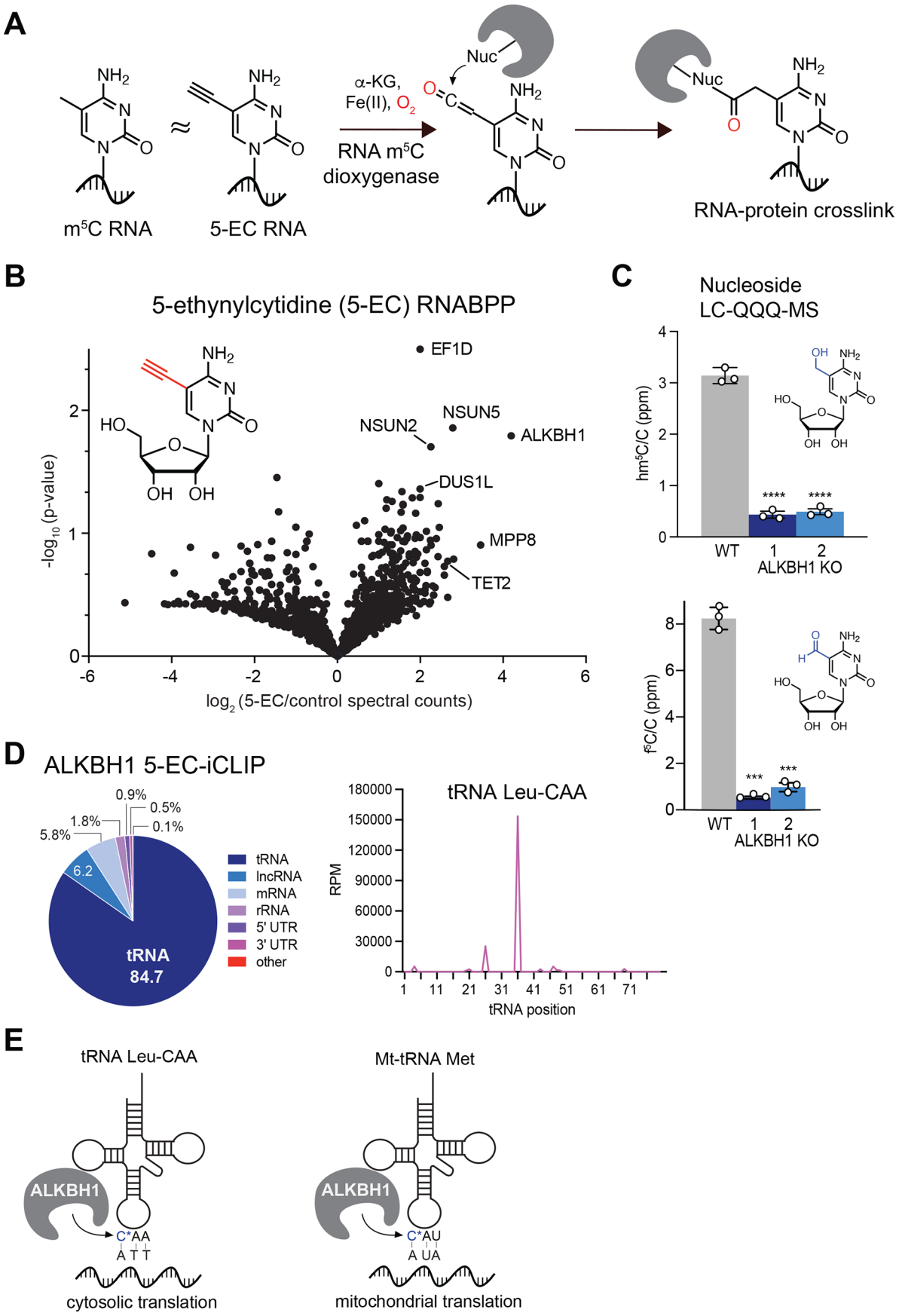

Figure 3. Profiling RNA m^{5}C dioxygenase enzymes using 5-ethynylcytidine (5-EC) as an RNABPP probe. (A) Covalent inhibition of RNA m^{5}C dioxygenases by 5-EC. (B) RNABPP experiment in HEK293T cells with 5-EC. (C) LC-QQQ-MS analysis of 5-hydroxymethylcytidine (hm^{5}C) and 5-formylcytidine (f^{5}C) in total RNA from WT and ALKBH1 KO cells. (D) Distribution of uniquely mapped reads from ALKBH1 5-EC-iCLIP

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Figure 3. continued

experiment. (E) Mechanisms for translational regulation by ALKBH1-dependent wobble base modifications. Data in B, C, and D were previously published in Arguello et al.[29] and adapted under terms of an open-access license.

NSUN5, NSUN6, and DNMT2, and our proteomic RNABPP experiment identified NSUN2, DNMT2, and NSUN5 as mRNA reactive hits (Figure 2B); follow-up work established that NSUN2 installs the majority of m^5C on human mRNA.

5-Fluoropyrimidines React with Human Dihydrouridine Synthase DUS3L. Interestingly, RNABPP with 5-FCyd captured proteins other than known m^5C methyltransferase enzymes. We identified TRMT2A/B[22−24] (Figure 2B), which are human homologues of the yeast tRNA 5-methyluridine (m^5U) methyltransferase TRM2. Separately, we established that 5-FCyd is partially deaminated to 5-fluorouridine (5-FUrd) in cells, accounting for the enrichment of TRMT2A/B, which are proposed to utilize a similar catalytic mechanism as m^5C methyltransferases (Figure 2C). Follow-up RNA mass spectrometry studies with TRMT2A KO cells demonstrated that this enzyme can install m^5U on tRNA and mRNA in human cells. Surprisingly, we also identified DUS3L, an uncharacterized human protein homologous to the yeast tRNA dihydrouridine synthase (DUS) DUS3[25] (Figure 2B). DUS enzymes convert uridine to dihydrouridine (D), which is an abundant tRNA modification conserved throughout evolution. D was discovered in the 1960s and found in the first tRNA structure;[26] however, its biological function has remained elusive and few methods for sequencing D sites have been described. It is found primarily in the eponymous "D loop" of tRNA, and eukaryotes also possess D47 in the tRNA variable loop. We established that DUS3L can react with 5-FUrd-containing RNA, likely through reduction of 5-FUrd to 5-fluorodihydrouridine[17] (Figure 2D), followed by nucleophilic attack of a conserved catalytic Cys396 residue upon the resulting α-fluoroamide modification at C5. With a tool in hand for cross-linking DUS3L to its RNA substrates, we performed 5-FUrd-iCLIP to map DUS3L modification sites across the transcriptome, revealing a major D site at position 47 in the tRNA variable loop as well as mRNA modification sites (Figure 2E). RNA nucleoside mass spectrometry confirmed that DUS3L can install D into human mRNA in addition to tRNA. Interestingly, independent studies performed in yeast also demonstrated the presence of D in yeast mRNA.[27,28] We also investigated cellular phenotypes in DUS3L KO cells to find that cell viability and bulk protein translation rate were both reduced (Figure 2F and 2G). Taken together, our work demonstrates that RNABPP can be used as a platform to discover RNA-modifying enzymes and characterize their native substrates. Further, we find that 5-FCyd is a versatile metabolic probe for multiple classes of RNA-modifying enzyme, including DUS enzymes, and characterize for the first time the substrates of human DUS3L.

RNABPP Probes for RNA m^5C Dioxygenases. As a second application of RNABPP, we investigated RNA m^5C dioxygenase enzymes.[29] Oxidation products of m^5C, 5-hydroxymethylcytidine (hm^5C) and 5-formylcytidine (f^5C), have been detected in RNA,[30−32] but the enzymes that generate these species have not been well characterized. To capture RNA m^5C oxidizing enzymes, we performed RNABPP with 5-ethynylcytidine (5-EC), which mimics m^5C but forms a reactive ketene upon oxidation[33] (Figure 3A). Profiling of 5-EC-reactive RNA-modifying enzymes revealed Fe(II), α-KG-dependent dioxygenase ALKBH1 as the major RNA m^5C oxidizing enzymes in human cells (Figure 3B). We characterized ALKBH1-dependent hm^5C and f^5C modifications using nucleoside LC-MS (Figure 3C) and modification-dependent sequencing to find two major sites of ALKBH1 modification at the wobble position of Mt-tRNA-Met and tRNA-Leu-CAA (Figure 3D). The role of f^5C on Mt-tRNA-Met in mitochondrial translation has been well established,[34−37] but ALKBH1-dependent modifications at the wobble base of tRNA-Leu-CAA do not have known function. Therefore, we investigated the role of hm^5C and f^5C on tRNA-Leu-CAA in translation, finding that decoding of noncognate TTA Leu codons is perturbed in cells depleted of ALKBH1 (Figure 3E).

Future Outlook. Moving forward, we envision that RNABPP will open several new lines of investigation. First, many organisms have functional nucleotide salvage pathways (required for metabolic activation of nucleosides), and therefore RNA metabolic labeling with modified nucleosides can be performed in a wide variety of biological contexts to characterize RNA-modifying enzymes. This includes diverse prokaryotes and eukaryotes, including model organisms such as yeast, flies, worms, and mouse. RNABPP can be used to reveal disease- or pathogen-specific RNA-modifying enzymes, which may motivate therapeutic targeting of these proteins. Similarly, tissue-specific RNA-modifying enzymes could be investigated to understand the role of these proteins in organismal physiology. After proteomic analysis, the substrates of uncharacterized RNA-modifying enzymes can be identified using the same modified nucleoside probes to cross-link RNA transcripts for high-throughput sequencing (analogous to CLIP methods).

Second, we demonstrate two distinct probes for RNABPP analysis, 5-FCyd/5-FUrd and 5-EC, but our method is compatible with any nucleoside chemistry that can be incorporated metabolically. Expanding the set of RNABPP chemical warheads will be important for application to other RNA-modifying enzymes. For example, mammals possess four DUS homologues predicted to employ similar catalytic mechanism, but 5-FUrd reacts primarily with DUS3L. It is likely that tuning the reactivity of our fluoropyrimidine probe, perhaps with different halogen substitutions, will enable the development of nucleoside probes for all four mammalian DUS enzymes.

Third, RNABPP in its current form relies upon metabolic labeling, but modified nucleotides can also be introduced into RNAs of defined sequence by chemical synthesis or in vitro transcription (IVT). These methods could be used to generate RNA activity-based probes with defined composition for biochemical and structural studies or for application in cells or lysate. Indeed, incorporation of 5-EC into an oligonucleotide mimic of the tRNA anticodon stem loop (ASL) enabled mechanism-based cross-linking with recombinant ALKBH1.[29] Further, these constructs could be the starting point for oligonucleotide-based inhibitors of RNA-modifying enzymes. Finally, our work has revealed new insights into RNA methyltransferases, dioxygenases, and DUS enzymes. Future work in our lab will focus on deepening our understanding of the biological role of these enzymes and associated RNA modifications in normal and disease physiology.

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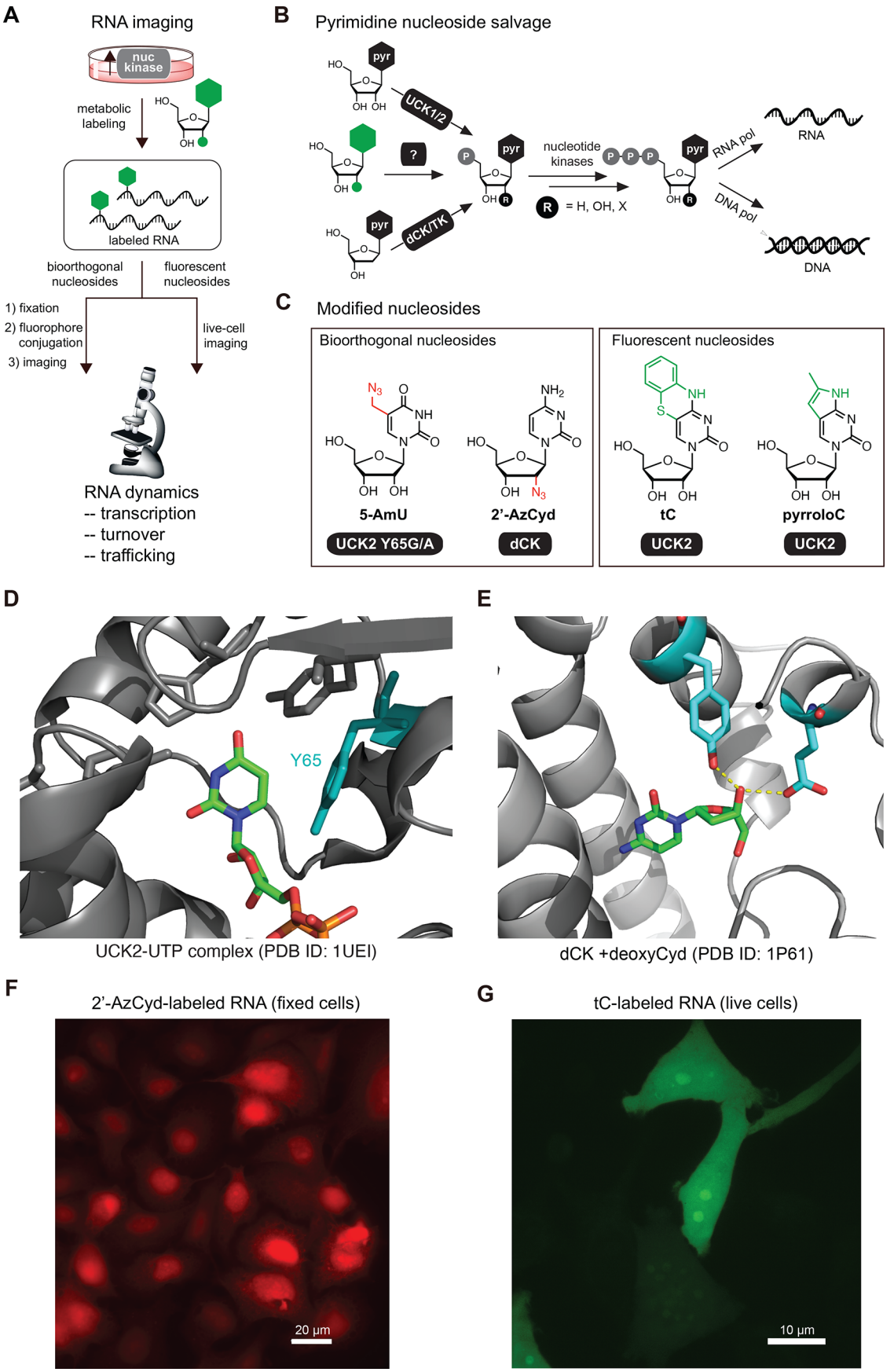

Figure 4. Imaging RNA dynamics with metabolically incorporated modified nucleosides. (A) Metabolic labeling workflow for cellular RNA imaging. Nucleoside kinase overexpression facilitates uptake of modified nucleosides. (B) Pyrimidine salvage pathways for native and modified nucleosides. (C) Modified nucleosides amenable to metabolic incorporation when indicated nucleoside kinase is overexpressed in cells. (D) Structure of UCK2 active

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Figure 4. continued

site with bound UTP.[78] Y65 residue in proximity to uracil C5 position is highlighted in teal. (E) Structure of dCK with deoxycytidine bound,[79] showing interactions with 3′-OH. (F) Imaging of 2′-AzCyd-labeled cells overexpressing dCK kinase after fixation and click chemistry with Cy3-alkyne. (G) Live-cell imaging of RNA with tC fluorescent nucleoside in HeLa cells overexpessing UCK2. Data in F were previously published in Wang et al.[45] Data in G were previously published in Wang et al.[46]

3. RNA IMAGING WITH METABOLICALLY INCORPORATED NUCLEOSIDES

Motivation. Cellular RNA is a highly dynamic molecule.[38] Transcripts are continually synthesized in the nucleus and undergo various degrees of processing, and then most are trafficked to the cytoplasm, where they are eventually degraded. Perturbations to cellular homeostasis, such as exposure to environmental stresses or cytotoxic drugs, can dramatically perturb the RNA transcriptome by inducing the transcription or turnover of specific transcripts, interfering with RNA synthesis and processing, or inducing the formation of specialized RNA-based structures such as biomolecular condensates and RNA granules.[39] Characterizing cellular RNA dynamics is critical to elucidate the molecular mechanisms regulating gene expression and homeostasis.

High-throughput RNA sequencing has provided unparalleled transcriptome-wide insights for RNA expression levels; however, sequencing measurements lack spatial and temporal information about RNA in cells and are best at quantifying relative changes rather than absolute RNA levels. In order to introduce a temporal dimension to the analysis, nascent RNA can be metabolically pulse-labeled with modified nucleosides that enable affinity enrichment or induce characteristic sequence mutations after chemical treatment. The most commonly used nucleosides for this purpose are 5-ethynyluridine (5-EU) and 4-thiouridine (4-SU).[40] 5-EU can be functionalized with the CuAAC reaction ("click chemistry") to characterize subcellular RNA localization by imaging. However, 5-EU-based imaging is only compatible with fixed cellular samples due to the toxicity of Cu(I) and its propensity to induce RNA degradation; therefore, new tools are needed to enable live-cell imaging of bulk RNA transcripts.

Engineering the Pyrimidine Salvage Pathway for RNA Metabolic Labeling. Our group has focused on developing new metabolic activation strategies to expand the set of modified nucleosides that can be utilized for metabolic RNA labeling. Our approach is based upon manipulating proteins in the nucleotide salvage pathway (Figure 4A and 4B). In particular, nucleoside kinases enzymes are responsible for phosphorylating nucleosides to nucleotide monophosphates;[41] this has been traditionally viewed as the rate-limiting step for uptake of anticancer and antiviral nucleosides. Our initial goal was to facilitate metabolic labeling of RNA with azido-nucleosides, as these molecules are substrates for SPAAC reaction[42] ("Cu-free" click chemistry), which would enable bioorthogonal conjugation with a fluorophore and subsequent live-cell RNA imaging. We chose 5-azidomethyluridine (5-AmU) because it is similar in structure to 5-EU (Figure 4C). Surprisingly, 5-AmU is not incorporated metabolically.[43] We hypothesized that 5-AmU is a poor substrate for uridine cytidine kinase 2 (UCK2), and indeed in vitro phosphorylation analysis with purified recombinant UCK2 demonstrated that 5-AmU was phosphorylated at very low efficiency.[44] Using structure-guided mutagenesis, we next introduced a single point mutation in the enzyme active site (a "hole"), to accommodate the bulky azidomethyl group (a "bump"). We focused on residue Y65 due to its proximity to uridine C5 (Figure 4D) and found that mutation of Tyr to smaller amino acids such as Ser, Ala, or Gly dramatically improved in vitro phosphorylation activity on 5-AmU. Further, expression of a Y65G or Y65A UCK2 mutant in human cells (but not WT UCK2) allowed metabolic incorporation of 5-AmU into cellular RNAs by RNA polymerase I and II, demonstrating that a single amino acid serves as the gatekeeper for pyrimidine nucleoside uptake.

In a similar strategy, we showed that metabolic labeling of RNA with 2′-azidocytidine (2′-AzCyd) can be enhanced by overexpression of deoxycytidine kinase (dCK)[45] (Figure 4C and 4E), highlighting the divergence in substrate tolerance between different nucleoside kinases. 2′-AzCyd is primarily incorporated by RNA polymerase I and is a specific label for rRNA (rRNA) (Figure 4F). Using 5-AmU RNA labeling, we also performed imaging of RNA in live cells through SPAAC conjugation of a BODIBPY−BCN fluorophore.[44] Although we could clearly visualize the 5-AmU-dependent fluorescence signal, high background fluorescence from the dye and the need for extensive washout made this method challenging for dynamic live-cell imaging.

Live-cell RNA Imaging with Metabolically Incorporated Fluorescent Nucleosides. To advance dynamic RNA imaging, we envisioned a fundamentally different approach to visualize the transcriptome in living cells. Rather than incorporation of a modified nucleoside containing functionality for bioorthogonal conjugation of a fluorophore, we proposed that a fluorescent nucleoside could be incorporated directly into RNA, allowing immediate cellular imaging without additional cell manipulation.[46] Not only would this approach be operationally simpler and enable rapid imaging, there would no longer be the requirement for bioorthogonal conjugation or need to extensively wash out free unconjugated fluorophore. The constraints to this strategy are the identification of a fluorescent nucleoside with suitable photophysical properties for live-cell imaging and compatibility with metabolic incorporation. Although fluorescent nucleotides have been described extensively in the literature,[47] these structures are used primarily for in vitro studies and to our knowledge no evidence exists that they can be incorporated metabolically. Further, the fluorescence of the nucleoside incorporated into RNA must exceed the fluorescence background generated by unincorporated fluorescent nucleotide metabolites in the cell.

We started by evaluating in vitro phosphorylation by UCK2 of a series of fluorescent cytidine analogues.[46] In collaboration with the Purse lab, we chose fluorescent nucleoside structures of modest size that still retained the ability to engage in Watson−Crick base pairing, including pyrrolocytidine (pyrroloC)[48] and derivatives of 1,3-diaza-2-oxophenothiazine (tC)[49] (Figure 4C). Surprisingly, given the poor tolerance of WT UCK2 for small acyclic subsitutions at the C5 position of pyrimidines,[44] phosphorylation of bicyclic pyrroloC and tricyclic parent tC (but not the methoxy or diethylamino-substituted analogues) proceeded efficiently. We next applied pyrroloC and tC in human cells for imaging and could detect cellular fluorescence in

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cells made to overexpress WT UCK2 by widefield or confocal fluorescence microscopy. We also expressed the previously investigated Y65G UCK2 mutant in cells but observed no benefit over the WT kinase for fluorescent nucleoside incorporation.

By confocal microscopy, fluorescence in cells fed either pyrroloC or tC could be observed in the nucleus and cytoplasm, with strong enrichment in structures consistent with nucleoli, the major sites for rRNA synthesis in cells (Figure 4G). Upon cotreatment with actinomycin D (an RNA Pol I/Pol II inhibitor), cellular fluorescence was dramatically decreased, indicating that the majority of the signal originated from labeled RNA rather than unincorporated fluorescent nucleotides. We applied our fluorescent metabolic labeling method to quantify RNA dynamics upon oxidative stress, conditions known to perturb RNA metabolism and trafficking, and observed increased turnover of bulk RNA, as well as the formation of small cytoplasmic foci. Using dual color live-cell imaging with mCherry-labeled proteins, we determined that these foci were neither canonical P bodies nor stress granules (which are both known to form upon oxidative stress) but colocalized with the RNA helicase DDX6. Moving forward, we plan to further investigate these oxidative-stress-induced "DDX6 granules" and understand their composition and significance to the integrated stress response.

Future Outlook. We have shown that overexpression of native and engineered nucleoside kinase enzymes can be used as a tool to expand the scope of modified pyrimidine nucleosides suitable for RNA metabolic labeling applications. These structures span modifications at the C5 position and 2′-ribose, as well as polycyclic modified nucleobases, and can be used to visualize bulk RNA dynamics in living cells. In addition, for modified nucleoside structures that are compatible with cells expressing endogenous nucleoside kinase levels, their incorporation can be greatly accelerated by overexpression of these proteins. For example, WT UCK2 overexpression increases 5-EU incorporation dramatically. This should be advantageous for labeling experiments that require high temporal resolution. Overall, our approach is attractive because metabolic RNA labeling is rapid and efficient with nucleosides (occurring within minutes), and pyrimidine nucleoside kinases (i.e., UCK2 and dCK) can be expressed at high levels in cells using plasmid vectors or stable transgenes without cytotoxicity. In addition, a variety of modified nucleoside structures are commercially available or can be readily accessed using synthetic methods. Alternative approaches for delivering modified nucleotide equivalents into cells rely upon either transfection-based methods to introduce the nucleotide triphosphate (NTP) directly[50] or chemical protection strategies that facilitate bypass of metabolic activation steps that are incompatible with the modified structure.[51] NTP transfection reagents can be toxic to cells, and chemically protected nucleotides are often challenging to synthesize and limited in their generality to cells with abundant levels of the key uncaging enzyme. We envision that nucleoside kinase overexpression and/or engineering (either rational structure-based design or a directed evolution approach) should be broadly applicable to incorporate a variety of chemical and biophysical probes into RNA including photo-cross-linkers, bioorthogonal groups, activity-based probes, and fluorophores, as well as modified epitranscriptomic nucleotides and nucleotides for synthetic biology. Separately, an outstanding question is the effect of UCK2/UCK1 expression levels on the metabolism of native modified and unmodified nucleosides. UCK2 is overexpressed in a variety of cancers,[52] but its importance for nucleotide biosynthesis (as compared to de novo biosynthesis pathways) has not been well characterized. Studies of UCK-dependent nucleotide metabolism are underway in our group.

Finally, our work has demonstrated that polycyclic cytidine-based nucleosides are promising scaffolds for live-cell RNA imaging. An important advantage of live-cell RNA imaging over analogous techniques in fixed cells is the ability to utilize dynamic approaches such as fluorescence recovery after photobleaching (FRAP). Moving forward, we plan to explore this modality to measure the dynamics of RNA in various cellular structures such as biomolecular condensates. To further push the utility of our approach, it will be critical to develop brighter and more photostable fluorescent nucleosides, environmentally sensitive and turn-on fluorescent nucleosides, and red-shifted fluorophores. Combining nucleoside chemistry and nucleoside kinase engineering should allow access to these structures for metabolic labeling applications and RNA imaging.

4. METHODS TO PROFILE RNA BINDING PROTEINS (RBPS) AND THEIR SUBSTRATES

Motivation. From birth to death, the RNA lifecycle is regulated by interactions with a large host of RNA-binding proteins.[4] Current estimates are that >1500 genes in the human genome encode RNA-binding proteins and regulate all aspects of RNA function including splicing and processing, nuclear export, translation, metabolism, and trafficking. Due to its vast complexity, there are still major gaps in our understanding of the RNA-binding proteome, the individual transcripts that are bound by each RBP, and the functional significance of these interactions. Further, dysregulation of RNA−protein interactions has been associated with human disease[5] and modulating these interactions with small molecules and antisense oligonucleotides (ASOs) is emerging as a promising, albeit largely unexplored, therapeutic modality.[53]

Identifying the transcripts targeted by an individual RBP is an important step toward understanding its function. Typically, this is accomplished using UV cross-linking combined with high-throughput RNA sequencing (CLIP-seq, HITS-CLIP, and many variations thereof).[8] CLIP methods are powerful but have limitations, including high input requirements, antibody availability, and the intensive and technically challenging nature of the protocol. As an alternative to CLIP, researchers have developed enzymatic "tagging" approaches that rely upon fusing an RNA-modifying enzyme to the RBP of interest to "mark" substrate transcripts with characteristic RNA modifications that can be detected by sequencing. Examples of this approach include TRIBE (targets of RNA-binding proteins identified by editing),[54] RNA tagging,[55] and STAMP (surveying targets by APOBEC-mediated profiling),[56] which use adenosine deaminase, poly(U) polymerase, and cytidine deaminase enzymes. These methods do not require biochemical purification steps or complex RNA-seq library assembly but lack the temporal resolution afforded by CLIP (as the RBP-enzyme fusion is expressed asynchronously in a population of cells), making them unsuitable for analyzing highly dynamic RNA−RBP interactions.

Profiling Dynamic RNA−RBP Interactions with Small-Molecule-Controlled RNA Editing. To extend RNA tagging methods to analyze dynamic RNA−RBP systems, we developed TRIBE-ID (targets of RNA-binding proteins identified by editing induced through dimerization)[57] (Figure 5A). TRIBE-

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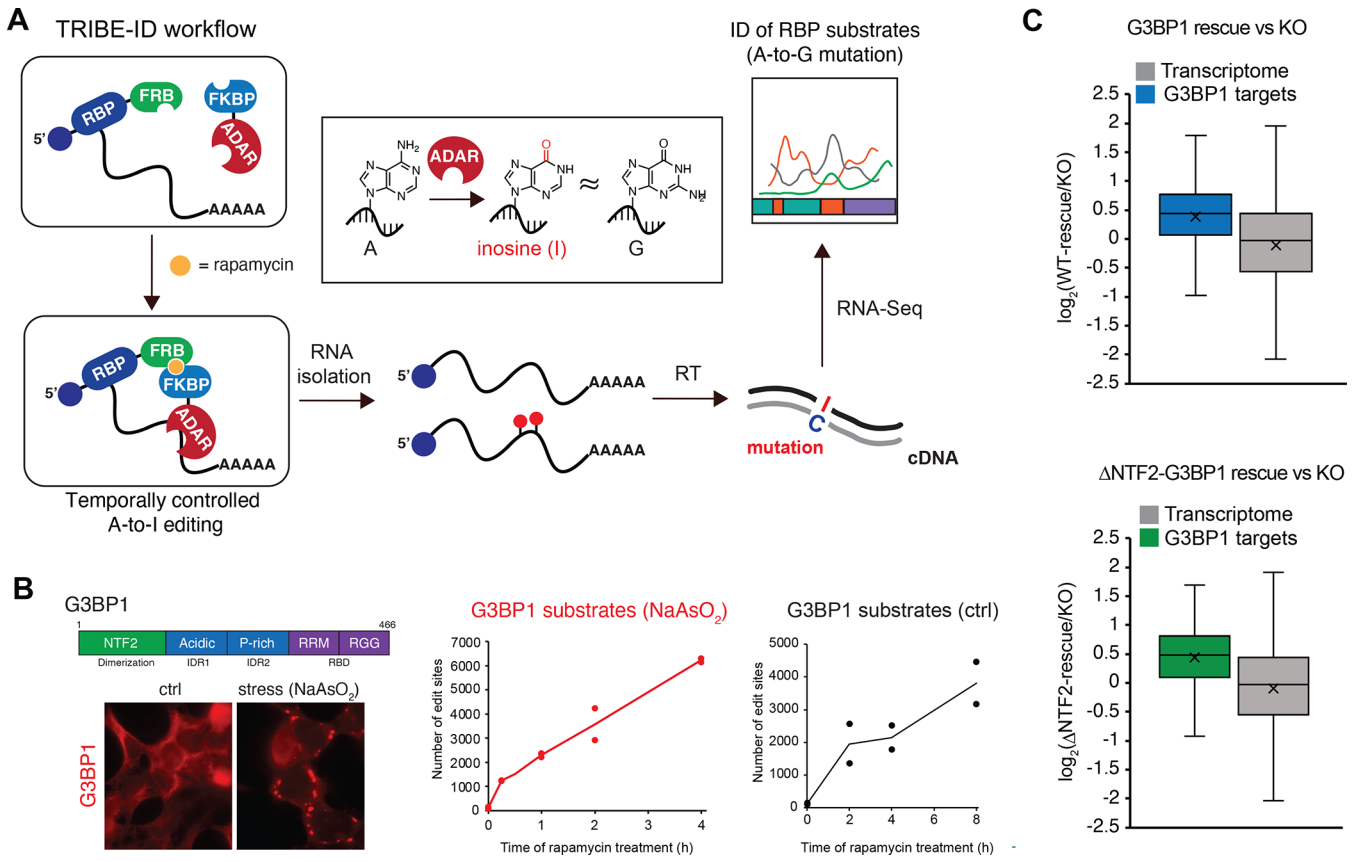

Figure 5. Studying RNA−protein interactions with TRIBE-ID. (A) Small-molecule-mediated RNA editing platform (TRIBE-ID) to investigate dynamic RNA−protein interactions. (B) Immunofluorescence analysis of G3BP1 accumulation in stress granules upon sodium arsenite exposure and time-dependent RNA editing as a function of rapamycin treatment. (C) RNA expression levels upon G3BP KO compared against rescue cell lines expressing full-length G3BP1 or deltaNTF2-G3BP1 constructs. Data in B and C were previously published in Seo and Kleiner[57] and are adapted with permission from Springer Nature.

ID combines ADAR-mediated adenosine-to-inosine (A-to-I) editing with rapamycin-induced FRB/FKBP chemical dimerization to enable small molecule control and therefore high temporal resolution. We applied TRIBE-ID to study RNA−RBP interactions during stress granule formation; stress granules are cytosolic biomolecular condensates that form rapidly upon translational arrest and contain numerous RNAs and RBPs.[58] The biophysical mechanisms governing stress granule condensation and their protein components have been studied; however, there is a major gap in our understanding of the function of these structures, particularly in how they relate to post-transcriptional RNA regulation. Our focus was on the RBP G3BP1 and its RNA substrates.[59] G3BP1 is required for the assembly of stress granules under diverse stresses (Figure 5B), but the effect of condensation on the function of G3BP1 RNA−protein complexes is unknown. In addition, CLIP-based data sets profiling G3BP1 substrates under nonstress conditions have not reached consensus.[60,61] Using our approach, we could specifically interrogate G3BP1−RNA interactions transcriptome-wide during normal conditions and during oxidative stress (which induces stress granules in minutes),[57] as TRIBE-ID can detect RNA−protein interactions in cells in as little as 15 min (Figure 5B). We found ∼2000 G3BP1−RNA binding sites distributed over ∼1000 transcripts under normal conditions, and about twice as many under sodium arsenite-induced stress, indicating more frequent G3BP1−RNA interactions in stress granules. In addition, the number of transcripts detected by TRIBE-ID is a direct function of rapamycin treatment time, and we could detect an increase in the accumulation of editing on individual transcripts over time. We performed the same analysis for the RBP YBX1, which also partitions into stress granules, and observed a similar trend indicating that RNA binding is generally increased by stress granule formation; application to YBX1 also established that TRIBE-ID can be applied more generally for RBP substrate analysis.

The role of biomolecular condensates (which often contain RNA and RBPs) in post-transcriptional regulation is largely an open question. Our finding that RNA binding is increased in stress granules suggests that they may amplify RBP function or extend it to other transcripts that are not engaged upon normal conditions. We examined these hypotheses by measuring RNA expression levels upon G3BP1/2 knockdown and rescue with WT G3BP1 or a G3BP1 construct lacking its oligomerization domain (G3BP1−deltaNTF2),[62] which can still bind RNA but cannot form stress granules. Knockdown of G3BP1/2 specifically reduced the expression of G3BP1 substrate transcripts but did not perturb global RNA expression levels (Figure 5C). Interestingly, RNA expression levels could be rescued by either expression of WT G3BP1 or G3BP1−deltaNTF2, and was equally efficient upon sodium arsenite stress, indicating that G3BP1-mediated post-transcriptional stabilization is largely independent of stress granule formation. Whether this concept applies to all stress-granule-localized RBPs and extends to all RNA substrates will require further investigation.

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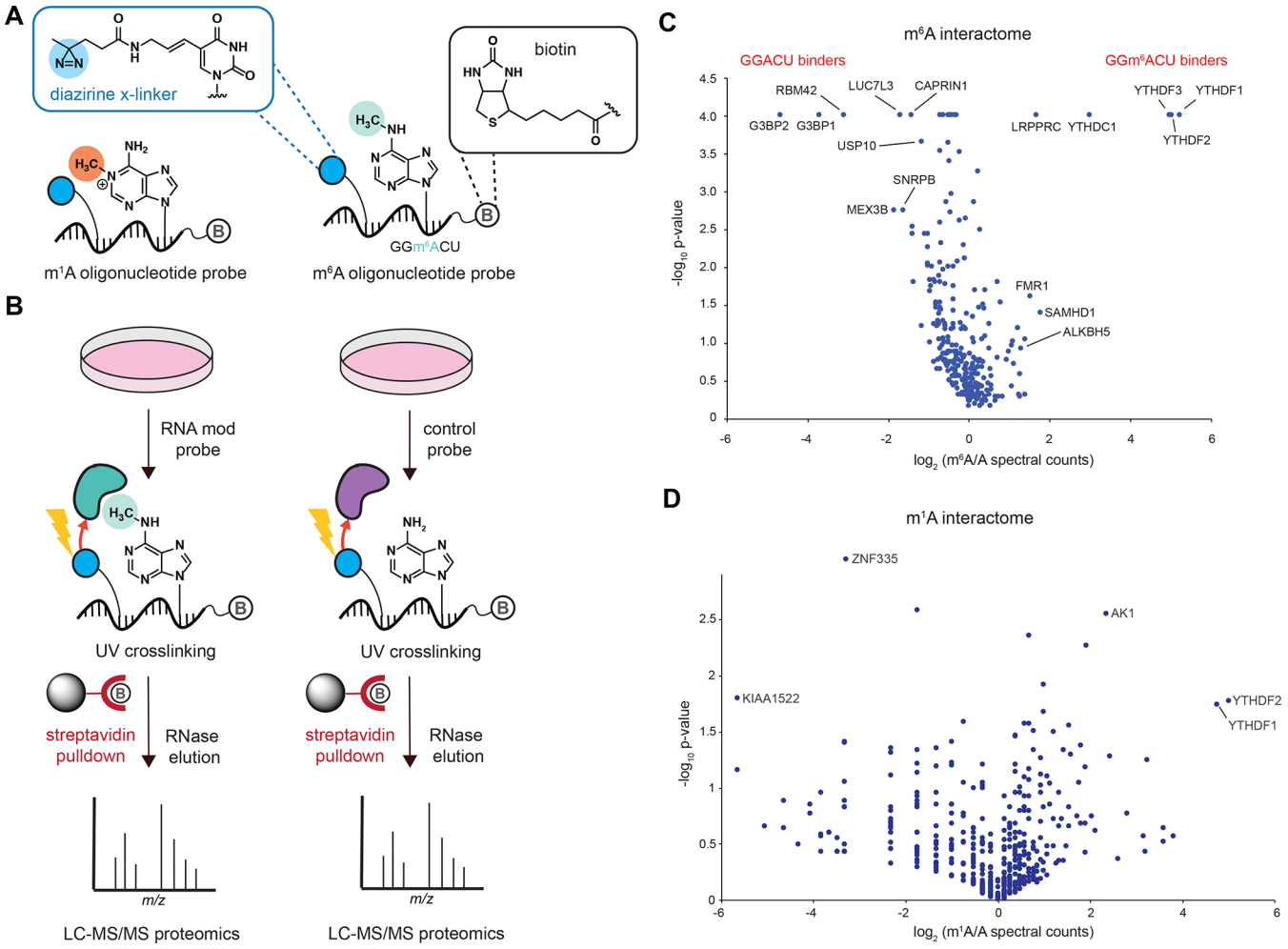

Figure 6. Profiling RNA modification readers using photo-cross-linkable diazirine-containing oligonucleotides and affinity proteomics.[66,69] (A) Design of diazirine-containing oligo probes. (B) Comparative proteomics workflow for identifying modification-specific reader proteins. (C) Proteomic profiling of m^{6}A-binding proteins. (D) Proteomic profiling of m^{1}A-binding proteins. Data in C were previously published in Arguello et al.[66] Data in D were previously published in Seo and Kleiner.[69]

Future Development and Application of TRIBE-ID. Our work with G3BP1 and YBX1 has established that small-molecule-induced RNA editing with ADAR can be a general and robust method to study RNA−protein interactions in cells with temporal control. We envision that TRIBE-ID will serve as a complementary method to CLIP and canonical RNA tagging approaches to enable RBP substrate analysis, particularly for investigation of dynamic cellular processes. Work to apply TRIBE-ID to other RBPs that rapidly partition into biomolecular condensates is currently ongoing in our group. Moving forward, we also plan to implement methodological improvements to the TRIBE-ID platform. RNA editing approaches for RBP substrate analysis are beholden to the specificity and efficiency of the RNA editing enzyme. Therefore, identifying enzymes with maximum substrate tolerance, predictable behavior, and low off-target activity is critical for extracting the most information from these experiments. Whereas human ADAR2 (E488Q) provided a good starting point for our experiments with stress-granule-localized RBPs, ADARs are known to prefer dsRNA substrates, and this may limit our ability to identify RNA−RBP binding sites at high resolution. Currently, several RNA-modifying enzymes have been proposed as possible RNA tagging reagents, but we lack head-to-head comparisons of their behavior in defined systems. Therefore, we plan to evaluate a panel of native and engineered RNA-modifying enzymes across different biochemical and cellular contexts.

The rapamycin/FKBP/FRB system is well established for chemical dimerization experiments but is limited by the effects of rapamycin on native cellular physiology through mTOR inhibition. Although this is unlikely to compromise experiments that rely upon short-term rapamycin treatment, longer term interaction analysis may be problematic. Therefore, evaluating chemical dimerizers that are maximally orthogonal to mammalian systems, such as abscisic acid (ABA) from plants,[63] artificial rapalogues,[64] and engineered dimerizer systems,[65] will be important. Finally, in addition to systematic characterization of RNA−protein interactions, RNA editing strategies should be adaptable to characterize a variety of biochemical and biophysical phenomena involving cellular RNA including RNA structure, lifetime, and subcellular localization. Such studies are currently underway in our group and will be reported in due course.

Profiling "Readers" of Epitranscriptomic RNA Modifications. Modifications on mRNA can modulate RNA behavior by serving as binding platforms for modification-specific RBPs or "readers".[12] Whereas transcriptome-wide sequencing methods such as CLIP and TRIBE are useful for

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profiling RBP substrates, they are less suitable for interrogating RNA modification-specific binding events, because the location of RNA modifications can rarely be determined from regular RNA-seq data, and modifications are often substoichiometric across transcripts. Therefore, studying modification-dependent RNA−protein interactions typically requires chemical synthesis of an RNA oligonucleotide containing the modification of interest at a specific position. Modified oligonucleotides can then be used with purified protein for biophysical binding measurements or in cellular lysate for affinity pulldowns to identify putative "reader" proteins. Pulldown experiments from lysate can be complicated by nonspecific binding of proteins to the resin/bait, which are often challenging to distinguish and separate from low affinity, but still biological relevant, protein−RNA interactions. Photo-cross-linking is an effective strategy to convert low affinity interactions into stable covalent bonds, enabling stringent isolation conditions; however, cross-linking between native nucleobases and proteins proceeds with low efficiency.

Photo-Cross-Linkable Oligonucleotide Probes To Identify RNA Modification "Readers". To simplify RNA affinity pulldowns, we introduced photo-cross-linkable oligonucleotide probes containing a diazirine-modified uridine nucleotide[66] (Figure 6A). Diazirines are efficient and modestly sized photo-cross-linkers used widely in chemical biology, but they have not seen much application to RNA, as incorporation into nucleic acids is not always straightforward. Our diazirine-modified oligonucleotides were generated by installing a C5-amino-modified U nucleotide (i.e., 5-aminoallyluridine) during solid-phase synthesis, followed by postsynthesis conjugation of NHS-diazirine to the amino-modified oligo. The C5-modified U allows placement of photo-cross-linker in close proximity to a specific nucleotide motif (rather than at the 5′ or 3′ end of the sequence). Other oligonucleotide modifications necessary for affinity pulldown of "reader" proteins such as biotin and the epitranscriptomic nucleotide can be incorporated during solid-phase synthesis using appropriate modified phosphoramidite building blocks.

To benchmark our strategy, we evaluated photo-cross-linking of our probes to m^{6}A reader proteins from the YTH-domain family.[66] We prepared diazirine-containing oligos with the sequence motif GG(m^{6}A/A)CU* (U* = C5-diazirine-modified U) as well as analogous probes with photo-cross-linkable nucleotides 5-iodouridine (5-IU) or 4-thiouridine (4-SU) in place of the diazirine-modified U residue. Irradiation at 365 nm of a reaction containing these oligo probes with purified YTH domain from YTHDF2 or YTHDC1 demonstrated efficient cross-linking with the m^{6}A-modified oligo probes and only minimal cross-linking to the corresponding oligo containing A instead of m^{6}A, as would be expected for an m^{6}A reader. Notably, cross-linking with our diazirine-modified m^{6}A oligonucleotide probe was considerably more efficient than the corresponding 4-SU or 5-IU oligonucleotides, with comparable specificity for the m^{6}A modification, validating our probe design. The m^{6}A/A oligonucleotide probes were next applied to discover novel m^{6}A readers in HeLa cell lysate using photo-cross-linking, affinity pulldown, and quantitative proteomics to identify proteins selectively enriched by one of the two probes (Figure 6B and 6C). Our comparative proteomics study identified 4/5 of all mammalian YTH-domain proteins (only YTHDC2 was missing in our proteomic data) as the most selective m^{6}A binders. In addition, LRPPRC and FMR1 showed modest preference for binding to the m^{6}A oligo over the A oligo. Interestingly, there were several proteins that displayed the opposite preference and bound more strongly to the A oligo than the m^{6}A. Our data predicts that these proteins are repelled by an m^{6}A methylation mark or that they are "antireaders". The most strongly enriched antireaders were G3BP1/G3BP2, which are the key stress-granule-forming RBPs. Interestingly, CAPRIN1 and USP10, which form a complex with G3BP1 were also repelled by m^{6}A. Using a complementary strategy, Vermeulen and co-workers used an oligonucleotide containing tandem repeats of the GG(m^{6}A/A)CU sequence to profile the m^{6}A interactome and arrived at similar findings.[61] The connection between G3BP1 and m^{6}A modification on mRNA suggests opposing roles in mRNA metabolism, because G3BP1 stabilizes mRNAs and m^{6}A induces their turnover. Further, m^{6}A modifications may play a role in mRNA trafficking to G3BP1-containing stress granules, although experiments in METTL3-deficient cells suggest that m^{6}A plays only a minor role in this process.[67]

Summary and Future Outlook. The characterization of epitranscriptomic reader proteins is an important step toward understanding the function of RNA modifications; however, few RNA modification readers other than the m^{6}A-YTH domain protein system have been described. Our photo-cross-linking strategy provides a powerful method to profile readers of diverse RNA modifications and should accelerate discovery in this area. Toward this end, we have used our method to profile m^{6}A readers in *Drosophila*[68] as well as readers of N^1-methyladenosine (m^{1}A) in human cells[69] (Figure 6D). Interestingly, our studies showed that YTH-domain proteins YTHDF1/2 can recognize m^{1}A on mRNA in addition to m^{6}A, consistent with a report from Wang and co-workers.[70] One of the limitations in applying probes of defined sequence is knowledge of the native and physiologically relevant sequence context within which a modification is found. As the accuracy of epitranscriptomic maps improves, integration of sequencing data with the generation of synthetic probes will enable site-specific interrogation of RNA modification function.

5. SUMMARY AND FUTURE PERSPECTIVE

In this Account, we describe our lab's efforts to apply chemistry and chemical biology to the study of RNA, focusing on the regulation of RNA through post-transcriptional modifications and interactions with RNA-binding proteins. A theme in our work is the installation of chemical probes and tags into RNAs to report on biochemical processes occurring in native environments (i.e., cells, lysate). As we have shown, manipulation of the chemical structure of RNA can be accomplished by exploiting a diversity of methods including synthetic chemistry and chemical reactivity, metabolic pathways, and RNA-modifying enzymes. Leveraging powerful advances in sequencing, proteomics, and imaging, these chemistry-inspired tools allow us to investigate RNA-associated processes with high molecular resolution across the transcriptome.

Our group and others have revealed the increasingly central and multifaceted role of RNA in biological processes. For example, some tRNA-modifying enzymes use primary metabolites as cofactors/substrates,[71−73] serving as a bridge between metabolic pathways and protein translation. In addition, the RNA-binding proteome is rapidly expanding to include protein families previously thought to operate independently of RNA, such as transcription factors, which appear to exhibit pervasive RNA binding.[74,75] Further, it is becoming increasingly apparent that cellular organization in the nucleus and cytoplasm relies

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upon RNA as a structural and regulatory scaffold.[76] As the field continues to advance, it is likely that additional biological roles for RNA will emerge, and continued innovation in chemical and biological methods for RNA analysis will be critical for probing these processes. Finally, industrial and academic groups are actively pursuing RNA as a drug target and as a therapeutic agent.[77] Understanding how RNA regulatory pathways operate in "normal" conditions and are altered in disease states should provide new opportunities for targeting these mechanisms, and new insights for leveraging RNA chemistry and biochemistry in next-generation RNA therapeutics. We are optimistic that our work, and that of our colleagues in the RNA chemistry and biology communities, will help accelerate these important efforts.

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Notes

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