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A molecular-level perspective on the frequency, distribution, and consequences of messenger RNA modifications

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Abstract

Cells use chemical modifications to alter the sterics, charge, and conformations of large biomolecules, modulating their biogenesis, function, and stability. Until recently post-transcriptional RNA modifications were thought to be largely limited to nonprotein coding RNA species. However, this dogma has rapidly transformed with the discovery of a host of modifications in protein coding messenger RNAs (mRNAs). Recent advancements in genome-wide sequencing technologies have enabled the identification of mRNA modifications as a potential new frontier in gene regulation—leading to the development of the epitranscriptome field. As a result, there has been a flurry of multiple groundbreaking discoveries, including new modifications, nucleoside modifying enzymes ("writers" and "erasers"), and RNA binding proteins that recognize chemical modifications ("readers"). These discoveries opened the door to understanding how post-transcriptional mRNA modifications can modulate the mRNA lifecycle, and established a link between the epitranscriptome and human health and disease. Despite a rapidly growing recognition of their importance, fundamental questions regarding the identity, prevalence, and functional consequences of mRNA modifications remain to be answered. Here, we highlight quantitative studies that characterize mRNA modification abundance, frequency, and interactions with cellular machinery. As the field progresses, we see a need for the further integration of quantitative and reductionist approaches to complement transcriptome wide studies in order to establish a molecular-level framework for understanding the consequences of mRNA chemical modifications on biological processes.

This article is categorized under:

RNA Structure and Dynamics > RNA Structure, Dynamics and Chemistry

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KEYWORDS

epitranscriptome, mRNA modification

Joshua D. Jones and Jeremy Monroe contributed equally to this study.

1 | INTRODUCTION

Chemical modifications have been studied as key modulators of RNA biogenesis, function, and stability for over half a century (Cohn & Volkin, 1951; F. F. Davis & Worthington Allen, 1957; Helm & Alfonzo, 2017; R. P. Perry, Kelley, Friderici, & Rottman, 1975; Rottman, Shatkin, & Perry, 1974). Until recently, post-transcriptional modifications were thought to be largely limited to noncoding RNAs (ncRNAs), as only three modifications, N7-methylguanosine (m^7G), N6-methyladenosine (m^6A), and inosine (I), were known in protein coding messenger RNAs (mRNAs) (Desrosiers, Friderici, & Rottman, 1974; Morse & Bass, 1997; Nachtergaele & He, 2017; Paul, 1998; R. P. Perry & Kelley, 1974). The discovery of over a dozen enzymatically incorporated modifications in mRNAs has shifted this paradigm and generated tremendous interest because mRNA modifications have the potential to control protein expression (Figure 1; Frye, Harada, Behm, & He, 2018; Gilbert, Bell, & Schaening, 2016).

It is still unclear if most modifications result from background off-target activities of ncRNA modifying enzymes, or if they represent a new layer of post-transcriptional control. Regardless, there are likely to be biological consequences for mRNA modifications, as these chemical tags can influence the interactions between mRNAs and the cellular machinery. The study of mRNA modifications (the epitranscriptome, Figure 2) is a rapidly emerging field as researchers seek to establish the influence of mRNA modifications on biology and human health (Gilbert et al., 2016; Hoernes, Huttenhofer, & Erlacher, 2016; Nachtergaele & He, 2017; Peer, Rechavi, & Dominissini, 2017; Roundtree, Evans, Pan, & He, 2017; Saletore et al., 2012). Initial correlative studies have revealed links between a subset of modifications and essential biological functions including development, sex determination and *circadian rhythm maintenance*, multiple cancers, and diseases (Angelova et al., 2018; Q. Cui et al., 2017; De Jesus et al., 2019; Fustin et al., 2018; Haussmann et al., 2016; L. J. Li, Fan, Leng, Pan, & Ye, 2018; Lin, Choe, Du, Triboulet, & Gregory, 2016; Roundtree, Evans, et al., 2017; Sibbritt, Patel, & Preiss, 2013; Yoon et al., 2017; Zhang et al., 2016; Zhong et al., 2018). However, key fundamental questions regarding the incorporation and molecular-level consequences of mRNA modification need to be investigated to understand how mRNA modification status contributes to discrete biological processes and disease states (Figure 3).

The mRNA epitranscriptome is chemically diverse, containing nucleoside isomers, methyl, acetyl, hydroxymethyl, and formyl modifications (Figure 2; Arango et al., 2018; Carlile et al., 2014; Delatte et al., 2016; Dominissini et al., 2016; Huber et al., 2015; Jia et al., 2011; Lovejoy, Riordan, & Brown, 2014; S. Schwartz et al., 2014; Squires et al., 2012; Tardu, Jones, Kennedy, Lin, & Koutmou, 2019; Xu et al., 2017). Modifications are present in eukaryotic, bacterial, and viral mRNAs (Deng et al., 2015; Kennedy, Courtney, Tsai, & Cullen, 2017). Advances in sequencing technologies enabled the development of techniques to identify the location of modifications transcriptome wide (Amort et al., 2017; Delatte

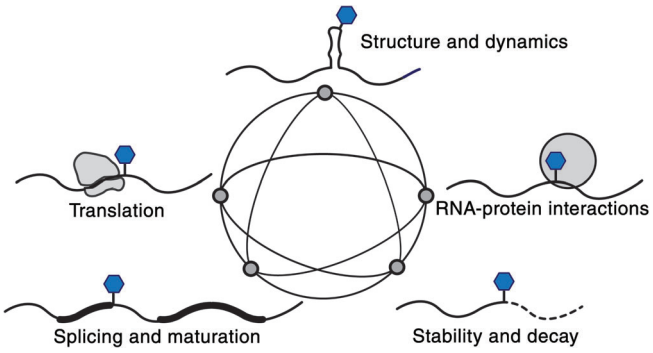

FIGURE 1 Chemical modifications have the potential to individually influence mRNA structure and dynamics, splicing and maturation, RNA–protein interactions, translation, and stability. The interconnected nature of the mRNA life cycle can intensify the effect of a modification through the modulation of downstream processes. For example, several mRNA modifications, m^6A, m^1A, m^1G, Ψ, and f^5C, have been shown to change the stability of RNA structures and would be predicted to redistribute the ensemble of mRNA secondary structures present in a cell (Charette & Gray, 2000; D. R. Davis, 1995; B. Liu et al., 2018; Roost et al., 2015; Spitale et al., 2015; R. Wang et al., 2016; Zhou et al., 2016). This alteration can modulate the ability to form RNA–protein interactions, which can in turn impact mRNA maturation, translation, and decay through pathways dependent on these interactions. Additionally, mRNA translation rates and mRNA decay rates are coupled, with poorly translated mRNAs being targeted more robustly for decay (Presnyak et al., 2015; Radhakrishnan et al., 2016). Thus, if an mRNA modification strongly impacts one step in an mRNA's life, this perturbation is likely to be observed in the outcome of related processes (e.g., modification induced perturbations in mRNA structure could slow translation, which in turn reduces the mRNA's half-life)

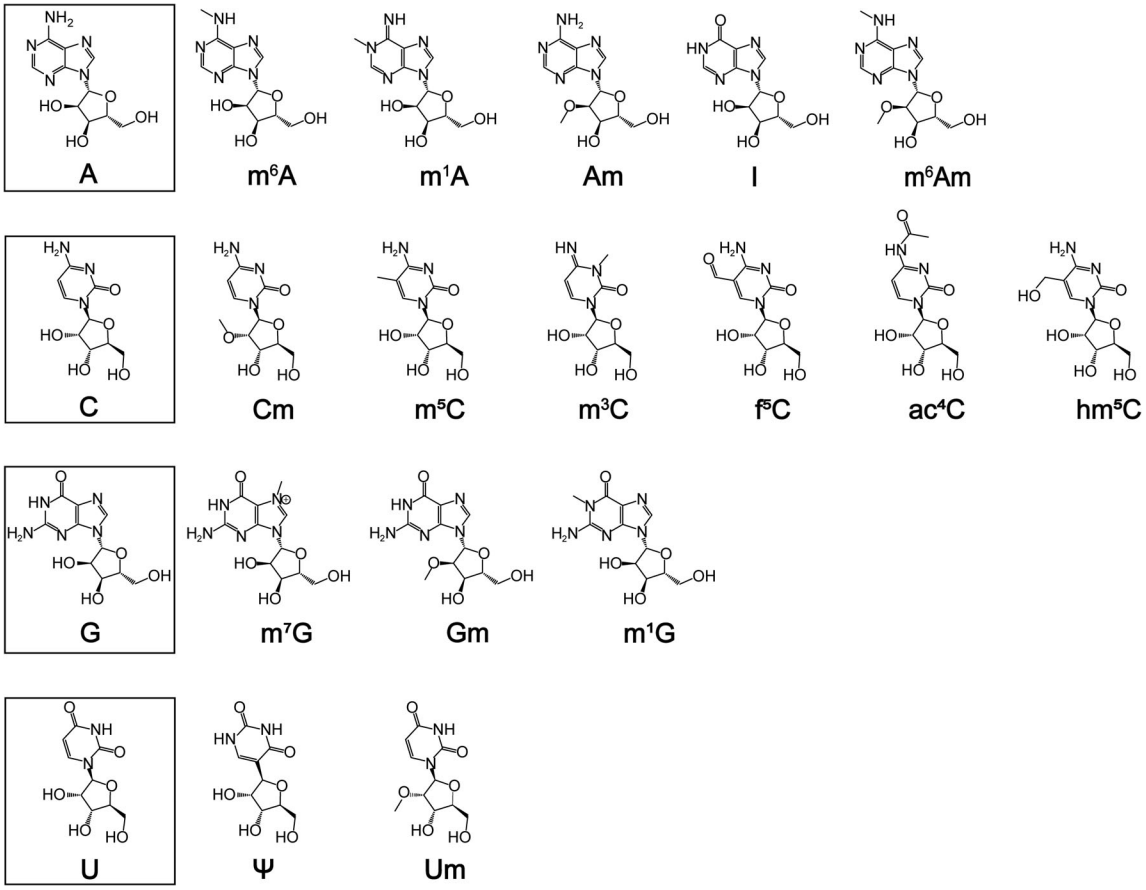

FIGURE 2 Reported messenger RNA (mRNA) modifications. Unmodified nucleosides are shown in boxes, while the modified nucleosides are unboxed. The full names of the nucleosides are: A, adenosine; m^6A, N^6-methyladenosine; m^1A, 1-methyladenosine; Am, 2′-O-methyladenosine; I, inosine; m^6Am, N^6, 2′-O-dimethyladenosine; C, cytidine; Cm, 2′-O-methylcytidine; m^5C, 5-methylcytidine; m^3C, 3-methylcytidine; f^5C, 5-formylcytidine; ac^4C, N^4-acetylytidine; hm^5C, 5-hydroxymethylcytidine; G, guanosine; m^7G, 7-methylguanosine; Gm, 2′-O-methylguanosine; m^1G, 1-methylguanosine; U, uridine; Ψ, pseudouridine; Um, 2′-O-methyluridine

et al., 2016; Motorin & Helm, 2019). These approaches have given us an expansive bird's-eye view of mRNA modifications by providing detailed maps of where 12 modifications can be incorporated into the transcriptome [N6-methyladenosine (m^6A) pseudouridine (Ψ), N4-acetylcytidine (ac^4C), N1-methyladenosine (m^1A), N7-methylguanosine (m^7G), 2′O-methyl modifications (Cm, Am, Gm, Um), 5-methylcytidine (m^5C), and 5-hydroxymethylcytidine (hm^5C), and inosine (I)] (Alon et al., 2015; Amort et al., 2017; Arango et al., 2018; Carlile et al., 2014; X. Cui et al., 2017; Danecek et al., 2012; Delatte et al., 2016; Dominissini et al., 2012; Garcia-Campos et al., 2019; Kim et al., 2004; Levanon et al., 2004; X. Li et al., 2015; Lovejoy et al., 2014; Meyer et al., 2012; Sakurai, Yano, Kawabata, Ueda, & Suzuki, 2010; S. Schwartz et al., 2014; Squires et al., 2012; Yang et al., 2017; You, Dai, & Wang, 2017; Yuan et al., 2019; L. S. Zhang et al., 2019).

Our best understanding of how mRNA modifications can influence gene expression comes from a long-standing body of work of inosine, and overlying the findings of transcriptome wide studies with genetic and biochemical investigations of m^6A (Bajad, Jantsch, Keegan, & O'Connell, 2017; S. Schwartz, 2016). Inosine can contribute to mRNA stability, splicing, and translational recoding (Bazak et al., 2014; Ferreira et al., 2016; Garrett & Rosenthal, 2012; Licht et al., 2019; Peng et al., 2018; Ramaswami et al., 2013; Rueter, Dawson, & Emeson, 1999). Similarly, the primary consequence of m^6A is mRNA degradation, though effects on transcript maturation and translation have also been reported (Ke et al., 2017; Pendleton et al., 2017; X. Wang et al., 2014; Xiao et al., 2016; X. Zhao et al., 2014). Eukaryotes possess a series of "reader" and "eraser" proteins that bind m^6A-containing transcripts to mediate these effects (Patil, Pickering, & Jaffrey, 2018; Rajecka, Skalicky, & Vanacova, 2019; S. Schwartz, 2016; H. Shi, Wei, & He, 2019). Inosine prevalence and the conservation of m^6A reader proteins across a variety of eukaryotic species suggest that at least some modifications could contribute to biological function. Despite our in-depth knowledge of where mRNA modifications can exist and

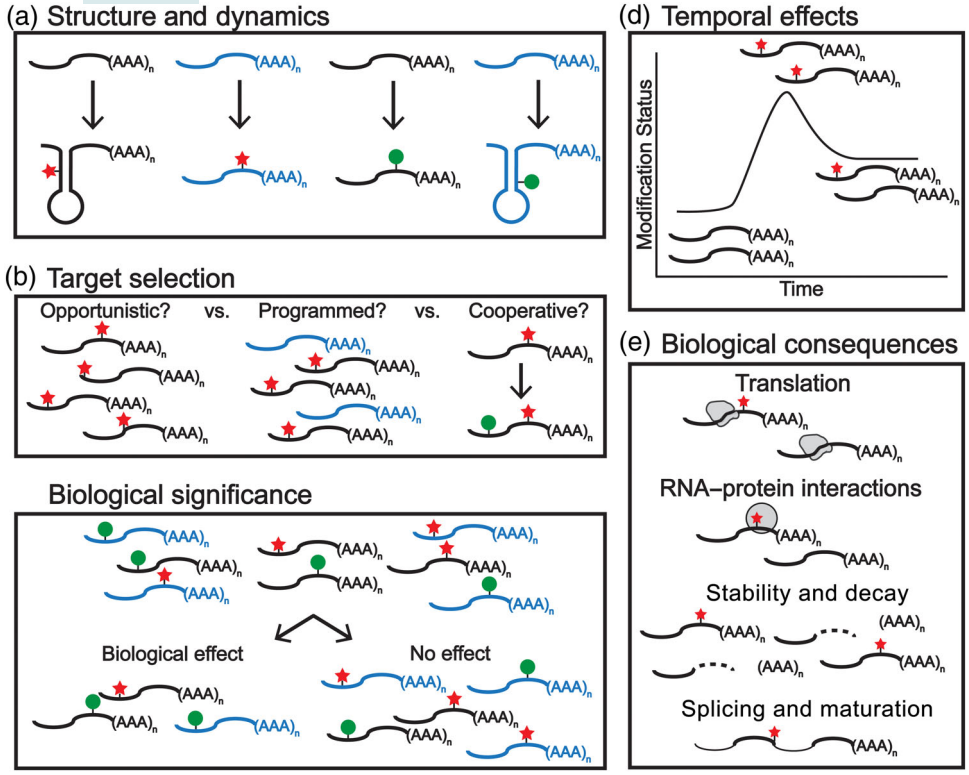

FIGURE 3 The implementation of quantitative approaches will allow us to critically assess some of the key questions in the epitranscriptome field and establish a molecular understanding of individual messenger RNA (mRNA) modifications. Here, we present several knowledge gaps that we think can be best filled using quantitative approaches: (a) Several mRNA modifications, m^6A, m^1A, m^1G, Ψ, and f^5C, affect the stability of RNA secondary structure, but limited knowledge is known about the effect of other chemical modifications (Charette & Gray, 2000; D. R. Davis, 1995; B. Liu et al., 2018; Roost et al., 2015; Spitale et al., 2015; R. Wang et al., 2016; Zhou et al., 2016). High-resolution structural biology and secondary structure-probing techniques are needed to uncover modification mediated structural changes. (b) Current transcriptome wide sequencing approaches have uncovered thousands of modification sites, but little is known about how modification insertion sites are selected. Modifications could be randomly incorporated on available sites, incorporated on specific locations of target transcripts, or there could be cross talk between sites on a single transcript (cooperative incorporation). Kinetic and thermodynamic investigations of modifying enzyme selectivity and broad analyses of the contributions of structure to selectivity (as in Carlile et al., 2019), coupled with measurements of the stoichiometry of multiple modifications on individual transcripts can help to distinguish between these models. (c) Targeted approaches will be required to discern which mRNA modification sites are biologically relevant. Measurements of modification stoichiometry, and assessment of how the stoichiometry at individual sites varies as a function of cell cycle, environment and disease is one example of experiments that could be done to identify significant sites of modification. (d) Occupancy of individual sites might be temporally controlled, and therefore the stoichiometry of individual sites need to be quantitatively assessed as a function of time. Without this information it is likely that biologically relevant sites may be overlooked. (e) It is difficult to deconvolute the impact of mRNA modifications on mRNA–protein, splicing, mRNA stability, and mRNA translation on protein output in cells (see Figure 1). Reconstituted systems are ideally suited to overcome this challenge by allowing researchers to dissect how each individual interaction is influenced by mRNA modifications. These sorts of studies can help to establish which biological processes are likely more impacted by particular modifications, and have the potential to suggest likely consequences of mRNA modifications

growing molecular-level insight into inosine and m^6A function, we have a limited (or no) picture of the biological role for the other 14 reported mRNA modifications.

Establishing a quantitative, biochemical framework for understanding individual mRNA modifications to complement existing transcriptome wide datasets is one of the next milestones for the epitranscriptome field. In particular, measurements of the frequency (stoichiometry) of individual modification sites under a variety of cellular conditions and disease states will be required to determine which sites are the most biologically meaningful. Additionally, in vitro structural, thermodynamic, and kinetic studies characterizing how interactions between proteins, ncRNAs, decay machinery, and the ribosome are changed by mRNA modifications will provide a deep understanding of how post-transcriptional modifications can influence protein production. Such work can also potentially reveal additional

functions of modifications that might not be immediately obvious from correlative studies. Together, these data will enable us to critically consider some of the key questions in the emerging epitranscriptome field—including assessing the potential biological significance/insignificance of individual modifications and modification sites (Figure 3). Here, we will discuss the pioneering studies starting to build a molecular foundation basis for understanding the epitranscriptome. We will focus on studies of modifications other than inosine, as adenosine to inosine (A to I) editing has been extensively reviewed elsewhere (Nishikura, 2016; Walkley & Li, 2017; Y. Wang, Zheng, & Beal, 2017). This review will emphasize the work conducted to quantify modification abundance, frequency of incorporation, and interactions with the cellular machinery.

2 | QUANTITATIVE APPROACHES FOR STUDYING mRNA MODIFICATION LEVELS AND CONSEQUENCES

We will begin by presenting and contextualizing some of the quantitative approaches used to characterize mRNA modifications. In particular, we will discuss methods that measure mRNA levels and stoichiometries to quantitatively describe the mRNA modification landscape of the transcriptome. Additionally, we will highlight a variety of approaches that provide insight into the biological consequences of mRNA modifications by evaluating the impact of modifications on mRNA:protein interactions, splicing, translation and decay.

2.1 | Liquid chromatography with tandem mass spectrometry (LC–MS/MS) measures total modification abundance

The overall abundance of modifications is an important metric for gauging how broadly a particular modification might influence mRNAs. Sequencing strategies have provided deep insight into where mRNA modifications can be localized. However, sequencing-based approaches cannot reliably report on absolute modification abundances because they rely heavily on the efficiency and specificity of the biochemical workflow as well as the bioinformatic parameters used to analyze the data (peak alignment, peak detection method, etc.; Helm & Motorin, 2017). Direct methods, such as LC–MS/MS, are better suited to measure the overall abundance of modifications in mRNAs. LC–MS/MS is a well-established approach extensively utilized to quantify post-transcriptional modifications in ncRNAs including tRNAs and rRNAs and is increasingly being applied to study mRNA modifications.

In order to measure modification levels by LC–MS/MS, mRNAs are purified and enzymatically hydrolyzed to mononucleosides (Chan et al., 2010; Kowalak, Pomerantz, Crain, & McCloskey, 1993; Pomerantz & Mccloskey, 1990; Russell & Limbach, 2013; Su et al., 2014). The resulting nucleosides are separated using liquid chromatography (LC) and absolutely quantitated by mass spectrometry using multiple reaction monitoring with an internal standard. Early studies used LC–MS/MS to confirm the presence of mRNA modifications found by RNA-seq, but more recently researchers have begun to use LC–MS/MS to identify new modifications and modifying enzymes (Chu et al., 2018; Tardu et al., 2019; Xu et al., 2017). Additionally, high-throughput applications of LC–MS/MS for mRNA modification discovery and characterization are emerging. These approaches are similar to mass spectrometry studies of tRNA modifications that characterize dozens of nucleosides in parallel and allow researchers to rapidly explore a broad chemical space (Chu et al., 2018; Su et al., 2014; Tardu et al., 2019; Xu et al., 2017). Nonetheless, the impact of current LC–MS/MS approaches is limited because they do not provide any sequence context for modified nucleosides and require large quantities of highly purified mRNA.

2.2 | Approaches for measuring modification occupancy

Determining the occupancy of discrete mRNA modification sites is imperative for uncovering the contributions of modifications to biological processes. If modifications serve as a gene regulatory mechanism, then the occupancy of controlled modification sites is likely to vary over the lifetime of an mRNA to alter its function (Figure 3). Transcriptome wide methodologies to measure site occupancy will allow us to form hypotheses about which modified sites may influence biological processes. This is especially true in the context of stress or diseased states, under which modification occupancy levels could dramatically fluctuate. Comparison of the absolute abundances of mRNA modifications with

the number of possible sites of modification indicates that the occupancy of most modification sites are likely sub-stoichiometric, similar to protein post-translational modifications (PTMs; Prus, Hoegl, Weinert, & Choudhary, 2019).

The occupancy of individual sites can be measured with site-specific cleavage and radioactive-labeling followed by ligation-assisted extraction and thin-layer chromatography (SCARLET) (N. Liu et al., 2013). SCARLET uses complementary oligos targeted to known modification sites to investigate the occupancy of the modification at discrete sites. This method takes advantage of the different chemical properties of modified and unmodified nucleosides—using thin-layer chromatography to separate radioactively labeled modified/unmodified RNA species in a manner that enables the quantification of the relative modification frequency (described in detail in Figure 4). Notably, because SCARLET does not rely on the specific recognition of a modification by an antibody or nuclease, it can be applied to all modifications. While SCARLET is a highly accurate method for quantifying the extent of modification incorporation in mRNAs, it requires researchers to know where modification sites exist, can only assess a single site at a time, and is challenging to implement. As such, despite being the first and arguably most reliable method of quantifying mRNA modification occupancy, SCARLET has only been applied to study a handful of m^6A sites, and a single Ψ-modified mRNA (Chen et al., 2015; Duan et al., 2017; X. Li et al., 2015; Linder et al., 2015; N. Liu et al., 2013; Y. Zhao, Karijolich, Glaunsinger, & Zhou, 2016).

Reliable approaches for measuring the transcriptome wide occupancy of mRNA sites only exist for studying m^6A and inosine (Sakurai et al., 2014; Zaccara, Ries, & Jaffrey, 2019). For the purposes of this review, we will focus on m^6A-related methods, m^6A-level and isoform-characterization sequencing (m^6A-LAIC-seq) and m^6A-sensitive RNA digestion and sequencing (MAZTER-seq; Figure 4), as inosine methods are well reviewed in Sakurai et al. (2014), Garcia-Campos et al. (2019), and Molinie et al. (2016). MAZTER-seq and m^6A-LAIC-seq use either an antibody or nuclease to identify modified sites in purified cellular RNA and synthetic mRNA controls. RNA-seq is subsequently used to measure the abundance of both mRNAs of interest and the controls to determine m^6A incorporation frequency. These methods, while powerful, both rely on indirect methods to detect the m^6A modification. The recent advent of direct nanopore ion sequencing technology may eventually permit researchers to directly measure the occupancy of all modifications on individual mRNA transcripts, but further advances in the computational analysis of nanopore data will be required before this approach can be widely implemented for this purpose (Jain, Olsen, Paten, & Akeson, 2016).

2.3 | Transcriptome wide studies of mRNA translation and half-life

Sequencing-based approaches have been used to assess both the half-life and translation of modified mRNAs transcriptome wide. Ribosome profiling is a powerful technique that allows researchers to take a snap-shot of where every ribosome sits on every mRNA in a cell at a given period of time (Ingolia, Ghaemmaghami, Newman, & Weissman, 2009). This approach enables the identification of well- and poorly-translated sequences in vivo, and can even provide insight into how distinct states of translation elongation are modulated (Lareau, Hite, Hogan, & Brown, 2014; C. C. Wu, Zinshteyn, Wehner, & Green, 2019). Similarly, the half-lives of all cellular mRNAs can be measured in parallel by using RNA-seq to observe the time-dependent reductions in mRNA levels following transcriptional shut-off by small molecules, such as actinomycin, or temperature sensitive RNA Polymerase II mutants (Lugowski, Nicholson, & Rissland, 2018). Ribosome profiling and transcriptome wide half-life measurements robustly report on the details of mRNA stability and translation and have greatly advanced our understanding of RNA biology.

Utilizing ribosome profiling and transcriptome wide decay studies to discern the function of a specific modification would ideally involve knocking-out (or knocking-down) an mRNA modifying enzyme, and comparing the translation and stability of modified and unmodified mRNAs. It is important to note that ribosome profiling and transcriptome wide half-life studies report on the average behavior of the overall population of mRNA transcripts containing a given sequence. Therefore, the heterogeneity of modification occupancy introduces challenges for interpreting data collected by these methods. The analysis of such data is further complicated by the fact that most enzymes that catalyze mRNA modification also catalyze the incorporation of the same functional groups into ncRNA species essential to protein translation (e.g., tRNAs, ribosomal RNA [rRNA]; Arango et al., 2018; X. Li et al., 2017; S. Schwartz et al., 2014; Tardu et al., 2019; Xu et al., 2017). Since translation and decay rates are coupled, these potential perturbations to the translation machinery can make complicate efforts to deconvolute the impacts of modifications on ncRNAs from those on mRNAs by these methods (Figure 1; Presnyak et al., 2015; Radhakrishnan et al., 2016). Ultimately, regardless of their limitations, ribosome profiling and transcriptome wide mRNA half-life studies will be useful for uncovering how

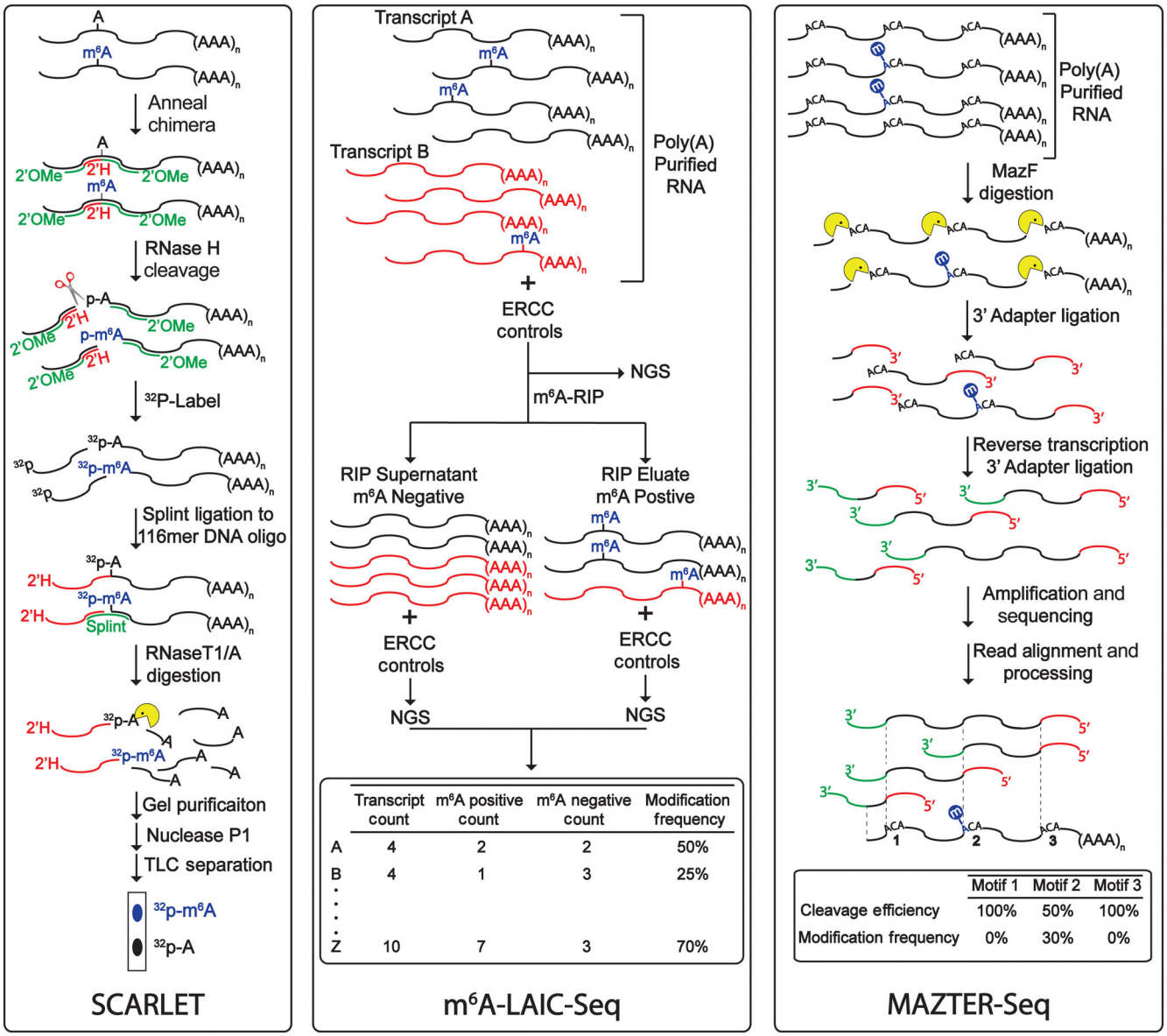

FIGURE 4 Methods to quantify messenger RNA (mRNA) m^6A modification stoichiometry. (a) Site-specific cleavage and radioactive-labeling followed by ligation-assisted extraction and thin-layer chromatography (SCARLET) is the most direct method developed to quantify m^6A frequency (N. Liu et al., 2013). During this process, a chimera is annealed to a specific mRNA where the DNA sequence is immediately upstream of the putative modification site. RNase H is used to cleave the mRNA to release an oligonucleotide containing the putative modification at the 5′ end. The 5′end of the oligonucleotide is ^{32}P-labeled using T4 polynucleotide kinase and is splint ligated to a 116mer DNA oligomer. RNase T1/A is used to digest the resulting chimera to contain a single A or m^6A at the 3′end of the 116mer DNA oligomer. The resulting oligonucleotide is gel purified, digested to nucleosides using nuclease P1, and analyzed using TLC. The stoichiometry is measure based on the relative intensity of the m^6A and A phosphorescence. (b) m^6A-level and isoform-characterization sequencing (m^6A-LAIC-seq; Molinie et al., 2016) utilizes m^6A modified external RNA controls consortium (ERCC) control RNAs to normalize the measured m^6A stoichiometries to increase the accuracy of a standard m^6A-RIP assay. ERCC controls are added before and after m^6A-RIP to normalize the efficiency of the immunoprecipitation and detection by next generation sequencing, respectively. The relative counts of m^6A positive and negative reads of the same transcript determine the stoichiometry. The occupancy levels measured by m^6A-LAIC-seq correlate well with modification levels of mRNA standards ($R = 0.995$) despite the lack of single nucleotide resolution. (c) MAZTER-seq also utilizes RNA-seq to characterize m^6A occupancy transcriptome wide (Garcia-Campos et al., 2019). However, MAZTER-seq does not rely on immunoprecipitation to isolate modified mRNAs, and instead identifies sites using the bacterial nuclease MazF to cleave immediately upstream of ACA sequences but not m^6ACA sequences. Purified mRNA is digested using MazF, and an adapter is ligated to the 3′end of the digested products. The resulting oligonucleotides are reverse transcribed, 3′ adapter ligated, amplified, and end-pair sequenced. Following read alignment and data processing, the stoichiometry is determined by calculating the cleavage efficiency of MazF at a specific ACA motif. While MAZTER-seq has the advantage of quantifying m^6A occupancy at single nucleotide resolution, there are some limitations to this approach. Namely, the lack of quantification at the ~50% of m^6A found outside ACA motifs (Pandey & Pillai, 2019), MazF only cuts at ACA sites with 70% efficiency and can cleave at other sequences resembling ACA, and the values measured by MAZTER-seq only modestly correlate with SCARLET measurements at similar sites (R values = 0.6–0.7; Garcia-Campos et al., 2019)

mRNA modifications change translation and mRNA stability, however, we anticipate that interpretation of transcriptome wide observations will benefit from synergistic in vitro studies.

2.4 | In vitro biochemistry and structural biology

For over 70 years high-resolution structural and functional studies with purified cellular components have proven invaluable for our understanding of how biology controls the production, function and degradation of biomolecules. One of the fundamental principles of biochemistry is that the 3D structure of molecules determines their function. In general, much less is known about the structures of RNAs relative to proteins. This is evidenced by the fact that structures of RNA and RNA-protein complexes represent <5% of the structures deposited in the Protein Data Bank (PDB) (Berman, Henrick, & Nakamura, 2003). High-resolution X-ray crystallography, cryo-EM and NMR studies of modified mRNAs, modified mRNA-protein, and modified mRNA-ribosome complexes will be vital for building a detailed understanding of how modifications can influence mRNA structure (Choi et al., 2016; Eyler et al., 2019; Svidritskiy, Madireddy, & Korostelev, 2016; Zhou et al., 2016). Additionally, lower resolution RNA-secondary structure mapping studies (e.g. using DMS or SHAPE reagents) comparing modified and unmodified mRNAs can provide additional insights information about how the shape of mRNAs is influenced by modifications (Mauger et al., 2019). Structural studies will prove useful not only for understanding how modifications impact the intrinsic properties of mRNAs to impact their function and stability, but also as researchers are seeking to develop therapeutics targeting m^6A-modified transcripts (Cross, 2019).

Changes in mRNA structure, charge, or base-pairing ability can reasonably be expected to alter the interaction of these molecules with other biomolecules. Therefore, in vitro kinetic and thermodynamic assays will be required to establish how modifications alter mRNA interactions with proteins, other RNAs, or the ribosome (Choi et al., 2016; Eyler et al., 2019; Vaidyanathan, AlSadhan, Merriman, Al-Hashimi, & Herschlag, 2017). Such studies are limited in their scale, but have the benefit of being readily interpretable. For example, in vitro translation assays on modified mRNAs have been used to directly report on how modifications alter the action of the translation machinery in a straightforward manner, without the needing to consider in rRNA or tRNA modification status or mRNA stability. Single molecule and bulk transient kinetic studies will allow us to establish how individual functional groups affect specific steps in the translation and decay pathways (Choi et al., 2016, 2018; Eyler et al., 2019). Given the critical contributions of fundamental biochemical and structural studies to our understanding essential biological processes, including post-translational protein modifications, we anticipate that such studies will be vital as researchers begin to understand the types and extent of consequences of mRNA modifications on biology.

3 | CURRENT QUANTITATIVE PERSPECTIVE ON mRNA MODIFICATIONS

In order to evaluate the significance of post-transcriptional mRNA modifications, there are several fundamental questions that remain to be critically investigated (detailed in Figure 3). Here, we discuss how quantitative measurements are allowing researchers to begin addressing some of the key gaps in our knowledge of mRNA modifications. We will examine the available quantitative measurements of mRNA modification levels and incorporation frequency, and consider these findings in the context of post-translational protein modification stoichiometries. Additionally, we will discuss the insights into how mRNA modifications impact interactions between mRNAs and the cellular machinery provided by structural biology, in vitro biochemistry, and quantitative transcriptome wide studies. Together, these data suggest that several mRNA modifications are reasonably abundant, affect mRNA structure, and can influence how proteins and the ribosome interact with transcripts.

3.1 | mRNA modification abundance and frequency

As we begin to investigate the consequences and potential biological functions of mRNA modifications, it seems reasonable to first consider where and how frequently they are present. The levels of different mRNA modifications vary widely; there is a > 1,000-fold range in concentrations for the mRNA modifications levels measured to date (Table 1 and Figure 5). m^6A is the most abundant modification across all organisms (Table 1), as might be expected given its

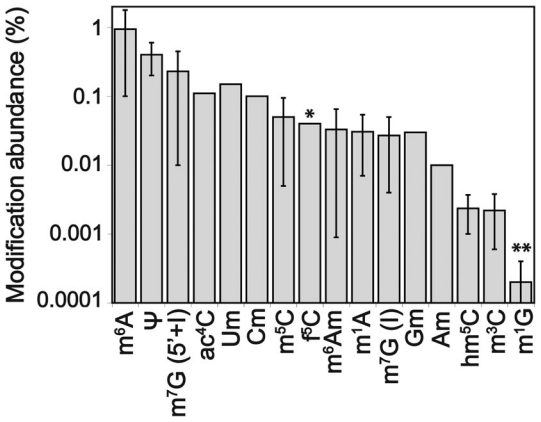

FIGURE 5 LC–MS/MS measurements of mRNA modification abundance. All values displayed are the average values for mammals unless indicated otherwise (* yeast, ** plant) (Table 1). The error bars reflect the range of values measured. Modifications without error bars have only one reported value in the literature

purported widespread role in the post-transcriptional regulation of a host of mRNAs. SCARLET, m^6A-LAIC-seq and MAZTER-seq established a wide range of m^6A frequencies (0–95%) at mapped sites (Chen et al., 2015; Duan et al., 2017; Garcia-Campos et al., 2019; X. Li et al., 2015; Linder et al., 2015; Y. Zhao et al., 2016). Transcriptome wide analyses of m^6A site occupancies revealed a nearly bimodal distribution of m^6A frequency (centered around ~10% and 50–60%), and demonstrate that m^6A modification frequencies differ on the same transcripts between GM12878, HEK293T, and hESC cells. m^6A modifications are enriched in mRNA 3′ UTRs and near stop codons (Dominissini et al., 2012; Meyer et al., 2012). Notably, the presence of m6A in 3′ UTRs opens up the possibility that m6A could be incorporated into some miRNA target sites to modulate miRNA-binding. Regardless of where they are localized, the data suggest that m^6A sites are essentially never fully occupied on any given transcript, making it unlikely that modifications serve as a binary switch to dictate the maturation, movement or behavior of the entirety of mRNA molecules of any particular sequence.

Several modifications, Ψ, ac^4C, Cm, and Gm, have abundances approaching those of m^6A (Arango et al., 2018; X. Li et al., 2015; Tardu et al., 2019; You et al., 2017). These modifications have been less well studied than m^6A, but their prevalence and localization in mRNA coding regions suggests that they might also play roles in regulating mRNA function (Arango et al., 2018; Carlile et al., 2014; X. Li et al., 2015; Lovejoy et al., 2014; Nakamoto, Lovejoy, Cygan, & Boothroyd, 2017; S. Schwartz et al., 2014; L. Sun et al., 2019; You et al., 2017). Like m^6A, the positions of some of these modifications appear to be conserved. For example Ψ is most commonly found in the second and third position of UUU and second position of UUC codons in protozoa, plant and human mRNA coding regions (Nakamoto et al., 2017; S. Schwartz et al., 2014; L. Sun, Xu, et al., 2019). Little is known about how often modifications other than m^6A or inosine are incorporated at particular mRNA sites. However, estimates of Ψ-frequency based on Ψ-seq experiments suggest that Ψ incorporated into mRNA by pseudouridine synthase 7 (Pus 7) is present at high levels; with a distribution occupancy comparable to ribosomal RNA (rRNA) levels (S. Schwartz et al., 2014). Furthermore, SCARLET measurements of Ψ occupancy in EEF1A1 mRNA are consistent with this conclusion—Ψ is present in 56% of the time in this transcript, a frequency comparable to many of the reported m^6A and inosine sites (X. Li et al., 2015; Sakurai et al., 2014). Together these data suggest that the incorporation frequency of Ψ, at least at some sites, is likely to be high.

Most modifications are present at levels 10- to 100-fold less than m^6A, and a handful of modifications (m^3C, m^1G, hm^5C) are even more rare, with levels at least 500-fold lower than m^6A (Figure 5, Table 1). The location of m^5C modifications have been mapped primarily to mRNA 5′ UTRs, and the relative occupancy of m^5C sites has also been estimated using a stringent bisulfite sequencing method (Amort et al., 2017; T. Huang, Chen, Liu, Gu, & Zhang, 2019). While incomplete conversion of cytidine and m^5C during bisulfite treatment limits the accurate quantification and location of m^5C throughout the transcriptome, control RNAs were utilized to tune treatment conditions and approximate m^5C modification frequency ($R^2 = 0.98$) and location. This study suggests that m^5C occupancy at most sites is low (<20%) relative to m^6A and Ψ, as might be expected from the modest levels of m^5C measured in mRNAs by LC–MS/MS (Figure 5). The modest levels of the majority of modifications suggest that the enzymes responsible for these modifications likely either target only a handful of specific mRNAs targets, as studies indicate is the case for m^1A, or might modify many different mRNAs at relatively low frequencies (Khoddami et al., 2019; Safra et al., 2017). Alternatively, it is possible that the levels of these modifications have just not been measured yet under conditions where the modification is most highly incorporated. We expect to find other relatively rare mRNA modifications, such as m^3C and m^1G, may also exhibit low occupancy at many (though perhaps not all) of their sites.

TABLE 1 Summary of modification abundances measured by targeted liquid chromatography with tandem mass spectrometry (LC–MS/MS)

mRNA modification	Abundance (Mod/main base)	References
N6-methyladenosine (m^6A)		
Mammal	0.1–1.79%	(X. Dai, Wang, Gonzalez, & Wang, 2018; Dominissini et al., 2016; Edupuganti et al., 2017; Jia et al., 2011; X. Li et al., 2016; Roundtree et al., 2017; H. Sun, Zhang, Li, Bai, & Yi, 2019; H. Wang et al., 2019; X. Wang et al., 2014; Wei et al., 2018; Xu et al., 2017; L. S. Zhang, Liu, et al., 2019; X. Zhang et al., 2019; Zheng et al., 2013; Zhu et al., 2017)
Yeast	0.14%	(Tardu et al., 2019)
Plant	0.35–0.7%	(Duan et al., 2017)
Virus	0.01–0.015%	(Martinez-Perez et al., 2017)
7-methylguanosine (m^7G)		
Internal:		
Mammal	0.0004–0.05%	Chu et al., 2018; L. S. Zhang, Liu, et al., 2019)
Plant	0.0032–0.0052%	(Chu et al., 2018)
5′ cap + internal:		
Mammal	0.01–0.45%	(Chu et al., 2018; L. S. Zhang, Liu, et al., 2019)
Plant	0.033–0.037%	(Chu et al., 2018)
1-methyladenosine (m^1A)		
Mammal	0.007–0.054%	(Dominissini et al., 2016; X. Li et al., 2016; Xu et al., 2017; L. S. Zhang, Liu, et al., 2019)
Plant	0.0001%	(Duan et al., 2017)
Pseudouridine (Ψ)		
Mammal	0.2–0.6%	(Dominissini et al., 2016; X. Li et al., 2015)
Yeast	0.11%	(Tardu et al., 2019)
2′-O-methylguansoine (Gm)		
Mammal	<0.03%	(You et al., 2017; L. S. Zhang, Liu, et al., 2019)
Yeast	0.13%	(Tardu et al., 2019)
2′-O-methyluridine (Um)		
Mammal	0.15%	(You et al., 2017)
2′-O-methyladenosine (Am)		
Mammal	0.012%	(You et al., 2017)
2′-O-methylcytidine (Cm)		
Mammal	0.1%	(You et al., 2017)
Yeast	0.2%	(Tardu et al., 2019)
5-methylcytidine (m^5C)		
Mammal	0.005–0.095%	(Huber et al., 2015; Ma, Ding, Ye, Yuan, & Feng, 2019; Xu et al., 2017)
Plant	0.00025–0.085%	(X. Cui, Liang, et al., 2017; Duan et al., 2017)
Yeast	0.1%	(Tardu et al., 2019)
3-methylcytidine (m^3C)		
Mammal	0.0006–0.0038%	(Xu et al., 2017)
1-methylguanosine (m^1G)		
Plant	0.00002%	(Duan et al., 2017)

(Continues)

TABLE 1 (Continued)

mRNA modification	Abundance (Mod/main base)	References
N6,2′-O-dimethyladenosine (m^6Am)		
Mammal	0.0009–0.065%	(Sendinc et al., 2019; H. Sun, Zhang, et al., 2019; Wei et al., 2018; You et al., 2017; X. Zhang, Wei, et al., 2019)
5-formylcytidine (f^5C)		
Yeast	0.04%	(Tardu et al., 2019)
N4-acetylcytidine (ac^4C)		
Mammal	0.11%	(Arango et al., 2018)
Yeast	0.14%	(Tardu et al., 2019)
5-hydroxymethylcytidine (hm^5C)		
Mammal	0.001–0.0037%	(Huber et al., 2015)

Notes: The values reported are the abundance of a modification with respect to its unmodified nucleoside (e.g., m^6A/A, m^7G/G, etc.) within purified messenger RNAs (mRNAs). The modification abundances range over 500-fold from m^6A (the most abundant modification), which displays the potential complexity of how different variants affect the mRNA life cycle. We have displayed the reported mRNA modifications that have been quantified in multiple cell types and tissues (mammalian, yeast, plant, and viral). Moreover, levels of both internal and 5′-cap plus internal m^7G have been quantified using alternative enzymatic digestion strategies.

It is important to note that just because sites are not well occupied in a given transcriptome wide snap-shot, this does not necessarily mean that they are insignificant. PTMs are also typically sub-stoichiometric, and their frequency varies from site to site and in response to cellular conditions (Prus et al., 2019). For example, protein acetylation occurs at a wide range of stoichiometries (<1–98%), with bulk of protein acetylation sites exhibiting very low modification frequencies (<1%) (Baeza et al., 2014; Hansen et al., 2019; Weinert et al., 2015). In contrast, sites of phosphorylation tend to be occupied at a modestly higher levels (>20%) (Carpy et al., 2014; Olsen et al., 2010; Tsai et al., 2015; R. Wu et al., 2011), while N-glycosylation sites are frequently occupied (>60%) (S. Sun & Zhang, 2015; Zielinska, Gnad, Wisniewski, & Mann, 2010). Despite the wide distribution of PTM stoichiometries, they each significantly impact the regulation of protein biology. This highlights the need to extensively characterize mRNA modifications despite the sub-stoichiometric modification of sites.

Furthermore, occupancy of sites might be temporally controlled so that they are only impactful at a particular time (Figure 3). In ncRNAs, nucleoside levels dynamically fluctuate in response to environmental stress, nutrient deprivation, stages in circadian rhythms, and cell cycle progression to alter RNA function (Baudin-Baillieu et al., 2009; Demirci et al., 2010; Helm & Alfonzo, 2017; Maraia & Arimbasseri, 2017; Sergiev, Aleksashin, Chugunova, Polikanov, & Dontsova, 2018). Analysis of mRNA modification abundances under different conditions may provide insight into modification-mediated mechanisms activated by environmental stress or disease. Consistent with this possibility, both the abundances and distributions of mRNAs modifications are dependent on environmental conditions, cell-type, disease, and organism (Table 1; Carlile et al., 2014; Chu et al., 2018; X. Cui, Liang, et al., 2017; Duan et al., 2017; X. Li et al., 2016; Ma et al., 2019; S. Schwartz et al., 2014; Tardu et al., 2019; Zhou et al., 2015). Nucleoside methylations and acetylations exhibit the largest changes in mRNA modification abundance as a result of shifting cellular environments (Tardu et al., 2019). In line with this observation, the metabolites used by enzymes to catalyze methyl- and acetyl- modifications (S-adenosylmethionine and acetyl-CoA) also fluctuate significantly in response to cellular conditions (Anstee & Day, 2012; Pietrocola, Galluzzi, Bravo-San Pedro, Madeo, & Kroemer, 2015; L. Shi & Tu, 2015). Comprehensive studies of modification frequency under varied cellular conditions, disease states, and time points will be necessary to uncover this information and link sites more directly to function (Figure 3).

3.2 | Consequences of mRNA modifications on translation

The functional role of mRNAs in the cell is to serve as blueprints for protein synthesis. As such the translational machinery surely encounters modified mRNAs. Most mRNA modifications have been observed throughout mRNAs,

and the preponderance of Ψ, ac^4C and 2′O-methyl modifications are found in mRNA coding regions (Arango et al., 2018; Carlile et al., 2014; Q. Dai et al., 2017; Nachtergaele & He, 2017; Nakamoto et al., 2017; S. Schwartz et al., 2014; You et al., 2017). Modifications have the potential to change how the ribosome interprets the mRNA blueprint because they can alter the hydrogen bonding interactions between codons and aminoacylated-tRNAs. This possibility is supported by decades of observations indicating that tRNA anticodon nucleobase modifications alter mRNA:tRNA interactions to influence ribosome speed and fidelity (Fan et al., 2017; Fan et al., 2015; Pan, 2018; Phizicky & Hopper, 2015; Ranjan & Rodnina, 2017; M. H. Schwartz & Pan, 2016). However, deciphering the impact of mRNA modifications on translation in biological systems has been challenging for a number of reasons. Foremost amongst these is the fact that most enzymes that catalyze mRNA modification also catalyze the incorporation of the same functional groups into ncRNA species essential to protein translation (e.g., tRNAs, ribosomal RNA [rRNA]; Arango et al., 2018; X. Li et al., 2017; S. Schwartz et al., 2014; Tardu et al., 2019; Xu et al., 2017). The shared origin of many coding and ncRNA modifications has limited the utility of classical genetics to discern mRNA modification function. Furthermore, the heterogeneity in modification occupancy makes it difficult for researchers to directly observe translation of modified transcripts in vivo. Last, it can be problematic to fully deconvolute the impact of translation from protein- and mRNA-stability on protein output in cells. Illustrative of this, reporter-based studies have reached conflicting conclusions regarding how several modifications influence translation (Dominissini et al., 2016; Grozhik & Jaffrey, 2018; Hoernes, Clementi, et al., 2016; Hoernes et al., 2019; Karijolich & Yu, 2011; Svidritskiy et al., 2016). Therefore, for the purpose of this review, we will focus on discussing in vitro studies with reconstituted translation systems that offer a way to directly assess how modifications impact ribosome function.

Initial in vitro studies of varying resolution on a limited set of mRNA modifications indicate that modifications can alter the overall rate and fidelity of protein synthesis (Choi et al., 2016, 2018; Eyler et al., 2019; Hoernes, Clementi, et al., 2016; Hoernes et al., 2018, 2019; Hudson & Zaher, 2015; You et al., 2017). In fully reconstituted *Escherichia coli* translation systems, naturally occurring enzymatic nucleoside modifications and damaged ribonucleobases change translation to varying degrees (Figure 6). The overall level of reporter peptides produced from mRNAs containing a single modified nucleobase is reduced by 2- to >50-fold for m^6A, Ψ, Cm, Am, m^1A, m^6G and m^1G, and is essentially unchanged for m^5C (Hoernes, Clementi, et al., 2016; You et al., 2017). The severity of the protein expression defect is highly dependent on the location of the modification within a codon - protein outputscan range by >25-fold depending on the position of a modification within a single codon (Hoernes, Clementi, et al., 2016; You et al., 2017). The effects observed in *E. coli* have been recapitulated in eukaryotic wheat germ extract translation assays, where m^6A, m^1A, and m^1G were shown to reduce the production of a reporter peptide in a position dependent manner (You et al., 2017). Notably, in contrast to findings in *E. coli*, m^6G also appeared to both impede and enhance protein output in wheat germ extacts depending on where it was localized within a modified codon.

Further elegant bulk and single-molecule mechanistic investigations of 2′ O-methyl, m^6A, Ψ, and m^6G modifications reveal that these modifications impact multiple steps in the ribosome kinetic pathway, reducing the rate of peptide bond formation and guanosine-5′-triphosphate hydrolysis by EF-Tu (Choi et al., 2016, 2018; Eyler et al., 2019; Hudson & Zaher, 2015). 2′ O-methylmodifications and m^6A have been further shown to impede tRNA accomodation—a crucial step in translation elongation (Choi et al., 2016, 2018). Crystal structures of the 30S *Thermus thermophilus* ribosome bound to m^6A-modified mRNAs, and the 70S ribosome on a Ψ-modified mRNA indicate that m^6A and Ψ can still form Watson-Crick base pairs with cognate tRNAs (Choi et al., 2016; Eyler et al., 2019). The structure of the 70S ribosome with $tRNA^{Phe}$ bound to ΨUU in the A site further demonstrates that despite the presence of correct mRNA:tRNA base-pairing interactions, the 3′ CCA of $tRNA^{Phe}$ is not properly positioned in the peptidyl-transfer center (PTC) of the ribosome, consistent with the kinetic observations suggesting that Ψ changes translation (Eyler et al., 2019). Together, these studies indicate that mRNA modifications tend to slow the ribosome and that the magnitude to which a modification perturbs translation depends strongly on the sequence context of the modification.

Ribosome profiling studies have reached slightly different conclusions regrding how modifications impact translation. These studies suggest that m^1A slows translation in mitochondria, and that m^6A and ac^4C enhance translation efficiency (Arango et al., 2018; Li et al., 2017; Wang et al., 2015). There could be several reasons for the differing conclusions reached by ribosome profiling and in vitro studies including that modification reader proteins in the cell might alter translation, and, given the substoichiometric occurance of mRNA modifications, the possibility that the population of mRNAs being well translated might lack the targeted modification. Further work will need to be done to reconcile the relationship between the differing observations between in vitro and ribosome profiling studies.

Since modifications can alter the fundamental properties of RNAs, including their secondary structures and base-pairing abilities, it has been proposed that one consequence of mRNA modification could be to promote the

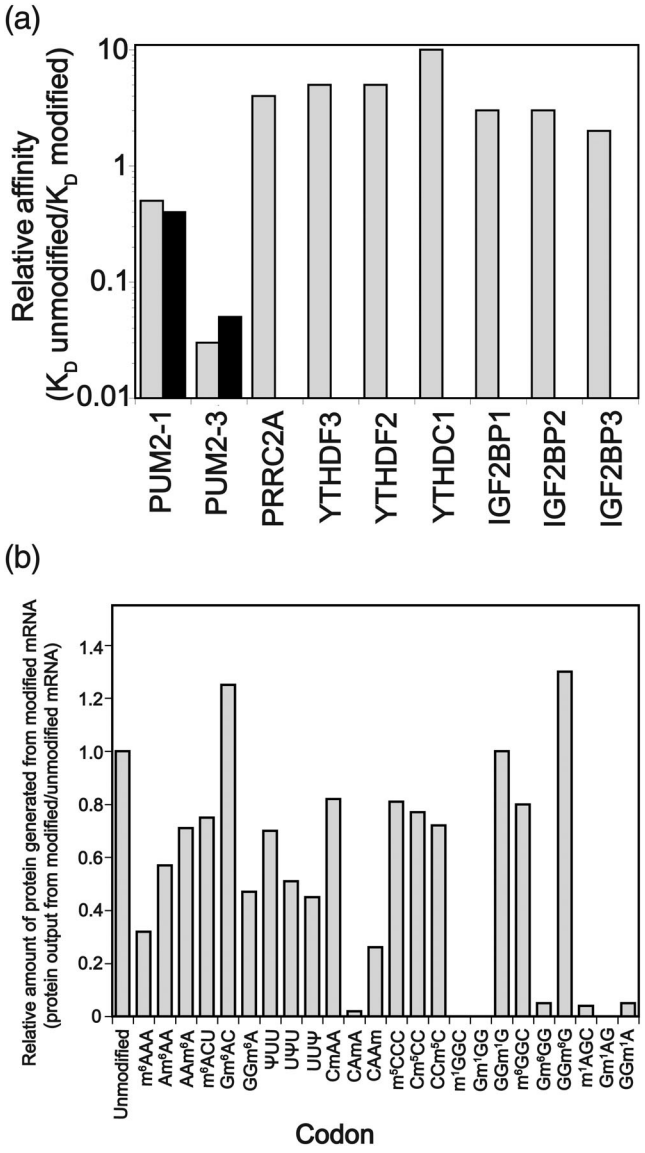

FIGURE 6 Impact of messenger RNA (mRNA) modifications on mRNA–protein interactions and protein synthesis. (a) mRNA-binding protein affinities are modestly altered by nucleoside modifications. The ratio of binding affinities (K_D) for proteins binding to m^6A (gray bars) and Ψ (black bars) unmodified and modified mRNA transcripts binding to a Pumilio proteins (PUM2), and the m^6A binding proteins proline-rich coiled-coil 2A (PRRC2A), YTHDF3, YTHDF2, YTHDC1, and insulin-like factor 2 mRNA binding proteins 1, 2, and 3 (IGF2BP1, IGF2BP2, IGF2BP3) (H. Huang et al., 2018; Vaidyanathan et al., 2017; X. Wang et al., 2014; R. Wu et al., 2019). The affinity of PUM2 was measured for model mRNAs containing 1 (PUM2_1) or 3 (PUM2_3) modifications. (b) Reporter proteins were expressed from mRNAs containing a single nucleotide modification in commercially available fully reconstituted bacterial translation assays. The plot displays the amount of protein produced from the modified mRNA relative to the amount of protein produced from an unmodified transcript as a function of codon. The values in this table were from studies by Hoernes, Clementi et al. (2016) and You et al. (2017). The magnitude of each modification's effect is depends not only on the identity of the modification, but also on the codon in which it is located, the position of the modification within that codon

incorporation of multiple amino acids on a single codon. Establishing if modifications alter tRNA selection on the ribosome is a timely question given that a wide range of modified nucleosides (Ψ, N1-methyl- Ψ, 2-thiouridine, 5-methylcytosine) are being routinely inserted into synthetic mRNAs at high stoichiometric ratios for therapeutic applications (Kariko et al., 2008). Multiple studies indicate that this is indeed possible for m^5C, m^1G, I, Ψ, and m^6G, but not for m^1A and m^6A (Eyler et al., 2019; Garrett & Rosenthal, 2012; Hoernes, Clementi, et al., 2016; Hudson & Zaher, 2015; Licht et al., 2019). Similar to their effects on translation rate, the magnitude of the decoding errors is highly dependent on the position of a modified within a codon. Additionally, Ψ-containing stop codons have been observed to direct the nonsense suppression of translation termination in both bacteria and yeast (Fernandez et al., 2013; Hoernes et al., 2019; Hoernes, Clementi, et al., 2016; Karijolich & Yu, 2011), though the impact of Ψ in stop codons remains an unresolved question, as a follow-up studies have not recapitulated these effects (Eyler et al., 2019; Hoernes et al., 2019; Svidritskiy et al., 2016).

Taken together, there is a growing body of in vitro translation and ribosome profiling studies suggesting that mRNA modifications have the ability to influence both the rate and fidelity of translation. How these alterations contribute to biology still remains to be established. Even if mRNA modifications are not used to directly regulate translation, their impact on translation may still have consequences for biological systems, for example under stress conditions where increased levels of amino acid substitution have been shown to increase cellular fitness (Fan et al., 2017, 2015; M. H. Schwartz & Pan, 2016). Further work will need to be done to determine the differential effects of individual

modifications on the translation mechanism, and identify situations (e.g., times in the cell cycle, environmental stress, or disease) in which more somewhat subtle impacts on translation could contribute to cellular health.

3.3 | mRNA–protein interactions modifications

In addition to understanding how mRNA modifications impact translation, it is also important to gain quantitative insight into the extent that modifications affect mRNA–protein interactions. Modulation of mRNA–protein interactions has the potential to be biologically significant because many RNA binding proteins interact with a multiple mRNA sequences and even small perturbations in affinity have the potential to shift the cellular environment, and thus fate, of a host of mRNAs (Castello, Hentze, & Preiss, 2015; Gerber, Herschlag, & Brown, 2004; Hentze, Castello, Schwarzl, & Preiss, 2018; Jarmoskaite et al., 2019; Lewis, Pan, & Kalsotra, 2017; Patil et al., 2018; Singh, Pratt, Yeo, & Moore, 2015; Ule et al., 2003; Vaidyanathan et al., 2017). Immunoprecipitation, pull-down, mass-spectrometry, and RNA-seq approaches have begun to identify proteins whose interactions with mRNAs are mediated by modifications. These studies reveal that m^6A, m^1A, and m^5C are specifically recognized by proteins that can either read or erase modifications to alter mRNA translation, localization, and stabilization (reviewed in X. Dai et al., 2018; X. Li et al., 2016; F. Liu et al., 2016; Peer et al., 2017; Roundtree, Evans, et al., 2017; S. Schwartz, 2016; Trixl & Lusser, 2019; Yang et al., 2017). To date, no "readers" or "erasers" of other mRNA modifications have been reported, though it has been proposed that the ribosome can serve as a universal reader of modifications in mRNA coding regions (Gilbert, Bell, & Schaening, 2016).

Modifications appear to modulate mRNA stability, suggesting that they impact, either directly or indirectly, interactions between mRNAs and the RNA degradation machinery. Notably, different modifications fine-tune stability in different directions—m^6A generally decreases stability, while ac^4C, Ψ and m^5C tend to increase mRNA half-life (Arango et al., 2018; Kariko et al., 2008; Ke et al., 2017; Nakamoto et al., 2017; Sibbritt et al., 2013). It is not entirely clear precisely how this is accomplished, though the observation that the YTHDC2 m^6A reader interacts with the major 5′→3′ exonuclease involved in mRNA decay, Xrn1, suggests that interactions between modified mRNAs and components of the mRNA degradation pathway may, at least sometimes, be facilitated by modification binding proteins (Kretschmer et al., 2018).

Despite the discovery of several proteins that interact with modified mRNAs, the extent to which modifications alter mRNA-protein interactions is less clear. Thermodynamic dissociation constants (K_D) have only been measured for a handful of the proteins reported to bind modified mRNAs (examples in Figure 6). Initial studies of modified mRNA–protein interactions demonstrate that m^6A and Ψ can both enhance and weaken RNA–protein interactions by ~2- to 20-fold (Figure 6). Members of the YTH-family of m^6A "reader" proteins appear to discriminate between methylated/unmethylated transcripts to a higher level (5- to 20-fold) than other mRNA binding proteins (two- to four-fold) (Figure 6). Interestingly, these studies find modifications only induce relatively modest changes in protein affinities for mRNAs. This suggests that modifications could be more likely to subtly, rather than drastically, shift the balance, and identity of cellular mRNA–protein pools.

4 | CONCLUSION

The epitranscriptome field is quickly opening a new chapter, advancing through modification discovery to investigate the biological roles and mechanisms of a broad set of mRNA modifications. The ground-breaking investigations that established this burgeoning field of study relied heavily on sequencing-based tools to map the location of discrete modifications across all RNAs in a cell. Such studies were a vital first step for the field to establish the existence and pervasiveness of mRNA modifications. More recently, researchers have begun skillfully mining transcriptome wide datasets to infer the biological function of modifications. The next horizon for the emerging mRNA modification field is to establish a molecular-level view of how modifications change interactions between mRNAs and the cellular machinery.

A detailed understanding of the consequences of modifications will be greatly enhanced by the biochemical and structural characterization of individual cellular components and how they interact with modified mRNAs. The quantitative data generated from such experiments (e.g., affinities, on and off rates, reaction rates, etc.) will facilitate the interpretation of existing and future transcriptome wide studies, as they will provide parameters for the mRNA modification community to refine their models of modification function. Structural biology and reductionist biochemistry approaches will provide more than mechanistic details—they have the potential to generate new insights into the function of modifications that cannot be immediately derived by correlative studies. For example, these techniques can

answer temporal questions, allow us to identify highly occupied modification sites, determine how specificity or promiscuity of modifying enzymes dictates target selection, and directly assess how modifications change interactions with the splicing, translation and decay machinery (Figure 3). The ability to compare interactions of fully modified/unmodified mRNAs with purified components will be particularly valuable in light of the challenges of interpreting transcriptome wide mapping, half-life, and ribosome profiling data for heterogenous populations of sub-stoichiometrically modified mRNAs. The initial quantitative studies described in this review demonstrate how biochemistry can reveal aspects of RNA–protein and mRNA–ribosome interactions that are masked by other approaches. The continued integration of quantitative, reductionist approaches combined and transcriptome wide studies will ultimately be required to identify the biological consequences of the epitranscriptome.

CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS

Joshua Jones: Writing-original draft, review, and editing. **Jeremy Monroe**: Writing-original draft, review, and editing. **Kristin Koutmou**: Conceptualization; funding acquisition; writing-original draft, review, and editing.

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