

## Cellular Dynamics of RNA Modification

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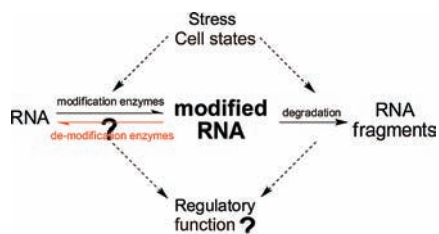
### CONSPECTUS

Five decades of research have identified more than 100 ribonucleosides that are post-transcriptionally modified. Many modified nucleosides are conserved throughout bacteria, archaea, and eukaryotes, while some are unique to each branch of life. However, the cellular and functional dynamics of RNA modification remain largely unexplored, mostly because of the lack of functional hypotheses and experimental methods for quantification and large-scale analysis.

Many RNA modifications are not essential for life, which parallels the observation that many well-characterized protein and DNA modifications are not essential for life. Instead, increasing evidence indicates that RNA modifications can play regulatory roles in cells, especially in response to stress conditions. In this Account, we review some examples of RNA modification that are dynamically controlled in cells. We also discuss some recently developed methods that have enhanced the ability to study the cellular dynamics of RNA modification.

We discuss four specific examples of RNA modification in detail here. We begin with 4-thio uridine ( $s^4U$ ), which can act as a cellular sensor of near-UV light. Then we consider queuosine (Q), which is a potential biomarker for malignancy. Next we examine  $N^6$ -methyl adenine ( $m^6A$ ), which is the prevalent modification in eukaryotic messenger RNAs (mRNAs). Finally, we discuss pseudouridine ( $\psi$ ), which is inducible by nutrient deprivation.

We then consider two recent technical advances that have stimulated the study of the cellular dynamics in modified ribonucleosides. The first is a genome-wide method that combines primer extension with a microarray. It was used to study the  $N^1$ -methyl adenine ( $m^1A$ ) hypomodification in human transfer RNA (tRNA). The second is a quantitative mass spectrometric method used to investigate dynamic changes in a wide range of tRNA modifications under stress conditions in yeast. In addition, we discuss potential mechanisms that control dynamic regulation of RNA modifications as well as hypotheses for discovering potential RNA demodification enzymes. We conclude by highlighting the need to develop new tools and to generate additional hypotheses for how these modifications function in cells. The study of the cellular dynamics of modified RNA remains a largely open area for new development, which underscores the rich potential for important advances as researchers drive this emerging field to the next level.



### Introduction

Naturally occurring RNA molecules contain various chemically modified nucleosides, which are derived from the four standard nucleosides, adenosine, guanosine, cytidine, and uridine. Since the initial discovery of modified nucleosides over five decades ago, more than 100 structurally distinct modified nucleosides have been identified to date in all three domains of life (<http://rna-mdb.cas.albany.edu/RNAmods/>).<sup>1</sup> RNA modifications are present in many different types of cellular RNAs, including ribosomal RNA (rRNA), transfer RNA (tRNA), messenger RNA (mRNA), small nuclear RNA (snRNA), and others. RNA modifications are formed by enzymatic processing of the corresponding

primary transcripts. A large number of enzymes and pathways that catalyze post-transcriptional RNA modifications are known and have been studied for many years and extensively reviewed previously.<sup>2,3</sup>

Renewed interests of RNA modifications also result from the recent discovery of modified nucleosides in regulatory RNAs including microRNA (miRNA), Piwi-interacting RNA (piRNA), and small interfering RNA (siRNA). For example, the conversion of adenosine to inosine, a process mediated by adenine deaminases, has been observed in several miRNAs and is believed to be crucial in regulating miRNA biogenesis.<sup>4,5</sup> piRNAs have 2'-O-methylation at their 3' ends.<sup>6</sup> Several 2'-O-methylation sites on rRNAs are long

known to confer antibiotic-resistance to bacteria,<sup>7</sup> and very recent advances include the discoveries that a 2'-O-methyl group added by bacterial proteins Pnkp and Hen1 to broken tRNAs prevents recleavage of the repaired tRNA by ribotoxins, and 2'-O-methylation of the viral mRNA cap evades host restriction by the IFIT family members.<sup>8,9</sup> Although the biological significance of such methyl modification on piRNAs remains unknown, related methyltransferases (Hen1 or PIMET in flies) have been identified and further studies are needed to elucidate its function and biological consequence.<sup>6</sup> siRNA can also be 2'-O-methylated in vivo, and this methylation could be the final step in assembly of the RNA-induced silencing complex, occurring after an Argonaute-bound siRNA duplex is converted to single-stranded RNA.<sup>10</sup> With the growing interests and efforts to convert siRNA into therapeutic agents for treatment of a variety of diseases (for instance, genetic and viral diseases and cancer), chemically modified siRNA involving modifications to the siRNA backbone, the sugar moiety, and the nucleotide bases of antisense and/or sense strands have been produced and investigated for improved stability, reduced immunostimulation, and increased potency.<sup>11,12</sup> These examples illustrate the importance of elucidating fundamental biological processes in expanding our ability to perturb and manipulate naturally occurring pathways for therapeutic purposes.

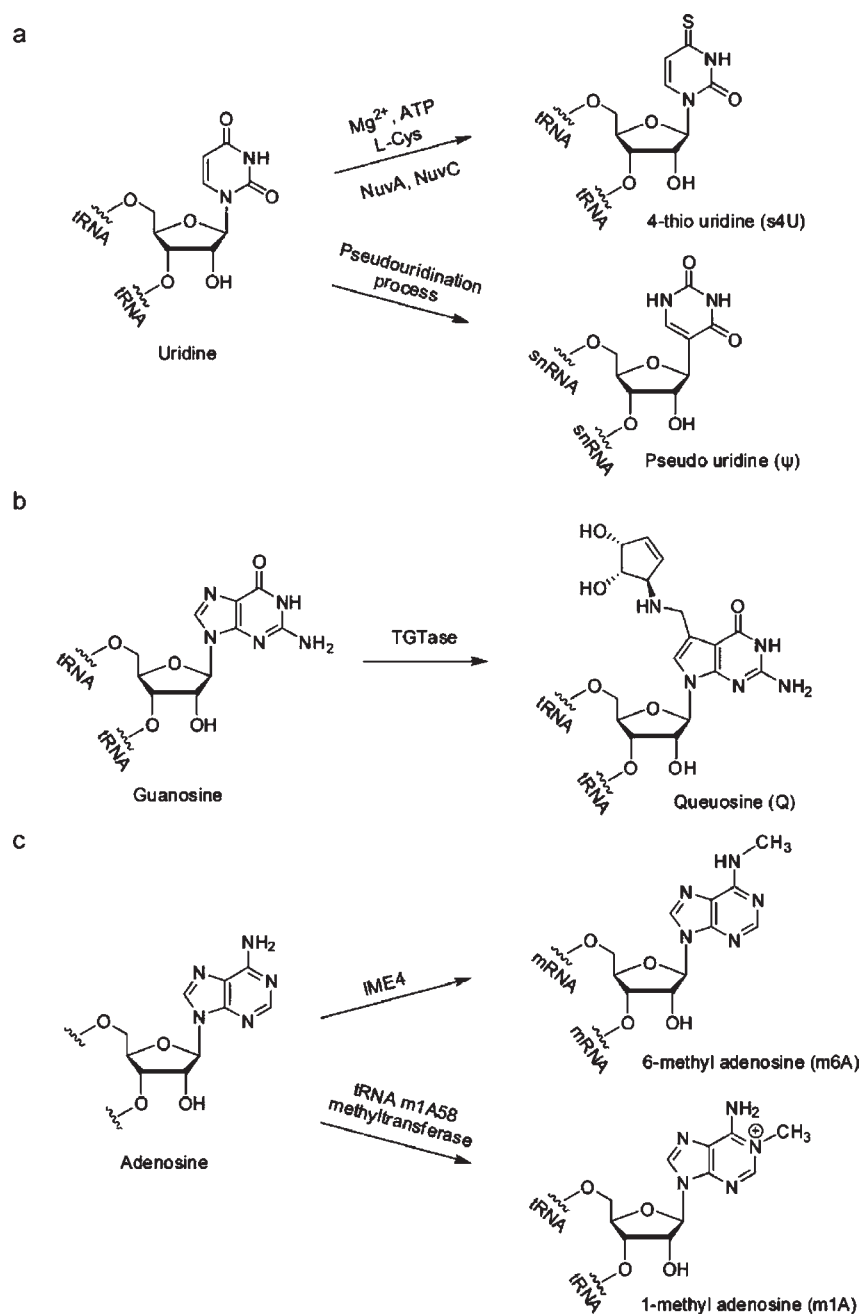
Instead of summarizing the rich knowledge of RNA modifications, related enzymes, and biological pathways which are well-described previously, the focus of this Account is to discuss the dynamic control of RNA modifications. We ask questions like: (1) Can the extent of RNA modifications be regulated when cells are challenged with environmental changes or when cells switch between different physiological states? (2) If dynamic regulation of RNA modification is indeed present, can changing patterns of RNA modification be regulatory for cell function and adaptation? Given that RNA-modifying enzymes are always present in cells, it was widely believed that RNA molecules are modified shortly after they are synthesized and perhaps stay modified thereafter. In the following sections, we describe several inducible RNA modifications to illustrate the dynamics of RNA modifications; we also discuss recent technological advances that enable the investigations of such questions. We hope our discussions will highlight this largely unexplored area and the urgent need for developing new, novel methods to analyze RNA modifications.

## Modified RNA Nucleosides as Possible Sensors and Regulators for Stress Response

Many RNA modifications are not essential for life. The nonessential feature of RNA modification may have been misunderstood and deemed less important because RNA modification is often presented as expanding the chemical repertoire of the nucleoside bases. However, such a "non-essential feature" is common for many biological features, considering that many DNA and protein modifications are not essential either. "Nonessential" modifications in proteins are often used in stress response of cells. One well-documented example is the phosphorylation of the eukaryotic initiation factor 2 (eIF2)  $\alpha$  subunit at Ser51 when cells are challenged with viral infection, amino acid deprivation, and other stresses.<sup>13,14</sup> This phosphorylation stabilizes the eIF2-GDP-eIF2 $\beta$  complex and inhibits the turnover of eIF2, thus leading to global down-regulation of protein synthesis under stress conditions. eIF2 $\alpha$  can also be dephosphorylated by both constitutive and stress-induced phosphatase complexes.

Chemical modifications on RNA can serve as cellular sensors. 4-Thio uridine ( $s^4U$ ) at position 8 of *E. coli* tRNA is formed by the tandem reactions of two enzymes, NuvA and NuvC (Figure 1a).<sup>15</sup> Unlike the four standard and most modified nucleosides, this thiolated uridine has an absorbance spectrum that extends into the near-UV range. *E. coli* with mutated *nuvA* are more easily killed by broadband near-UV light compared to the wild type strain, suggesting a possible role of tRNA  $s^4U$  in protecting cells against UV stress.<sup>16</sup> Both in vitro and in vivo  $s^4U$  can be cross-linked to the nearby nucleoside C13 upon irradiation. Such cross-linked tRNA was shown to cause the accumulation of nucleotide ppGpp, which is the key small molecule regulator of bacterial stress response.<sup>17</sup> Accumulation of ppGpp inhibits cell growth and rRNA synthesis, whereas *E. coli* mutants unable to accumulate ppGpp are more sensitive to near-UV light. Moreover, the formation of the  $s^4U$ -C13 adduct may directly trigger the induction of several ppGpp-inducible proteins, oxidative stress proteins, and the dinucleotide ApppGpp, another small molecule regulator of stress response.<sup>16</sup> Thus,  $s^4U$  acts as a sensor for near-UV light and may up-regulate the synthesis of certain stress-response proteins, through the regulated synthesis of signaling molecules ppGpp and ApppGpp.

Queuosine (Q) is a guanosine analogue often found in the first position of anticodon of tRNAs that read NAY codons (N stands for any of the four bases, Y for U and C). Q modification of tRNA is evolutionarily conserved across all



**FIGURE 1.** RNA modification discussed in this Account. (a) s<sup>4</sup>U and  $\psi$  from uridine. (b) Q modification from guanosine. (c) m<sup>1</sup>A and m<sup>6</sup>A from adenosine.

three domains of life. Interestingly, only prokaryotes, but not eukaryotes, can synthesize queuine (the base of Q) de novo through a multistep synthesis pathway whereas eukaryotes salvage queuine from their diet or from intestinal microflora.<sup>18</sup> Eukaryotic tRNA is modified to Q-containing tRNA via a single step of base-exchange reaction by tRNA-guanine transglycosylase (TGTase) (Figure 1b).<sup>15,19</sup> TGTase can be inhibited by endogenous compounds such as N<sup>7</sup>-methyl G (m<sup>7</sup>G) and pteridine; thus, different

mechanisms (availability of exogenous queuine, presence of endogenous inhibitors and level of TGTase) control the levels of Q modification in tRNA. The presence of Q at the wobble position of several tRNAs affects their codon preference: tRNA<sup>His</sup><sub>(GUG)</sub> prefers CAC<sub>2</sub> over CAU<sub>2</sub>, whereas tRNA<sup>His</sup><sub>(QUG)</sub> has little preference for CAC<sub>2</sub>.<sup>20</sup> In addition, Q-tRNAs have been reported to suppress stop codons as tRNA<sup>Tyr</sup><sub>(GUA)</sub> allows read-through of stop codons while tRNA<sup>Tyr</sup><sub>(QUA)</sub> prevents read-through.<sup>19</sup> However, the

precise physiological role of Q modification has not been established to date. It was reported that lung cancer cells have higher Q deficiency than normal tissue; in fact, the degree of Q hypomodification is related to the severity of malignancy in human lymphoma, leukemia, lung cancer, ovarian carcinoma, and brain tumor.<sup>18</sup> Such correlation could be due to perturbations to any of the mechanisms that affect the Q modification level described above. When a queuine-free diet was fed to germ-free mice in a stress free environment, they showed no pathological symptoms, indicating a possible role of queuine and Q-modified tRNA in stress response.<sup>21</sup> Taken together, although the precise biological function and molecular mechanism of queuine and Q-modified tRNA awaits further study, the correlation between the degree of Q hypomodification and severity of malignancy has been observed in numerous occasions, making Q modification a potential biomarker for malignancy.

### Potential Regulatory Roles of Inducible RNA Modifications

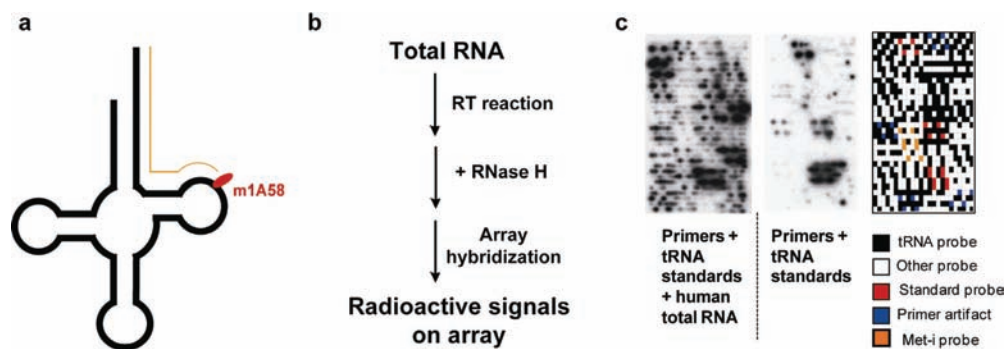
If RNA modifications play regulatory roles, then the extent of certain modifications could vary depending on cellular environment and physiological state. We describe several cases where inducible RNA modifications have been demonstrated in mRNA, spliceosomal RNA, and tRNA to illustrate the possibility of RNA modification having a potential regulatory function.

N<sup>6</sup>-methyl adenine (m<sup>6</sup>A) is present in viral RNAs that replicate in the nucleus and is also an abundant base modification in mRNAs of multicellular organisms (Figure 1c). Unicellular eukaryotes such as yeast were initially thought to lack m<sup>6</sup>A in mRNA; however, it has now been estimated that approximately 50% mRNA of *S. cerevisiae* contain m<sup>6</sup>A during meiosis.<sup>22</sup> In higher eukaryotes including plants, insects, and mammals, m<sup>6</sup>A is present in readily detectable amounts in mRNA. For example, on average there are 3–5 m<sup>6</sup>A sites in HeLa mRNAs, making it the most abundant nucleoside modification in mRNA.<sup>23</sup> Because the presence of a methyl group at N<sup>6</sup> position does not alter its Watson–Crick base-pairing property, detection methods using cDNA sequencing cannot map the m<sup>6</sup>A sites in mRNA, thereby significantly hindering the functional study of m<sup>6</sup>A modifications in mRNA. m<sup>6</sup>A is nonrandomly distributed: using a combination of ribonuclease digestion and chromatography, a consensus sequence RRACH for m<sup>6</sup>A modification was proposed, where R represents purine, the underlined A is the methylation site, and H is a nonguanine base.

In a single site mapped to a nonviral mammalian mRNA, m<sup>6</sup>A is localized to an AGACU sequence in the 3'-untranslated region of bovine prolactin mRNA, and the extent of methylation at this site was estimated to be around 20%. Therefore, in addition to the unknown function of m<sup>6</sup>A in mRNA, its partial modification status adds another layer of difficulty and excitement in studying its biological function.

Another approach to study RNA modifications is to investigate the RNA-modification enzymes. After extensive efforts, the mRNA m<sup>6</sup>A methyltransferase activity in HeLa has been attributed to a 70 kD subunit protein (MT-A70) in a 200 kD protein complex.<sup>23</sup> MT-A70 is shown to localize to speckled domains in the human interphase nuclei and colocalize with U2B', a snRNP protein, indicating that MT-A70 may be associated with nuclear pre-mRNA splicing components and m<sup>6</sup>A on HeLa mRNAs could potentially act as a signal for efficient splicing. Bioinformatics analysis of MT-A70 demonstrates that MT-A70 is a prototype of a previously uncharacterized class of RNA adenosine methyltransferases in a variety of organisms, including *S. cerevisiae* IME4, which is one of the key regulators of yeast meiosis pathways. It is now clear that the IME4 protein is responsible for the formation of m<sup>6</sup>A under sporulating conditions.<sup>24</sup> A very recent study by Fray and co-workers has revealed that a substantial amount of internal m<sup>6</sup>A sites are present in the GpA, but not CpA or UpA context in mRNAs of sporulating cells, consistent with the earlier proposed consensus sequence.<sup>22</sup> Using an m<sup>6</sup>A-specific antibody, the transcripts of three key early meiotic regulators, *IME1*, *IME2*, and *IME4*, were found to contain m<sup>6</sup>A modifications. However, the primary function of m<sup>6</sup>A on mRNAs of sporulating yeast was not interpreted as a splicing signal since none of the three methylated transcripts is spliced. In addition, m<sup>6</sup>A seems to be present in polyA-containing RNAs of all sizes. Genome-wide analysis of methylated transcriptomes using the newly developed m<sup>6</sup>A antibody is expected to provide more insights into the functional role of m<sup>6</sup>A modification.

Pseudouridine ( $\psi$ ) is a C-glycoside rotation isomer of uridine catalyzed in either a guide RNA-independent ( $\psi$  synthases) or guide RNA-dependent (H/ACA box snoRNP and scaRNP) pseudouridination process (Figure 1a).<sup>25</sup>  $\psi$  is found in all species and is particularly common in tRNAs, rRNAs, and spliceosomal snRNAs, but not in mRNAs. It has been estimated that there are about 100  $\psi$ 's in mammalian rRNAs and over 20  $\psi$ 's in vertebrate spliceosomal snRNAs. When in the *anti* conformation with respect to the ribose,  $\psi$  maintains the Watson–Crick interface to pair with adenine like a uridine. The nitrogen atom at position 1, no longer



**FIGURE 2.** Genome-wide microarray method to investigate  $m^1A58$  hypomodification in human tRNA. (a)  $m^1A58$  in the secondary structure of a tRNA. The orange line represents the primer for RT reaction. (b) Brief schematic of the microarray method. (c) Microarray data and array layout.

forming a glycosidic bond to the ribose, is protonated at physiological pH and resides in the major groove. This  $N^1$  proton can be involved in hydrogen bonding with a phosphate group from the same or neighboring nucleotide,<sup>26</sup> contributing to the increased stability of the structure. Furthermore, the presence of  $N^1$  proton enables the retention of a water molecule at the site of  $\psi$  modification.<sup>26–28</sup> Regarding function, certain  $\psi$  residues in rRNA have been shown to play a role in ribosome biogenesis and in protein synthesis; several  $\psi$  sites in U2 spliceosomal snRNA contribute to pre-mRNA splicing.<sup>25,29</sup> There are three “constitutive”  $\psi$  sites in *S. cerevisiae* U2 snRNA:  $\psi35$ ,  $\psi42$ , and  $\psi44$ .  $\psi35$  is formed by pseudouridine synthase Pus7p,  $\psi42$  by snR81 RNP (a H/ACA box snRNP), and  $\psi44$  by protein pseudouridylylase Pus1.<sup>30</sup> In early 2011, Yu and colleagues identified two new, inducible  $\psi$  sites in *S. cerevisiae* U2 snRNA,  $\psi56$  and  $\psi93$ , when cells are grown under nutrient deprived conditions.<sup>30</sup> Formation of  $\psi56$  is catalyzed by Pus7p, while snR81 RNP is responsible for the formation of  $\psi93$ . The presence of  $\psi93$  affects pre-mRNA splicing, suggesting that this inducible modification is a stress-response-related mechanism of the cells. Detailed mutagenesis data have demonstrated that the inducibility of the two new  $\psi$  sites can be attributed to their imperfect consensus sequences for modification. Inducible pseudouridylation could be a lot more widespread than these two sites currently reported.

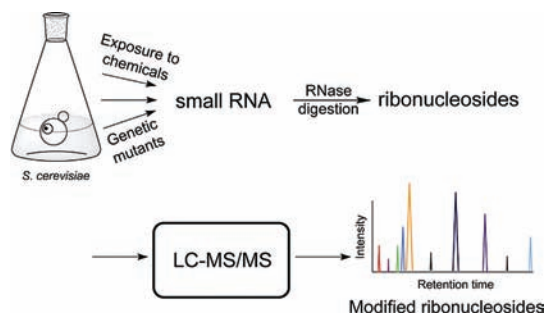
## RNA Modification Studies Stimulated by New Techniques

Discoveries of inducible RNA modifications open up many possibilities in the biological study of RNA modification: Besides the few cases described above, can other RNA modifications vary depending on physiological conditions? What are the molecular mechanisms and regulatory

functions for variation? What is the extent of variations in RNA modifications and what are the biological implications of variable RNA modification patterns? Clearly, experimental methods have to catch up to make such studies possible.

One significant challenge to investigate dynamic RNA modifications is the ability to quantify the extent of modifications. Ideal methods would allow the detection of small changes in the fraction of modified nucleosides for highly abundant cellular RNA such as tRNA or rRNA as well as for low abundant cellular RNA such as mRNA or long intergenic noncoding RNA (lincRNA). In addition, methods that are genome-wide, yet with reasonable specificity toward single modification site would be desirable. This section reviews some of the techniques developed very recently and their applications in the studies of cellular dynamics of RNA modifications.

A genome-wide, microarray based approach was developed in our lab to investigate hypomodification of  $N^1$ -methyl adenine ( $m^1A$ ) at position 58 of human tRNAs (Figure 2).<sup>31</sup>  $m^1A$  is a conserved modification in the  $T\psi C$  loop in most eukaryotic tRNAs. The presence of the methyl group at the  $N^1$  position disrupts the Watson–Crick interface of adenine, but still allows formation of a Hoogsteen base pair with uridine or thymine. In the tRNA tertiary structure,  $m^1A58$  base-pairs to T54 and also introduces a positive charge in the elbow region.<sup>32</sup>  $m^1A58$  stabilizes initiator-tRNA<sup>Met</sup> in yeast and thus is an essential modification; however, it is not required for the stability of some other tRNA species in yeast.<sup>33</sup> This differential response prompted us to examine the possibility of  $m^1A58$  hypomodification in human tRNA and how tRNA function may be affected by this hypomodification. We made use of the extremely low efficiency of reverse transcriptase (RT) to read-through  $m^1A$ , so cDNA for a tRNA beyond A58 is only synthesized



**FIGURE 3.** Application of LC-MS/MS method to study tRNA modifications in yeast. 23 out of the 25 known tRNA modifications in yeast can be quantified with this method.

in the absence of  $m^1A58$  modification (Figure 2a).<sup>34,35</sup> Total RNA isolated from five human cell lines was subjected to primer extension reactions performed with dNTP mixture and [<sup>32</sup>P]TTP (Figure 2b). The amount of the radioactive RT products corresponding to each tRNA directly reflects the level of  $m^1A58$  modification of individual tRNA species. Subsequently, the RNase H treated RT product mixtures were analyzed using tRNA specific microarrays containing DNA probes complementary for human tRNA genes (Figure 2c). Of the five human cell lines examined, hypomodified  $m^1A58$  was detected in approximately one-quarter of all tRNA species, and the  $m^1A58$  hypomodification pattern is quite similar among all five cell lines. siRNA knockdown of the  $m^1A58$  methyltransferase increased the level of hypomodification of a majority of the already hypomodified tRNA species, while the tRNA abundance remained unchanged. A slow-growth defect was observed for the siRNA treated cells. We further demonstrated that  $m^1A58$  hypomodified tRNAs can generally associate with the polyribosome. One possible hypothesis for cells to maintain a unique pattern of  $m^1A58$  hypomodified tRNAs is to readily respond to different cell states or stress conditions. Alternatively, the presence of hypomodified tRNA increases the cellular diversity of tRNA species, and some of the hypomodified tRNAs may perform functions unrelated to translation. In any case, the ability to measure hypomodification of  $m^1A58$  at the genomic scale demonstrates our capability to investigate other RNA modifications that interfere with RT reaction, for example, N<sup>1</sup>-methyl guanine or 2'-O-methylations by microarray methods.

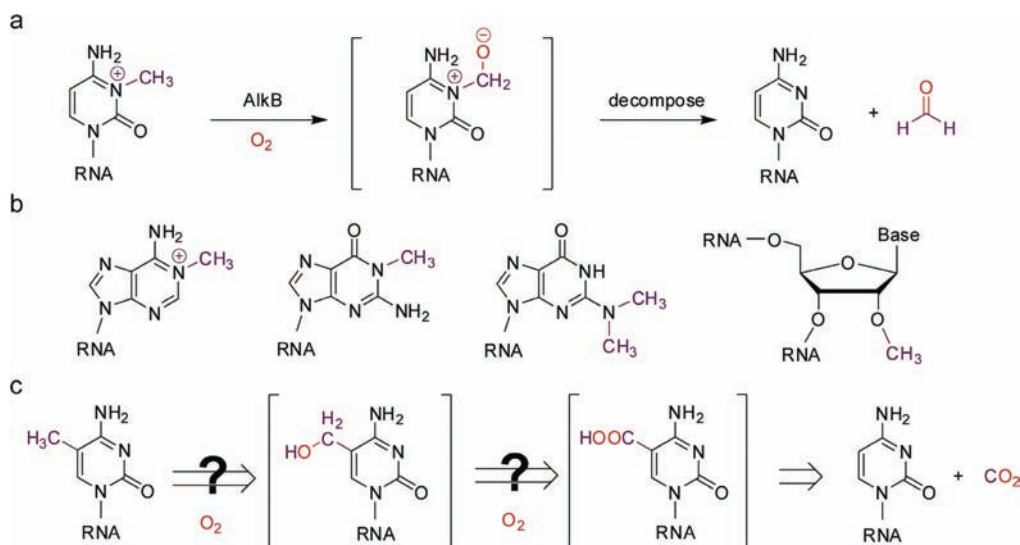
Very recently, Begley, Dedon, and co-workers have developed a highly accurate method for quantification of full set of tRNA modifications in *S. cerevisiae* using liquid chromatography coupled, tandem-quadrupole mass spectrometry (LC-MS/MS) (Figure 3).<sup>36</sup> Earlier works by the Begley group have demonstrated that Trm9, a *S. cerevisiae* tRNA

methyltransferase which methylates the uridine wobble base of tRNA<sup>Arg</sup><sub>(UCU)</sub> and tRNA<sup>Glu</sup><sub>(UUC)</sub>, enhances codon-specific translation elongation and increases the levels of key damage response proteins.<sup>37</sup> In this recent study, they explored the dynamics of tRNA modifications by challenging the cells, either wild type or single gene deletion strains lacking one tRNA modification enzyme at a time, with four chemicals that cause methylation stress (methylmethane sulfonate) or oxidative stress through different mechanisms (hydrogen peroxide, arsenite, and hypochlorite).<sup>36</sup> In wild type yeast, the modification levels of 2'-O-methyl cytidine (Cm), 5-methyl cytidine ( $m^5C$ ), and N<sup>2,2</sup>-dimethyl guanosine ( $m^2_2G$ ) increase upon treatment of hydrogen peroxide, but are either unaffected or decreased when cells are exposed to the other three chemicals. Mutant yeast cells lacking the enzymes responsible for the biogenesis of these three modifications are hypersensitive to hydrogen peroxide, indicating that these particular tRNA modifications and their related biosynthetic pathways could be a key feature of the cellular response toward oxidative stress induced by hydrogen peroxide. Both wobble and nonwobble tRNA modifications exhibit dynamic regulation; they seem to be involved in a complex network of translational control presumably to enable better adaptation to stress. Such a LC-MS/MS method should be applicable to the study of dynamics of other types of RNA modifications; it will also be interesting to determine how RNA modifications in other cellular RNA are regulated during stress conditions.

### Potential Mechanisms of Dynamic RNA Modifications

The hypomodification patterns in  $m^1A58$  in human tRNAs or changes in tRNA methylations under stress in yeast could be achieved through the control of the expression or the activity of (i) the modification enzymes; (ii) the RNA synthesis or degradation machinery; (iii) the demodification enzymes. Control of the modification enzyme can lead to persistent hypomodification patterns for RNA substrates that are not optimized for the modification reaction. Control of the RNA degradation machinery would reduce the amount of hypomodified RNA while synthesis of new tRNA could temporarily increase this amount.

The most intriguing hypothesis for regulatory control of RNA modifications is through the removal of specific modifications by demodification enzymes. Many post-translational modifications of proteins such as phosphorylation, methylation, and acetylation have been shown to be reversible. For DNA, the most important and well-studied



**FIGURE 4.** Possible enzyme mechanisms for demethylation of modified RNA. (a) AlkB-mediated demethylation of N-alkylated nucleic acid bases. (b) Several other N- and O-methylated nucleosides that could be demethylated via an AlkB-like mechanism. (c) Potential demethylation scheme to convert a C5-modified cytosine to a standard cytosine.

epigenetic marker is m<sup>5</sup>C. Recent discoveries of 5-hydroxymethyl cytosine (hm<sup>5</sup>C) in DNA raise the possibilities that, for some sites, the presence of hm<sup>5</sup>C may be a potential route to completely demethylate m<sup>5</sup>C.<sup>38,39</sup>

If demodification enzymes for cellular RNAs exist, what could be the potential mechanisms of action for these enzymes? The *E. coli* AlkB family dioxygenases are recently discovered enzymes that perform oxidative demethylation of m<sup>1</sup>A and m<sup>3</sup>C DNA bases, thus protecting the cells against alkylation DNA damage (Figure 4a).<sup>40,41</sup> Among the nine identified AlkB human homologues, ABH8 has been very recently demonstrated to be a tRNA modification enzyme that carries out oxidation of a methylene group in a specific tRNA.<sup>42,43</sup> ABH8 contains an N-terminal RNA recognition motif, a central AlkB homologous domain and a C-terminal methyltransferase domain. The ABH8 enzyme first adds a methyl group to the wobble base 5-carboxymethyl uridine of tRNA<sup>Gly</sup><sub>(U<sup>•</sup>CC)</sub> using the methyltransferase domain, and the corresponding methyl ester can then be hydroxylated to form 5-methoxycarbonylhydroxymethyl uridine through the action of the AlkB-like domain. The hydroxylated form of this glycy-tRNA was shown in silk worm to significantly affect the affinity of tRNA<sup>Gly</sup><sub>(U<sup>•</sup>CC)</sub> to GGA and GGG codons, and thus probably alters the translational efficiency of mRNAs containing these two glycine codons.<sup>44</sup> ABH8 is localized in the cytoplasm, is abundant in normal human spleen and pancreas tissues, and is highly expressed in bladder cancer cells.<sup>45,46</sup>

Other members of the human AlkB-homologue proteins may be involved in RNA demethylation. In fact, the AlkB protein, in addition to its DNA repair function, was shown to restore the biological function of mRNA and tRNA molecules that were first inactivated through chemical methylation in vitro.<sup>47</sup> Two human homologues, ABH3 and FTO, are both capable of removing methylated bases in DNA or RNA oligonucleotides in vitro, although the biological functions of these two enzymes remain elusive.<sup>48,49</sup> Thus, it is tempting to speculate that the human members of the AlkB proteins have endogenous cellular, methylated RNA substrates, and they carry out oxidative RNA demethylation using the same mechanism characterized for AlkB. Recently, JMJD6, a Jumonji C (JmjC) domain-containing hydroxylase which was thought to be a histone arginine demethylase or U2AF65 lysyl hydroxylase, has been shown to bind efficiently to single-stranded RNA but not to DNA or double-stranded RNA.<sup>50</sup> Another JmjC-domain-containing protein, TYW5, is a RNA hydroxylase in the biosynthesis of a hypermodified nucleoside, hydroxywybutosine, in tRNA<sup>Phe</sup><sub>51-53</sub>. It is possible that other JMJD family dioxygenases, some of them with no known functions currently, could also be RNA demodification enzymes.

### Future Directions: RNA Epigenetics?

In this Account, we describe the possibility of dynamic regulation of RNA modifications. We discuss known examples of inducible RNA modification and their potential biological function, and explore specific mechanisms on how

biological events could be affected by the extent of RNA modification. We envision that breakthroughs in several areas would significantly facilitate the growth of this emerging field:

**1. New Methods.** A primary bottleneck to advance this area is the lack of high throughput and quantitative methods. Although a couple of genome-wide studies have been described here, these methods are very laborious and have the ability to examine just a limited numbers of modifications. Most limiting for all existing methods is that only abundant cellular RNA (tRNA, rRNA, and snRNA) can be studied. For many RNA modifications present in low abundant cellular RNA, for example, mRNA, no genome-wide method exists to enable their investigations. This is because amplification is often required for studies of low-abundant RNA; the current amplification method, RT-PCR, erases the signature of many types of RNA modification.

In the past few years, DNA sequencing has become a major tool in the studies of RNA biology. However, sequencing after RT-PCR amplification cannot provide a direct view of RNA modification types or extents. Single molecule sequencing of nucleic acids in real time offers some hope in achieving this goal. For example, a recent report by Pacific Biosciences shows that m<sup>6</sup>A, m<sup>5</sup>C, and hm<sup>5</sup>C modification in DNA can be directly recorded during sequencing due to their distinct chemical features in DNA polymerase reaction.<sup>54</sup> One hopes that this or other methods would be sufficiently advanced in the future to enable genome-wide detection, quantification, and characterization of RNA modification.

**2. Discovery of RNA Demodification Enzymes.** Identification of new biological enzymes with demodification functions on endogenous RNA substrates and elucidation of the corresponding biological pathways would fully establish and reveal the regulatory roles of dynamic RNA modification. As discussed above, oxidative demethylation offers one potential route to reverse RNA methylation (Figure 4); however, RNA modifications are present in many chemical types and other enzymes may be found that remove other types of modifications.

**3. New Functional Hypotheses for Cellular Dynamics of RNA Modification.** The field currently tends to focus on the obvious functions modified RNAs perform. For example, most RNA modification functions are examined and discussed in the context of translation for tRNA and rRNA and splicing for snRNA. However, tRNAs have many extratranslational functions for which modifications could make a difference. mRNA modifications in mammals have been suggested to play a role in immune response.<sup>55,56</sup> Once the cellular pathways and the enzymes involved for dynamic RNA modifications are better described, new

functional hypotheses will be necessary to fully understand the biology of RNA modification.

Due to their diverse and complex nature, post-transcriptional RNA modifications are difficult to study and this field has lagged behind other areas in RNA biology. The combination of new technologies, new ideas and new results could some day indicate that RNA modification is highly dynamic and is involved in a wide range of biological processes. Like the epigenetic states of histone protein modification and DNA methylation, the pattern and selectivity of RNA modification could also be regulated and maintained in distinct cell types and physiological states. RNA modification may represent a fundamental source of epigenetic diversity in biological regulation.

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#### BIOGRAPHICAL INFORMATION

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#### FOOTNOTES

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