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Review

Sequencing methods and functional decoding of mRNA modifications

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ABSTRACT

More than 160 types of post-transcriptional RNA modifications have been reported; there is substantial variation in modification type, abundance, site, and function across species, tissues, and RNA type. The recent development of high-throughput detection technology has enabled identification of diverse dynamic and reversible RNA modifications, including N6,2′-O-dimethyladenosine (m6Am), N1-methyladenosine (m1A), 5-methylcytosine (m5C), N6-methyladenosine (m6A), pseudouridine (Ψ), and inosine (I). In this review, we focus on eukaryotic mRNA modifications. We summarize their biogenesis, regulatory mechanisms, and biological functions, as well as high-throughput methods for detection of mRNA modifications. We also discuss challenges that must be addressed in mRNA modification research.

1. Introduction

The first chemical modification of RNA was discovered in the middle of the last century [1]. More than 160 RNA modifications have been identified thus far [2]. Diverse modifications are present in different types of RNA; these modifications are associated with various aspects of the RNA life cycle, and they have considerable impacts on the regulation of gene expression. Studies of RNA modifications and their regulatory functions have led to a new field of research known as “RNA epigenetics” or “epitranscriptomics” [3,4]. Studies of the stoichiometry, distribution, and function of RNA modifications can expand the broader understanding of transcriptomics. In the past 70 years, most studies have focused on modifications of highly abundant RNAs such as ribosomal RNA (rRNA), transfer RNA (tRNA), and small nuclear RNA (snRNA); multiple studies have demonstrated the functional importance of these modifications in translation and splicing [5,6]. Chemical modifications are also present in mRNA, and the most abundant of chemical modification is N6-methyladenosine (m6A). N6,2′-O-dimethyladenine (m6Am) is a cap-adjacent modification, which has a chemical structure similar to m6A but displays different functions. Additionally, the development of high-throughput detection technology has enabled the identification of many chemical modifications in mRNA, including N1-methyladenine (m1A), 5-methylcytosine (m5C), pseudouridine (Ψ), N7-methylguanine (m7G), 2′-O methylated nucleotide (Nm), and N4-acetylcytidine (ac4C). In this review, we focus on these six common mRNA modifications. We summarize their distribution and relevant high-throughput sequencing methods; introduce their “writers,” “erasers,” and “readers”; and discuss their functions in the regulation of gene expression.

2. Abundance and biogenesis of mRNA modification

2.1. N6-methyladenosine (m6A)

The m6A modification was first identified in viral RNA in 1974, and since then it has been identified in yeast, various plants, and mammals [7]. As the post-transcriptional modification, m6A is the highest abundance in mRNA and long non-coding RNA (lncRNA). The estimation of m6A content in human polyadenylated RNA, according to liquid chromatography-tandem mass spectrometry (LC-MS/MS), revealed an m6A/A ratio of 0.4–0.7% (Fig. 1) [8,9]. The context sequence of m6A modification sites on mRNA is highly conserved, forming a DRACH motif (D: G/A/T; R: A/G; H: A/C/T).

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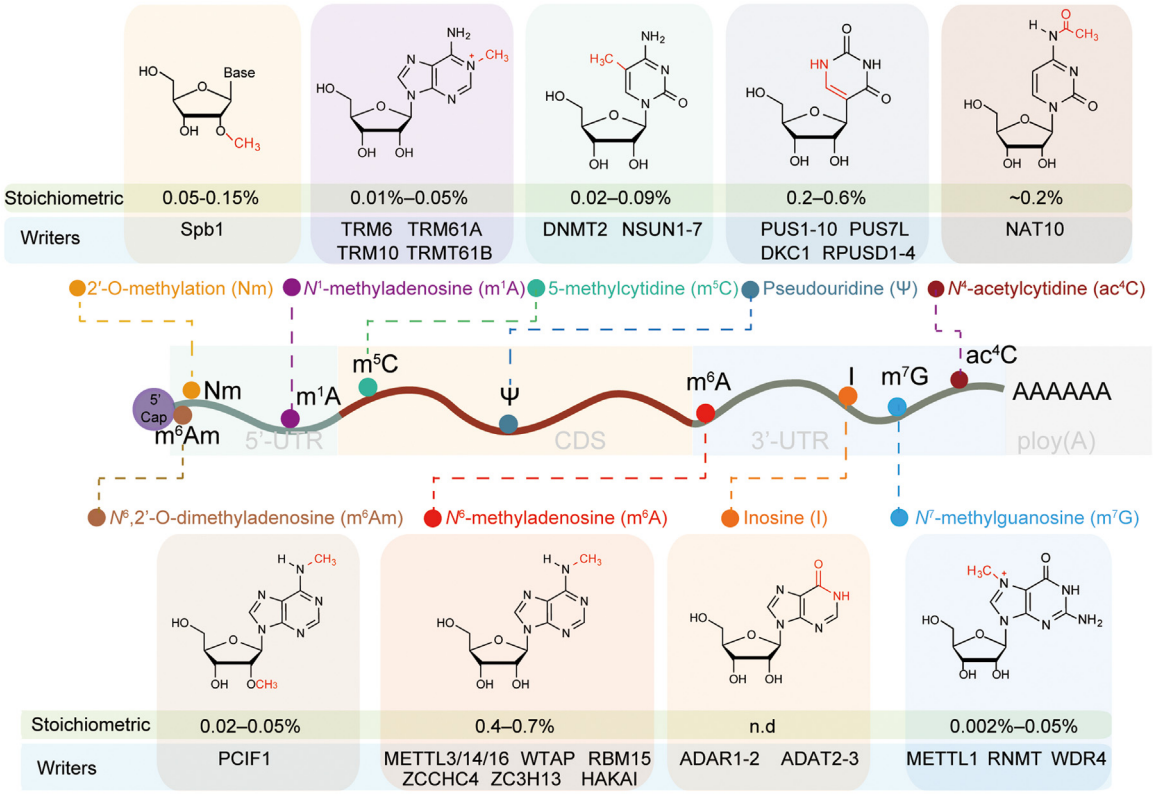

Fig. 1. Distribution, chemical structures, and regulatory proteins of mRNA modifications.

Unmodified adenine in mRNA is catalyzed to m6A by the m6A methyltransferase complex, which includes the proteins Methyltransferase 3, N6-Adenosine-Methyltransferase Complex Catalytic Subunit (METTL3), Methyltransferase 14, N6-Adenosine-Methyltransferase Subunit (METTL14), WT1 Associated Protein (WTAP), vir like m6A methyltransferase associated (KIAA1429), Zinc Finger CCCH-Type Containing 13 (ZC3H13), and RNA Binding Motif Protein 15 (RBM15) [8,10-13]. Recent studies have revealed that E3 ubiquitin ligase Hakai (HAKAI) also displays m6A methyltransferase activity [14]. The core of the catalytic complex is a heterodimer complex composed of METTL3 and METTL14; METTL3 is the main catalytic subunit, whereas METTL14 serves as an RNA-binding platform. The m6A methyltransferase was discovered decades ago; in 2010, *Jia* et al. discovered that fat mass and obesity-associated (FTO) exhibits efficient m6A demethylase activity [15]. Thus, m6A constitutes the first dynamic reversible mRNA modification discovered that can be oxidized and "erased" by demethylases such as FTO and alkB homolog 5, RNA demethylase (ALKBH5) [9,15].

2.2. N6,2′-O-dimethyladenosine (m6Am)

Discovered in 1975, m6Am is a 5′-terminal modification located at the first nucleotide after the mRNA cap; this modification is commonly present on mRNA in mammalian cells. The estimated m6Am/A ratio in human mRNA is 0.02–0.05%, according to LC-MS/MS (Fig. 1); its relative content is approximately one-tenth of the m6A content [16,17]. Because m6Am is specifically located at the first base of mRNA, its high "local concentration" suggests that m6Am may regulate translation.

In 2019, we and several other groups independently identified phosphorylated CTD interacting factor 1 (PCIF1) as the m6Am methyltransferase in humans and mice [16,18-20]. Additionally, PCIF1 modifies m6Am in the RNA cap; it cannot directly catalyze the generation of m6A from adenine. During our investigation of m6A demethylases, we found that the m6A demethylase FTO also has m6Am demethylation activity; it can demethylate m6Am to Am, both in vivo and *in vitro* [17,21]. Moreover, there is evidence that the m6Am demethylation activity of FTO depends on an intact mRNA cap structure. When the 5′ cap structure is degraded, the m6Am-demethylating activity of FTO is reduced [16]. In 2018, researchers conducted a detailed study regarding the mechanism of FTO substrate regulation; they found that FTO demethylated m6Am and m6A modifications on mRNA, and the type of FTO activity was determined by FTO localization in the cell. FTO mediated the demethylation of m6A in the nucleus; it mediated the demethylation of both m6Am and m6A in the cytoplasm [17]. In addition to m6Am on mRNA, FTO demethylates m6Am on snRNA [17].

2.3. N1-methyladenosine (m1A)

Methylation of adenine base N1 site forms m1A modification (Fig. 1). Under physiological conditions, m1A has a positive charge. The presence of the N1 methyl group modifies the structure, altering the free energy, method of pairing with other bases, and mechanism by which pairing occurs. m1A can undergo Hoogsteen base pairing with other bases, resulting in mismatches during reverse transcription; this type of pairing is unstable. Current detection methods indicate that most m1A modification sites are located in cytoplasmic and mitochondrial tRNA; m1A modification sites are highly conserved on tRNA among different species [22,23]. The estimated m1A/A content in mRNA is approximately 0.01–0.05%, according to high-sensitivity mass spectrometry; this is one-tenth of the m6A content [24,25]. An important chemical feature of m1A is that, under alkaline conditions, the methyl group at position N1 undergoes Dimroth rearrangement to position N6, leading to the formation of m6A.

Multiple mechanisms can establish m1A modifications. The m1A sites on tRNA and some mRNA are catalyzed by the tRNA methyltransferase 6 non-catalytic subunit (TRMT6)-TRMT61A complex; these m1A sites correspond to GUUCNA motifs [*22, 23*]. tRNA methyltransferase 61B (TRMT61B) and tRNA methyltransferase 10C (TRMT10C) catalyze m1A on mitochondrial mRNA. [22,23]. ALKBH1, ALKBH3, and FTO

demethylate m1A [17,23-25], indicating that m1A modifications are reversible and suggesting that m1A has a regulatory effect on gene expression.

2.4. Inosine (I)

There are various chemical modifications of adenosine; the deamination of adenosine involves conversion to inosine (I) (Fig. 1). In eukaryotes, inosine is the most common RNA modification. Adenosine deaminase RNA specific (ADAR) proteins can deaminate adenine to I within double-stranded RNA (dsRNA). In humans, the ADAR protein family has three members: ADAR1, ADAR2, and ADAR3. ADAR1 and ADAR2 are universally expressed, whereas ADAR3 is only expressed in the brain but lacks deamination activity [26]. Adenine recognition by ADAR does not involve a sequence motif; it depends on RNA secondary structures upstream and downstream of modification sites.

2.5. 5-methylcytosine (m5C)

50 years ago, m5C was first identified in RNA and most m5C modifications are located on tRNA and rRNA [27]. The m5C/C content in mRNA, according to mass spectrometry, is approximately 0.02–0.09% (Fig. 1). The use of modified RNA bisulfite treatment, combined with high-throughput sequencing and subsequent sequencing, has allowed researchers to identify thousands of m5C modification sites in HeLa cells. The m5C is mainly distributed in the coding sequence (CDS) region and particularly enriched at translation initiation sites [28]. Not only display dynamic changes among species and cell lines, but m5C also shows tissue and cell specificity. The m5C methyltransferases NSUN1 and NSUN2 reportedly interact with eukaryotic mRNA; these proteins can regulate the development of cancer in an m5C-mediated manner [28–30].

2.6. N4-acetylcytidine (ac4C)

Early investigations suggested that ac4C was a conserved chemical tRNA and rRNA modification [31]. Recent studies have revealed numerous ac4C modifications in human and yeast mRNA. The estimated ac4C/C ratio in human mRNA is 0.2%, according to LC-MS/MS [32]. It is found that ac4C contributes to codon reading accuracy during translation; it can improve mRNA translation efficiency and RNA stability. N-acetyltransferase 10 (NAT10) and its co-factors can catalyze the formation of ac4C. Additionally, ac4C has been implicated in the development, progression, and prognosis of multiple human diseases, particularly cancer [31].

2.7. N7-methylguanosine (m7G)

There are conserved m7G modification sites in rRNA and tRNA. Additionally, mature mRNA caps contain m7G; thus, cap-m7G is a very common modification on mRNA. Recent high-throughput sequencing studies demonstrated that mRNA also contains internal-m7G, mainly in the 3′-untranslated regions (3′-UTR) [33,34]. The estimated ratio of internal-m7G/G in human mRNA is 0.002–0.05% (Fig. 1) [33,34]. The methyltransferase 1, tRNA methylguanosine (METTL1)-WD repeat domain 4 (WDR4) complex is an m7G methyltransferase that can methylate m7G on tRNA and some internal-m7G within mRNA. m7G is reportedly associated with numerous human diseases, but its specific mechanism requires further investigation.

2.8. Ribose methylation

Methylation is the most common RNA modification. Most methylation occurs on RNA bases, but some methylation occurs on ribose; methylation of the 2′-OH group is most common (Fig. 1). Nm (where N refers to A, U, C, or G) is mainly found in tRNA, rRNA, and snRNA; however, recent studies have revealed that Nm is also present in low-abundance RNAs, such as mRNA [35]. In RNA, Nm is formed by two enzymatic mechanisms: either by a fibrinogenic protein (FBL) alone or by a ribonucleoprotein complex that includes an enzyme and C/D-box snoRNA as guide RNA for the formation of small nucleolar ribonucleoproteins (snoRNPs) that perform catalytic functions [36]. Importantly, proximal mRNA cap N1m and N2m use another methylation system, which involves independent catalysis by the methyltransferases cap methyltransferase 1 (CMTR1) and CMTR2 [37,38]. However, FBL only displays methylation activity for the Nm site on rRNA. It is unclear whether this nucleoprotein complex can catalyze Nm modification sites in mRNA. The ratio of internal-Nm/N in human mRNA varies from 0.012% to 0.15% [35].

2.9. Pseudouridine (Ψ)

As the second most abundant modification in mRNA, ψ is regarded as the fifth base in RNA. Produced by rotational isomerization of the C–C glycoside of uridine, ψ is common in tRNA, rRNA, and snRNA. The development of highly sensitive detection techniques has revealed that mRNAs also contain ψ [*39–41*]. The ratio of ψ/U in mammalian mRNAs, according to LC-MS/MS, is approximately 0.2–0.6% [41]; its abundance is comparable with the abundance of m6A (Fig. 1).

In mammals, 13 pseudouridine synthases (PUSs) have been identified. These PUSs can be divided into independent (e.g., PUS1, PUS7, and TRUB1) and RNA-dependent (e.g., Dyskerin Pseudouridine Synthase 1, DKC1) groups; each PUS catalyzes a specific type of ψ in mRNA [39–42]. Thorough analyses of the biochemical functions of ψ have revealed its effects on tRNA and rRNA functions in translation and snRNA function in splicing. However, the genetic manipulation of PUS proteins can directly affect RNA metabolism and influence cellular phenotype; thus, the functions of endogenous ψ in mRNA require further investigation [43]. However, the pseudouridylation of artificial RNA had substantial effects on translation and innate immunity, indicating that ψ may regulate gene expression.

3. mRNA modification sequencing methods

Consistent progress in high-throughput detection technology has led to important advances in epigenetic transcriptomics. High-throughput sequencing technologies have been developed for m6A, m6Am, m1A, I, m5C, ψ, m7G, Nm, and ac4C. High-throughput methods for modification detection use the following strategies: (1) RNA modification enrichment via specific antibodies; (2) recognition of RNA modifications via proteins or enzymes; (3) RNA modification labeling via chemical reactions; and (4) utilization of unique base-pairing characteristics present in RNA modifications.

3.1. Sequencing methods of m6A

In 2012, m6A-seq/MeRIP, a high-throughput sequencing method for RNA modification, was developed to map m6A modifications at the transcriptome level [44,45]. MeRIP/m6A-seq is a representative antibody enrichment-based method for high-throughput detection of RNA modifications (Fig. 2a). MeRIP/m6A-seq is widely used; corresponding "MeRIP" sequencing methods have been developed for m6Am, m1A, m5C, m7G, and ac4C. MeRIP is highly dependent on antibodies; its resolution (~150–200 nt) is limited by the length of mRNA fragments [44,45]. Therefore, it is challenging to use MeRIP for accurate modification site mapping. Additionally, because of the low efficiency of antibody enrichment, MeRIP and other antibody-based methods require large amounts of RNA.

Based on the MeRIP approach, a UV light cross-linking strategy was introduced for the higher-resolution analysis of the m6A modification landscape. Currently, two common sequencing technologies can be combined with UV cross-linking. PA-m6A-seq incorporates 4SU into

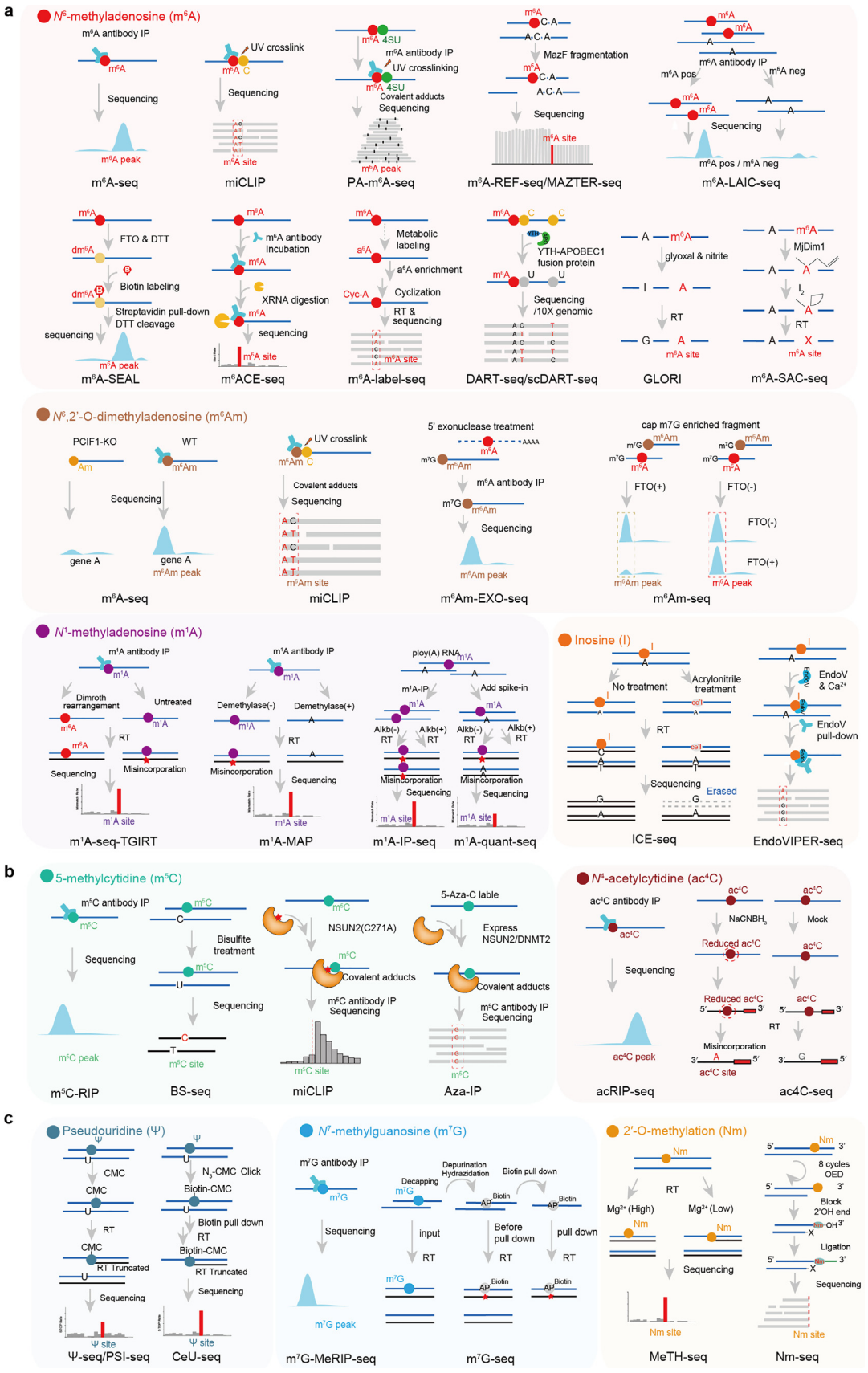

Fig. 2. Methods for detection of RNA modifications. Sequencing methods for (a) adenosine modifications, (b) cytosine modifications, and (c) other modifications.

RNA upon the addition of 4-thiouridine (4SU) to the cell culture medium; m6A-containing RNA is enriched using an antibody, and the RNA is cross-linked with antibodies via UV irradiation (365 nm). Because 4SU produces a T-to-C mutation at each cross-linking site after sequencing, the resolution of m6A detection is considerably improved (Fig. 2a) [46]. Another strategy involves the direct use of 254 nm UV irradiation to induce cross-linking between m6A antibody and RNA, thereby forming covalent sets. Covalent binding sites generate mismatches and stop signals during reverse transcription. The m6A individual-nucleotide-resolution cross-linking and immunoprecipitation (miCLIP), developed using this strategy, can identify m6A at single-base resolution (Fig. 2a) [47]. Because m6A antibodies do not distinguish between m6A and m6Am, miCLIP can also be used to identify m6Am [47].

In 2016, m6A-LAIC-seq was developed to quantitatively detect m6A at the transcriptome level [48]. This method involved the enrichment of full-length mRNA using an m6A antibody (Fig. 2a). Additionally, External RNA Controls Consortium (ERCC) standards can be added into both immunoprecipitated and supernatant samples; these standards are used to correct the RNA content in each sample. Calculation of the expression level of a single transcript in both types of samples allows analysis of m6A modifications at the transcriptome level (Fig. 2a).

Because antibody-based methods are hindered by non-specific binding and sequence bias, antibody-independent m6A sequencing methods have been sought. In 2019, two research groups developed antibody-independent single-base m6A sequencing methods: MAZTER-seq and m6A-REF-seq (Fig. 2a). MAZTER-seq and m6A-REF-seq use MazF, an endonuclease that recognizes an ACA motif, to identify m6A sites by analyzing the rate of cleavage at ACA sites [49,50]. The use of a MazF-derived endonuclease restricts these two methods to the identification of m6-ACA sites alone; accordingly, these methods cannot detect m6A throughout the transcriptome.

Researchers have also developed multiple m6A sequencing techniques that involve chemical labeling. In 2020, *Wang* et al. [51] developed m6A-SEAL, an FTO-assisted chemically labeled m6A sequencing method. In this method, m6A is oxidized to hm6A by treatment with FTO, then converted to dm6A through a dithiothreitol-mediated thiol-addition reaction, followed by the final addition of biotin to dm6A (Fig. 2a). This method identifies m6A by streptavidin pull-down of biotin dm6A, avoiding the false-positive result that can occur during antibody-based detection. In 2020, *Shu* et al. [52] developed a metabolic-labeling m6A sequencing method: m6A-label-seq. In this method, cells must be cultured with Se-allyl-L-selenohomocysteine; accordingly, m6A is replaced with a6A, and m6A sites are identified using the mismatches generated by a6A during reverse transcription (Fig. 2a).

In 2019, *Koh* et al. [53] developed m6ACE-seq, a sequencing method that uses exonuclease activity to identify m6A. In this method, an m6A antibody is initially used to identify m6A, and an XRN1 exonuclease is used to digest RNA from the 5′ ends; this protects the antibody binding site and achieves single-base identification of m6A (Fig. 2a). In contrast, DART-seq, developed in 2019 [54], uses an APOBEC1-YTH fusion protein to recognize m6A and deaminate cytosine around m6A; the resulting C-to-T mutations allow identification of m6A sites (Fig. 2a). Although the DATR-seq method requires a small amount of RNA and can be used within a long-read sequencing platform, this method requires the construction of a cell line expressing the APOBEC1-YTH fusion protein. In 2022, *Tegowsk* et al. [55], applied DART-seq to the 10X Genomics sequencing platform, achieving the first instance of m6A detection at the single-cell level (Fig. 2a).

Until recently, there was no quantitative method for the detection of m6A modifications. Development in 2022, m6A-SAC-seq enables the quantitative detection of m6A modifications through oligonucleotide sequence correction (Fig. 2a). In m6A-SAC-seq, the authors use dimethyltransferases to label m6A to a6m6A by means of an allylic-SAM cofactor; a6m6A is cyclized after iodine treatment. The reaction products (N1, N6-ethanoladenine, and N1, N6-propanadenine homologs) are mismatched during reverse transcription and can be used to identify m6A sites[56]. The reaction efficiency of this method differs according to the motif surrounding each m6A site; thus, a spike-in sequence is required for correction. In 2022, *Liu* et al. developed GLORI [57], a transcriptome-level sequencing method for absolute quantification of m6A. GLORI converts unmodified A-to-I through efficient deamination, which is read as guanine during sequencing; m6A does not undergo deamination and is thus read as adenine (Fig. 2a). This glyoxal and nitrite-mediated deamination convert 99% of A-to-I in an unbiased manner, enabling absolute quantification of m6A.

3.2. Methods for sequencing other mRNA modifications

The MeRIP/m6A-seq can also use to identify m6Am by comparing peaks between wild-type and PCIF1 knockout samples (Fig. 2a) [16]. For direct identification of m6Am, m6Am-Exo-seq [19] retains the mRNA cap using an exonuclease method; it identifies m6Am sites using an m6A antibody (Fig. 2a). The m6Am-seq sequencing method uses FTO processing to selectively erase the m6Am modification. Comparison of FTO(+) samples and FTO(-) samples peaks allows direct identification of the m6Am modification site (Fig. 2a) [58,59].

The antibody enrichment strategy can also be used to detect m1A. Multiple groups have established m1A high-throughput sequencing methods (m1A-ID-seq, m1A-seq-TGIRT, and m1A-MAP) (Fig. 2a) [22–25]. Because m1A contains a positive charge, which would lead to termination or mismatch during reverse transcription, the m1A site can be identified at single-base resolution via detection of stop or mismatch signals after reverse transcription. To improve m1A site detection reliability, the enriched m1A-containing RNA was subjected to demethylation or Dimroth rearrangement in conjunction with the removal of the original m1A; this served as a verification step. To improve detection sensitivity, *Zhou* et al. [60] conducted directed evolution of reverse transcriptase, which enhanced its sensitivity to m1A. The resulting reverse transcriptase allowed direct identification of m1A sites via reverse transcription (m1A-quant-seq); alternatively, m1A sites can be enriched with an m1A-specific antibody, then identified via reverse transcription with the new reverse transcriptase (m1A-IP-seq) (Fig. 2a). These new m1A sequencing methods have shown that, in addition to tRNA and rRNA, cytoplasmic mRNA and mitochondrial mRNA contain large amounts of m1A modifications, which display dynamic reversibility [22].

The detection of inosine (I) in RNA is based on the principle of mismatches caused by base modifications. During reverse transcription, I is read as guanine, which results in an A-to-G mutation in the cDNA. Comparison of the RNA sequence with the reference genome allows the identification of I modification sites [61]. Additionally, a chemical-label-based sequencing method has been developed for I detection. ICE-seq uses acrylonitrile-mediated specific labeling of I to form N1-cyanoethylinosine (CE^1 I), a reaction product that causes cDNA truncation during reverse transcription [62]. I sites can be identified at the whole transcriptome level by comparing RNA samples between treated and untreated groups (Fig. 2a). In 2020, *Knuston* et al. [63] developed the EndoVIPER-seq sequencing method. In this method, RNA fragments are treated with glyoxal; EndoV is used to bind and enrich I-containing RNA fragments for sequencing. Analyses of the RNA modification landscape in different tissues across species revealed that species (rather than tissue type) was the main factor leading to differences in I modification profiles.

Bisulfite transformation is the most common method for analysis of 5mC in DNA. Based on the BS-seq approach in DNA, modified bisulfite sequencing can be used to detect m5C in RNA (Fig. 2b) [64]. Additionally, multiple other high-throughput sequencing methods for m5C have been reported. In contrast to m6A, which uses antibodies, miCLIP uses covalent binding between the C271A mutant NSUN2 and the substrate

RNA to detect m5C sites (Fig. 2b) [30]. In the Aza-IP method, m5C sites are initially labeled with 5-Aza-C; the labeled product forms a covalent adduct with methyltransferase. The m5C-labeled product can be enriched and then sequenced (Fig. 2b) [65]. In m5C-RIP, m5C-specific antibodies are used for direct enrichment of m5C in bacteria, yeast, and plants (Fig. 2b). The antibody enrichment approach has also been used for ac4C sequencing; more than 4000 ac4C peaks have been identified in the human transcriptome using acRIP-seq (Fig. 2b) [66]. Additionally, because ac4C can react with NaCNBH3 to generate C-to-A misincorporation (Fig. 2b), ac4C-seq can identify ac4C modification sites at a single-base resolution [67].

Unlike other RNA modifications such as methylation and acetylation, Ψ and uridine have the same molecular formula and base-pairing properties; thus, Ψ is difficult to detect with common RNA modification sequencing methods. Fortunately, the compound N-cyclohexyl-N'-β-(4-methylmorpholinium) ethylcarbodiimide (CMC) was discovered in 2014. This compound specifically labels Ψ, and the resulting product (CMC-ψ) can cause premature termination at Ψ modification sites during reverse transcription; thus, Ψ can be detected at single-base resolution. ψ-seq, Pseudo-seq, and PSI-seq (Fig. 2c) [39,40], have been developed using this strategy. These methods enabled the identification of several hundred Ψ sites in human and yeast mRNA. In 2015, *Li* et al. [41] developed CeU-seq, which uses azido-CMC (N3-CMC; a derivative of CMC), to label the Ψ site and then enrich the ψ-containing RNA via biotin pull-down (Fig. 2c). Through the enrichment of Ψ sites, *Li* et al. identified more than 2000 single-base resolution Ψ sites in mammalian mRNAs via CeU-seq.

Antibody-based and chemical labeling-based m7G detection methods have been developed. The m7G-MeRIP-seq identified more than 2000 internal-m7G peaks in the mammalian transcriptome by high-throughput sequencing involving the enrichment of m7G-containing RNA fragments via specific antibodies (Fig. 2c) [33]. Utilizing truncation and mutation signals generated by UV crosslinking, m7G-miCLIP-seq can detect m7G at single-base resolution (Fig. 2c) [34]. Furthermore, m7G-seq allows determination of the m7G modification landscape through reduction reactions (Fig. 2c). In this method, m7G is initially reduced by NaBH4 to generate an AP site, which is subsequently labeled with biotin and pulled down using streptavidin beads. Biotin-AP sites cause mismatches during reverse transcription, enabling the identification of internal-m7G sites at single-base resolution [33].

Nm is a modification of ribose. The 2′,3′-vicinal diol of unmethylated ribose can be oxidized and degraded by periodic acid, whereas 2′-O methylated ribose is resistant to periodic acid oxidation. This distinction served as the basis for the development of Nm-seq to identify Nm at single-base resolution (Fig. 2c) [35]. Recently, MeTH-seq was reported; it uses a low concentration of Mg^{2+} to stop reverse transcription at the 3′ ends of the Nm site (Fig. 2c). In MeTH-seq, eight oxidation-elimination-dephosphorylation cycles are conducted to remove unmodified 3′-terminal nucleotides. Therefore, only RNA fragments with Nm at the 3′ end can be linked to an adapter and sequenced. This method was used to identify Nm sites on yeast mRNA with single-base resolution.

4. Functions of mRNA modifications

Chemical modifications of RNA can directly affect its physicochemical properties, which influence its functions and interactions [4]. RNA modifications can also affect RNA function via the recruitment of reader proteins. There is increasing evidence that RNA modifications play key regulatory roles in various physiological and pathological processes. For example, m6A has regulatory roles in embryonic development, sex determination, spermatogenesis, cancer progression, and viral infections [68,69]. RNA modifications in transcripts are recognized by reader proteins and translated into downstream molecular events, which regulate the activities of life. Here, we introduce known reader proteins and functions of m6A, m5C, and I.

4.1. Functions of m6A

The most important m6A reader proteins are YTH family proteins, including YTH N6-methyladenosine RNA binding protein C1 (YTHDC1), YTHDC2, and YTHDF1–3 (Fig. 3a), which function in the nucleus and cytoplasm, respectively. YTHDC1, a highly conserved YTH family protein, is localized in the nucleus. YTHDC1 can inhibit splicing factors to regulate mRNA splicing [70]. Studies in HeLa cells have revealed that YTHDC1, serine and arginine rich splicing factor 3 (SRSF3), and nuclear RNA export factor 1 (NXF1) interact to promote the nuclear export of m6A-labeled mRNA [71]. YTHDC1 also regulates translation: its C-terminal YTH domain can bind m6A-modified mRNA, whereas its N-terminal domain can promote translation initiation by recruiting the translation initiation complex via elF3 binding. Heterogeneous nuclear ribonucleoprotein A2/B1 (HNRNPA2B1), another nuclear reader of m6A, mediates m6A-dependent pri-miRNA processing [72] and regulates alternative splicing of pre-mRNA [73]. Biochemical evidence suggests that the regulatory function of HNRNPA2B1 is mediated by an m6A switch mechanism.

In the cytoplasm, m6A-labeled mRNA is regulated by YTHDF1, YTHDF2, YTHDF3, and YTHDC2 (Fig. 3a). YTHDF1 binding sites are distributed near stop codons in mRNA, which are closely linked with m6A sites. Mechanistic studies have shown that YTHDF1 can improve RNA translation efficiency through effects on translation initiation [74]. Under physiological conditions, YTHDF2 can accelerate the degradation of m6A-labeled mRNA via the recruitment of C–C motif chemokine receptor 4 (CCR4)- negative on TATA-less (NOT) complexes [75]. Under heat shock conditions, YTHDF2 expression is significantly increased; YTHDF2 then enters the nucleus to protect m6A-labeled mRNA from degradation by FTO [76]. YTHDF3 can regulate RNA translation through interactions with YTHDF1 [77]. Additionally, YTHDF3 can interact with YTHDF2 to mediate mRNA degradation. YTHDC2, the largest protein in the YTH family, can recognize modification sites to improve the translation efficiency of m6A-containing transcripts. It was recently reported that METTL3 also promotes the translation of m6A-labeled mRNA [78]. Finally, METTL3 can add m6A to pri-miRNA, and m6A can promote the specific recognition of pri-miRNA by DGCR8, thereby facilitating miRNA processing [79].

There is increasing evidence that m6A has a regulatory role in viral life cycles. DNA and RNA viruses contain RNA modifications. Recent studies have shown that HIV-1 viral RNA contains m6A, which can recruit YTHDF1–3 and promote further expression of viral mRNA. YTHDF knockdown can downregulate the expression of HIV-1 RNA and inhibit its proliferation in CD4+ T cells [80,81]. Similarly, multiple genome segments in the EV71 virus contain m6A; modifications of m6A methylase, demethylase, and reading protein expression patterns can substantially influence viral proliferation. Moreover, METTL3, an m6A methylase, can interact with the RNA-dependent RNA polymerase (RdRp) of EV71 virus to increase RdRp stability by enhancing the levels of sumoylation and ubiquitination on RdRp. These interactions improve translation efficiency, facilitating EV71 virus proliferation [82]. Various steps in viral life cycles are regulated by m6A. First, m6A can enhance viral mRNA translation efficiency. Second, m6A prevents retroviral replication by inhibiting reverse transcription. Third, m6A can decrease or increase viral mRNA stability. Fourth, m6A affects viral RNA splicing and nuclear export. Fifth, viral RNA encapsulation is regulated by m6A. Finally, recent studies have revealed that severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) contains an m6A modification that can inhibit viral replication [83].

4.2. Functions of other mRNA modifications

Recently, multiple m5C readers have been identified, including Aly/REF export factor (ALYREF), Y-box binding protein 1 (YBX1), and Y-box binding protein 2 (YBX2) (Fig. 3b). ALYREF can recognize m5C-containing mRNA and promote its nuclear export [28]. YBX1, a cyto-

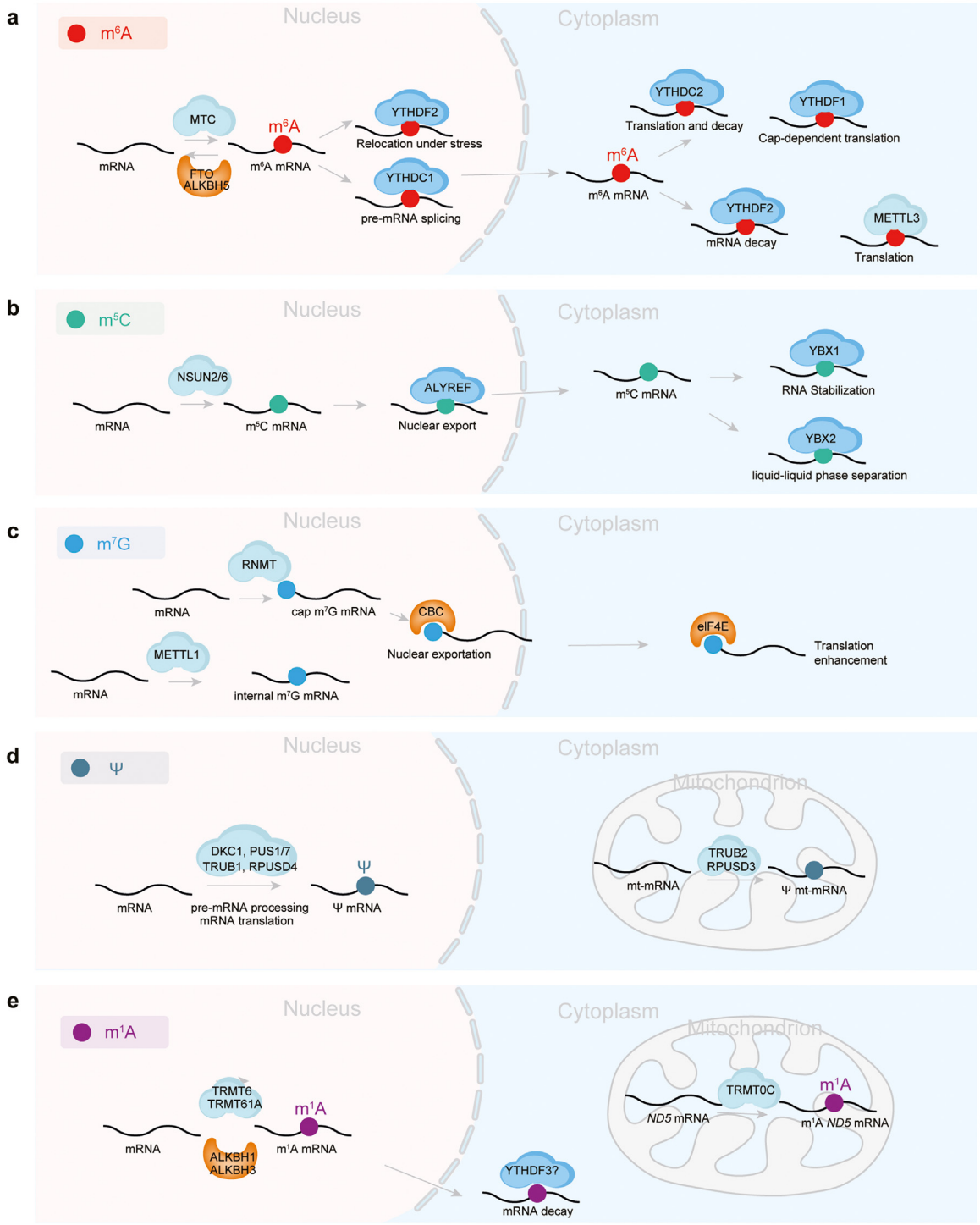

Fig. 3. Regulatory roles of RNA modifications. (a) YTHDC1 in the nucleus can promote pre-mRNA splicing; YTHDF1 in the cytoplasm promotes cap-dependent translation. YTHDC2 and METTL3 promote mRNA translation, whereas YTHDC2 and YTHDF2 promote mRNA decay. (b) ALYREF in the nucleus facilitates m5C-labeled mRNA nuclear export, YBX1 in the cytoplasm improves mRNA stability, and YBX2 contributes to liquid-liquid phase separation. (c) m7G is catalyzed by RNMT and METTL1; m7G-labeled mRNA can interact with elF4E to promote translation. (d) In the nucleus, ψ is catalyzed by DKC1, PUS1/7, TRUB1, and RPUSD4, thus promoting mRNA processing and translation. mt-mRNA in mitochondria is catalyzed by TRUB2 and RPUSD4. (e) m1A in the nucleus is catalyzed by TRMT6/TRMT6A, *ND5* mRNA in mitochondria is catalyzed by TRMT0C, and m1A-labeled mRNA in the cytoplasm is bound by YTHDF3 to promote mRNA decay.

plasmic m5C reader, regulates RNA stability by recruiting ELAVL1 [84]. In human urothelial bladder cancer, YBX1 targets m5C in the oncogene *HDGF* to increase the half-life of the oncogene transcript; thus, it has a carcinogenic role [84]. YBX2 was recently identified as a reader of m5C. YBX2 promotes liquid-liquid phase separation of m5C-labeled RNA, both in vivo and in vitro [85]. W100, the m5C-binding domain of YBX2, plays an important role in mediating YBX2 phase separation.

I is recognized by the p54nr^b and hEndoV proteins. p54nr^b, an I-specific RNA-binding protein, maintains RNA modifications in the nucleus[86]. endonuclease V (hEndoV), a specific ribonuclease that preferentially hydrolyzes I-containing RNA, facilitates the degradation of I-containing transcripts [87].

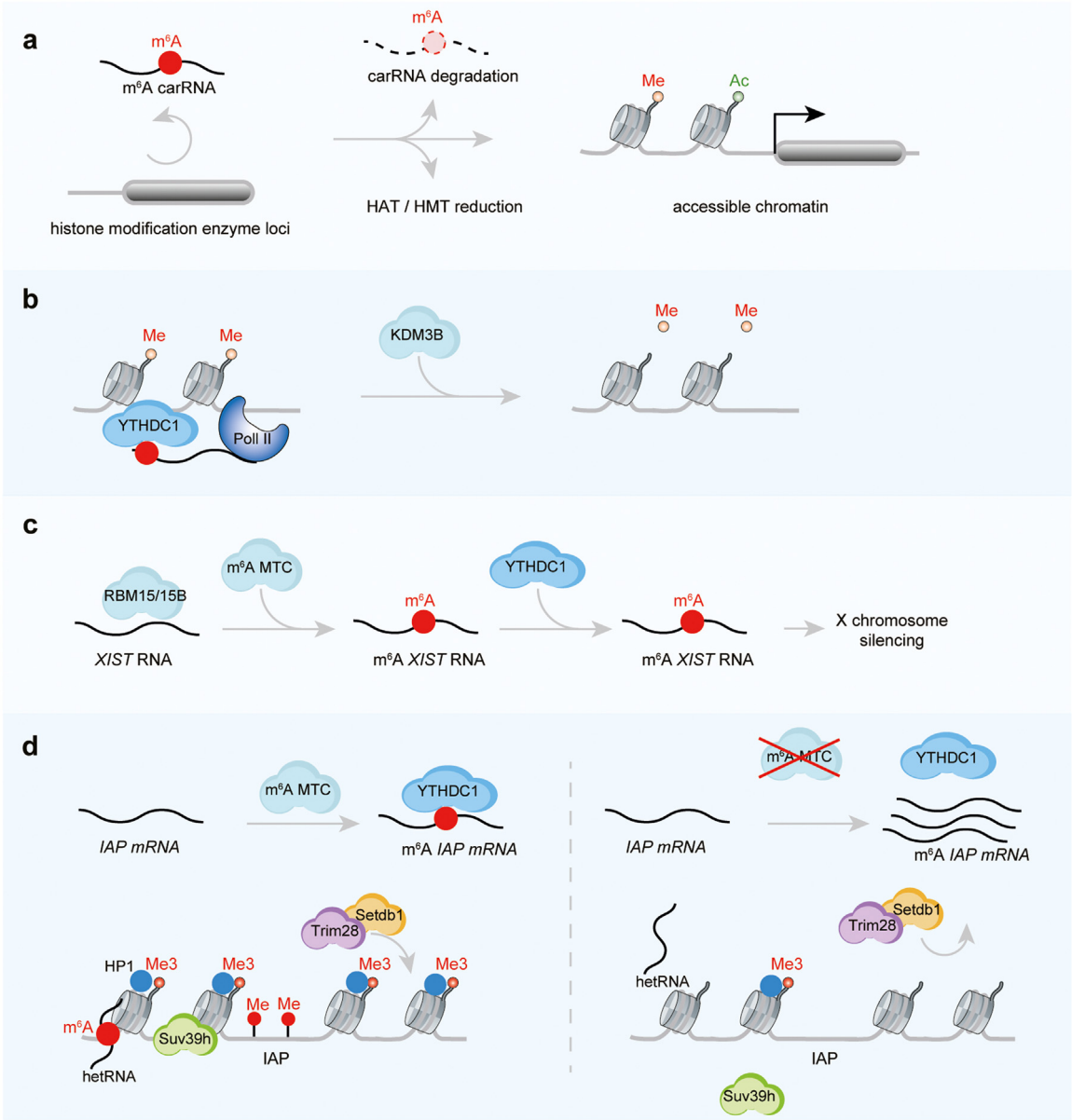

Fig. 4. Crosstalk between RNA modifications and histone modifications. (a) Chromosome-associated regulatory RNAs (carRNAs) are methylated and labeled for degradation, resulting in a decrease in carRNAs and histone acetyltransferases (HATs) or histone methyltransferases (HMTs); this process ultimately leads to altered chromatin accessibility. (b) KDM3B is recruited by m6A to promote histone methylation. (c) RBM15/RBM15B-mediated deposition of m6A and recruitment of YTHDC1 facilitates XIST-induced silencing of the X chromosome. (d) The m6A methyltransferase complex (MTC) promotes m6A methylation of intracisternal A particle (IAP) mRNA and heterochromatic RNA (hetRNA). Ythdc1 binding to m6A is essential for H3K9me3 deposition downstream of Setdb1/Trim28. Methylated hetRNA binds to chromatin and promotes the retention of HP1 and Suv39h proteins.

5. Messenger RNA modifications mediate epigenetic crosstalk

Chemical modifications on RNA have an important effect on gene expression; epigenetic modifications also play important regulatory roles in gene expression. The synchronous and precise regulation of gene expression is critical for the maintenance of life; its core is composed of complex crosstalk among biological macromolecules that are involved in transcriptional regulation. In mammalian cells, m6A is the most abundant mRNA modification. There is increasing evidence of crosstalk between m6A and epigenetic mechanisms. These interactions affect translation, histone modification, and the selection of m6A methyltransferase complex binding sites. Here, we summarize the crosstalk between m6A and histone modifications, as well as its impact on the regulation of gene expression.

5.1. m6A regulates chromatin accessibility

Chromosome-associated regulatory RNA (carRNA) helps to maintain genomic structure and preserve transcriptional regulation. *Liu* et al. [88] demonstrated that m6A on carRNA regulates chromatin accessibility and downstream transcription (Fig. 4a). *Liu* et al. found that Mettl3 knockdown in mESCs enhanced carRNA stability, leading to open chromatin and active transcription. A decrease in the m6A content of carRNA promoted the recruitment of histone acetyltransferase E1A binding protein P300 (EP300) and the transcription factor YY1. Thus, m6A-labeled carRNA stabilizes closed chromatin by recruiting inactivating factors and inhibiting activating factors [88]. Additionally, the expression of LINE1 increased after the Mettl3 knockdown, thereby promoting chromatin accessibility. Overall, m6A regulates chromatin accessibility and downstream transcription by interactions with carRNA.

5.2. m6A and histone modifications

A recent study showed that METTL3/METTL14 can suppress H3K9me2 through m6A (Fig. 4b) [89]. The direct effects of m6A on histone modifications were investigated using a tetracycline-induced reporter in human Flp-In HEK293 cells. Histone modifications were measured after tetracycline treatment; the level of H3K9me2 was significantly decreased, compared with controls. *Li* et al. verified that the loss of METTL3 or METTL14 increased the overall level of H3K9me2; this upregulation could be reversed by inducing the expression of wild-type METTL3. Additional experiments showed that m6A could trigger KDM3B-mediated demethylation of H3K9me2 via YTHDC1. Additionally, KDM3B-binding sites overlapped with m6A sites; KDM3B-ChIP data showed that many KDM3B-binding sites contained METTL3-binding sites. These findings suggest a mechanism by which m6A is linked to histone modifications.

5.3. m6A regulates X-chromosome silencing

lncRNAs are key regulators of epigenetic mechanisms. A recent study demonstrated that X (Inactive)-Specific Transcript (XIST) function is regulated by m6A (Fig. 4c). Numerous m6A sites have been identified on XIST RNA [*12*]. Knockdown of RBM15 or RBM15B resulted in the failure of XIST to silence target genes. Additionally, the knockdown of Rbm15/Rbm15b significantly reduced the m6A level on XIST; METTL3 knockdown yielded similar results. Co-immunoprecipitation analysis showed that RBM15/RBM15B and METTL3 interact in a WTAP-dependent manner. Finally, YTHDC1 is essential for m6A recognition and downstream transcriptional silencing on XIST [*12*]. These findings suggest that m6A is a dynamic regulator of X-chromosome silencing; moreover, lncRNA methylation status has a critical effect on m6A function.

5.4. m6A affects heterochromatin

Heterochromatin is an important part of the genome that helps to maintain genome integrity and regulate gene expression. The main function of heterochromatin involves inhibiting the activities of satellites and transposable elements. In mammals, endogenous retroviruses (ERVs) are a class of retrotransposons that can disrupt genomic structure; they must be silenced. Recent studies demonstrated that m6A regulates ERVs in mESCs; it specifically interacts with the intracisternal A particle (IAP) element (Fig. 4d) [90–92]. MeRIP revealed that m6A was enriched in the 5′-untranslated region of IAP mRNA; it recruited YTHDF proteins to degrade IAP mRNA [92]. Mettl3 knockdown significantly reduced H3K9me3 levels on IAP elements. In contrast, the knockdown of the m6A demethylase Alkbh5 significantly increased H3K9me3 levels on IAP elements. These results showed that IAP mRNA and protein levels were negatively correlated with the IAP m6A level. Mettl3-catalyzed IAP m6A sites are recognized by Ythdc1; they contribute to heterochromatin formation. Mechanistic studies revealed that Ythdc1 is a guide protein for Mettl3, which facilitates Mettl3 interactions with chromatin, as well as Setdb1 and Trim28 proteins; it subsequently regulates H3K9me3 deposition in IAPs [92].

Another mechanism for stabilizing heterochromatin involves m6A-mediated regulation of major satellite repeats (Fig. 4d). Major satellite repeats are transcribed by RNA polymerase II (Pol II) to form heterochromatic RNA (hetRNA) [93]. This hetRNA can form RNA-DNA duplexes that facilitate the recruitment of histone lysine methyltransferases HP1 and Suv39h, thereby maintaining histone methylation. In Mettl3/Mettl14-knockdown mESCs, the m6A level in hetRNA decreases, resulting in reduced formation of RNA-DNA hybrids; this process hinders heterochromatin maintenance [94].

6. Conclusion and outlook

RNA is the core of the central dogma. Chemical modifications of RNA have important roles in the regulation of RNA metabolism, and they add a new dimension to the regulation of gene expression. For example, the most studied modification, m6A, can influence mRNA splicing, translation efficiency, RNA stability, and other aspects. Therefore, m6A plays a regulatory role in physiological processes such as gametogenesis, sex determination, stem cell status maintenance and differentiation, viral infection, and cancer progression. In addition to m6A, other RNA modifications such as m6Am, m1A, m5C, m7G, Nm, and ψ on mRNA reportedly have various regulatory effects in mammalian cells. Technological developments in modification detection have allowed increasing numbers of mRNA modification sites to be identified; nevertheless, the functions and potential regulatory mechanisms of RNA modifications remain incomplete.

First, there is a lack of detection methods for various new modifications. Whereas more than 160 modifications have been identified in RNA, only nine modifications have been characterized in mRNA. Additionally, analyses of modification functions are limited by detection methods; thus, high-sensitivity detection methods are needed. Previous studies have demonstrated extensive crosstalk among multiple types of RNA modifications, as well as between RNA modifications and histone modifications. Therefore, sequencing methods that can simultaneously detect multiple RNA modifications will provide key insights concerning the complex functions of RNA. Additionally, the increasing attention to cellular heterogeneity in physiology and pathology has led to a need for single-cell and spatial transcriptome modification detection methods that can characterize the functions of RNA modifications at the single-cell scale.

Second, the proteins that regulate RNA modifications are unknown. Functional studies of RNA modifications rely on writer and reader proteins. Writer proteins have not been identified for many modifications, such as m1A; thus far, known writer proteins only interact with a few modification sites on mRNA. With respect to the ψ modification, 13 candidate writer proteins have been reported; however, these writer proteins have significant functional redundancy in human mRNA[41], hindering functional analyses of ψ. New writers have been discovered for m6A modifications that have been extensively characterized, such as the recently discovered HAKAI and ZC3H13 have regulatory roles in unique physiological processes. Additionally, writer proteins for some RNA modifications (e.g., m1A, m5C, m7G, and ac4C) can act on different types of RNA; this property has made functional studies challenging. Moreover, numerous modifications (e.g., m6Am, m7G, Nm, ψ, and ac4C) do not clearly correspond to reader proteins, which are responsible for initiating RNA modifications. The lack of studies focused on reader proteins has impeded research concerning gene expression regulation in the context of mRNA modifications.

Third, numerous reports have shown that human diseases are associated with RNA modifications. Accordingly, there is an urgent need to develop RNA modification tools that accurately edit disease-related modification sites. In summary, research regarding mRNA modifications remains in an early stage; studies thus far have simply focused on detecting mRNA modifications. In the future, research should focus on in-depth studies concerning the functions of RNA modifications, as well as the development of tools to manipulate those modifications.

Declaration of competing interest

The authors declare that they have no conflicts of interest in this work.

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that they could not cite all publications on this topic because of space limitations within the journal.

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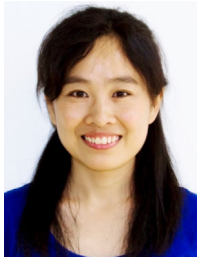

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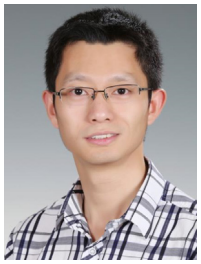

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