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Epitranscriptomic code and its alterations in human disease

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Abstract

Innovations in epitranscriptomics has resulted in identification of more than 160 RNA modifications till date. These developments together with the recent discovery of writers, readers and erasers of modifications occurring across a wide range of RNA and tissue types, led to a surge in integrative approaches for transcriptome-wide mapping of modifications and protein-RNA interaction profiles of epitranscriptome players. RNA modification maps and cross-talk between them have begun to unfold the role of modifications as signaling switches, opening the notion of epitranscriptomic code as a driver of post-transcriptional fate of RNA. Emerging single molecule sequencing technologies and development of antibodies specific to various RNA modifications could enable charting transcript specific epitranscriptomic marks across cell types and their alterations in disease.

Keywords

RNA modifications; post-transcriptional regulation; regulatory networks; next generation sequencing; RNA metabolism

Emergence of an expanded view of RNA alphabet

Genomic studies over the last two decades have enabled us to have a comprehensive understanding of both coding (mRNA) and non-coding RNAs (ncRNA) as well as to uncover their role as active and passive players in governing the functional outcomes of a cell [1–4]. However, the molecular players mediating and controlling the transition from RNA to protein i.e, post-transcriptional regulation, which governs expression patterns,

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localization, splicing, stability and structure of RNA, has been relatively under-appreciated. Recent research has led to the discovery of dynamic chemical modifications of nucleotide bases on RNA and are increasingly witnessed to be key switches in its metabolism [5–8]. A variety of such chemical modifications are now known to be the result of RNA-binding proteins [9], which can be broadly classified as writers (enzymes responsible for installation of the modification), readers (RNA-binding proteins which can recognize and bind to the sequence upon modification of the RNA), and erasers (enzymes responsible for the removal of the modification) (Figure 1). A multitude of such chemical switches have been observed to be regulated and catalyzed by modification enzymes (Table 1).

Although, modifications like pseudouridine (ψ) and internal N^6-methyladenosine (m^6A) in mRNAs have been known for decades [10], lack of efficient detection and analysis techniques limited their profiling for a long time. However, recent technological advancements and the availability of high throughput detection methods have enabled the documentation of more than 160 types of RNA modifications (Table 1) with increasing evidence for their role in gene regulation, cell development, translation, metabolism and stress responses [11, 12]. Further, the discovery of m^6A erasers like Fat mass obesity-associated protein (FTO) and alkB homologue 5 (ALKBH5) that can demethylate the target loci, has pushed the idea that RNA modifications are dynamic and reversible similar to DNA modifications and are likely to contribute to a complex epitranscriptomic code, revealing their significance in multiple human diseases [13–15]. Studies also support that mutations associated with more than half of the known RNA modifications and RNA modifying enzymes are involved in major human diseases like cancer, neurological disorders, cardiovascular diseases, metabolic diseases, genetic birth defects and mitochondrial-related defects [16]. Such developments together with significant evolutionary conservation of RNA modification enzymes and their target sites across domains of life [17], rapid discovery of new RNA modification writers and erasers along with their functions in regulating RNAs and their functional outcomes, has led to the birth of Epitranscriptomics [18].

A survey of available RNA modification databases [11, 12] reveals that RNA modifications are abundant in tRNAs, rRNAs, snoRNAs and snRNAs, but their diversity is most widespread among tRNAs, rRNAs and mRNAs followed by snRNAs and snoRNAs. Nevertheless, given the rapid development in detection methods which can scale to whole transcriptomes, our understanding of the dynamic epitranscriptomes and resulting contribution to cellular phenotypes is expected to improve dramatically. In this review, we summarize few of the extensively studied RNA modifications that are observed to be abundant in mRNAs and tRNAs, with substantial roles in RNA metabolism and human diseases. For brevity, we focus on RNA modifications and omit the discussion on the role of RNA editing events in disease [19].

Types of major RNA modifications

The first RNA modification Pseudouridine (ψ) identified in the early 1950s [20], is an isomer of C^5-glycoside from the nucleoside uridine and is considered to be the most abundant cellular RNA modification with a ψ/U ratio of 0.2–0.6% [21, 22], observed mainly in rRNAs and tRNAs; although the rapid growth in high-resolution detection techniques has

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also led to the discovery of pseudouridine within the mRNAs of eukaryotes [23, 24]. Pseudouridine formation is dynamically controlled by environmental stress stimuli and is considered an irreversible modification due to the formation of an inert C-C bond. It is known to play a crucial regulatory role in RNA stabilization, structural alteration and mRNA metabolism during stress conditions [24–26]. High-resolution detection techniques like Pseudo-seq where pseudouridine specific chemical agents like CMC (N-cyclohexyl-N'-(2-mor-pholinoethyl)carbomiimide metho-p-toulenesulfonate) are used to block reverse transcription one nucleotide downstream, has enabled unfolding its significance in human diseases like prostate cancer, dyskeratosis congenital, and pituitary tumorigenesis [23, 24, 27].

Adenosine modifications like N6-methylAdenosine (m^6A), discovered in the 1970s [28] are thought to be the most abundant mRNA modification accounting for 0.1–0.5% of all adenosines, with a crucial role in regulating RNA stability, expression, and localization [5, 29, 30]. Although the precise location of m^6A on mRNA is still under debate, new high-throughput detection techniques point to their enrichment near 3' untranslated regions (UTRs) and at stop codons in long exons [31–33]. Studies carried out by Meyer and co-workers on cancer and tissue-specific cell lines using immunoblotting techniques also revealed that m^6A modifications are tissue-specific with a high degree of variability in their occurrence profiles between brain, heart and kidney [31]. In addition, Molinie and coworkers also reported the differential m^6A levels among mammalian embryonic and B-cell lymphoblastoid cell lines using the m^6A-LAIC-seq detection method [34]. m^6A specific immunoprecipitation techniques like m^6A-Seq, also called MeRIP-Seq, revealed their widespread prevalence among 25% of the known transcripts and their extensive evolutionary conservation [31, 35]. However, the major shift in the study of m^6A modifications and their role in human diseases ascended after the discovery of Fat mass obesity-associated protein (FTO) and alkB homologue 5 (ALKBH5) due to their ability to erase m^6A modifications [13, 15]. Mutations related to m^6A erasers and writers like FTO, ALKBH5, METTL14, and METTL3 are observed to be associated with major diseases like cancer, type 2 diabetes, leukemia, infertility and some major neuropsychiatric behavioral and depressive disorders, signifying the importance of m^6A in major human diseases [36, 37].

Recently, N^1-methyladenosine (m^1A) another adenosine modification, known to be originally abundant in non-coding RNAs has been reported to be prevalent in mRNA [30, 38, 39]. Comprehensive analysis carried out on human embryonic kidney cells (HEK293T) using m^1A specific immunoprecipitation techniques (m^1A-ID-seq) [40], revealed the abundant distribution of m^1A at 5' untranslated regions (UTR) of mRNA in the vicinity of start codons and also known to be associated with novel sequence motifs. Studies on ALKBH3 (DNA/RNA demethylase) knockout cell lines also revealed the reversible nature of m^1A modifications similar to m^6A modification and tend to be dynamically responsive towards physiological stress stimuli [39]. Although, m^1A modifications are observed to be associated with human diseases like obesity and neurodevelopmental regression, more phenotypic studies are required to uncover the regulatory and functional dynamics of N^1-methyladenosine in the context of human diseases.

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Modifications to cytosines on RNA include N^5-methylcytidine (m^5C), which was originally observed in tRNAs and rRNAs. It is now known to play a key role in controlling the secondary structure conformation and translation of RNAs [41]. The first global transcriptome mapping analysis carried out by Squires and colleagues in 2012 using a sodium bisulfite sequencing technique, unveiled more than 10000 m^5C modification positions in human mRNA enriched around annotated untranslated regions (UTRs) [42]. Recently, Yang et. al mapped the m^5C sites across multiple tissues in mice, to demonstrate its enrichment in CG-rich locations and in regions immediately downstream of translation initiation sites [43]. The study also provided evidence for a conserved and tissue-specific m^5C epitranscriptome with NSUN2 as a methytransferase and ALYREF as a novel m^5C reader with the ability to shuttle mRNAs between the nucleus and cytoplasm [43]. However, although several m^5C methyl transferases like NSUN2, DNMT1, NSUN3 and TRDMT1 have been reported, unlike m^6A, so far no erasers have been established for m^5C leaving the debate on the reversible nature of this modification wide open.

N^3-methylcytdine, another cytosine modification, was initially reported in tRNAs [44, 45] and is known to be catalyzed by TRM140, TRM141 in yeast [46–48] and by their homologs METTL2b, METTL6 in mammals [45, 49–51]. However, recent gene knock out studies carried out by Xu and co-workers on mice and human cell lines reported the existence of m^3C modification in human mRNAs and observed to be catalyzed by METTL8 [51, 52]. Mutations in the enzymes METTL2, METTL6 and METTL8 known to catalyze m^3C modification, were found to be associated with diseases like asthma, lung and breast cancer in humans [16].

A comprehensive list of RNA modifications and their human regulators is presented in Table 1, since a detailed discussion of all the known modifications is beyond the scope of this review. However, it is now clear from these emerging studies, that a deeper understanding of each of these modifications to uncover their functional and regulatory dynamics as well as to unfold their cross-talk with other modifications and layers of regulation is required, to dissect the role of epitranscriptomes in health and disease.

Experimental and integrative approaches for detecting RNA modifications

Detecting RNA modifications on the transcriptome level has been challenging because many of the known RNA modifications are either reverse transcription (RT) silent or cannot inherit the modification marks onto the cDNA. In addition, lack of efficient high throughput detection methods has poised the field of epitranscriptomics for a long time. However, the past decade has seen a tremendous increase in high throughput detection and sequencing-based techniques for transcriptome-wide identification and mapping of RNA modifications. Currently, the available detection techniques mainly depend on chemical and antibody-based detection methods followed by sequencing analysis (Table 2). However, fourth generation sequencing technologies like Oxford Nanopore Technologies (ONT), which promise to provide long read sequencing of the native full-length RNA transcripts, open new frontiers for RNA modification detection, similar to the developments made on DNA modification detection on native DNA fragments in the recent past [53, 54].

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Alteration of modification enzymes and their marks in diseases

Several recent studies have provided a link between RNA modifications and their interactions with post-transcriptional regulatory machinery, implicating this cross-talk in modulating the metabolism of RNA (Table 3). However, our understanding of the importance of these modifications in disease biology is only beginning to emerge. For instance, patients with myotonic dystrophy type 2 (DM2), a neuromuscular disease characterized by neuronal loss and impairment [55], have increased binding of Muscleblind-like 1 protein (MBNL1) to CUG repeats in non-coding regions of the target transcripts [56]. A recent report by deLorimier et. al [57] provided a link between pseudouridine modification and the efficiency of binding by Muscleblind-like 1, 2, and 3 group of proteins, which are known to be sequestered to CUG repeat containing regions in DM2 patients. Using thermal melt and gel shift binding assays, the authors demonstrated that modification of U to ψ in CUG repeats results in reduced RNA flexibility and inhibition of binding to these repeats by MBNL proteins and hence could be a promising therapeutic approach to modulate the activity of the targets directly regulated by MBNL proteins [57]. Li and coworkers [58] demonstrated an increase of ~40–50% in mRNA pseudouridylation levels in HEK293T, HEK293, A549, DU145, Hela, HT29, HepG2, H1299, WPMY-1 cells and mESCs cells upon exposure to acute oxidative stress by H2O2 treatment, suggesting another direct link between global ψ levels and increased cellular stress. In addition, mutations in human PUS1, a member of the TRUA family of pseudouridylating enzymes, are documented to lead to Mitochondrial myopathies, Lactic Acidosis and Sideroblastic Anaemia (MLASA) like symptoms [59–61], while a truncated form of PUS3 detected in patients with intellectual disability (ID), resulted in reduced levels of ψ at positions 38 and 39 in tRNA of patients with PUS3 truncation suggestive of the prominent role of PUS3 enzyme and its target sites, in cognition and neurodevelopmental pathways [62]. Although these studies report the role of PUS enzymes in disease due to their ability to alter translation by pseudouridylation of RNA [59–62], caution is required in evaluating the impact of PUS enzymes in disease prognosis, considering the complex factors like structural variations among nuclear and cytoplasmic PUS enzyme isomers, sensitivity of pseudouridylation to environmental stimuli and the impact of other pseudouridylation targets in diseases [27, 59–62].

N^6-methyladenosine (m^6A) levels have been shown to be high in the developing mouse brain with increasing levels of methylation in adulthood and similarly, high m^6A levels in the nervous system have also been observed in adult flies [31, 63]. These studies have been further confirmed by a recent study which detected high m^6A levels in various normal adult mouse brain regions, with cerebellum exhibiting significantly higher m^6A levels compared to cerebral cortex [64]. The authors observed high m^6A methylation levels for the targets of the RBP, fragile X mental retardation protein (FMRP) and suggested that m^6A is likely used for selective recognition of targets in the synapse where FMRP is expressed, acting as a dynamic expression switch. m^6A modification has also been shown to be required for timely decay of transcripts involved in stem cell maintenance and cell cycle regulation in cortical neuronal progenitors [65]. Such timely decay of transcripts allows for accurate progression of the cell cycle and to induce spatiotemporal formation of different neuronal subtypes.

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Comparison of the m^{6}A methylomes in human and mouse Neuronal Progenitor Cells (NPCs) during cortical neurogenesis revealed an m^{6}A enrichment in human gene transcripts compared to mouse gene transcripts, many of these human genes are associated with genetic risk for human brain disorders like schizophrenia and autistic spectrum disorder [65]. m^{6}A mRNA modification is essential for survival as mice lacking the writer METTL3 die at E6.5 [66]. Depletion of METTL3 in human embryonic stem cells (hESC) was shown to impair neuronal differentiation [67] and formation of mature neurons [66]. Loss of components of the m^{6}A methyltransferase complex in flies has been shown to result in locomotion defects while METTL3 mutants displayed alterations in walking speed and orientation [63, 68]. In addition, conditional KO (cKO) of METTL14 in neurons of METTL14 WT and cKO mouse cortical neural progenitor cells (NPCs) revealed an essential role of m^{6}A in embryonic cortical neurogenesis [65, 69]. METTL14 WT and cKO mouse showed decreased proliferation and premature differentiation of Neural Stem Cells (NSC) [69], as well as a delayed specification of neuronal subtypes during brain development [65]. In addition to its recently established role in neuronal development, m^{6}A modification has also been shown to play a critical role in the process of axon regeneration in mature mouse neurons [70]. The authors demonstrated that upon nerve injury m^{6}A levels of many transcripts encoding for regeneration and translation machinery in dorsal root ganglion were elevated, which led to increased translation during the time of axon regeneration, via the m^{6}A specific reader protein YTHDF1 [70].

METTL3 has also been reported as a key player in human cancer metastasis with knockdown studies in lung cancer cell lines (A549, H1299, H1792) and Hela cells reporting a strong positive influence of METTL3 in promoting growth, survival and invasion of human lung cancer cells [71]. Recent studies carried out by Li et al. on renal cell carcinoma (RCC) cell lines (CAKI-1, CAKI-2 and ACHN) and a normal human renal tubular epithelial cell line HK-2, reported the crucial role of METTL3 in the regulation of tumor progression through PI3K/Akt/mTOR signaling pathway [72]. In addition, studies on immune-deficient mice by Barbieri et al. also demonstrated the effect of METTL3 down-regulation on leukaemic cell cycle arrest and inhibition of cell differentiation [73]. These observations support a cancer-specific regulatory role for METTL3, indicating the need for an indepth investigation of m^{6}A methylation in oncogenesis and progression across cancer types.

So far, two proteins in humans have been reported to act as m^{6}A erasers: FTO and ALKBH5 – with the ability to catalyze the removal of the methyl group of m^{6}A by oxidation. Although ALKBH5 is moderately expressed in the brain, Du et al. found polymorphisms in ALKBH5 gene in a Chinese Han cohort of 738 patients with major depressive disorder (MDD) and 1098 controls, suggesting ALKBH5 as a significant risk loci for MDD [37]. Although the study lacked a replication cohort and functional evidence, it is tempting to speculate that the loss/gain of function of this eraser in MDD patients could result in aberrant post-transcriptional outcomes. In contrast, FTO is highly expressed in the human brain and displays dynamic expression patterns during postnatal neurodevelopment [74, 75]. Although several genome-wide association studies have linked SNPs in the FTO loci to a multitude of human diseases including obesity [76], cancer [77, 78], infertility [79], Attention-deficit/hyperactivity disorder (ADHD) [80] and Alzheimer's disease [81, 82] with varying levels of statistical significance and case-control cohort sizes, evidence from these

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studies is largely causal and the precise functional role of FTO in contributing to these phenotypes is debatable and needs further investigation [76]. Association analysis with replication cohorts linked nonsynonymous mutations in the FTO enzymatic domain of adolescent French Canadian founder population with brain malformation and impaired brain function while intronic SNPs have been associated with abnormal brain volumes with additional functional evidence using a FTO knock-out mouse model directly linking FTO loss with decreased brain size and body weight [83, 84]. RNA hypermethylation of FTO was found to be associated with increased levels of target mRNAs but decreased protein levels in FTO knock-out mice [36]. Deletion of FTO in dopaminergic neurons of mice revealed impaired dopamine receptor signaling as well as abnormal locomotion and reward stimulatory actions in response to cocaine [36]. Widagdo et. al [85], showed that fear conditioned mice compared to footshock unconditioned stimulus exhibited a significant increase in m^6A intensity on several learning-induced neuronal loci from prefrontal cortex, and knockdown of FTO further enhanced memory by an accompanying reduction in the stability of the target mRNAs. Loss of FTO was also shown to reduce the proliferation and neuronal differentiation, leading to impaired learning and memory suggesting a more complex locus and brain region-specific effects of m^6A levels likely resulting from rewiring the post-transcriptional recognition landscape of the target RNAs [84]. A more recent study by Yu et. al [86] shows that FTO is enriched in mouse dorsal root ganglia and is specifically expressed in axons, influencing translation of axonal mRNAs providing a means for just in time local translation regulation for axon specific transcripts.

Furthermore, recently FTO and ALKBH5 were found to play a regulatory role in human cancer development. Studies carried out by Liu et al. on breast cancer cell lines (MCF-7, MDA-MB-231 and HCC1937) demonstrated that overexpression of FTO regulates PI3K/AKT signaling pathway promoting glycolysis in breast cancer cells [87]. In another study carried out by Li et al, high expression of FTO with t(11q23) rearrangements has been observed to regulate the expression of ASB2 and RARA, leading to cell transformation and leukemogenesis in acute myeloid leukemia (AML) [88]. In addition, recently two different association studies by Salgado-Montilla et al in a cohort of Puerto Rican men and Akbari et al. from literature survey, also reported causal link between FTO and cancer onset as well as progression [89, 90]. In a breast cancer biopsies study carried out by Zhang et al, a concordance of ALKBH5 and HIF-1a expression has been reported, suggesting hypoxia dependent regulation of ALKBH5.

Knockdown of ALKBH5 in MDA-MB-231 breast cancer cells showed decreased metastasis from breast to lungs in immunodeficient mice [91].

DNMT2 is a member of highly conserved cytosine-5-DNA methyltransferase protein family among eukaryotes and known to catalyze as a tRNA methylase [92, 93]. It has been shown that double knockout of Dnmt2 and NSun2 in mice will trigger lethal phenotypes such as severe developmental defects and impair cellular differentiation [94]. In addition, deletion of NSun2 [95, 96] or Dnmt2 [97] alone has been reported to cause cellular differentiation damage in zebra fish and mice skin, testis and brain. Furthermore, association studies in families of Iranian, Kurdish and United Arab Emirates origin linked multiple mutations in NSun2 with Intellectual Disability (ID) and Dubowitz-like syndrome, with additional

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functional evidence from a NSun2 knock-out model in flies resulting in severe short-term-memory (STM) which could be rescued by re-expression of the wildtype protein along with ID and facial dysmorphism phenotypes in flies, suggesting functional conservation of the phenotypes in human brains [98–100]. These observations are further substantiated using a NSun2 knock-out mouse model by Blanco et. al where loss of NSun2 mediated methylation on tRNAs was shown to result in angiogenin-mediated endonucleolytic cleavage of transfer RNAs (tRNA), leading to an accumulation of 5' tRNA-derived RNA fragments, which results in reduced protein translation rates and activates stress pathways leading to reduced cell size and increased apoptosis of cortical, hippocampal and striatal neurons in mice [101]. In addition, a recent study in human and mice NPCs showed that m5C deposited by NSUN2 regulates NSC differentiation and motility [102]. These studies thus provide a link between the failure of m^5C deposition and brain development. Hence, several methyl transferases including NSUN2, DNMT1, NSUN3, and TRDMT1, as well as their corresponding marks are being recognized as clinically important due to their significance in human disease [98, 99, 103, 104].

One of the best characterized associations between ID in human and mutations in a gene encoding for 2′-O-methylation (Nm) writer, are those recorded on FTSJ1 gene providing a link between Nonsyndromic X-Linked Intellectual Disability (NSXLID) and Nm [105]. Two Nm events Cm32 and Gm34 were found to be completely lost in tRNA(Phe) obtained from two genetically independent lymphoblastoid cell lines of NSXLID patients with loss-of-function FTSJ1 mutations [105]. TRMT44 is another putative 2′-O-methyluridine methyltransferase in which non-synonymous coding sequence mutations were identified to be enriched in Partial Epilepsy with Pericentral Spikes (PEPS) subjects compared to control population, providing causal link between mutations in the enzyme and this mendelian idiopathic epilepsy [106]. However, considering the rapidly growing number of epitranscriptome maps and availability of new techniques for detection and identification of novel modifications and their cognate enzymes, more extensive functional studies which can uncover the role of individual epitranscriptomic marks and their impact on human disease are needed.

Emerging role of epitranscriptome readers in disease

Epitranscriptome readers are the enzymes that are recruited at modification sites on RNA, regulating the splicing, degradation, localization and translation of the RNA. Given the dynamic nature of m^6A and its enrichment in mRNA, m^6A modification readers were among the most extensively studied. Members of the YTH domain family proteins (YTHDF1, YTHDF2, YTHDF3, YTHDC1, and YTHDC2) and HNRNP proteins (HNRNPA2B1 and HNRNPC) were the first known direct readers of m^6A modifications, with roles in translation regulation and degradation of RNA [107–114]. Recently, a new set of m^6A readers IGF2BP1, IGF2BP2, IGF2BP3 and eIF3 were identified to regulate mRNA stability and translation [115, 116]. Consistent with the increasing appreciation for the role of readers, polymorphisms in ZC3H13, a recently discovered m^6A reader [117], have been associated with schizophrenia [118]. Interestingly, cytoplasmic METTL3, known to be a writer of m^6A has also been recently reported to serve as an m^6A reader, promoting the translation of oncogenic mRNAs in lung cancer due to elevated levels of METTL3 [71].

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YTH family members known to recognize m6A RNA, were found to suppress HIV-1 infection [119] and METTL3 has been reported as a potential therapeutic target due to its regulatory role in the maintenance of leukemic state of myeloid leukemia, through a chromatin mediated pathway wherein METTL3 localizes to the transcriptional start sites of active genes, resulting in their enhanced translation by relieving ribosome stalling [73]. Such studies support the potential of human m6A readers as future drug targets. However, more in-depth studies are required to unveil the full potential of other known and novel modification readers as therapeutic targets.

Concluding Remarks

Although recent developments in epitranscriptomics approaches have enabled the transcriptome-wide mapping of several modifications, several limitations still exist. For instance, like much of the research to date, using antibody-based immunoprecipitation approaches that cannot distinguish between m^6A and m^1A or more generally between the different intermediate products of the modification marks on the full-length transcripts, remains a significant challenge.

Moreover, methods based on crosslinking and immunoprecipitation are known to be inefficient, resulting in the identification of only a small fraction of all the target sites due to low yields, along with significant differences in the identified targets depending on the specific crosslinking protocols employed [120, 121]. Although, more recent advanced CLIP methods like enhanced CLIP (eCLIP) [122] and DO-RIP-seq [123] were able to address some of these limitations such as reproducibility, coverage and quantification of RBP binding events [124], utility and repurposing of these methods for efficient quantification of RNA modification sites at single nucleotide resolution still needs exploration. This is especially a challenge for the field since most of these post-transcriptional modifications are not present at high levels on RNA and have differential abundance between the types of RNA in a spatiotemporal fashion. Hence, highly sensitive and accurate approaches are needed to identify, quantify and monitor RNA modifications that occur at low abundance across classes of RNAs: rRNAs, tRNAs, snoRNAs, miRNAs, mRNAs, and lncRNAs, on individual transcript isoforms to uncover the role of epitranscriptome in modulating the spatio-temporal cellular regulatory networks (Figure 2, see Clinician's Corner and Outstanding Questions box). For instance, as discussed above, RNA m^6A methylation has been shown to drive region-specific post-transcriptional regulatory networks in mouse brain, by selectively dictating the binding of FMRP target RNAs in synapse, due to their increased methylation status [64]. Given the limitations and scalability of current short read sequencing technologies to delineate such associations, a detailed understanding of epitranscriptome code is still not possible at present. However, state-of-the-art long read direct RNA sequencing technologies [53] could shape our improved understanding of such epitranscriptome codes similar to how epigenetic codes for gene transcription have evolved over the last decade (Figure 2). Improvements in such single molecule techniques to identify the modifications marks on full length transcripts, together with the inclusion of various complementary omics profiling datasets like RNA-seq, ribosome profiling and proteomic analysis of the respective normal and diseased states, would enable delineation of the specific roles of the modifications and their combinations in controlling the fate of gene

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expression. Such developments together with additional layers of information on RNA localization and structure would enable a deeper understanding of the cross-talk and interplay between various modifications and regulatory networks, to facilitate using reference epitranscriptome maps in individual tissues for therapeutic benefit in disease contexts.

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Glossary:

snoRNAs — Small nucleolar RNA (snoRNAs) are a class of small non-coding RNAs and are involved in guiding chemical modification of other RNAs. They are classified into two groups - C/D box snoRNAs involved in methylation processes and H/ACA box snoRNAs associated with pseudouridylation.

snRNA — Small nuclear RNA (snRNA) are found within splicing speckles and cajal bodies of the cell nucleus in eukaryotic cells. They are known to play a significant role in RNA splicing and frequently cooccur with snRNPs.

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Highlights

- A survey of available literature on RNA modifications reveals that RNA modifications are abundant in tRNAs, rRNAs, snoRNAs and snRNAs, but their diversity is prominent in tRNAs, rRNAs and mRNAs followed by snRNAs and snoRNAs
- Increasing evidence provides a link between RNA modifications and post-transcriptional regulatory processes
- Gene knock out and functional studies report the importance of RNA modifications in human health and disease
- Emerging long read direct RNA sequencing technologies and development of antibodies specific to RNA modifications could be promising venues for charting the combinatorial epitranscriptomic code across cell types
- Locus and cell type specific combination of epitranscriptome marks could drive the post-transcriptional regulatory fate of an RNA molecule

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Outstanding questions

- Is there a cross-talk between modification marks, to control the fate of RNA transcripts?

  Most current work is focused on identifying individual modification types; however, the interplay between modification marks on individual transcripts is unclear. Future work could uncover the combinatorial regulatory play between a multitude of RNA modifications and their relevance for gene regulation in specific cellular contexts.

- Do isoforms of a gene exhibit different epitranscriptomic marks across tissues?

  Current high-throughput technologies provide a catalogue of modification sites on a transcriptome-wide scale. However, due to the limitations of existing short read sequencing technologies, which are commonly employed as a downstream approach, deconvolution of these modification marks at individual isoform level has been difficult.

- Could emerging single molecule sequencing technologies decipherthe epitranscriptomic code and its relevance for human health and disease?

  Single molecular sequencing technologies like nanopore, which can simultaneously identify both isoforms and their modification marks in their native state could be a key to generating high resolution tissue-specific epitranscriptome maps.

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Clinician's Corner

- Increasing evidence points to the diversity of RNA alphabet, resulting from covalent modification of canonical RNA bases, across a multitude of RNA types occurring in the cell
- Multiple studies provide a link between RNA modifications and disease via altered modulation of post-transcriptional regulatory processes
- Improvements in available antibodies specific to RNA modifications and corresponding tailored experimental protocols, are making it feasible to chart an atlas of RNA modifications in specific cell types and their alterations in disease contexts
- Emerging technologies like single molecule direct RNA sequencing of full length transcripts are likely to yield high resolution locus and cell type specific epitranscriptome maps in the near future

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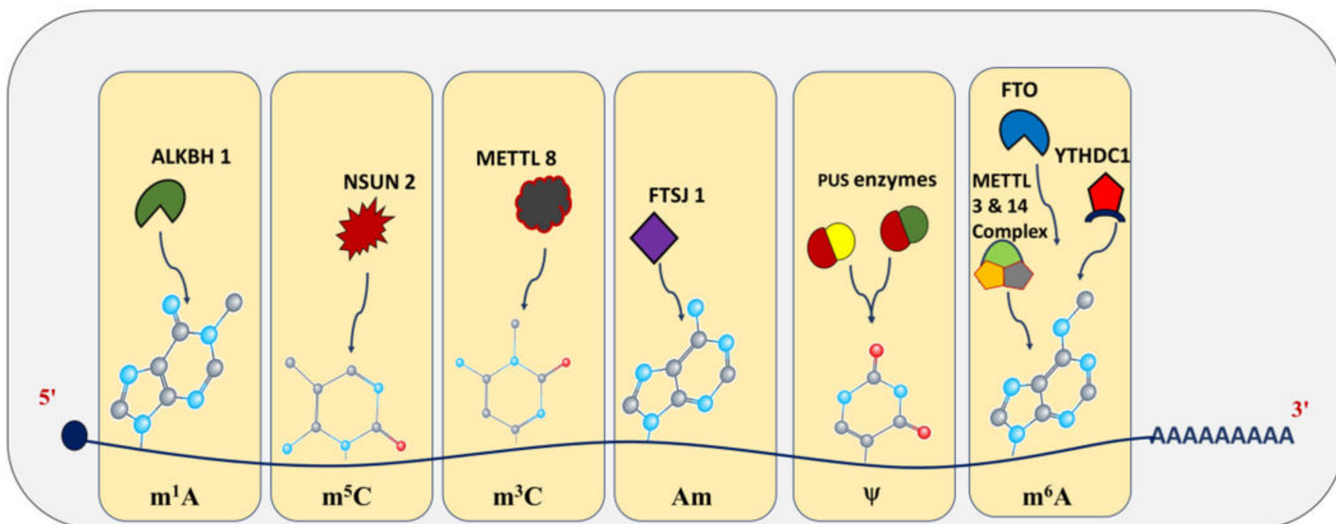

Figure 1: Frequently occurring chemical modifications in mRNA and their currently known writers, readers, and erasers.

Readers and eraser proteins are only listed for m^6A modification type. A comprehensive list of RNA modifications along with their currently known enzymes are listed in Table 1.

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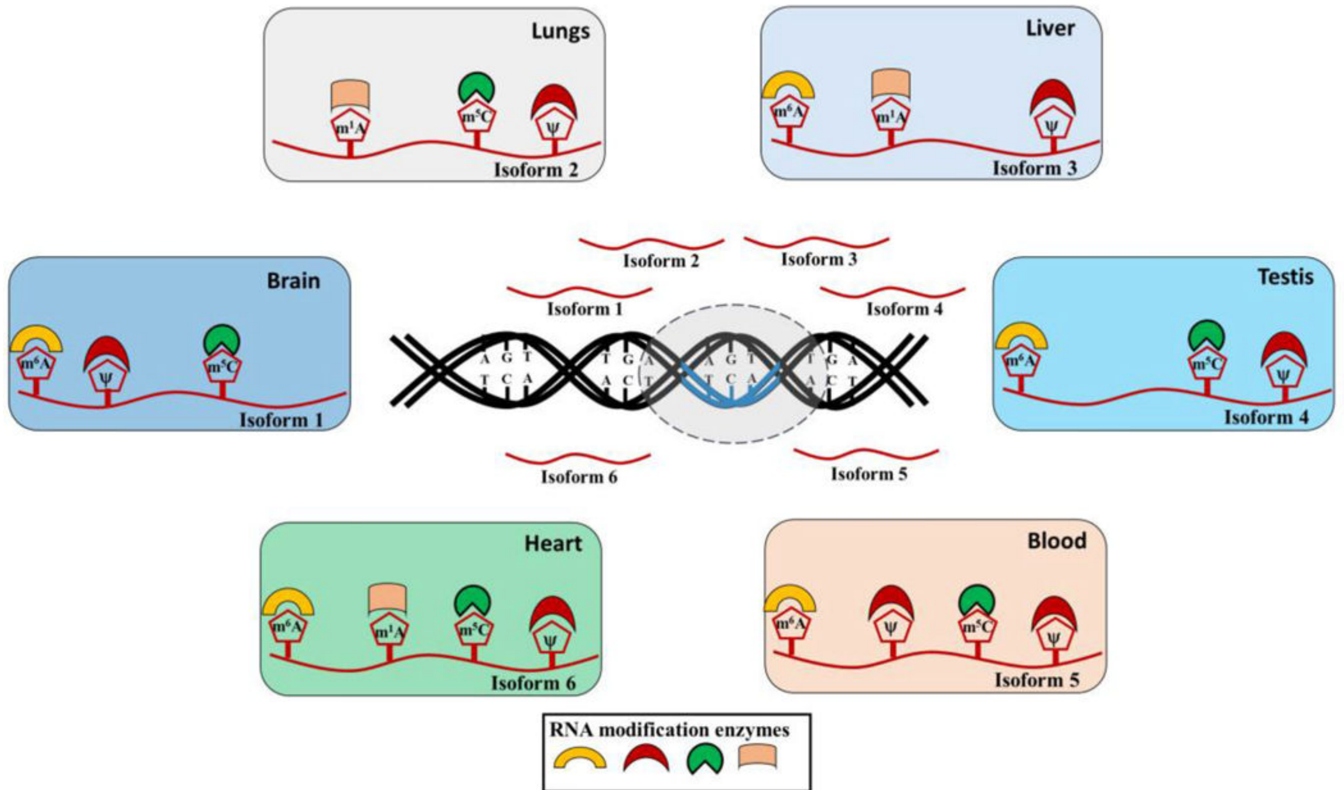

Figure 2: Emerging concept of the epitranscriptome code governing the fate of different transcript isoforms of a gene across tissue types.

Although a combination of transcript isoforms of a gene are expressed in a tissue their dynamic tissue/cell-type specific regulation by different modification enzymes (Writers, Readers and Erasers) via epitranscriptome code, determines the differential post-transcriptional regulatory fate of an RNA molecule resulting from a loci. Such combinatorial epitranscriptome marks specific to an RNA transcript originating from a genic locus, can dictate its splicing, stability, localization as well as translation status, providing a precise cell-type specific spatiotemporal context for regulation.

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Table 1:

Comprehensive list of currently known RNA modifications and their corresponding modification enzymes in the human genome. Listed human enzyme abbreviations stand for ACA13: small nucleolar RNA/ H/ACA box 13, EMG-1: N1-specific pseudouridine methyltransferase, NEP1: N1-specific pseudouridine methyltransferase, TRM6: tRNA methyltransferase 6, TRMT10C/RG9MTD1: tRNA methyltransferase 10C, TRMT61A: tRNA methyltransferase 61A, TRMT61B: tRNA methyltransferase 61B, NML: ribosomal RNA processing 8, hRRP8: Human Ribosomal RNA processing 8, SDR5C1: hydroxysteroid 17-beta dehydrogenase 10, ALKBH1: alkB homolog 1, TRMT5: tRNA methyltransferase 5, TRMT112: tRNA methyltransferase 112, TRMT1L: tRNA methyltransferase 1L, TRMT10A/RG9MTD2: tRNA methyltransferase 10A, TRMT10B/ RG9MTD3: tRNA methyltransferase 10B, CDK5RAP1: CDK5 regulatory subunit associated protein 1, CDKAL1: CDK5 regulatory subunit associated protein 1 like 1, TRMU: tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase, FTSJ1: FtsJ RNA methyltransferase homolog 1, FTSJ2: FtsJ RNA methyltransferase homolog 2, FTSJ3: FtsJ RNA methyltransferase homolog 3, CCDC76: tRNA methyltransferase 13 homolog, TARBP1: TAR (HIV-1) RNA binding protein 1, MRM1: mitochondrial rRNA methyltransferase 1, RNMTL1/MRM3: mitochondrial rRNA methyltransferase 3, METTL2B: methyltransferase like 2B, METTL2A: methyltransferase like 2A, METTL8: methyltransferase like 8, METTL6: methyltransferase like 6, TYW1: tRNA-yW synthesizing protein 1 homolog, TYW3: tRNA-yW synthesizing protein 3 homolog, ALKBH8: alkB homolog 8, ELP3: elongator acetyltransferase complex subunit 3, ELP4: elongator acetyltransferase complex subunit 4, IKBKAP/ELP1: elongator complex protein 1, NSUN3: NOP2/Sun RNA methyltransferase family member 3, GTPBP3: GTP binding protein 3, mitochondrial, CTU1: cytosolic thiouridylase subunit 1, NSUN2: NOP2/Sun RNA methyltransferase family member 2, NSUN1: NOP2/Sun RNA methyltransferase family member 1, NSUN4: NOP2/Sun RNA methyltransferase family member 4, NSUN5: NOP2/Sun RNA methyltransferase family member 5, NSUN6: NOP2/Sun RNA methyltransferase family member 6, p120: RAS p21 protein activator 1, WDR4: WD repeat domain 4, TGS1: trimethylguanosine synthase 1, NAT10: N-acetyltransferase 10, DIMT1L: DIM1 dimethyladenosine transferase 1 homolog, FTO: alpha-ketoglutarate dependent dioxygenase, TRMO: tRNA methyltransferase O, DUS1L: dihydrouridine synthase 1 like, DUS2: dihydrouridine synthase 2: DUS3L: dihydrouridine synthase 3 like, DUS4L: dihydrouridine synthase 4 like, ADAT2: adenosine deaminase, tRNA specific 2, ADAT3: adenosine deaminase, tRNA specific 3, ADAT1: adenosine deaminase, tRNA specific 1, ADAR1: adenosine deaminase, RNA specific, ADAR2: adenosine deaminase, RNA specific B1, PUS1: pseudouridylate synthase 1, PUS3: pseudouridylate synthase 3, PUS7: pseudouridylate synthase 7, RPUSD2: RNA pseudouridylate synthase domain containing 2, THG1L: tRNA-histidine guanylyltransferase 1 like.

Short Name	New Nomenclature	Name	Human Enzyme
m1Am	01A	1,2′-O-dimethyladenosine	
m1Gm	01G	1,2′-O-dimethylguanosine	
m1Im	019A	1,2′-O-dimethylinosine	
m1acp3Y	1309U	1-methyl-3-(3-amino-3-carboxypropyl)pseudouridine	ACA13, EMG1, NEP1
m1A	1A	1-methyladenosine	TRM6, TRMT10C, TRM61A, TRM61B, Nucleomethyin, NML, hRRP8, SDR5C1, ALKBH1

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Short Name	New Nomenclature	Name	Human Enzyme
m1G	1G	1-methylguanosine	TRMT5, TRMT10A TRMT10B, TRMT10C RG9MTD2, RG9MTD1, RG9MTD3, SDR5C1
m1I	19A	1-methylinosine	
m1Y	19U	1-methylpseudouridine	EMG1, NEP1
m2,8A	28A	2,8-dimethyladenosine	
msms2i6A	N/A	2-methylthiomethylenethio-N6-isopentenyl-adenosine	
ges2U	21U	2-geranylthiouridine	
k2C	21C	2-lysidine	
m2A	2A	2-methyladenosine	
ms2ct6A	2164A	2-methylthio cyclic N6-threonylcarbamoyladenosine	
ms2io6A	2160A	2-methylthio-N6-(cis-hydroxyisopentenyl) adenosine	
ms2hn6A	2163A	2-methylthio-N6-hydroxynorvalylcarbamoyladenosine	
ms2i6A	2161A	2-methylthio-N6-isopentenyladenosine	CDK5RAP1
ms2m6A	621A	2-methylthio-N6-methyladenosine	
ms2t6A	2162A	2-methylthio-N6-threonylcarbamoyladenosine	CDKAL1
se2U	20U	2-selenouridine	
s2Um	02U	2-thio-2′-O-methyluridine	TRMU
s2C	2C	2-thiocytidine	
s2U	2U	2-thiouridine	TRMU
Am	0A	2′-O-methyladenosine	FTSJ1, FTSJ2, FTSJ3
Cm	0C	2′-O-methylcytidine	FTSJ1, FTSJ2, FTSJ3, CCDC76
Gm	0G	2′-O-methylguanosine	TARBP1, FTSJ1, MRM1, RNMTL1
Im	09A	2′-O-methylinosine	
Ym	09U	2′-O-methylpseudouridine	
Um	0U	2′-O-methyluridine	FTSJ1, FTSJ2, FTSJ3
mcmo5Um	0503U	2′-O-methyluridine 5-oxyacetic acid methyl ester	
Ar(p)	00A	2′-O-ribosyladenosine (phosphate)	
Gr(p)	00G	2′-O-ribosylguanosine (phosphate)	
(pN)2′3′>p	3377N	2′3′-cyclic phosphate end	
m3Um	03U	3,2′-O-dimethyluridine	
acp3D	308U	3-(3-amino-3-carboxypropyl)-5,6-dihydrouridine	
acp3Y	309U	3-(3-amino-3-carboxypropyl)pseudouridine	

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Short Name	New Nomenclature	Name	Human Enzyme
acp3U	30U	3-(3-amino-3-carboxypropyl)uridine	
m3C	3C	3-methylcytidine	METTL2B, METTL2A, METTL8, METTL6
m3Y	39U	3-methylpseudouridine	
m3U	3U	3-methyluridine	
imG-14	4G	4-demethylwyosine	TYW1
s4U	74U	4-thiouridine	
m5Cm	05C	5,2′-O-dimethylcytidine	
m5Um	05U	5,2′-O-dimethyluridine	
mchm5Um	0522U	5-(carboxyhydroxymethyl)-2′-O-methyluridine methyl ester	ALKBH8
mchm5U	522U	5-(carboxyhydroxymethyl)uridine methyl ester	
inm5s2U	2583U	5-(isopentenylaminomethyl)-2-thiouridine	TRMU
inm5Um	0583U	5-(isopentenylaminomethyl)-2′-O-methyluridine	
inm5U	583U	5-(isopentenylaminomethyl)uridine	
nm5ges2U	21510U	5-aminomethyl-2-geranylthiouridine	
nm5se2U	20510U	5-aminomethyl-2-selenouridine	
nm5s2U	2510U	5-aminomethyl-2-thiouridine	
nm5U	510U	5-aminomethyluridine	
nchm5U	531U	5-carbamoylhydroxymethyluridine	ALKBH8
ncm5s2U	253U	5-carbamoylmethyl-2-thiouridine	
ncm5Um	053U	5-carbamoylmethyl-2′-O-methyluridine	FTSJ1, FTSJ3
ncm5U	53U	5-carbamoylmethyluridine	ELP3, ELP4, IKBKAP
chm5U	520U	5-carboxyhydroxymethyluridine	
cm5s2U	2540U	5-carboxymethyl-2-thiouridine	
cmnm5ges2U	2151U	5-carboxymethylaminomethyl-2-geranylthiouridine	
cmnm5se2U	2051U	5-carboxymethylaminomethyl-2-selenouridine	
cmnm5s2U	251U	5-carboxymethylaminomethyl-2-thiouridine	TRMU
cmnm5Um	051U	5-carboxymethylaminomethyl-2′-O-methyluridine	
cmnm5U	51U	5-carboxymethylaminomethyluridine	Protein MTO1 homolog mitochondrial isoform
cm5U	52U	5-carboxymethyluridine	
cnm5U	55U	5-cyanomethyluridine	
f5Cm	071C	5-formyl-2′-O-methylcytidine	
f5C	71C	5-formylcytidine	NSUN3

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Short Name	New Nomenclature	Name	Human Enzyme
ho5C	50C	5-hydroxycytidine	
hm5C	51C	5-hydroxymethylcytidine	
ho5U	50U	5-hydroxyuridine	
mcm5s2U	2521U	5-methoxycarbonylmethyl-2-thiouridine	ALKBH8, CTU1, ELP3, ELP4, IKBKAP, TRMU
mcm5Um	0521U	5-methoxycarbonylmethyl-2′-O-methyluridine	ALKBH8, ELP3, ELP4, IKBKAP, KIAA1456/TRM9L
mcm5U	521U	5-methoxycarbonylmethyluridine	
mo5U	501U	5-methoxyuridine	
m5s2U	25U	5-methyl-2-thiouridine	TRMU
mnm5ges2U	21511U	5-methylaminomethyl-2-geranylthiouridine	
mnm5se2U	20511U	5-methylaminomethyl-2-selenouridine	GTPBP3
mnm5s2U	2511U	5-methylaminomethyl-2-thiouridine	TRMU
mnm5U	511U	5-methylaminomethyluridine	
m5C	5C	5-methylcytidine	NSUN2, DNMT2, NSUN1, NSUN3, NSUN4, NSUN5, NSUN6 WBSCR20, hNOP2, NOL1, p120, TRDMT1
m5D	58U	5-methyldihydrouridine	
m5U	5U	5-methyluridine	TRMT2A, TRMT2B1
tm5s2U	254U	5-taurinomethyl-2-thiouridine	TRMU
tm5U	54U	5-taurinomethyluridine	GTPB3
CoA(pN)	455N	5′ (3′ -dephospho-CoA)	
acCoA(pN)	4155N	5′ (3′ -dephosphoacetyl-CoA)	
malonyl-CoA(pN)	4255N	5′ (3′ -dephosphomalonyl-CoA)	
succinyl-CoA(pN)	4355N	5′ (3′ -dephosphosuccinyl-CoA)	
p(pN)	552N	5′ diphosphate end	
5′-OH-N	550N	5′ hydroxyl end	
(pN)	N	5′ monophosphate end	
NAD(pN)	255N	5′ nicotinamide adenine dinucleotide	
pp(pN)	553N	5′ triphosphate end	
yW-86	47G	7-aminocarboxypropyl-demethylwyosine	
yW-72	347G	7-aminocarboxypropylwyosine	TYW3
yW-58	348G	7-aminocarboxypropylwyosine methyl ester	
preQ1tRNA	101G	7-aminomethyl-7-deazaguanosine	
preQ0tRNA	100G	7-cyano-7-deazaguanosine	

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Short Name	New Nomenclature	Name	Human Enzyme
m7G	7G	7-methylguanosine	WBSCR22/TRMT112, WDR4, METTL1 (tRNA)
m7Gpp(pN)	79553N	7-methylguanosine cap (cap 0)	
m8A	8A	8-methyladenosine	
m2Gm	02G	N2,2′-O-dimethylguanosine	
m2,7Gm	027G	N2,7,2′-O-trimethylguanosine	TGS1
m2,7G	27G	N2,7-dimethylguanosine	
m2,7Gpp(pN)	279553N	N2,7-dimethylguanosine cap (cap DMG)	
m2,2Gm	022G	N2,N2,2′-O-trimethylguanosine	TRMT1, TRMT1L (C1ORF25)
m2,2,7G	227G	N2,N2,7-trimethylguanosine	TGS1
m2,2,7Gpp(pN)	2279553N	N2,N2,7-trimethylguanosine cap (cap TMG)	
m2,2G	22G	N2,N2-dimethylguanosine	
m2G	2G	N2-methylguanosine	
m4Cm	04C	N4,2′-O-dimethylcytidine	
m4,4Cm	044C	N4,N4,2′-O-trimethylcytidine	
m4,4C	44C	N4,N4-dimethylcytidine	
ac4Cm	042C	N4-acetyl-2′-O-methylcytidine	
ac4C	42C	N4-acetylcytidine	NAT10 U13
m4C	4C	N4-methylcytidine	
m6Am	06A	N6,2′-O-dimethyladenosine	
m6,6Am	066A	N6,N6,2′-O-trimethyladenosine	
m6,6A	66A	N6,N6-dimethyladenosine	DIMT1L
io6A	60A	N6-(cis-hydroxyisopentenyl)adenosine	
ac6A	64A	N6-acetyladenosine	
f6A	67A	N6-formyladenosine	FTO
g6A	65A	N6-glycinylcarbamoyladenosine	
hm6A	68A	N6-hydroxymethyladenosine	FTO
hn6A	63A	N6-hydroxynorvalylcarbamoyladenosine	
i6A	61A	N6-isopentenyladenosine	
m6t6A	662A	N6-methyl-N6-threonylcarbamoyladenosine	TRMO
m6A	6A	N6-methyladenosine	ALKBH5, FTO, Mettl14, Mettl3
t6A	62A	N6-threonylcarbamoyladenosine	
Qbase	10G (base)	Qbase	
A	A	adenosine	
C+	20C	agmatidine	

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Short Name	New Nomenclature	Name	Human Enzyme
mm(pN)	2551N	alpha-dimethylmonophosphate cap	
m(pN)	1551N	alpha-methylmonophosphate cap	
G+	103G	archaeosine	
ct6A	69A	cyclic N6-threonylcarbamoyladenosine	
C	C	cytidine	
D	8U	dihydrouridine	DUS1L, DUS2, DUS3L, DUS4L, PP35
oQtRNA	102G	epoxyqueuosine	
galQtRNA	104G	galactosyl-queuosine	
mpp(pN)	1553N	gamma-methyltriphosphate cap	
gluQtRNA	105G	glutamyl-queuosine	
G	G	guanosine	
pG(pN)	GN	guanosine added to any nucleotide	
Gpp(pN)	9553N	guanylylated 5′ end (cap G)	
ht6A	2165A	hydroxy-N6-threonylcarbamoyladenosine	
OHyW	34830G	hydroxywybutosine	
I	9A	inosine	ADAT2-ADAT3, ADAT1, ADAR2, ADAR1
imG2	42G	isowyosine	
manQtRNA	106G	mannosyl-queuosine	
OHyWy	3480G	methylated undermodified hydroxywybutosine	
mimG	342G	methylwyosine	
o2yW	34832G	peroxywybutosine	
preQ0base	100G (base)	preQ0base	
preQ1base	101G (base)	preQ1base	
Y	9U	pseudouridine	PUS1, PUS3, RPUSD2, PUS7
QtRNA	10G	queuosine	
OHyWx	3470G	undermodified hydroxywybutosine	
Xm	0X	unknown methylated base	
xX	X	unknown modification	
xA	?A	unknown modified adenosine	THG1L
xC	?C	unknown modified cytidine	
xG	?G	unknown modified guanosine	
xU	?U	unknown modified uridine	
N	N/A	unknown nucleotide residue	
U	U	uridine	
cmo5U	502U	uridine 5-oxyacetic acid	

Short Name	New Nomenclature	Name	Human Enzyme
mcmo5U	503U	uridine 5-oxyacetic acid methyl ester	
yW	3483G	wybutosine	TRMT12
imG	34G	wyosine	

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Table 2:

Antibody and chemical based detection methods commonly used for transcriptome-wide identification of chemical modifications.

Detection Technique	Modification	Antibody or chemical based	Antibody or chemical used
MeRIP-Seq[31]	m^6A	Antibody	anti-m6A antibody
m^6A-Seq[125]]	m^6A, m^6Am	Antibody	anti-m6A polyclonal antibody
miCLIP[95, 126, 127]	m^6A, m^6Am, m5C	Antibody	anti-m6A polyclonal antibody, anti-m5C monoclonal antibody
PA-m^6A-Seq[128]	m^6A	Antibody	anti-m6A antibody
m^6A-CLIP[126]	m^6A	Antibody	anti-m6A antibody
SCARLET[33]	m^6A	Chemical	^{32}P-labeling
m^6A-LAIC-Seq[34]	m^6A	Antibody	anti-m6A antibody
RNA-BisSeq[129]	m5C	Chemical	Sodium bisulphite
Aza-IP[130]	m5C	Antibody and Chemical	anti-V5 antibody, 5-azacytidine(5-aza-C)
m5C-RIP[131]	m5C	Antibody	anti-m5C monoclonal antibody
hMeRIP-Seq[132]	hm5C	Antibody	anti-hm5C antibody
Pseudo-Seq[133]	ψ	Chemical	carbodiimide N-cyclohexyl-N′-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate (CMC)
PSI-Seq[134]	ψ	Chemical	1-cyclohexyl-(2-morpholinoethyl)carbodiimide metho-p-toluene sulfonate (CMCT)
CeU-Seq[58]	ψ	Chemical	N3-CMC
m^1A-Seq[39]	m^1A	Antibody	anti-m1A antibody
m^1A-ID-Seq[40]	m^1A	Antibody	anti-m1A antibody

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Table 3:

List of frequently observed RNA modifications on RNA transcripts and their reported crosstalk with post-transcriptional regulatory processes.

Modification	Regulatory mechanisms affected due to RNA modification
ψ	RNA stability[24, 135–138], splicing [23]and translation efficiency [139].
m^6A	mRNA stability[109], splicing [112], microRNA processing[107], RNA secondary structure[108], and translation[110, 116, 140]
m^5C	RNA processing[95], mRNA stability [127, 141], mRNA export[43], tRNA cleavage & translation [92, 94, 142]
m^1A	Translation [143], structural stability and/or folding of tRNA [144].
2′-Ome	RNA structure and stability [145]