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Review

The rise of epitranscriptomics: recent developments and future directions

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The epitranscriptomics field has undergone tremendous growth since the discovery that the RNA N^6-methyladenosine (m^6A) modification is reversible and is distributed throughout the transcriptome. Efforts to map RNA modifications transcriptome-wide and reshape the epitranscriptome in disease settings have facilitated mechanistic understanding and drug discovery in the field. In this review we discuss recent advancements in RNA modification detection methods and consider how these developments can be applied to gain novel insights into the epitranscriptome. We also highlight drug discovery efforts aimed at developing epitranscriptomic therapeutics for cancer and other diseases. Finally, we consider engineering of the epitranscriptome as an emerging direction to investigate RNA modifications and their causal effects on RNA processing at high specificity.

Highlights

Advanced detection of RNA modifications, especially N^6-methyladenosine, paves the way for transcriptome-wide, single-cell, and stoichiometric modification profiling at single-nucleotide resolution.

RNA modification databases, tumor atlases, drug-like compound libraries, and machine learning have accelerated therapeutic development focused on epitranscriptomics.

Novel RNA modification engineering tools based on the CRISPR/Cas technology or endogenous RNA modification machinery can be applied to uncover causal relationships between individual modification sites and phenotypic outcomes.

The rise of epitranscriptomics

That RNA molecules are decorated with modified nucleotides, or RNA modifications, has been known since the 1950s [1]. Early characterization of highly abundant RNA species, such as ribosomal RNA (rRNA) and transfer RNA (tRNA), revealed that extensive post-transcriptional modification of these RNAs is required to support their higher-order structure [2,3]. However, the lack of methods to detect and map RNA modifications occurring on low-abundance RNAs and their elusive functional relevance had rendered the field of **epitranscriptomics** (see Glossary) dormant for several decades. In 2011, the discovery that an internal RNA modification, m^6A, was reversible, and that the fat mass and obesity-associated protein (FTO) mediated its removal, revealed the potential dynamic nature of m^6A modification and hinted at its functional relevance [4].

Efforts to characterize the distribution, dynamics, and roles of m^6A have since followed, propelling the field of epitranscriptomics into the limelight [5]. Sophisticated protocols for high-throughput profiling of RNA modifications have been developed and have revealed dynamic distribution of m^6A and other RNA modifications across the transcriptome [5–7]. Today, it is well established that over 170 types of RNA modifications dynamically decorate different RNA species and regulate almost every aspect of RNA metabolism [8]. In addition to m^6A, modifications such as pseudouridine (Ψ), N^1-methyladenosine (m^1A), 5-methylcytosine (m^5C), and others are widespread on RNA and have been under intense investigation in recent years [5]. Importantly, aberrant expression of RNA modification machinery and dysregulation of the epitranscriptome in cancer have been exploited to develop novel therapeutic agents that could reverse modification-dependent pro-tumorigenic gene expression programs [9]. Streamlined detection of RNA modifications has also facilitated the study of epitranscriptomics in other complex biological processes, such as development, homeostasis, and diseases beyond cancer. Pioneering research by the Hungarian–American biochemist Katalin Karikó and the American immunologist

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Drew Weissman on utilizing RNA modifications to suppress mRNA immunogenicity and promote its translation led to the development of mRNA-based vaccines against coronavirus disease 2019 (COVID-19) [10–12]. Karikó and Weissman were subsequently awarded the 2023 Nobel Prize in Physiology or Medicine.

In this review, we discuss key developments in the epitranscriptomics field, including advancements in transcriptome-wide modification detection methods and drug discovery efforts. With a focus on the most-studied RNA modification, m^{6}A, we consider the biological insights gained from mapping RNA modifications transcriptome-wide at varying resolutions and identify the areas that are yet to be explored using recently developed technologies. We also discuss drug discovery focused on epitranscriptomics, including various strategies and tools that have been implemented to develop novel therapeutic agents that target different epitranscriptomic components. Finally, we highlight RNA modification engineering as a powerful approach to characterize functional relevance of the epitranscriptome at a much higher specificity than is possible by modifying the expression or activity of the RNA modification machinery.

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Box 1. RNA modifications and effector proteins

RNA molecules are highly decorated by chemical modifications that comprise the epitranscriptome and impart structural and functional properties to the modified transcripts [5,7]. N^6-methyladenosine (m^{6}A) is the most-studied RNA modification that dynamically and reversibly decorates multiple RNA species and plays important roles in downstream RNA processing [5]. Dedicated m^{6}A modification machinery composed of m^{6}A writer, eraser, and reader proteins orchestrates m^{6}A installation, removal, and integration into downstream biological processes, respectively (Figure I). The m^{6}A modification is installed by methyltransferase-like 3/14 (METTL3/14) and METTL16 writer proteins that use S-adenosylmethionine (SAM) as a methyl donor, whereas demethylation of m^{6}A is mediated by FTO and alkB homolog 5 (ALKBH5) oxygenases [5]. Several m^{6}A reader proteins – including those of YT521-B homology (YTH) domain, insulin-like growth factor (IGF), and heterogeneous nuclear ribonucleoprotein (HNRNP) families – recognize m^{6}A and regulate RNA processing, such as nuclear export, translation, and decay [5,89]. The m^{6}A epitranscriptome is dynamically regulated in response to environmental stimuli and cellular stress, thus fine-tuning gene expression in a changing environment. Furthermore, m^{6}A has been implicated in cardinal biological processes, including development, tissue homeostasis, and disease [5]. For example, the m^{6}A epitranscriptome becomes dysregulated in cancer, contributing to pro-tumorigenic gene expression programs that drive tumor progression [9,41]. Therapeutic targeting of the m^{6}A modification machinery is an emerging approach to treat various types of cancer, such as leukemia and glioblastoma [9,41,53]. Beyond m^{6}A, over 170 RNA modifications have been described across different RNA species [7]. Rapid development of RNA modification detection methods has enabled us to probe the roles of numerous RNA modifications in cell biology. For example, pseudouridine (Ψ), an isomer of uridine, is often considered to be the fifth RNA nucleotide, given its widespread presence on RNA, and is installed by Ψ synthases (PUS) [1]. However, no Ψ eraser proteins have been identified, and thus it remains unclear whether Ψ is reversible. Nonetheless, Ψ affects multiple aspects of RNA processing, such as splicing and translation, whereas dysregulation of the Ψ epitranscriptome is evident in cancer [1]. For example, we have recently shown that increased expression of *PUS7* leads to aberrant pseudouridylation of tRNA in glioblastoma stem cells (GSCs), promoting their proliferation and tumor growth [46]. Pharmacological inhibition of PUS7 with a small-molecule compound C17 suppresses GSC proliferation *in vitro* and glioblastoma progression *in vivo* [46]. Overall, the dynamic epitranscriptome regulates most aspects of RNA biology to influence gene expression.

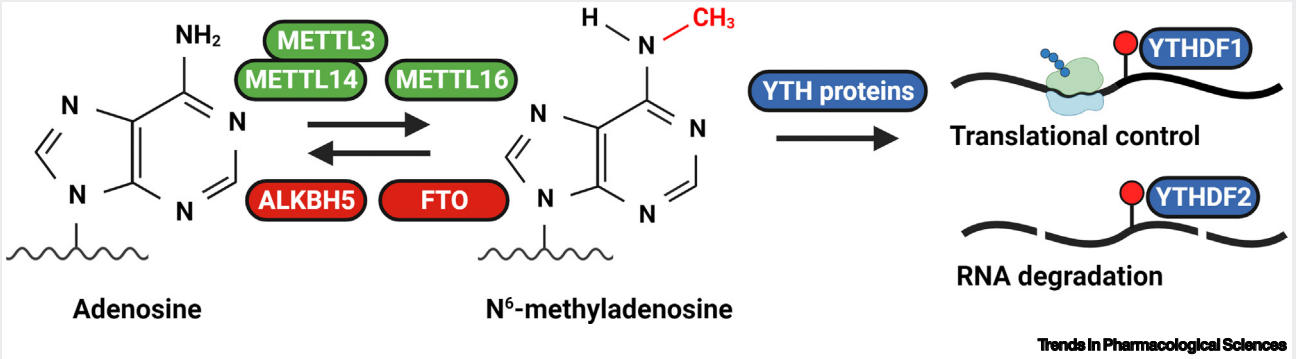

Figure I. The dynamics of N^6-methyladenosine (m^{6}A) RNA modification. Adenosine moieties of RNA are methylated by m^{6}A writer proteins, methyltransferase-like 3/14 (METTL3/14) and METTL16. The modified m^{6}A can be either erased by the oxygenases fat mass and obesity-associated protein (FTO) and alkB homolog 5 (ALKBH5) or recognized by reader proteins that in turn mediate downstream RNA processing. For example, an m^{6}A reader protein YTH N^6-methyladenosine RNA binding protein F1 (YTHDF1) has been shown to promote m^{6}A-decorated messenger RNA (mRNA) translation, whereas YTHDF2 facilitates mRNA degradation. Figure created with BioRender.com.

Advancements in transcriptome-wide detection of RNA modifications

RNA modifications can be characterized in different aspects, such as their location and distribution across the transcriptome, dynamics in response to changing cellular or environmental conditions, heterogeneity across different cells and tissues, and **stoichiometry**. Each of these characteristics can provide distinct insights into the roles of the epitranscriptome in RNA processing and cell biology. Indeed, continuous advancements in methods for mapping RNA modifications have been the driving force of scientific discovery in the epitranscriptomics field. In this section, we focus on m^{6}A to discuss the evolution of transcriptome-wide RNA modification detection methods and consider the biological insights gained from charting the epitranscriptome.

Location and distribution of RNA modifications

The discovery that m^{6}A was reversible and identification of its dedicated modification machinery (Box 1) were major clues that m^{6}A played regulatory roles in RNA processing [4,5,13,14]. However, determining how dynamic the m^{6}A methylome is and whether it can influence global RNA biology requires reliable transcriptome-wide approaches for mapping m^{6}A. The first transcriptome-wide m^{6}A modification landscapes were obtained by RNA immunoprecipitation-based m^{6}A detection (**MeRIP-seq** and m^{6}A-seq protocols) using m^{6}A-specific antibodies in 2012 (Figure 1A) [15,16]. These studies revealed that m^{6}A decorated multiple RNA species, whereas the global m^{6}A landscape changed dynamically in response to changing cellular conditions [15,16]. Antibody-based m^{6}A detection remains the most commonly used and facile approach to map m^{6}A today.

One important limitation of MeRIP-seq and m^{6}A-seq protocols is their limited resolution, because m^{6}A-decorated RNA is enriched as 100–200 nucleotide-long fragments [6,17]. Therefore, subsequently developed protocols for m^{6}A mapping have been aimed at achieving single-nucleotide resolution, so that individual m^{6}A sites could be identified. Cross-linking of m^{6}A-specific antibodies to RNA has improved resolution of antibody-based m^{6}A detection protocols because a mutational signature is introduced around the m^{6}A site during reverse transcription [18]. Several methods for introducing mutations at the m^{6}A site without the use of m^{6}A-specific antibodies have also been developed. For example, enzymatic deamination at sites adjacent to RNA modification targets (DART-seq) is based on the recruitment of a cytidine deaminase APOBEC1 to m^{6}A-decorated sites via a fused m^{6}A reader YTH domain, leading to C-to-U conversion in the vicinity of m^{6}A residues (Figure 1B) [19]. Chemical and metabolic labeling of m^{6}A can also be used to introduce mutations or deletions at the m^{6}A site or enrich m^{6}A-decorated RNA (Figure 1C) [20–22]. For example, the **m^{6}A-SAC-seq** protocol is based on enzymatic labeling of m^{6}A sites by a dimethyltransferase MjDim1 followed by chemical modification, which leads to incorporation of a mutational signature during reverse transcription [20]. An m^{6}A-sensitive RNase MazF that cleaves RNA at ACA motifs but fails to do so in the presence of m^{6}A has also been applied to map m^{6}A (Figure 1D) [23,24]. Detection of m^{6}A at single-nucleotide resolution has generated comprehensive maps of the m^{6}A methylome that will serve as a rich resource for future mechanistic studies. Concurrently, protocols for antibody- and labeling-based detection of other RNA modifications have been developed, enabling the study of epitranscriptomics beyond m^{6}A [25].

Cellular heterogeneity of RNA modifications

Single-cell RNA sequencing (scRNA-seq) has revolutionized transcriptomic profiling, revealing immense spatiotemporal heterogeneity of gene expression in individual cells. Protocols that combine m^{6}A detection methods with single-cell sequencing technology, including scm^{6}A-seq and scDART-seq, can be used to map m^{6}A at single-cell resolution and uncover cellular heterogeneity of the m^{6}A epitranscriptome [26,27]. Single-cell profiling of m^{6}A has revealed many lowly modified m^{6}A sites that are present in only a subpopulation of cells, as well as variable m^{6}A stoichiometry across individual cells. Interestingly, these distinct m^{6}A methylation patterns can be

Glossary

Directed evolution: a methodology to engineer desired traits of biological molecules by introducing mutations and evaluating their effects on functionality. Directed evolution is often applied to improve enzyme activity or endow enzymes with novel functions.
Epitranscriptomics: a field of genetics that encompasses RNA modifications and their function.
m^{6}A-SAC-seq: a methodology for mapping m^{6}A by selective allyl chemical labeling of m^{6}A sites followed by next-generation sequencing. During reverse transcription, a mutational signature is introduced at chemically labeled sites, enabling m^{6}A detection.
Machine learning (ML): a field of computer science and artificial intelligence that encompasses computational techniques to perform a certain task without explicitly providing an algorithm.
MeRIP-seq: a methodology for mapping m^{6}A transcriptome-wide by immunoprecipitation of m^{6}A-decorated RNA fragments followed by next-generation sequencing.
Nanopore sequencing: a third-generation sequencing approach based on detection of ionic current changes when a nucleic acid strand passes through a nanopore. Nanopore sequencing can be performed on native nucleic acids, thus preserving nucleic acid modifications and enabling their direct detection.
Native RNA: in sequencing, 'native' indicates that RNA has not been subject to copy DNA (cDNA) synthesis and polymerase chain reaction (PCR) during library preparation, and thus RNA modifications are preserved.
Omics: a term used to describe fields of biology, such as genomics, that encompass the entirety of a large-scale biological system, such as the genome.
Rational drug design: knowledge- and data-driven development of therapeutic candidates based on their chemical properties and interactions with a target.
Stoichiometry: in the context of RNA modifications, stoichiometry defines the level of modification at a given site across multiple copies of the same RNA transcript. For example, if 70 out of 100 molecules of a given mRNA transcript are m^{6}A modified at a specified position, the level of m^{6}A at that position is 70%.
Supervised learning: a type of machine learning that uses labeled data

used to group cells into clusters based on the global m^{6}A signature [26,27]. More recently, simultaneous profiling of m^{6}A and gene expression at single-nucleus resolution has been developed to probe the interplay between epitranscriptome and transcriptome [28]. Although the protocols for single-cell detection of RNA modifications are just emerging, we anticipate that future studies will reveal as yet unappreciated spatiotemporal heterogeneity of the epitranscriptome across human development and diseases.

to train algorithms for outcome prediction or data classification of unseen datasets.
Tumor atlas: an assembly of large-scale omics data about different types and states of cancer.
Virtual drug screening: a computational approach to identify drug-like compounds that are likely to bind to a specific protein target.

Stoichiometry of RNA modifications

Unlike DNA, RNA transcripts can be present in dozens or hundreds of copies in a single cell. RNA modification stoichiometry describes what fraction of these RNA copies is modified at a given time. Yet the mechanisms of regulation and the functional relevance of modification stoichiometry are largely unknown. One way to quantify RNA modification stoichiometry is to include spike-in calibration probes modified at different levels into the modification detection pipeline, so that modification levels can be normalized during data analysis. Using m^{6}A-SAC-seq with spike-in calibration, Hu *et al.* were able to document dynamic changes in m^{6}A stoichiometry during hematopoietic stem and progenitor cell (HSPC) differentiation [20]. These changes in m^{6}A stoichiometry were shown to be accompanied by alternative splicing events, indicating functional relevance of m^{6}A stoichiometry [20]. Recently, eTAM-seq and GLORI protocols based on selective adenine but not m^{6}A deamination were shown to achieve high adenine conversion, enabling quantification of m^{6}A stoichiometry based on the ratio of intact (m^{6}A) and deaminated adenines (Figure 1E) [29,30]. Quantitative detection of m^{6}A by deamination revealed a median methylation level of 40% across all m^{6}A sites and its functional relevance: m^{6}A stoichiometry is dynamic under changing cellular conditions and has a significant effect on mRNA translation efficiency [29]. Protocols for stoichiometric quantification of other RNA modifications, such as Ψ, are also emerging. For example, PRAISE [31] and BID-seq [32] protocols for quantitative detection of Ψ are based on bisulfite-induced deletion and have revealed variable Ψ stoichiometry across different modification sites. Although biological relevance and regulatory mechanisms of RNA modification stoichiometry have yet to be elucidated, quantitative detection of RNA modifications is likely to reveal yet another layer of complexity of the epitranscriptome and facilitate new discoveries in the field.

Insights gained from third-generation sequencing approaches

Alongside next-generation sequencing (NGS)-based protocols discussed earlier, third-generation sequencing approaches and **machine learning (ML)** play an increasingly important role in charting the epitranscriptome. Specifically, **nanopore sequencing** can be performed on **native RNA**, thus preserving RNA modifications during library preparation, and has been widely applied to detect m^{6}A and other RNA modifications (Figure 1F) [33,34]. In nanopore sequencing, computer algorithms are used to infer RNA modifications from biophysical parameters recorded as the RNA molecule passes through a nanopore [33,35]. Comparative methods, such as DRUMMER [36] and ELIGOS [37], are based on comparative analysis of test and modification-depleted control samples to infer RNA modifications between two conditions [33,35]. Although comparative methods do not require training datasets, modification-depleted control samples can be challenging to prepare and increase the complexity of a given experiment. Alternatively, **supervised learning**-based tools, such as m6Anet [38] and DENA [39], are trained with labeled datasets and used to predict RNA modifications in unseen datasets without using control samples [35]. ML can also be applied to predict RNA modifications *de novo* based on sequence-specific or genomic feature information [35]. As with NGS approaches, nanopore sequencing-based detection of RNA modifications has the potential to achieve single-nucleotide resolution and enable stoichiometric quantification [38]. Nanopore sequencing should also enable simultaneous detection of different RNA modifications because all modifications are preserved on native RNA. Indeed, a neural network-based algorithm CHEUI has recently been applied to infer m^{6}A and m^{5}C modifications simultaneously,

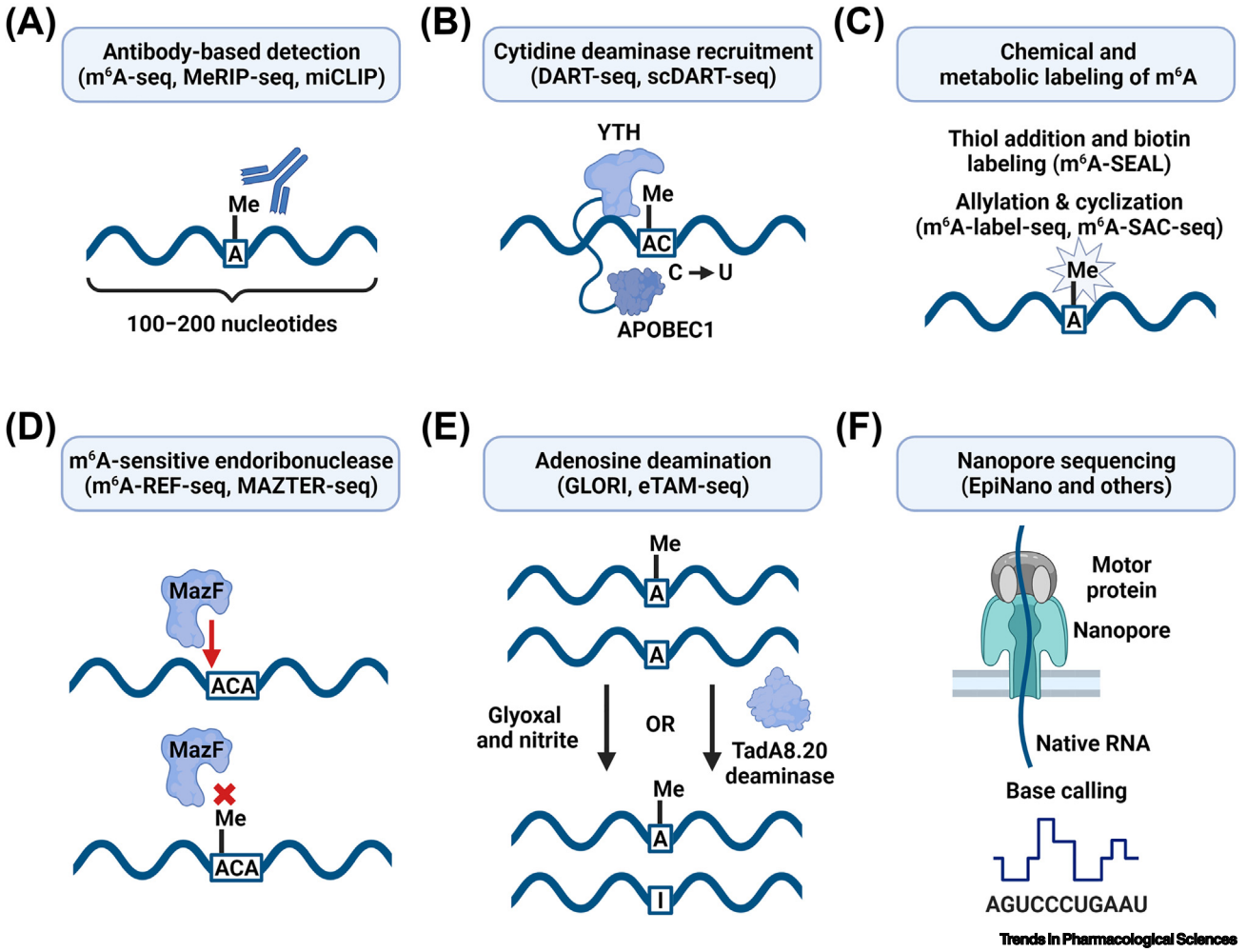

Figure 1. Methods for mapping N^6-methyladenosine (m^6A). (A) Protocols for m^6A detection using m^6A-specific antibodies (m^6A-seq and MeRIP-seq) were first reported in 2012. Whereas m^6A-seq and MeRIP-seq achieve a resolution of 100–200 nucleotides in mapping m^6A, higher resolution can be achieved by cross-linking m^6A-specific antibodies to their RNA targets using a modified protocol (miCLIP). (B) Antibody-free m^6A detection using deamination at sites adjacent to RNA modification targets (DART-seq) is based on recruitment of a cytidine deaminase to m^6A sites to induce a mutational signature that can later be used to infer the m^6A position. Recently, DART-seq has also been combined with single-cell sequencing technology for detecting m^6A in single cells (scDART-seq). (C) Chemical and metabolic labeling of m^6A can be used to enrich m^6A-decorated RNA fragments (m^6A-SEAL) or induce a mutational signature around the m^6A site during reverse transcription (m^6A-label-seq and m^6A-SAC-seq). (D) m^6A-sensitive endoribonuclease MazF digests RNA at the ACA but not the m^6ACA motif and is used in m^6A-REF-seq and MAZTER-seq protocols of m^6A detection. (E) Recently developed protocols for selective adenosine but not m^6A deamination enable transcriptome-wide stoichiometric m^6A detection, reminiscent of bisulfite sequencing of DNA methylation. (F) Third-generation direct RNA sequencing can be used to detect m^6A and other RNA modifications on native RNA by comparative analysis of modified and unmodified transcripts or by supervised learning. Abbreviation: APOBEC1, apolipoprotein B mRNA editing enzyme catalytic subunit 1. Figure created with BioRender.com.

revealing nonrandom co-occurrence of the two modifications [40]. It will be interesting to determine whether different RNA modifications can be installed in a coordinated manner, and whether co-occurring RNA modifications have synergistic effects on downstream RNA processing.

Drug development focused on epitranscriptomics

Widespread adoption of high-throughput methods for mapping RNA modifications has revealed dysregulation of the epitranscriptome in human diseases, especially cancer [9,41]. Subsequently, mechanistic studies have indicated that disease-associated phenotypes, such as tumor growth, can be reversed by manipulating the expression or activity of the RNA modification machinery [9,41]. Accordingly, the motivation to uncover novel therapeutically targetable pathways of the

epitranscriptome for treating human diseases has been another driving force in the epitranscriptomics field. Strategies for drug discovery focused on epitranscriptomics include direct targeting of the RNA modification machinery and targeting of RNA modification-dependent vulnerabilities in cancer and other diseases.

Targeting the RNA modification machinery

Tumor atlases [42,43] have been instrumental in uncovering protein effectors of the RNA modification machinery that become dysregulated in cancer and play oncogenic roles (Figure 2A, Key figure) [1,9]. Accordingly, small-molecule inhibitors can be developed to suppress the activity of oncogenic RNA modification machinery. **Virtual drug screening** is an efficient and cost-effective approach to screen drug-like compound libraries and has been applied to identify hit compounds targeting various protein effectors of the RNA modification machinery, including the m^6A eraser FTO [44], the m^6A reader insulin-like growth factor 2 mRNA binding protein 2 (IGF2BP2) [45], and the Ψ writer pseudouridine synthase 7 (PUS7) [46] (Figure 2B). Alternatively, classical drug screening using cell-free assays has been used to identify inhibitors of the m^6A writer methyltransferase-like 3 (METTL3) [47] and the m^6A reader YTH N^6-methyladenosine RNA binding protein F2 (YTHDF2) [48]. Cell-free assays are also often used to validate the inhibitory activity of the identified hit compounds (Figure 2C). For example, *in vitro* RNA m^6A methylation and demethylation assays have been used to evaluate METTL3 [47] and FTO [44,49,50] inhibitors, respectively. Target selectivity and pharmacokinetics of therapeutic candidates can be further improved by **rational drug design**, as exemplified by chemical optimization of METTL3 [51] and FTO [49,50,52] inhibitors (Figure 2D).

Having identified and optimized hit compounds targeting the RNA modification machinery, their effects on cancer cell growth and tumor progression can be assessed *in vitro* and *in vivo*. In addition to standard *in vitro* cell proliferation assays, limiting dilution analysis can be performed to evaluate cancer stem cell activity that is often affected by the dysregulated epitranscriptome (Figure 2E) [44–46,52,53]. *In vivo* models enable evaluation of pharmacokinetics, immune cell infiltration, and other aspects of tumor progression that cannot be evaluated *in vitro* (Figure 2F). For example, Su *et al.* found that an FTO inhibitor CS1 robustly suppressed proliferation of leukemia cells *in vitro*, but it exhibited low efficacy *in vivo* due to its limited bioavailability [44]. To overcome the hydrophobicity of CS1 and promote its uptake, the authors packaged CS1 into polyethylene glycol-polylactide (PEG-PLA) micelles or coated CS1 with β-cyclodextrin, which substantially improved CS1 uptake and antileukemic activity *in vivo* [44]. *In vivo* models have also revealed synergistic effects of epitranscriptomic drugs and cancer immunotherapy; for example, pharmacological inhibition of YTHDF2 [48] or FTO [50] synergizes with anti-PD-L1 immunotherapy and limits tumor immune evasion. Overall, efforts to develop drugs that target the RNA modification machinery have resulted in a new class of epitranscriptomic therapeutics that hold promise to alleviate cancer burden in patients.

Targeting RNA modification-dependent tumor vulnerabilities

An alternative approach for therapeutic development in the epitranscriptomics field is to identify tumor vulnerabilities based on modification-dependent dysregulation of gene expression. This approach entails a comparative analysis of the epitranscriptome and gene expression between cancer cells and healthy control tissues to identify cellular pathways that are dysregulated in cancer because of modification-dependent changes in gene expression. For example, Lv *et al.* found that dysregulated m^6A methylation of *OPTN* mRNA, encoding a mitophagy regulator optineurin, promoted *OPTN* degradation and dysfunction of mitophagy in glioblastoma stem cells [54]. Pharmacological inhibition of METTL3 and mitophagy synergistically suppressed glioblastoma progression *in vivo* and prolonged survival, revealing the METTL3-mitophagy axis as a potential therapeutic target in glioblastoma [54]. Notably, identification of modification-dependent tumor vulnerabilities is reliant on accurate

Key figure

Therapeutic development focused on epitranscriptomics

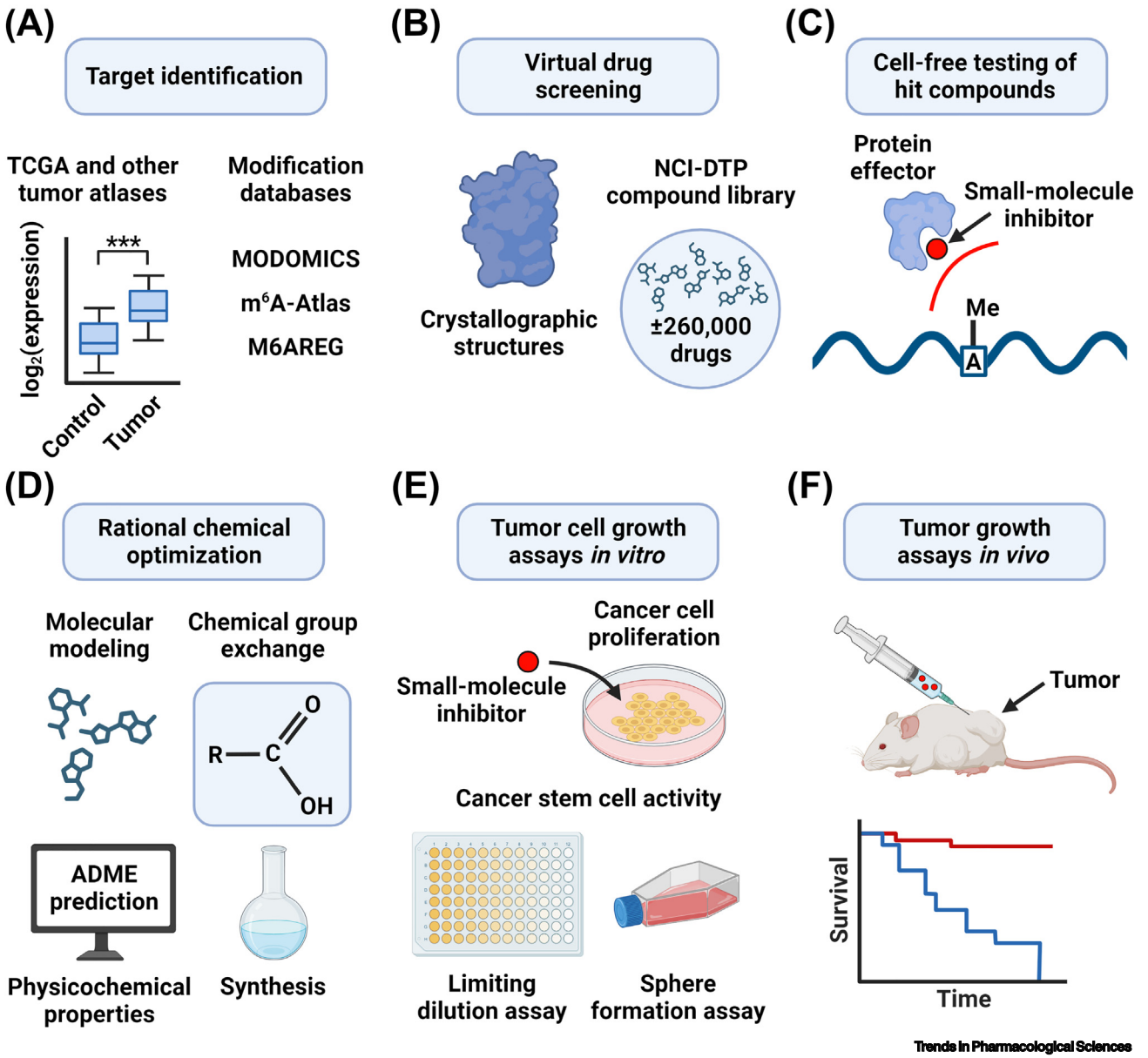

Figure 2. Dysregulation of the epitranscriptome in cancer and other diseases often stems from aberrant expression and function of associated RNA modification enzymes that can be targeted pharmacologically to reverse disease-associated phenotypes. (A) Therapeutic development begins at the stage of target identification, which has been greatly facilitated by tumor atlases that can be used to identify aberrantly expressed RNA modification machinery in cancer. RNA modification databases – such as MODOMICS, N^6-methyladenosine (m^6A)-Atlas, and M6AREG – can be used to generate testable hypotheses regarding the mode of action of a particular RNA modification enzyme. (B) Having identified a target of interest, virtual drug screening is performed to uncover potential hit compounds. Drug-like compound libraries – such as the library curated by the National Cancer Institute Developmental Therapeutics Program (NCI-DTP) – facilitate virtual screening and subsequent acquisition of selected compounds. (C) Identified hit compounds are tested using cell-free assays to characterize target–ligand interactions and evaluate drug inhibitory activity.

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transcriptome-wide detection of RNA modifications, highlighting the importance of continuously advancing RNA modification detection methods for therapeutic development in the field. RNA modification databases – such as MODOMICS [8], m^6A-Atlas [55], and the m^6A-centered regulation of disease development and drug response database (M6AREG) [56] – also facilitate mechanism-driven drug discovery by enabling data compilation and sharing (Figure 2A). Finally, ML can be applied to process large-scale **omics** data to uncover hidden crosstalk between distant pathways and predict novel targets involved in the regulation of the epitranscriptome to accelerate drug discovery [57–59].

Therapeutic development for diseases other than cancer

Although drug development for targeting dysregulated RNA modification machinery or modification-dependent vulnerabilities has been focused mostly on cancer, the expanding mechanistic understanding of epitranscriptomics paves the way for developing epitranscriptomic therapies to treat various other human diseases. Accumulating evidence of epitranscriptomic dysregulation in cardinal human diseases (such as diabetes, heart disease, and neurodegeneration) indicates the potential for targeting dysfunctional pathways to restore tissue homeostasis [60]. Furthermore, the knowledge of functional implications of RNA modifications has already been applied to derive remarkably effective mRNA-based vaccines against COVID-19 [12]. In particular, incorporation of a modified Ψ (N^1-methylpseudouridine) into mRNA vaccines has been pivotal in circumventing the intrinsic immunogenicity of unmodified mRNA and ensuring success of these vaccines against COVID-19 [10,11,61]. With the immunogenicity hurdle of mRNA-based vaccines finally overcome, it can be expected that such vaccines will be developed against other infectious [62] and, potentially, chronic diseases. Future work may also reveal how RNA modifications can be used to improve the stability of mRNA vaccines and prolong their shelf-life.

Engineering the epitranscriptome for mechanistic studies

Dysregulation of the RNA modification machinery in human cancers has enabled relatively straightforward investigation of these protein effectors: experimental manipulation of their expression can be used to determine whether a specific protein acts as an oncogene or a tumor suppressor [9,41]. However, perturbation of a protein effector, such as the m^6A writer METTL3, results in changes at hundreds or thousands of modification sites, making it challenging to decipher the effects of individual modification sites on phenotypic outcomes. Furthermore, RNA modifications play regulatory roles in various biological processes, such as human brain development and function (Box 2), that do not necessarily involve dysregulation of the RNA modification machinery but rather depend on correct gene expression programs modulated by the epitranscriptome. Defining causal effects of RNA modifications on RNA processing and downstream phenotypic outcomes thus necessitates targeted manipulation of modification sites, which can be achieved by introducing point mutations into the primary DNA sequence. However, targeted mutagenesis is a laborious process that cannot recapitulate important features of RNA modification, such as its reversibility or stoichiometric information. Alternative approaches for inducible and reversible manipulation of individual RNA modification sites are highly desired to uncover specific roles of these chemical moieties and facilitate the next phase of modification-focused scientific discovery in the epitranscriptomics field. In this section, we discuss epitranscriptomic engineering as an emerging approach to manipulate RNA modifications in a targeted manner. We highlight state-of-the-art molecular tools used for engineering the epitranscriptome, their applications in mechanistic studies, and associated challenges.

(D) The hit compound can be further optimized by rational chemical optimization and synthesized for testing *in vitro* and *in vivo*. (E,F) Tumor-cell growth assays can be used to assess the effects of the hit compound on cancer-cell proliferation and cancer-stem-cell activity *in vitro*, whereas pharmacokinetics of the hit compound and its effects on tumor growth and survival can be assessed *in vivo*. Abbreviations: ADME, absorption, distribution, metabolism, and excretion; M6AREG, m^6A-centered regulation of disease development and drug response database; TCGA, The Cancer Genome Atlas. Figure created with BioRender.com.

Box 2. New frontiers: brain epitranscriptomics

Streamlined detection of RNA modifications, especially m^6A, has fueled investigation into their roles in human development, homeostasis, and diseases beyond cancer. Brain epitranscriptomics is an excellent case study exemplifying the expansive growth of the RNA modification field and the importance of the epitranscriptome in complex biological processes [90–92]. Given the incredible complexity of the human brain, studying the epitranscriptome provides an opportunity to identify novel regulators of brain function and dysfunction. Indeed, the field of brain epitranscriptomics has attracted substantial interest in recent years, and RNA modifications have been implicated in neurogenesis [93–95], gliogenesis [96], learning and memory [84], neuronal injury [97,98], gliomas [46,53,99], neurodegenerative diseases [100], and other processes (reviewed in [90–92]).

The emerging tools for advanced detection and engineering of RNA modifications discussed in this review can be applied to gain new insights into brain epitranscriptomics. For example, spatiotemporal profiling of RNA modifications at single-cell resolution may reveal as yet uncharacterized heterogeneity of the developing human brain, improve neuron cell subtyping, and clarify the mechanisms of neurogenic-to-gliogenic transition. Similarly, characterizing how the epitranscriptome deteriorates in aging may reveal distinct contributions of RNA modifications to the onset and progression of neurodegenerative diseases, such as Alzheimer's disease, and expose targetable cellular pathways for therapeutic development [100,101]. Accumulating evidence also indicates a prominent role that RNA modifications play in faithful RNA trafficking and localization to distal synapses during neurogenesis, synaptogenesis, and memory formation [102–104]. CRISPR/Cas-based tools for RNA tracking can be applied to study RNA trafficking in these processes to uncover modification-dependent regulatory mechanisms.

Finally, the study of brain epitranscriptomics can benefit from using cellular models of human origin, given substantial species-specific divergence between human and rodent brains [105]. Human pluripotent stem cells (hPSCs), including embryonic stem cells (ESCs) and induced PSCs (iPSCs), can be used to derive neuronal and non-neuronal human brain cells to study human-specific regulatory mechanisms and phenotypes affected by RNA modifications *in vitro* [106,107]. Furthermore, hPSCs can be differentiated into 3D brain organoids, multicellular assemblies of neural cells that partially recapitulate human brain development and diseases [108]. Brain organoids have successfully been applied to study the roles of m^6A [95] and m^3C [93] in human brain development, revealing a critical role of RNA modifications in neurogenesis. Overall, scientific discovery in the field of brain epitranscriptomics fueled by technological developments is poised to reveal new insights into the complexity of the human brain.

Molecular tools for engineering RNA modifications and their applications

The development and rapid adaptation of CRISPR/Cas technology have enabled versatile genetic engineering by targeted recruitment of a Cas endonuclease using a sequence-specific single guide RNA (sgRNA) [63]. Catalytically inactive Cas (dCas) proteins can be used to recruit other protein modalities to the desired genomic loci without introducing DNA strand breaks [64]. Similarly, RNA-targeting dCas13 can be fused to various protein effectors that modify RNA, thus enabling RNA modification engineering (Figure 3A) [65,66]. In this way, RNA modifications can be introduced or removed at specific sites without the need for mutating the primary DNA sequence. Indeed, successful targeted installation and erasure of m^6A using dCas13-METTL3 and dCas13-ALKBH5, respectively, have been reported [67–69]. Similarly, dCas13 fused to a deaminase domain of adenosine deaminase acting on RNA type 2 (ADAR2) can induce targeted A-to-I RNA editing [66,70]. RNA can also be targeted using dCas9 if a single-stranded DNA molecule containing a protospacer adjacent motif (PAM) is supplied, an approach that has been applied to write and erase m^6A [71–73]. In addition to CRISPR/Cas-based molecular tools, endogenous RNA modification machinery can be recruited by providing a sequence-specific guide RNA. For example, two groups have recently demonstrated that endogenous pseudouridylation machinery can be recruited to install Ψ on desired RNA transcripts (Figure 3B) [74,75]. Furthermore, endogenous ADAR proteins can be recruited for A-to-I editing by supplying exogenous circular guide RNA [76]. Molecular tools can also be tailored to achieve temporal control of RNA modification engineering. For example, Shi *et al.* developed a system for inducible m^6A modification, where the release of photo-caged abscisic acid upon UV light exposure is required for tethering dCas13 with METTL3 or ALKBH5 via adaptor proteins (Figure 3C) [77]. We anticipate that the toolkit for RNA modification engineering will continue to expand as our understanding of diverse RNA modifications and their modification machinery advances.

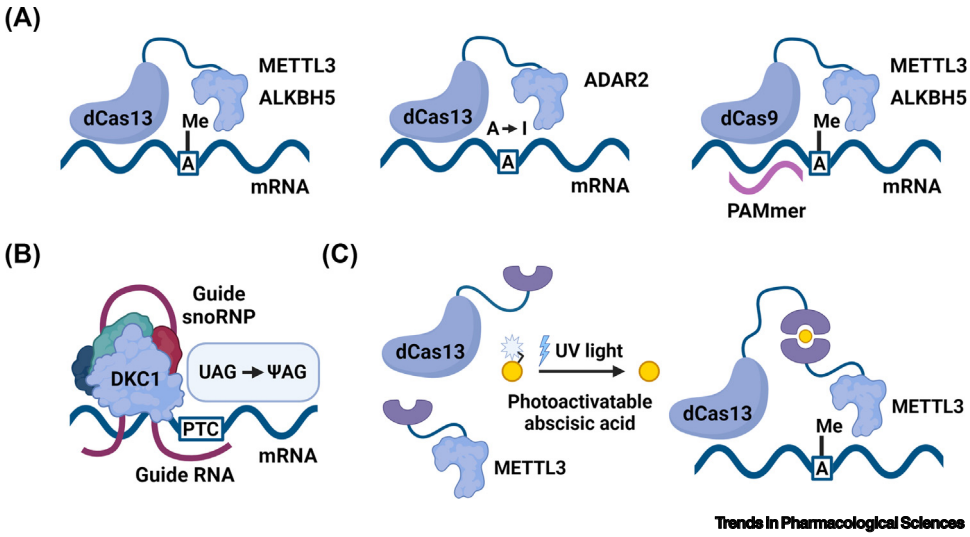

Figure 3. Molecular tools for engineering RNA modifications. (A) CRISPR/Cas technology enables sequence-specific targeting of RNA molecules by Cas endonucleases that recruit protein modalities for RNA modification. Catalytically inactive Cas13 (dCas13) or the RCas9 system that combines dCas9 and a single-stranded DNA monomer containing a protospacer adjacent motif (PAMmer) can be fused to catalytic domains of methyltransferase-like 3 (METTL3) or alkB homolog 5 (ALKBH5) to induce N^6-methyladenosine (m^6A) methylation and demethylation of RNA targets, respectively. dCas endonucleases can also be used to induce other RNA modifications, such as A-to-I editing, highlighting the versatility of the RNA modification engineering technology. (B) In addition to the CRISPR/Cas-based tools, endogenous RNA modification machinery can be recruited using tailored guide RNAs. For example, recruitment of the Ψ synthase dyskerin 1 (DKC1) complex by supplying a specific guide RNA enables targeted pseudouridylation. (C) Temporal control of RNA modification engineering can be achieved by photoinducible dimerization of a dCas endonuclease and an RNA modification machinery component. Abbreviations: ADAR2, adenosine deaminase acting on RNA type 2; PTC, premature termination codon; snoRNP, small nucleolar ribonucleoprotein. Figure created with BioRender.com.

RNA modification engineering studies have revealed causal effects of RNA modifications on RNA processing, including splicing [68,69], nuclear export [69], interaction with RNA-binding proteins [73], and stability [67,68] of RNA transcripts (Figure 4A). For example, targeted methylation of an alternatively spliced exon of the *Brd8* mRNA promotes exon skipping, whereas targeted methylation of the *Actb* mRNA leads to a substantial decrease (up to 70%) in *Actb* mRNA levels [68]. These findings indicate that even individual m^6A sites have a major influence on RNA processing and stability. Engineering Ψ at premature termination codons (PTCs) leads to PTC-readthrough and translation of a full-length protein, confirming the role of Ψ in PTC suppression [74,75]. Importantly, RNA engineering can reveal causal relationships between RNA modifications and human diseases. Targeted m^6A methylation of *CDCP1* mRNA promotes translation of the CUB domain-containing protein 1 (CDCP1) and progression of bladder cancer (Figure 4A) [71]. Furthermore, correcting the W437X mutation of the PTEN-induced kinase 1 (PINK1) implicated in Parkinson's disease by targeted A-to-I editing rescues impaired mitophagy [78]. Therefore, RNA modification engineering can be applied to model modification-dependent disease phenotypes and establish causal relationships between the epitranscriptome and disease-associated phenotypes.

In addition to engineering RNA modifications, targeted recruitment of reader proteins can be used to study their roles in downstream processing of modified RNAs (Figure 4B). Targeted recruitment of YTHDF1 and YTHDF2 promotes mRNA translation and degradation, respectively, reflecting the roles of these m^6A readers documented in other studies [79]. Furthermore, tagging dCas13 with green fluorescent protein (GFP) enables live tracking of mRNA transcripts, which can be applied to clarify the roles of RNA modification reader proteins in RNA localization and trafficking [65]. For example, YTHDF proteins have been proposed to guide m^6A-decorated mRNAs

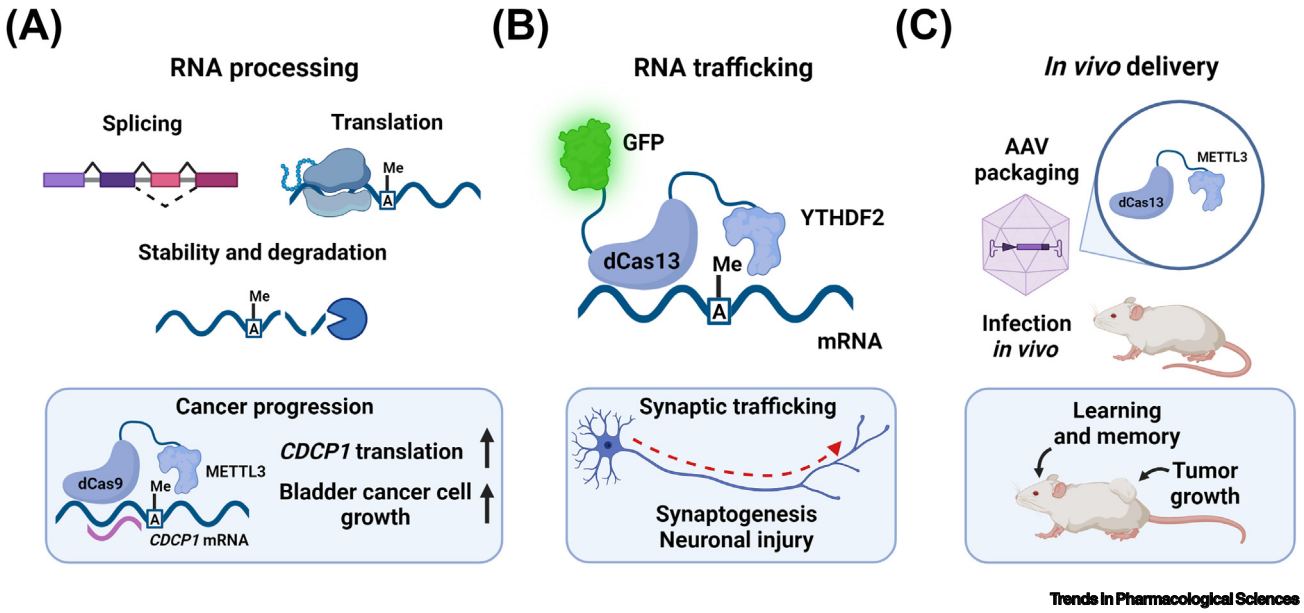

Figure 4. Applications of molecular tools to study RNA modifications in various biological processes. (A) RNA modification engineering facilitates the elucidation of causal relationships between individual RNA modification sites and their downstream effects on RNA processing and phenotypic outcomes. For example, targeted methylation of *CDCP1* mRNA enhances its translation and bladder cancer progression. (B) Fluorescence-labeled inactive Cas (dCas) endonucleases fused to N^6-methyladenosine (m^6A) reader proteins can be used to study how m^6A reader proteins orchestrate RNA trafficking and localization in highly complex and elongated cells, such as neurons. (C) Molecular tools for RNA engineering can be packaged into adeno-associated virus (AAV) vectors for delivery *in vivo* to study the roles of RNA modifications in complex biological processes, such as learning and memory formation or tumor growth and metastasis. Abbreviations: METTL3, methyltransferase-like 3; YTHDF2, YTH N^6-methyladenosine RNA binding protein F2. Figure created with BioRender.com.

into stress granules, but this observation remains controversial and requires further investigation [80–82]. Similarly, tracking RNA dynamics in cells that depend on highly orchestrated RNA trafficking, such as neurons, may reveal novel roles of RNA modifications and reader proteins in complex biological processes, such as synaptogenesis (Figure 4B). The roles of reader proteins in the onset and progression of human diseases can also be clarified using these molecular tools.

RNA modification engineering tools may also be packaged into adeno-associated virus (AAV) vectors for delivery *in vivo* (Figure 4C) [83]. RNA modification engineering *in vivo* will facilitate the study of complex phenotypes that cannot be modeled *in vitro*, such as synaptic plasticity, learning, and memory. For example, m^6A has been shown to enhance mRNA translation and promote learning and memory in response to neuronal stimulation [84], but the exact RNA modification-dependent mechanisms of synaptic plasticity remain to be elucidated. Similarly, the effects of individual RNA modifications on tumor growth, metastasis, and tumor immune microenvironment can be assessed *in vivo*. Finally, *in vivo* models can be used to evaluate clinical relevance of RNA modification engineering, given its potential to reverse disease-associated phenotypes [71,75,78].

Challenges and limitations of RNA modification engineering

Although RNA modification engineering is a powerful approach to pinpoint individual contributions of RNA modifications to RNA processing, challenges associated with this technology should be carefully considered. Important considerations include on-target editing efficiency and off-target activity, complicated by the fact that RNA molecules are present in dozens of copies and undergo dynamic turnover. It is also unclear whether endogenous modification stoichiometry can be reproduced using RNA engineering tools. On-target editing efficiency and the levels of the

engineered modification can be assessed using targeted m^{6}A-SAC-seq [85] or eTAM-seq [30] for m^{6}A and BID-seq for Ψ [32], whereas off-target activity can be evaluated by transcriptome-wide detection of the RNA modification of interest. The optimal footprint for modification engineering should also be considered; for example, Liu *et al.* reported a ten-nucleotide window for m^{6}A engineering directed by a dCas9 fusion construct with METTL3/14 catalytic domains [73]. A narrow engineering window may be sufficient for editing individual modification sites, whereas recruitment of several units of the RNA modification machinery by peptide scaffolds may be needed to engineer m^{6}A clusters. Finally, whether RNA modification engineering tools should be directed for nuclear or cytoplasmic localization, and whether they physically interfere with RNA processing, such as translation, should be determined [86].

Concluding remarks and future perspectives

The epitranscriptomics field has undergone explosive growth since the discovery in 2011 that m^{6}A is a reversible modification [4]. Continuously improving RNA modification detection methods have enabled in-depth mechanistic studies of the epitranscriptome, whereas drug screening and rational drug design strategies have yielded potent small-molecule inhibitors of the RNA modification machinery. Although mostly focused on m^{6}A and cancer, these developments have harnessed substantial interest in the wider field of epitranscriptomics, prompting investigations into other RNA modifications and in the context of a multitude of complex biological processes of human development, homeostasis, and disease (Box 2).

As the field moves forward, increasing access to advanced technologies of mapping and engineering RNA modifications will be critical to gain new insights into modification-dependent regulation of RNA biology and downstream phenotypic outcomes (see Outstanding questions). For example, defining the roles and regulation of recently described m^{6}A clusters [29,30] may reveal as yet unappreciated interplay between individual RNA modifications and their concerted influence on downstream RNA processing. Likewise, the roles and interplay of less thoroughly examined modifications across different RNA species will further advance our understanding of RNA metabolism and regulation. Improvements in detection methods will facilitate mapping of RNA modifications at ever increasing resolution; for example, long-read nanopore sequencing may enable detection of RNA modifications at isoform resolution [35]. As exemplified by the TadA8.20 deaminase variant used in eTAM-seq, protein engineering by **directed evolution** will play an important role in the development of novel enzymes that selectively label various RNA modifications, aiding their detection [30,87]. Refined maps of the epitranscriptome will, in turn, facilitate training of supervised learning algorithms to advance nanopore-based detection methods and *de novo* prediction of RNA modifications. ML will also be instrumental in propelling cancer therapeutic development as it can be widely applied in target discovery and drug design efforts [58,59,88]. We anticipate that therapeutic strategies focused on the epitranscriptome will also be developed for human diseases other than cancer as our understanding of RNA modifications in complex biological processes continues to advance. Accessible mapping of RNA modifications combined with ML will drive the discovery of RNA modification-based tissue and blood biomarkers of human diseases, especially cancer, to accelerate diagnosis and prognosis. Finally, the expanding toolkit of RNA modification engineering will enable precise examination of the mechanisms governing modification-dependent regulation of diverse biological processes with the focus on the RNA modification itself.

Outstanding questions

How is m^{6}A stoichiometry regulated at individual modification sites, and how does it affect RNA processing?

What are the functional roles of m^{6}A clusters?

Are all m^{6}A sites equally important on mRNAs that contain multiple m^{6}A sites?

How do different RNA modifications interplay to regulate downstream RNA processing?

How are RNA modifications distributed across different transcript isoforms?

What are the species-specific differences in the RNA epitranscriptome of rodents and humans?

Can single-cell profiling of RNA modifications reveal as yet uncharacterized heterogeneity of developmental programs or tumor evolution?

Can RNA modification engineering be applied therapeutically for targeted correction of detrimental modification sites?

Can RNA modification engineering be applied to influence intracellular mRNA trafficking for targeted delivery of mRNA transcripts to distinct cellular compartments?

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Declaration of interests

C.H. is a scientific founder, member of the scientific advisory board, and equity holder of Aferna Bio Inc. and AccuaDX Inc.; a scientific co-founder and equity holder of Accent Therapeutics Inc.; and a member of the scientific advisory board of Rona Therapeutics. No interests are declared by the remaining authors.

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