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Review article

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RNA modifications in physiology and disease: towards clinical applications

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Abstract

The ability of chemical modifications of single nucleotides to alter the electrostatic charge, hydrophobic surface and base pairing of RNA molecules is exploited for the clinical use of stable artificial RNAs such as mRNA vaccines and synthetic small RNA molecules — to increase or decrease the expression of therapeutic proteins. Furthermore, naturally occurring biochemical modifications of nucleotides regulate RNA metabolism and function to modulate crucial cellular processes. Studies showing the mechanisms by which RNA modifications regulate basic cell functions in higher organisms have led to greater understanding of how aberrant RNA modification profiles can cause disease in humans. Together, these basic science discoveries have unravelled the molecular and cellular functions of RNA modifications, have provided new prospects for therapeutic manipulation and have led to a range of innovative clinical approaches.

Sections

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Introduction

Naturally occurring chemical modifications of RNA — such as 5-methylcytidine (m^5C) and pseudouridine (Ψ) — were first discovered around 1960 (refs. 1,2), and more than 150 distinct RNA modifications have been described since[3] (prominent examples of which are summarized in Table 1). Most, if not all, types of RNA carry chemical modifications at some point in their life cycle, which affect stability, structure and RNA–protein interactions[4]. How nucleotide modifications regulate RNA metabolism is best understood for the three main types of RNA involved in protein synthesis — mRNA, tRNA and ribosomal RNA (rRNA). Modifications of these RNAs are deposited during or after transcription and modulate gene transcription as well as RNA processing, nuclear export, cellular localization and mRNA translation[5]. Together, RNA modifications function as a molecular bridge between gene transcription and protein output, with downstream effects on cell function (Fig. 1). The development of high-throughput detection methods to map RNA modifications in a transcriptome-wide manner has markedly advanced our understanding of their functional roles (Box 1).

How RNA modifications regulate cell functions has been studied in detail during acute stress responses to oxidative stress, DNA damage or anticancer drugs[6–9]. In response to external stress stimuli, RNA-modifying proteins (Table 1) can cause the rapid degradation or stabilization of their target RNA and can thereby rewire the proteome of a cell before transcriptional changes are established[7–11]. Thus, RNA modifications decode changes in the external environment to initiate appropriate cellular responses. This ability to efficiently translate external cues into cellular functions such as cell division, differentiation or migration is particularly important during development[6], when the ability to adapt to a changing microenvironment is crucial for correct cell fate decisions[12]. Abnormal deposition of RNA modifications causes severe human diseases such as neurological deficits[13] and metabolic diseases, including mitochondrial disorders, obesity and diabetes[14–16].

Similarly, cancer cells must continually adapt to often deleterious microenvironments such as exposure to oxidative or nutrient stress and chemotherapeutic drugs[17]. In response to these factors, the transcriptional and translational machineries of cancer cells are temporarily decoupled, allowing for dynamic and rapid adaptations to a changing environment. For example, during the acute stress response to ultraviolet radiation-induced DNA damage, global protein synthesis is inhibited and cells switch to transcript-specific translation of genes involved in the stress response and cell type-specific survival[18].

In this Review, we describe the molecular and cellular functions of chemical modifications found in mRNA, tRNA and rRNA that link transcription with translational output, focusing on those modifications for which there is direct genetic evidence that they are functionally important for human health. We then discuss how these molecular mechanisms affect metabolic plasticity and cellular flexibility in response to external cues in metabolic disorders and cancer. We highlight the diversity of chemical modifications rather than focusing on single, particularly well-studied modifications, such as inosine (I) and N^6-methyladenosine (m^6A), in depth (reviewed elsewhere[19,20]). Finally, we describe how the chemical and molecular properties of nucleotide modifications are exploited in advanced RNA-based therapies. The discussion illustrates how basic science discoveries explaining how RNA modifications regulate normal cell functions and how their dysregulation causes human diseases, combined with a comprehensive understanding of their chemical and molecular functions, can be used to develop novel and innovative therapies.

Types of RNA modification

Biochemical modifications occur in all four nucleosides: adenosine (A), guanosine (G), cytidine (C) and uridine (U). The most common types of purine or pyrimidine base modifications found in coding and non-coding RNAs are methylation, pseudouridylation and adenosine-to-inosine (A-to-I) editing. Modifications of the ribose moiety of nucleosides are limited in terms of chemical diversity, but ribose methylation is frequently found in rRNA, tRNA and small nuclear RNA (snRNA). Through effects on RNA processing, stability and structure, nucleotide modifications regulate gene expression at transcriptional, post-transcriptional and translational levels.

Modifications in coding RNAs

At least ten types of nucleotide modification have been reported in eukaryotic mRNAs (Table 1), which ensure their correct transcription, processing, subcellular localization and translation[6,21] (Fig. 2a). Internal m^6A modifications and the 5′ cap are the most prevalent modifications in mRNA, and their correct deposition requires at least 20 regulatory proteins (Fig. 2b).

The deposition of RNA modifications is highly sensitive to changes in the environment, and external cues cause widespread changes in mRNA modification profiles[4,11]. This is often achieved by supressing the respective modifying enzymes, but m^6A can also be actively removed by the demethylases ALKBH5 and FTO[22–24] (Fig. 2b). FTO demethylates m^6A and the 5′ cap N^6,2′-*O*-dimethyladenosine (m^6Am), depending on its subcellular distribution, which alters transcription and RNA processing[25,26]. Whether m^6A modifications increase or decrease gene expression often depends on the binding of specific reader proteins including the YTH domain family and HNRNP[27] (Fig. 2b). YTHDF proteins mediate general mRNA degradation but can also promote transcript-specific translation[28–31]. The nuclear reader protein YTHDC1 regulates mRNA splicing and localization[32,33], and modulates chromatin accessibility and silences retrotransposons in an m^6A-dependent manner[34,35].

Functions of the 5′ cap include protecting newly synthesized mRNAs from degradation, promoting translation initiation and marking mRNAs as 'self' to guide the selective recognition of foreign nucleic acids by the immune system[36–39]. The precise molecular roles of m^6Am modifications of the 5′ cap remain unclear as modification enhances mRNA stability[40,41] but can increase[42] or decrease[43] cap-dependent translation. In response to stress, canonical cap-dependent translation is disrupted, and the translation of essential mRNAs is maintained by internal ribosome entry sites and cap-independent translational enhancers, or through circularization of mRNAs facilitated by METTL3-bound 3′ untranslated regions (UTRs)[44–50].

The classical view of the 5′ cap as being a rigid structure that enhances mRNA stability has been challenged by the identification of a large number of competing capping and decapping enzymes, leading to non-canonical cap structures and the generation of potentially deleterious intermediate metabolites[51] (Fig. 2b). Non-canonical mRNA caps are mostly derived from metabolites and RNA-related cofactors such as NAD, FAD, CoA, uridine diphosphate glucose (UDP-glucose), UDP-*N*-acetylglucosamine and dinucleotide polyphosphates, and their generation affects RNA stability, mitochondrial functions and, possibly, mRNA translation[51].

Other modifications reported in eukaryotic mRNAs include Ψ, inosine and, more rarely, N^1-methyladenosine (m^1A), m^5C, 5-hydroxymethylcytidine (hm^5C) and N^4-acetylcytidine (ac^4C)[52–59] (Fig. 2a). Although their molecular functions are less well understood, these

Table 1 | Key examples of RNA modifications and their respective modifying proteins as discussed in the text

Type of RNA	Modification	RNA-modifying proteins	Molecular functions
mRNA	m^6A	METTL3–METTL14 complex, VIRMA, HAKAI, ZC3H13, WTAP, RBM15, RBM15B	Transcription, processing, localization and mRNA translation
	m^7G	RNMT	Cap-dependent mRNA translation and self versus non-self recognition
	2′-O-Me	CMTR1, CMTR2	Protection from mRNA decapping and degradation
	m^6Am	PCIF1	mRNA stability and translation
	m^1A	TRMT6, TRMT61A, ALKBH3	mRNA decay, stability and translation
	m^5C	NSUN6, NSUN2	mRNA stability, localization and translation
	hm^5C	TET enzymes	mRNA nuclear export
	ac^4C	NAT10	mRNA translation and stability
	Ψ	PUS enzymes	Immune response evasion, mRNA translation
	I	ADAR1, ADAR2	Gene expression, mRNA structure, protein diversity
lncRNA	m^6A	METTL3–METTL14 complex, VIRMA, HAKAI, ZC3H13, WTAP, RBM15, RBM15B	lncRNA splicing modulation and nuclear export
	m^5C	NSUN2	lncRNA stability and localization
	Ψ	PUS1, PUS3, PUS7	lncRNA stability, localization and splicing
	I	ADAR1, ADAR2	Pro-inflammatory gene expression, lncRNA stability
tRNA	Ψ	PUS3, PUS7	tRNA stability
	2′-O-Me	FTSJ1	Cytosolic translation decoding
	m^5C	NSUN2	Protection from cleavage and decoding
	$m^{2,2}G$	TRMT1	tRNA folding
	m^7G	METTL1, WDR4	mRNA decoding
	m^5C	NSUN3	Mitochondrial mRNA decoding
	f^5C	ALKBH1	Mitochondrial mRNA decoding
	m^1G	TRMT5	Stability of tRNA–ribosome interaction
	ms^2t^6A	CDKAL	Mitochondrial mRNA decoding
	τm^5U	TRMU, MTO1, GTPBP3	Mitochondrial translation decoding
	τm^5s^2U	TRMU, MTO1, GTPBP3	Mitochondrial translation decoding
	Q	QTRT1, QTRT2	Protection from cleavage and mRNA decoding
	cm^5U, mcm^5U, mcm^5s^2U, ncm^5U	ELP1, ELP2, ELP3, ELP4, ELP5, ELP6, CTU1–CTU2 complex, ALKBH8	Cytosolic translation decoding
	yW	TYW2	Stability of tRNA–ribosome interaction
	m^1A, m^3C	ALKBH3	tRNA cleavage
	I	ADAT2, ADAT3	mRNA decoding
rRNA	m^5C	NSUN5	Maintenance of protein synthesis level
	$m^6{}_2A$	TFB1M	Mitochondrial ribosome assembly and maturation
	2′-O-Me	FBL-containing snoRNAs	Folding and stability of rRNA
	Ψ	H/ACA box snoRNAs with GAR1, NHP2, NOP19 and DKC1	Binding and decoding of mRNA, ribosome biogenesis

ac^4C, N^4-acetylcytidine; cm^5U, 5-carboxymethyluridine; f^5C, 5-formylcytidine; hm^5C, 5-hydroxymethylcytidine; I, inosine; lncRNA, long non-coding RNA; m^1A, N^1-methyladenosine; m^6A, N^6-methyladenosine; $m^6{}_2A$, N^6-dimethylation of adenosine; m^6Am, N^6,2′-O-dimethyladenosine; m^3C, 3-methylcytidine; m^5C, 5-methylcytidine; m^1G, N^1-methylguanosine; $m^{2,2}G$, N^2,N^2-dimethylguanosine; m^7G, N^7-methylguanosine; mcm^5U, 5-methoxycarbonylmethyluridine; mcm^5s^2U, 5-methoxycarbonylmethyl-2-thiouridine; ms^2t^6A, 2-methylthio-N^6-threonylcarbamoyladenosine; ncm^5U, 5-carbamoylmethyluridine; 2′-O-Me, 2′-O-methylation (also known as Nm); Ψ, pseudouridine; τm^5U, 5-taurinomethyluridine; τm^5s^2U, 5-taurinomethyl-2-thiouridine; PUS, pseudouridine synthase; Q. queuosine; rRNA, ribosomal RNA; snoRNA, small nucleolar RNA; yW, wybutosine.

modifications have similarly been linked to RNA processing, cellular localization and mRNA translation.

The mRNA 3′ poly(A) tail directly interacts with 5′ cap structures to synergistically enhance translation in eukaryotes[60]. The circularization of mRNAs is achieved through protein–protein interactions between PABPC1 bound to the 3′ poly(A) tail and the eukaryotic translation initiation factor 4F (eIF4F) complex — consisting of the DEAD-box RNA helicase eIF4A, the cap-binding protein eIF4E and the large scaffold

protein eIF4G (ref. 61) – bound to the 5′ cap[60,61]. This mRNA loop formation is thought to promote translation through ribosome recycling. The m^{6}A methyltransferase METTL3 promotes mRNA looping by binding to eIF3h to enhance oncogene-specific translation[44,45,50]. Alternatively, METTL3 can directly bind PABPC1 in an RNA-independent manner to stabilize the PABPC1–eIF4F complex[47].

In summary, distinct sets of regulatory proteins shape mRNA modification profiles both co-transcriptionally and post-transcriptionally to stabilize or destabilize mRNAs before or during translation[62]. Whether the modifying proteins responsible for 5′ cap and m^{6}A modifications directly cooperate to modulate translation is unclear.

Modifications in non-coding RNAs

Similar to mRNAs, long non-coding RNAs (lncRNAs) are often transcribed by RNA polymerase II, 5′ capped with N^7-methylguanosine (m^{7}G), spliced and polyadenylated at their 3′ ends[63]. Accordingly, lncRNAs are likely to be decorated with similar modifications to mRNAs, except for those solely required for translation. So far, m^{6}A, m^{5}C and Ψ have been found in lncRNAs such as MALAT1, vault RNAs, HOTAIR, TERRA, 7SK and XIST[64–69]. Similarly, rRNAs are extensively modified during their transcription and subsequent maturation (discussed later).

However, the most extensively modified type of RNA is tRNA, which can contain more than 20 distinct modifications mediated by approximately 40 proteins in humans. The human genome contains more than 600 nuclear tRNA genes[70] and 22 mitochondria-encoded tRNAs[71] for cytosolic and mitochondrial translation, respectively. A human nucleus-encoded tRNA molecule carries, on average, 13 modifications (affecting ~17% of the total residues)[72] (Fig. 3a), and a mitochondrial tRNA molecule contains, on average, five modifications (~8.7% of total residues)[73] (Fig. 3b). The precise function of individual modifications strictly depends on their location within the tRNA molecule, with effects on transcription, processing, splicing, stability and subcellular localization[74,75].

Modifications of anticodon nucleotides, in particular at the wobble position – which is involved in decoding of multiple codons by the same tRNA molecule – are the most structurally diverse (Fig. 3a,b). Wobble position modifications can regulate mRNA translation efficiency and fidelity by compensating for codon biases in genes containing an uneven distribution of synonymous codons[76]. Most modifications in the anticodon loop but outside the anticodon sequence stabilize tRNA–mRNA codon interactions and thereby also enhance decoding fidelity and the translation speed[77].

Distinct tRNA modifications can be further modified by single enzymes or multi-enzyme cascades, creating modifications such as 5-formylcytidine (f^{5}C), 5-methoxycarbonylmethyl-2-thiouridine (mcm^{5}s^{2}U), 5-taurinomethyluridine (τm^{5}U) and 5-taurinomethyl-2-thiouridine (τm^{5}s^{2}U) (Fig. 3c–e). Not all of the functional details of the intermediate modifications created by these multi-enzyme cascades have been fully elucidated as yet.

Modifications such as queuosine (Q) or m^{5}C located in the anticodon or the variable loop of a tRNA can protect from endonucleolytic cleavage[8,78–80] (Fig. 3a). The fragmentation of tRNAs is a conserved response to oxidative stress in eukaryotes[81] but the precise role of fragmented tRNAs in regulating protein synthesis is not fully understood.

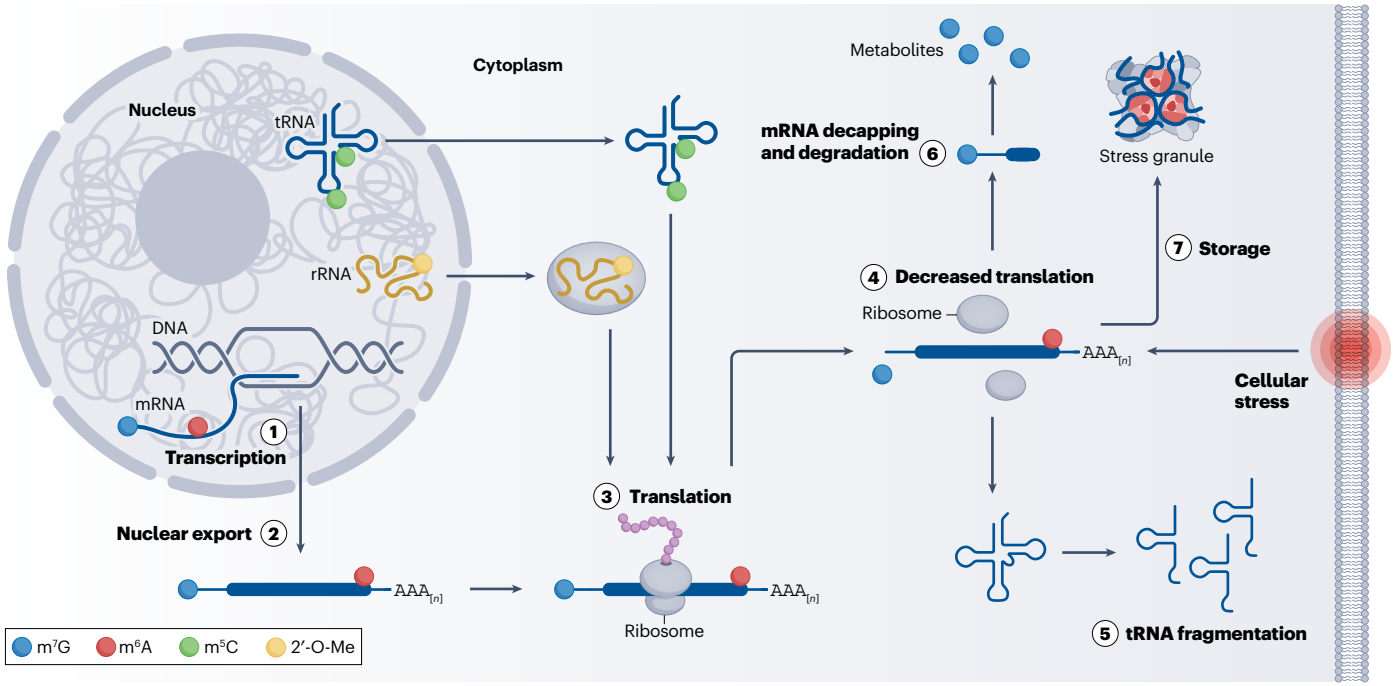

Fig. 1 | RNA modifications affect all steps of gene expression. The life cycle of an mRNA molecule starts with gene transcription in the nucleus (**1**), where modifications such as N^7-methylguanosine (m^{7}G) and N^6-methyladenosine (m^{6}A) are co-transcriptionally installed. These modifications regulate mRNA transcription, processing and nuclear–cytoplasmic export (**2**), and – together with distinct sets of modified tRNA and ribosomal RNA (rRNA) molecules, carrying, for example, 5-methylcytidine (m^{5}C) or 2′-O-methylation (2′-O-Me; also known as Nm) modifications – often stabilize mRNA translation at ribosomes (**3**). In response to external cues (such as cellular stress), changes to RNA modifications can lead to a general reduction in mRNA translation (**4**), through fragmentation of hypomodified tRNAs (**5**) and decapping and degradation of mRNAs (**6**), releasing several metabolites, or storage in membrane-less organelles such as stress granules (**7**).

Some tRNA-derived fragments compete with mRNAs for ribosome binding and, thus, regulate translation during stress[82]; other tRNA fragments associate with Argonaute proteins and regulate gene expression post-transcriptionally[83]. The stability and function of tRNA-derived fragments are also determined by specific nucleotide modifications. For example, Ψ-modified tRNA fragments impose selective protein synthesis programmes in stem cells and cancer cells[84,85].

Other types of small non-coding RNA that contain nucleotide modifications are microRNAs (miRNAs), PIWI-interacting RNAs (piRNAs), snRNAs and small nucleolar RNAs (snoRNAs)[86]. The biogenesis and maturation of miRNAs was reported to involve m^6A, mono-uridylation, m^7G and 2′-O-methylation (2′-O-Me; also known as Nm) modifications[87–90]. 2′-O-Me in piRNAs silences transposons in the germ line, improves stability by protecting from non-templated nucleotide addition and shields piRNAs from decay[90]. Modifications such as Ψ, 2′-O-Me, m^6A and m^6Am in snRNAs regulate RNA splicing. For example, m^6A at position 43 in mammalian U6 snRNA, which is catalysed by METTL16, regulates the efficient recognition of splice sites[91,92]. snoRNAs guide post-transcriptional modifications (Ψ and 2′-O-Me) of rRNAs and also themselves contain several Ψ sites[55].

Physiological roles of RNA modification

The most energy-consuming process in all cells is protein synthesis, which must be regulated by strictly balancing anabolic and catabolic activities in response to external factors such as oxygen availability. RNA modifications have key roles in regulating protein synthesis, and the RNA-modifying enzymes themselves are directly regulated by metabolites, nutrients and cellular metabolic pathways[93].

Box 1

Quantifying RNA modifications at single-nucleotide resolution

The discovery of novel functions of RNA modifications requires their mapping at high resolution across the whole transcriptome. The most reliable method to measure levels of RNA modifications is liquid chromatography coupled to mass spectrometry (LC–MS), which quantifies nucleosides with high specificity and sensitivity. Although LC–MS can, in principle, detect all modifications of an RNA molecule simultaneously[268], the sequence positions of the modified nucleosides are lost because the RNA molecule is digested into individual nucleosides before analysis.

The sequence-specific detection of RNA modifications is possible with an ever-increasing number of high-throughput, next-generation sequencing methods, but these methods are currently only applicable to a comparatively small number of RNA modifications: *N*^{6}-methyladenosine (m^6A), 3-methylcytidine (m^3C), 5-methylcytidine (m^5C), 5-hydroxymethylcytidine (hm^5C), *N*^{6},2′-*O*-dimethyladenosine (m^6Am), inosine (I), *N*^{7}-methylguanosine (m^7G), pseudouridine (Ψ) and 2′-O-methylation (2′-O-Me; also known as Nm)[269–271]. Next-generation sequencing methods exploit the chemical and molecular properties of modified nucleotides, and can broadly be divided into direct sequencing, chemically assisted sequencing, antibody-based sequencing and enzyme-assisted sequencing.

Direct sequencing methods for RNA modifications are based on truncation and misincorporation signatures, caused by stalling of the reverse transcriptase at the modified site (for example, Ψ, *N*^{1}-methyladenosine (m^1A), m^6A, *N*^{4}-acetylcytidine (ac^4C), 2′-O-Me and m^7G)[269] or by selective reverse transcriptases that preferentially install mutations at distinct modified sites (for example, m^1A)[272]. Truncation and misincorporation detection methods in tRNAs often include treatment with alkylation B-derived enzymes to remove m^1A, m^3C, *N*^{1}-methylguanosine (m^1G) or *N*^{2},*N*^{2}-dimethylguanosine ($m^{2,2}G$) modifications, which then allows for full-length parallel sequencing and direct comparison with methylated RNAs[273,274].

Several sequencing strategies are based on the chemical conversion of modified nucleotides, causing misincorporations during reverse transcription. For example, bisulfite sequencing methods convert cytidines into uridines but m^5C sites are protected[275]. Ψ forms CMC-Ψ when treated with 1-cyclohexyl-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate (CMCT), causing stalling during reverse transcription[276]. This principle has been further adapted in Ψ-seq, Pseudo-seq and PSI-seq sequencing methods for single-base resolution mapping of Ψ modifications[56,277]. Bisulfite-induced deletion sequencing (BID-seq) is a recently developed quantitative method that maps Ψ modifications transcriptome-wide using a bisulfite-mediated reaction to convert Ψ stoichiometrically into deletion upon reverse transcription without cytidine deamination[278].

Antibody-based sequencing techniques are available for m^6A, m^5C, m^1A, ac^4C and m^7G modifications[269]. RNA fragments containing nucleotide modifications are enriched by immunoprecipitation using antibodies specific for the modifications themselves or for the respective modifying enzymes (in the case of RIP-seq and CLIP-seq methods). Specificity is often enhanced when enzymatic activity of the RNA-modifying enzymes is incorporated into the assay. For example, generation of mutant enzymes of the m^5C methyltransferases NSUN2 and NSUN6 allows for covalent binding to the targeted nucleotide so that the RNA can be isolated without further fixation (methylation iCLIP method)[57,65,279]. A fusion protein of the cytidine deaminase APOBEC1 with the m^6A-binding YTH domain of YTHDF2 is used in DART-seq to add a cytidine-to-uridine deamination site adjacent to the m^6A-modified base[280].

However, the only high-throughput sequencing technique that can, in principle, detect all types of RNA modification simultaneously and within their sequence context is nanopore sequencing, which allows for direct sequencing of RNA molecules without reverse transcription or amplification[281]. The RNA is passed through a protein nanopore that monitors changes to electrical currents specific to modified and unmodified nucleotides. The resulting signal is then decoded to provide the specific RNA sequence. Nanopore sequencing has been used to detect m^6A and Ψ or to simultaneously detect all modified nucleotides in ribosomal RNA[281–284]. However, owing to its high error rate, the use of nanopore sequencing to map RNA modifications globally for functional analyses remains limited.

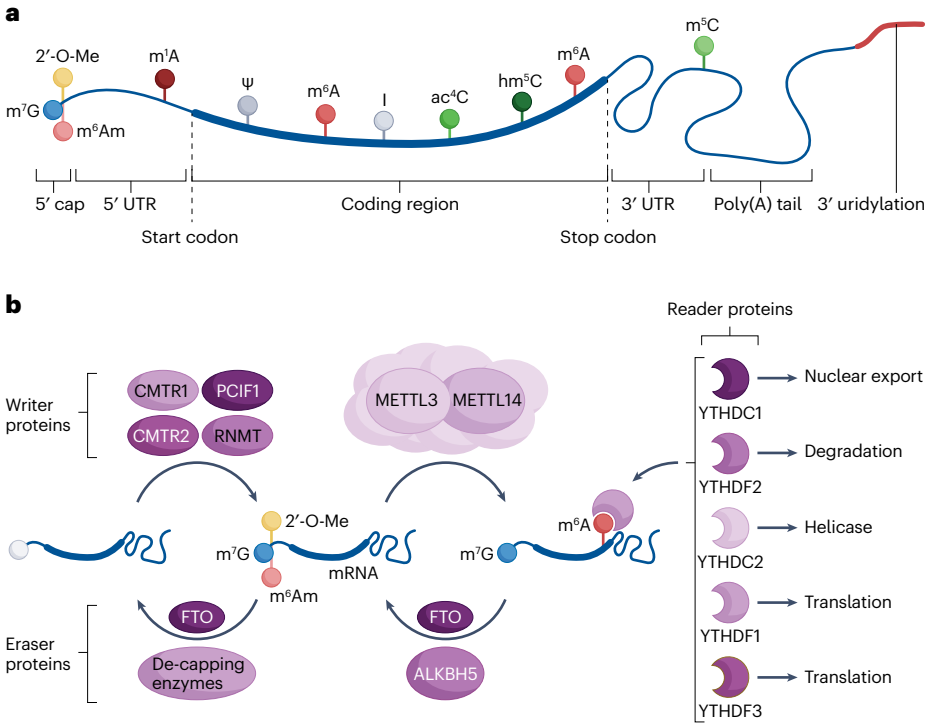

Fig. 2 | Nucleotide modifications in coding RNA. a, Example modifications in an mRNA molecule consisting of 5′ and 3′ untranslated regions (UTRs), the coding region flanked by start and stop codons, and a poly(A) tail. The 5′ cap consists of a single N^7-methylguanosine (m^7G), and mammalian RNA caps are further decorated with 2′-O-methylation (2′-O-Me; also known as Nm) at the two cap-proximal nucleotides[259,260]. If the first transcribed nucleotide is an adenosine, the N^6,2′-*O*-dimethyladenosine (m^6Am) mark is installed[42,43]. Internal adenosines within consensus DRACH motifs are converted into N^6-methyladenosine (m^6A) unless they are close to a splice site, where exon junction complexes prevent methylation[261–263]. Other modifications in eukaryotic mRNAs include pseudouridine (Ψ) and the rarer N^1-methyladenosine (m^1A), 5-methylcytidine (m^5C), 5-hydroxymethylcytidine (hm^5C), N^4-acetylcytidine (ac^4C) and inosine (I). 3′ uridylation of mRNA is indicated in red. **b**, The 5′ cap and internal m^6A modifications depend on the actions of multi-protein, RNA-modifying enzyme complexes. 2′-O-Me at the two cap-proximal nucleotides is mediated by CMTR1 and CMTR2 (refs. 259,260); m^7G is installed by RNMT; and the m^6Am mark is installed by PCIF1 (refs. 42,43). The m^6A methyltransferase writer complex consists of the METTL3–METTL14 heterodimer[264], which is at the core of a multi-protein complex composed of WTAP, VIRMA, CBLL1, ZC3H13, RBM15 and RBM15B (refs. 68,265,266). Cap m^6Am and internal m^6A modifications can both be removed by FTO. The demethylase ALKBH5 erases m^6A modifications. Different members of the YTH domain protein family bind to m^6A modifications in the nucleus or cytoplasm with distinct functions in regulating mRNA molecules.

In response to environmental stressors such as reduced oxygen availability, global protein synthesis and transcription are repressed, and cells switch to the targeted translation of stress-specific regulatory proteins[94–96]. Thus, the acute cellular stress response is entirely regulated at the level of translation. RNA-modifying proteins regulate this response by, for example, removing the m^7G cap of translationally repressed mRNAs, causing mRNA degradation and assembly into mRNA ribonucleoproteins, which leads to accumulation in processing bodies and stress granules[97,98]. Stress granules are membrane-less organelles in which mRNAs can be stored until active translation is resumed[99]. Internal m^6A modifications in mRNAs also enhance their phase separation into stress granules in vitro, although the direct function of these modifications in partitioning mRNAs into stress granules in vivo is unclear[100–102].

tRNA modifications also have multiple regulatory roles in the adaptation of protein synthesis to cellular stress. Modifications of tRNAs and levels of charged tRNAs can change rapidly in response to stress[72]. For example, nutritional stress induced by amino acid starvation can lead to selective or reduced charging of tRNAs by aminoacyl-tRNA synthetases, resulting in a slowdown in protein synthesis or altered patterns of codon usage[72]. Changes to tRNA modifications at the wobble position can have transcript-specific effects on translation and enhance cellular fitness[103]. For example, cells with unmodified uridine at the wobble position (U34) in tRNAs are deficient in resolving stress-induced protein aggregates, which then trigger acute proteotoxic stress[104].

How RNA modifications connect stress pathways with protein synthesis to regulate cell fate decisions has been studied during stem cell differentiation. Slow-cycling or quiescent stem cells in adult tissues often have low rates of protein synthesis to save energy and avoid premature exhaustion[7,105,106]. Loss of m^5C in the anticodon and variable loop of tRNAs causes a global reduction in protein synthesis owing to increased cleavage of unmodified tRNAs by the endonuclease angiogenin, and this reduction in mRNA translation can maintain stem cell functions and delay the differentiation of stem cells[7,8,79,107]. Similarly, exposure to oxidative stress represses m^5C formation at specific tRNA sites, reduces protein synthesis and results in a distinct catabolic state of cells[10]. The importance of adapting protein synthesis rates to cell fate changes is not restricted to stem cells. The transition of resting T cells into active

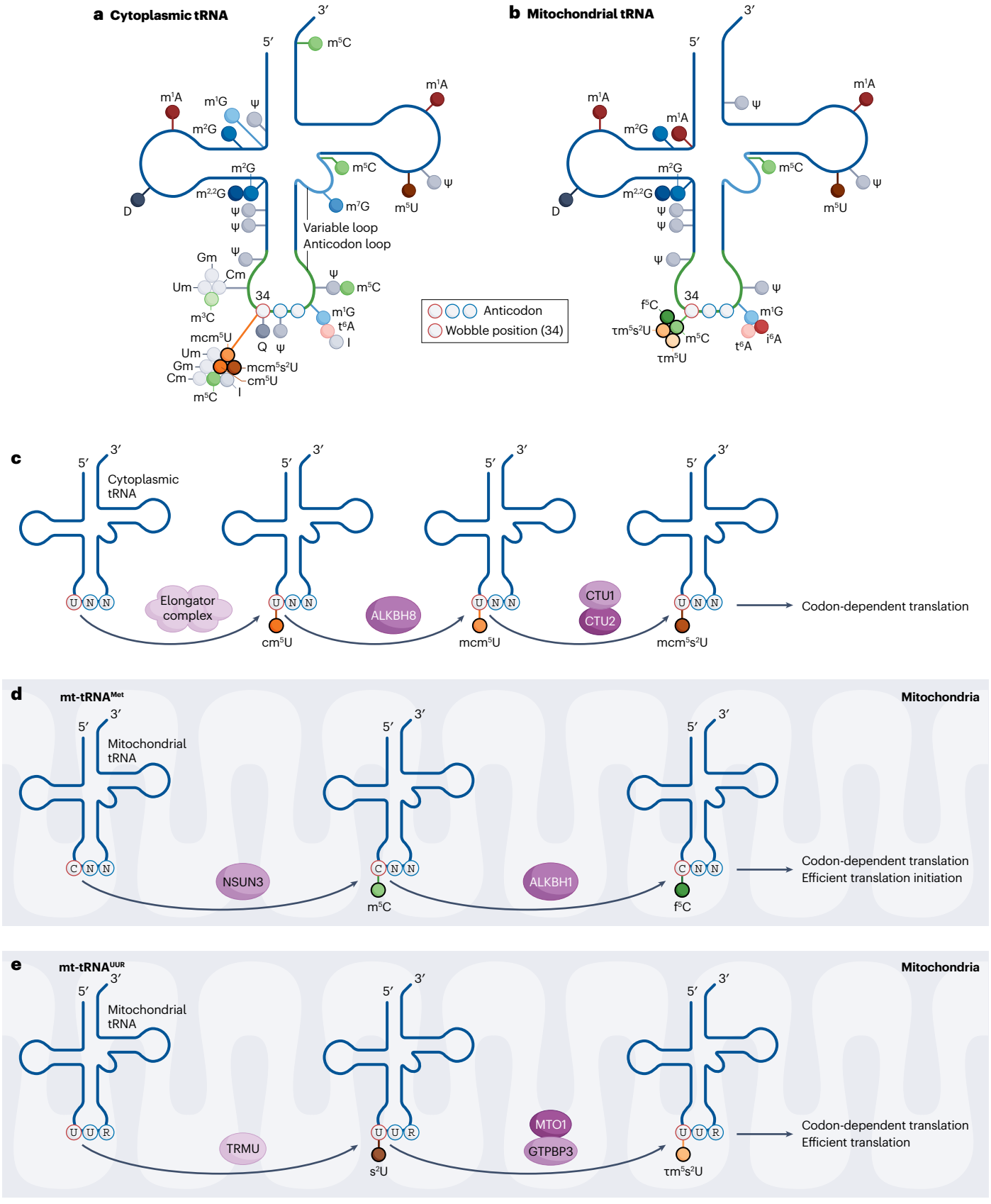
a Cytoplasmic tRNA
b Mitochondrial tRNA
Variable loop
Anticodon loop
Anticodon
Wobble position (34)
c
Cytoplasmic tRNA
Elongator complex
ALKBH8
CTU1
CTU2
Codon-dependent translation
d
mt-tRNAMet
Mitochondria
Mitochondrial tRNA
NSUN3
ALKBH1
Codon-dependent translation
Efficient translation initiation
e
mt-tRNAUUR
Mitochondria
Mitochondrial tRNA
TRMU
MTO1
GTPBP3
Codon-dependent translation
Efficient translation

Fig. 3 | Nucleotide modifications in the anticodon sequence of tRNAs regulate efficient translation and allow optimal codon use. a,**b**, Mammalian cytoplasmic (part **a**) and mitochondrial (part **b**) tRNA molecules containing examples of modifications. Modifications outlined in black are generated by multi-protein complexes. The anticodon loop (green) and variable loop (light blue) are highlighted. The anticodon position with the red outline represents the wobble position (34) within the triplet nucleotide sequence. **c**, Multi-enzyme cascade reactions form modifications at the wobble position uridine (U34) of cytoplasmic tRNAs. U34 in cytoplasmic tRNA is modified by the elongator complex, consisting of two copies of each of its six subunits (ELP1–ELP6). The acetyltransferase elongator complex generates 5-carboxymethyluridine (cm^5U) at position 34, which is further methylated by ALKBH8. The resulting 5-methoxycarbonylmethyluridine (mcm^5U) residue is thiolated by the CTU1–CTU2 complex to form 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U), which is required for codon-dependent translation. **d**, In the mitochondria, NSUN3 mediates the formation of 5-methylcytidine (m^5C) at position 34 in the mitochondrial tRNA for methionine (mt-$tRNA^{Met}$). This is then further oxidized by ALKBH1 to 5′-formylcytidine (f^5C), which is required for efficient decoding of AUA and AUU as methionine during mitochondrial translation[149,150,267]. **e**, Synthesis of the 5-methyluridine (m^5U) derivatives 5-taurinomethyluridine (τm^5U) and 5-taurinomethyl-2-thiouridine (τm^5s^2U) in the anticodon of human mt-$tRNA^{UUR}$ is mediated by TRMU, MTO1 and GTPBP3 to maintain mitochondrial translational decoding of UUA and UUG codons[171]. Cm, 2′-*O*-methylcytidine; D, dihydrouridine; Gm, 2′-*O*-methylguanosine; I, inosine; i^6A, N^6-isopentenyladenosine; m^1A, N^1-methyladenosine; m^3C, 3-methylcytidine; m^1G, N^1-methylguanosine; m^2G, N^2-methylguanosine; $m^{2,2}G$, N^2,N^2-dimethylguanosine; m^7G, N^7-methylguanosine; Ψ, pseudouridine; Q, queuosine; s^2U, 2-thiouridine; t^6A, taurine-6-adenosine; Um, 2′-*O*-methyluridine.

effector cells requires protection from tRNA cleavage as T cell activation involves increased synthesis of reactive oxygen species (ROS). To maintain protein synthesis in the presence of ROS, SLFN2 binds to tRNAs and protects them from stress-induced cleavage by angiogenin[108], showing that tRNAs that are not protected by modifications or otherwise undergo increased cleavage.

Global repression of translation during stress responses also involves the inhibition of rRNA processing, and the unprocessed rRNAs are stored within nucleoli until the stress resolves[109]. The storage of unprocessed rRNA in the nucleolus perturbs nucleolar dynamics[109], and disruption of the nucleolus induces several cellular responses including stabilization of the tumour suppressor protein p53 and activation of stress signalling pathways[109–111]. Both transcription and subsequent maturation of rRNAs are modulated by rRNA modifications[112]. For example, deletion of the RNA-modifying protein NSUN5 removes m^5C at position C3782 in 28S rRNA, which decreases protein synthesis, promotes adaptive translational programmes for survival under stress and, notably, enhances organismal longevity[113,114].

In summary, RNA modification profiles are altered in response to changes in the microenvironment, which can initiate cellular stress responses through targeted translation. Stress response pathways commonly stimulate stem cell division and differentiation to ensure correct cell numbers during development, adult homeostasis and the response to injury[115]. Consequently, the dysregulation of RNA modification patterns in mRNAs, tRNAs and rRNAs can cause human disease.

Pathological aberrant RNA modification

Loss-of-function mutations in genes encoding RNA-modifying proteins, and point mutations that prevent an RNA molecule from being modified, can both cause human disease. Aberrant RNA modification patterns affect gene expression by, for example, causing RNA degradation or structural changes. Altered structures of rRNA or tRNA molecules can affect ribosome binding or codon–anticodon interactions, and thereby reduce the efficiency and/or accuracy of protein synthesis. Dysregulated mRNA translation commonly dampens the metabolic plasticity required for cellular adaptations to the environment, which often underlies neurological and metabolic disorders and can contribute to cancer development.

Neurological disorders

The human brain seems to be particularly sensitive to aberrant tRNA modification profiles[16,116]. Mutations in human genes encoding tRNA-modifying enzymes are frequently linked to neurological disorders and cause microcephaly, intellectual disability and other neurodevelopmental deficits[16]. Additional disease symptoms can include short stature, aggressive behaviour or depression and anxiety-related behaviour when the tRNA-modifying genes *PUS7* and *PUS3* (responsible for Ψ modification), *FTSJ1* (2′-O-Me modification) or *NSUN2* (m^5C modification) are mutated[8,117–119]. It is likely that at least some of the behavioural changes are directly caused by aberrant deposition of tRNA modifications. For example, overexpression of NSUN2 in the mouse prefrontal cortex is sufficient to cause depressive-like behaviour, and its ablation resulted in an anti-depressive phenotype in mice[117]. Although it remains largely unexplored whether RNA modifications might be new drug targets in depression, distinct A-to-I RNA-editing variants can be used as biomarkers to differentiate between patients with bipolar disorder or unipolar depression[120].

Animal models and human stem cell differentiation assays have provided insight into how the functions of brain cells change when tRNAs are hypomodified. Mouse embryos lacking NSUN2 have reduced brain size owing to delayed differentiation and reduced motility of neurons[8,121]. The smaller brain size can be rescued in vivo by inhibiting tRNA cleavage or by treatment with *N*-acetylcysteine to inhibit oxidative stress[8]. Ablation of NSUN2 or FTSJ1 in adult mice reduces the codon-specific translation of proteins that are crucial for glutamatergic neurotransmission or for the organization and functions of neuronal synapses[117,122]. Depletion of TRMT1 (responsible for N^2,N^2-dimethylguanosine ($m^{2,2}G$) modification) in human neural cells alters their sensitivity to oxidative stress and causes perturbations in redox homeostasis[123]. Knockout of METTL1 (responsible for m^7G modification) in mouse embryonic stem cells causes ribosome stalling at m^7G-modified tRNA-dependent codons during mRNA translation of genes required for self-renewal and neural differentiation[124].

Although these studies confirm that tRNA modifications adapt mRNA translation to the cell context, they do not indicate why the brain seems to be particularly affected by defects in mRNA translation. Several stages during brain development, at which cell fate choices such as neuronal subtype differentiation are made, are primarily regulated at the level of translation and depend on mitochondrial metabolism[125–129], which may offer a partial explanation for this sensitivity. Furthermore, the complexity of dendrites and axons requires that protein synthesis occurs at locations remote from the cell body — more than 800 mRNAs have higher rates of translation in the dendritic–axonal compartment than in the cell body[130] — thus also requiring regulation primarily at the level of translation.

YTHDF proteins contribute to the subcellular localization of m^{6}A-containing mRNAs to neurites[131]. In *Drosophila*, Ythdf interacts with Fmr1, the fly homologue of FMRP. FMRP is a multifunctional RNA-binding protein that is required for the plasticity of neuronal synapses by regulating RNA synthesis and translation[132]. FMRP has effects on the stability, translation and nucleus-to-cytoplasm export of m^{6}A-containing RNAs[133–135]. Similarly, FMRP associates with ADAR, an RNA-editing enzyme that converts adenosine into inosine in double-stranded RNA, in mice, fish and *Drosophila*[136–138]. Epigenetic silencing of human *FMR1* (which encodes FMRP) causes the neurodevelopmental disorder Fragile X syndrome[139]. Notably, most RNAs are hypoedited in post-mortem brain tissue of individuals with Fragile X syndrome and autism[140].

Metabolic disorders

Mitochondrial diseases are the most common group of inherited metabolic disorders and amongst the most common forms of inherited neurological diseases[141]. Mitochondrial diseases are characterized by defects in oxidative phosphorylation (OXPHOS) and are caused by mutations in genes involved in mitochondrial functions[141]. Mutations in the nuclear genes encoding enzymes that modify mitochondrial tRNAs or rRNAs[16,142], as well as mutations in the mitochondrial genome that affect these modifications[143–145], cause mitochondrial disorders by dysregulating mitochondrial protein synthesis. The mitochondrial genome contains 22 tRNAs, 2 rRNAs and 13 mRNAs encoding proteins of the OXPHOS system[146]. Translation of mitochondria-encoded genes is repressed when a single NSUN3-mediated m^{5}C modification is missing at position 34 in the anticodon sequence of the mitochondrial tRNA for methionine (mt-tRNA^{Met})[147]. Patients with loss-of-function mutations in *NSUN3* present with combined mitochondrial respiratory chain complex deficiency and early onset of mitochondrial encephalomyopathy[148–150]. Thus, tightly controlled communication between mitochondrial and nuclear gene expression ensures optimal mitochondrial function and energy production to efficiently synthesize all proteins required for a given cell state.

Mutations in human *TRMT5* also cause multiple mitochondrial respiratory chain complex deficiencies[151]. In eukaryotes, TRMT5 is responsible for the formation of N^1-methylguanosine (m^{1}G) at position 37 in the anticodon loop of mitochondrial and cytosolic tRNAs[152,153]. m^{1}G at position 37 stabilizes the interaction between tRNAs and the ribosome, and loss of this modification leads to +1 frameshifting, which triggers global and mitochondrial defects in translation[154,155]. Whether TRMT5 directly coordinates mitochondrial translation with cytosolic translation is unknown. In the cytoplasm, TRMT5-mediated m^{1}G formation can be further modified to wybutosine (yW) at position G37 of the tRNA for phenylalanine (tRNA^{Phe}) — requiring at least four enzymes (TYW1–TYW4) — which stabilizes codon–anticodon interactions during protein synthesis[156,157]. Hypomodification of G37 in tRNA^{Phe} caused by repression of TYW2 increased ribosome frameshifting and dysregulated mRNA abundance via nonsense-mediated decay[158].

In addition to regulating the translation of mitochondria-encoded genes, RNA modifications also control the processing of mitochondrial long polycistronic transcripts that include sequences of mRNAs, rRNAs, tRNAs and other non-coding RNAs. ALKBH7 can demethylate m^{2,2}G and m^{1}A within mitochondrial pre-tRNA regions to decrease polycistronic mitochondrial RNA processing and reduce steady-state mitochondria-encoded tRNA levels and protein translation, which decreases mitochondrial activity[159]. Translocation of ALKBH7 to the mitochondria in response to extensive DNA damage is required for programmed necrosis induced by alkylation and oxidation[160].

Mitochondrial dysfunction can cause type 2 diabetes by altering pancreatic β-cell function and insulin resistance[161]. Human *CDKAL1*, which encodes a threonylcarbamoyladenosine tRNA methylthiotransferase, is a susceptibility gene for type 2 diabetes that regulates mitochondrial function in adipose tissue[162]. Loss of 2-methylthio-N^6-threonylcarbamoyladenosine (ms^{2}t^{6}A) in mt-tRNA$^{Lys(UUU)}$ in *Cdkal1*-depleted mice resulted in type 2 diabetes owing to misreading of lysine (Lys) codons in proinsulin, leading to a reduction of glucose-stimulated proinsulin synthesis[163]. Furthermore, the release of insulin from pancreatic β-cells is controlled by the mitochondria, which couples the metabolism of nutrients with energy output leading to insulin release. Mitochondrial dysfunction therefore underlies β-cell failure and the development of diabetes. Mutations in TFB1M, which mediates N^6-dimethylation of adenosine (m$^6{}_2$A) in mitochondrial 12S rRNA, similarly result in mitochondrial dysfunction, impaired insulin secretion and diabetes in mice and humans[164–167].

The above examples illustrate how RNA modifications can regulate cellular metabolism in response to dietary intake; in turn, some modifications also directly depend on specific nutritional factors to be generated. For example, queuine — a nitrogenous base obtained from the gut microbiota — is required to form queuosine at wobble positions of tRNAs with GUN anticodons[168,169]. Queuine depletion slows the translation of queuosine-decoded codons, leading to the accumulation of misfolded proteins that activate the unfolded protein response in cells and mice[170]. Accordingly, nutritionally determined tRNA modification levels directly control translation speed and fidelity.

Meat, dairy and fish are the main sources of taurine, which is one of the metabolic substrates required for the synthesis of τm^{5}U and τm^{5}s^{2}U in human mitochondrial tRNAs[171]. Taurine deficiency is associated with cardiomyopathy, renal dysfunction, developmental abnormalities and severe damage to retinal neurons, and drives ageing in mice, monkeys and humans[172,173]. Taurine supplementation has been shown to increase the health span (the period of healthy living) of mice and monkeys[173]. Taurine-dependent modification of mitochondrial tRNAs is catalysed by MTO1 in mammals, and *Mto1* deficiency severely impaired mitochondrial translation and respiratory activity in mice[174]. Similarly, loss of taurine-dependent modifications in mitochondrial tRNAs causes rare diseases in humans including hypertrophic cardiomyopathy, lactic acidosis, encephalopathy and infantile liver failure[175–178].

Mitochondrial myopathies are often caused by loss of taurine modifications resulting from mutations in the mitochondrial tRNAs[144,171]. In a phase III clinical trial, oral taurine supplementation effectively increased taurine-dependent modification in mt-tRNA$^{Leu(UUR)}$ and reduced the recurrence of stroke-like episodes in patients with MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) syndrome[179]. Mitochondrial activity of cells from patients with MELAS can also be rescued by overexpressing MTO1 to restore τm^{5}U modification[180].

Under diabetic conditions, cap-dependent translation of certain cytosolic mRNAs is decreased owing to the low cap-binding activity of eIF4E (ref. 181). Although the m^{6}Am cap modification does not modulate binding of eIF4E (ref. 42), polymorphisms in the m^{6}Am demethylase FTO have been associated with various metabolic diseases, including obesity and type 2 diabetes in humans[182–184]. *Fto*-knockout mice show resistance to high-fat diet-induced obesity, and FTO overexpression in mice results in obesity[185–188]. The precise role of m^{6}Am in regulating obesity remains obscure because FTO targets both internal m^{6}A

modifications and m^6Am cap modifications. However, in obese mice, upregulation of FTO leads to downregulation of its m^6Am-modified target genes, which are highly enriched in metabolic processes[189].

In summary, genetic loss of RNA modifications or the respective RNA-modifying proteins results in diverse human diseases; a common denominator of these diseases is often the impaired coordination of anabolic and catabolic processes with disrupted global and transcript-specific translation.

Cancer

Most aspects of tumorigenesis, including initiation, progression, metastasis and drug resistance, require cellular metabolic reprogramming events to ensure the survival and growth of cancer cells[190]. Given that RNA modifications regulate cell functions in response to external cues, it is not surprising that many of the 150 currently known RNA-modifying proteins in humans[191] are misregulated in cancer and are implicated in the responses of tumour cells to oxidative stress, DNA damage and drug exposure occurring, for example, during radiotherapy and chemotherapy[6–9].

One of the first mechanistic examples showing that inhibition of RNA modifications sensitizes tumour cells to chemotherapy in vivo was the inhibition of m^5C formation in tRNAs by depleting NSUN2 in squamous cell carcinoma in mice[7]. Loss of NSUN2-mediated m^5C alone is not lethal for cells, but increases tRNA fragmentation, reduces global protein synthesis and enforces quiescence in tumour-initiating cells at the expense of cellular flexibility. The inability to upregulate m^5C modification renders tumour-initiating cells hypersensitive to cytotoxic stress, and tumour regeneration is blocked after treatment with 5-fluorouracil or cisplatin[7]. Inhibition of m^5C formation in RNA and DNA by 5-azacytidine is often used to treat myelodysplastic syndromes[192–194]. The clinical efficacy of 5-azacytidine is likely to be, at least in part, due to loss of m^5C in RNA. Thus, the removal of RNA modifications can affect the ability of cancer cells to adapt to their environment, a feature that can be exploited during cancer treatment regimens.

Similarly, uridine modifications of the wobble site in cytosolic tRNAs are implicated in tumorigenesis by modulating oncogene-specific translation (Fig. 4). 5-Carboxymethyluridine (cm^5U), 5-methoxycarbonylmethyluridine (mcm^5U), mcm^5s^2U and 5-carbamoylmethyluridine (ncm^5U) modifications of the U34 wobble site — which are mediated by the elongator complex — are required for WNT-driven intestinal tumour initiation and breast cancer metastasis[195,196]. These modifications also enhance codon-dependent translation of glycolysis-related genes and promote the resistance of melanoma cells to chemotherapy[197]. Because elongator complex-mediated modifications promote transcript-specific translational programmes, they function in a strict cell type-specific manner. Deletion of individual members of the elongator complex (ELP1–ELP6) can promote or inhibit cancer cell survival dependent on cell-specific oncogenic mutations and signalling pathways. For example, loss of ELP3 causes bone marrow failure by activating p53-dependent checkpoints, but simultaneous inactivation of ELP3 and p53 promotes leukaemia development[198]. Loss of ELP5 decreases sensitivity to the chemotherapeutic drug gemcitabine, which is used to treat gallbladder cancer, by reducing codon-dependent translation of a *TP53 trans*-activator[199]. Germline mutations leading to biallelic inactivation of ELP1 predispose to childhood medulloblastoma in the presence of somatic alterations in PTCH1 (ref. 200). Tumours from these patients have loss of elongator complex-dependent tRNA modifications, altered codon-dependent translational reprogramming and induction of the unfolded protein response[200]. Thus, tRNA modifications at the U34 wobble site regulate cell context-specific translational programmes.

Together, these studies show that the inhibition of RNA-modifying proteins in cancer therapy may be effective only in specific oncogenic settings (Fig. 4). For the most part, it is unknown how the RNA modification pathways are interconnected with oncogenic signalling pathways, but this knowledge will be required to exploit the inhibition of RNA-modifying proteins in stratified therapeutic approaches because individual RNA modifications can have highly selective cellular functions. For example, NSUN3 is required for the formation of m^5C and its derivative f^5C at a single site (C34) in mt-$tRNA^{Met}$ (ref. 73). NSUN3-deficient human oral cancer cells have reduced mitochondrial translation of OXPHOS components, which is associated with increased glycolysis and adaptations to mitochondrial function that do not affect cell viability or primary tumour growth in vivo; however, the metabolic plasticity of these cells is severely impaired and mitochondrial m^5C-deficient tumours fail to metastasize[147]. Inhibiting mitochondrial translation in metastasizing tumour cells is a promising therapeutic strategy[201] that is currently being tested in multiple clinical trials[202].

In addition to tRNA modifications, rRNA modifications can also support transformed cell phenotypes by rewiring gene expression at the level of translation. Human 80S ribosomes contain approximately 130 individual rRNA modifications[203], the most abundant in eukaryotes being 2′-O-Me and Ψ[112]. Some rRNA modifications are essential for ribosome biogenesis, whereas others facilitate efficient and accurate protein synthesis[112,203–205]. Differential deposition of 2′-O-Me on rRNAs can affect how the genomic template is translated into functional proteins. For example, distinct 2′-O-Me rRNA signatures promote malignant self-renewal in patients with acute myeloid leukaemia[204,206–208] by redirecting leukaemic stem cells towards optimal codon usage for the translation of amino acid transporter mRNAs and subsequently increased intracellular amino acid levels[208]. Targeting amino acid metabolism is a promising form of cancer therapy because it directly interferes with the demand of tumour cells for biomass accumulation and energy production[209]. 2′-O-Me modification is catalysed by the C/D box small nucleolar ribonucleoprotein (snoRNP) complex, which contains the methyltransferase FBL, several scaffold proteins and the guiding C/D box snoRNA[210,211]. This offers the possibility to specifically disrupt the gene expression programme of leukaemic stem cells by targeting single 2′-O-Me sites using, for example, snoRNA antisense oligonucleotides (ASOs).

A biological function of internal m^6A modifications of mRNA has been reported for most cancers (extensively reviewed elsewhere[19]). The first m^6A inhibitor (STM-2457) targeting METTL3 reached phase I clinical trials in 2022 (ClinicalTrials.gov identifier NCT05584111)[212]. However, METTL3 is often also essential for the survival of non-transformed cells and its inhibition is unlikely to selectively target cancer cells. Targeting m^6A reader proteins (YTHDF1–YTHDF3), which mediate the functions of m^6A in the cytoplasm through transcript-specific translation, is a promising alternative approach. For example, inhibition of YTHDF2 selectively eradicates leukaemic stem cells in vivo[213]. In the absence of YTHDF2, stability of the m^6A-modified mRNA encoding the tumour necrosis factor receptor TNFR2 is increased, leading to upregulation of TNF-induced apoptosis in leukaemic but not normal haematopoietic stem cells. However, deletion of YTHDF2 leads to the expansion of functional haematopoietic stem cell populations, which

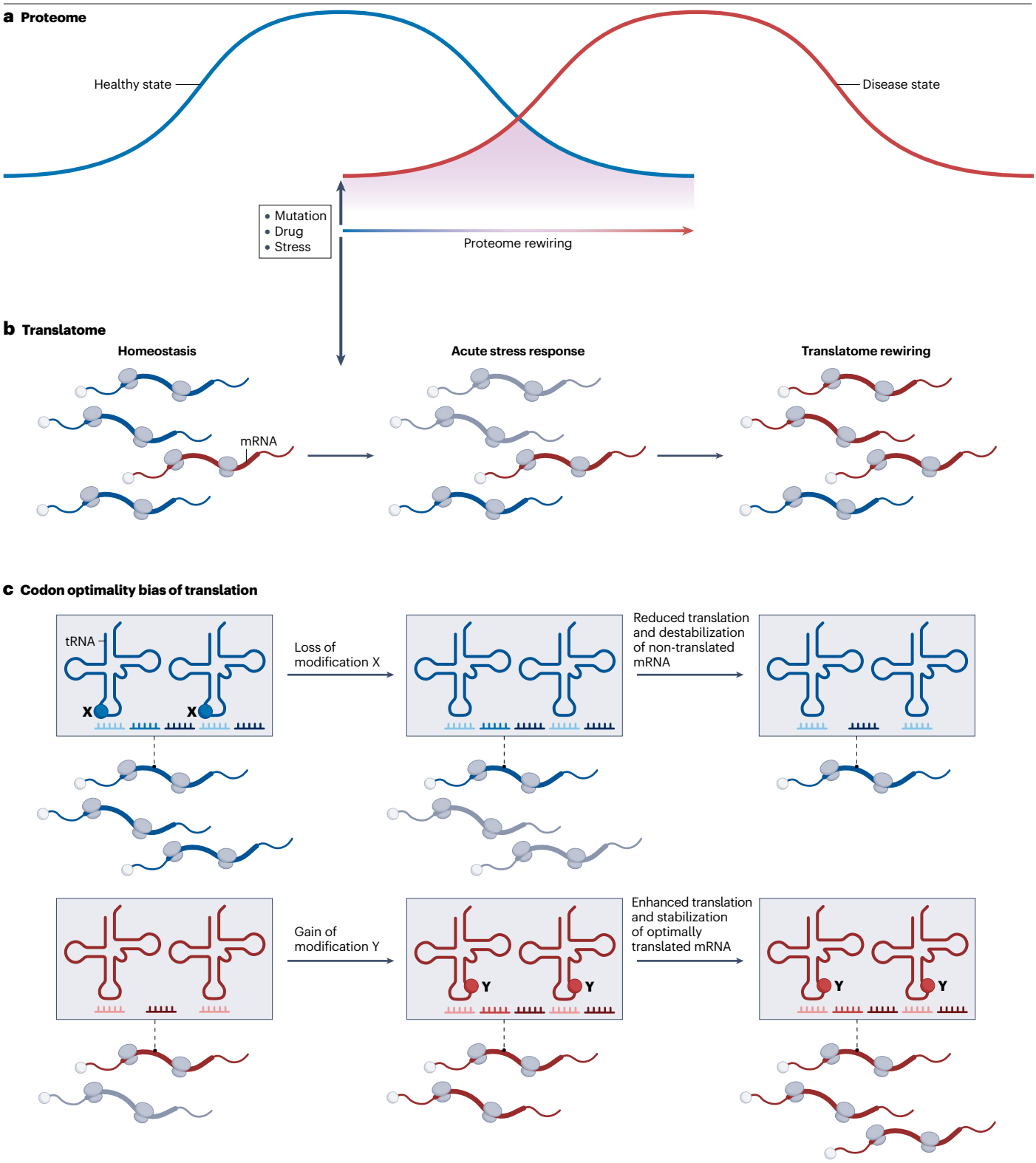
a Proteome
Healthy state
Disease state
• Mutation
• Drug
• Stress
Proteome rewiring
b Translatome
Homeostasis
Acute stress response
Translatome rewiring
mRNA
c Codon optimality bias of translation
tRNA
X
X
Loss of modification X
Reduced translation and destabilization of non-translated mRNA
Gain of modification Y
Y
Y
Enhanced translation and stabilization of optimally translated mRNA
Optimal codons for modification X
Optimal codons for modification Y
Translation efficiency, fidelity and speed

Fig. 4 | Cell context-dependent functions of anticodon tRNA modifications. **a**, Changes to disease-associated phenotypes can be caused by, for example, mutations in RNA-modifying enzymes, cytotoxic drug exposure or stress factors that can rewire the proteome through tRNA modifications. **b**,**c**, Examples of how the acute stress response can induce translatome rewiring (part **b**) and how tRNA anticodon modifications can contribute to this effect by enhancing the translation of mRNAs enriched with optimal codons (part **c**). In homeostasis (left-hand panels), more-abundant mRNA (blue) is efficiently translated because it contains a greater number of optimal codons efficiently recognized by tRNAs carrying modification X, whereas mRNA with fewer optimal codons and more non-optimal codons (red) is not efficiently recognized, resulting in reduced translation and mRNA destabilization. During the acute stress response (middle panels), loss (modification X) or gain (modification Y) of tRNA anticodon modifications can lead to reduced or enhanced mRNA translation resulting in destabilization (blue mRNA) or stabilization (red mRNA), respectively. Enhanced translation is shown for mRNAs (red) containing a greater number of optimal codons efficiently recognized by tRNAs carrying modification Y. Re-balancing the mRNA translation speed and fidelity towards mRNAs of a different gene set (from blue to red) can rewire the translatome (right-hand panels) in the acute stress response without requiring transcriptional changes in the nucleus. Transcript-specific enhanced translation can thereby determine the outcome of a cellular response.

in the long term causes stem cell exhaustion, owing to the upregulation of pro-inflammatory genes[214].

The accumulation of other rarer mRNA modifications such as m^1A and m^5C can also increase the translation of specific transcripts in cancer cells[215,216]. However, the simultaneous occurrence of these mRNA modifications in, for example, tRNAs with higher stoichiometry[217,218] hampers our understanding of the precise underlying molecular mechanisms.

In summary, detailed functional studies identifying how specific RNA modification profiles affect the fate of normal and malignant stem cells have improved our understanding of the tumorigenic process, identified novel promising anticancer drug targets and called attention to potential side effects. This knowledge could also help to advance diagnostic methods to increase the certainty of clinical decision-making (Box 2).

Clinical use of RNA modifications

The growing number of studies explaining how naturally occurring RNA modifications affect coding and non-coding RNAs at the molecular level has provided therapeutic opportunities to correct pathological aberrant RNA modifications, but also has led to improvements of clinically used RNAs by incorporating natural and synthetic modifications (Box 3).

Coding RNAs

The ability of specific nucleotide modifications to stabilize mRNAs and enhance their translation is now widely exploited for vaccination or therapeutic purposes to deliver engineered proteins through mRNA molecules. Currently, more than 450 clinical studies using mRNA vaccines are listed in the ClinicalTrials.gov database, including personalized mRNA vaccines against infections, cancer and genetic disorders. For example, together with cancer immunotherapy, mRNA vaccines can be used to activate antigen-presenting cells and stimulate the immune system by expressing tumour antigens in these cells[219].

Many rare genetic diseases are caused by the loss of one protein and can, in principle, be treated by protein replacement therapies[220]. To use in vitro-transcribed RNA for therapeutic purposes, it should mimic naturally occurring, mature cytosolic mRNA as closely as possible; in other words, the coding sequence should be marked by start and stop codons and flanked by UTRs, and the mRNA should ideally have a 5′ cap and poly(A) tail[221] (Fig. 1a). The addition of internal nucleotide modifications can further enhance translation and help to identify the in vitro-transcribed RNA as self rather than foreign to protect it from degradation in the cytosol. Foreign RNAs often derive from invading microbial pathogens, such as bacteria or viruses, and are recognized by the innate immune system through specific pattern-recognition receptors (PRRs)[222]. Several nucleotide modifications affect the recognition of RNAs by PRRs and modulate the release of inflammatory cytokines accordingly. For example, inosine can prevent immune signalling through the PRR MDA5 (ref. 223), and certain ribose methylations inhibit cytokine production downstream of the PRR TLR7 (refs. 224,225). The latter pathway is also silent for mRNAs with quantitative substitution of uridine by ψ[226] or N^1-methylpseudouridine ($m^1ψ$)[227] (Box 3).

Thus, mRNAs containing ψ or $m^1ψ$ have low immunogenicity via TLR7 and less efficiently activate protein kinase R (PKR). In the absence of phosphorylation by PKR, eIF2α promotes formation of the preinitiation translation complex and the mRNAs are more stably translated[226,228–231]. Increased translation results in increased ribosome density on mRNAs, and ψ and $m^1ψ$ modifications further help to resolve stalled ribosomes and prevent premature termination of translation[232]. Together, these RNA modifications have enabled the successful and rapid development of mRNA vaccines against coronavirus disease 2019 (COVID-19)[221]. In principle, any of the modifications that affect RNA structure and function could be applied to the development of mRNA

Box 2

RNA modifications as biomarkers

A currently underexplored field is the use of the epitranscriptome as a biomarker signature to guide clinical decision-making and predict treatment outcome. Global levels of RNA modifications in tissues or biological fluids (such as blood or urine) can serve as diagnostic biomarkers for human diseases. For example, tumour grades were accurately discriminated and predicted by profiling 12 distinct RNA modifications in glioma samples using mass spectrometry-based approaches combined with statistical multivariate analysis and machine learning[285]. Using combined expression profiles of 25 RNA modification writer proteins correctly predicted the prognosis of patients with non-small-cell lung cancer who received neo-adjuvant PD1 inhibitor therapy[286]. Construction of gene signatures related to ten RNA-modifying proteins predicted the therapeutic response of patients with bladder cancer to commonly used chemotherapies (cisplatin, vinblastine, paclitaxel and methotrexate)[287]. Therefore, assessing the RNA modification landscape in patients with cancer can identify differential therapeutic regimens and predict response to therapy, and could lead the way for personalized treatment.

vaccines or protein replacement therapy, but information about their efficacy and safety is very limited[233,234].

Small non-coding RNAs

Approved therapies involving small non-coding RNAs include the use of ASOs to reduce or increase protein levels or to improve protein function[235]. ASO therapeutics are always chemically modified. Modifications of the phosphorothioate backbone, as well as various ribose and base modifications, improve the pharmacological properties of ASOs and increase their binding affinity for target RNAs or proteins[236,237]. A particular type of ASOs are splicing modulators such as nusinersen. Nusinersen is used for the therapy of spinal muscular atrophy (SMA), a fatal genetic disease affecting children and adults, and was approved in 2016 (ref. 238). SMA is caused by reduced expression of the survival motor neuron (SMN) protein, which is encoded by two linked paralogues, *SMN1* and *SMN2*. Homozygous deletion or mutation in *SMN1* cannot be compensated fully by *SMN2* because it is expressed as a truncated protein isoform lacking exon 7. In ASO therapy for SMA, *SMN2* intronic splicing silencer elements are blocked and exon 7 inclusion is promoted[239]. Nusinersen and other ASOs used for the treatment of SMA are composed of 2′-*O*-methoxyethyl-modified oligonucleotides with a phosphorothioate backbone and contain fully methylated cytidines (m^5C) and uridines (5-methyluridine (m^5U))[237]. ASOs have also been approved for the therapy of Duchenne muscular dystrophy and polyneuropathy caused by hereditary transthyretin amyloidosis[240], and more than 130 clinical trials of ASOs in various diseases are currently registered.

Programming of the RNA interference pathway using small interfering RNAs (siRNAs), which are short, double-stranded and heavily modified RNAs, is another new therapeutic approach. Patisiran and givosiran are approved siRNA drugs for the treatment of hereditary amyloidogenic transthyretin and acute hepatic porphyria, respectively[241,242]. Therapeutic siRNAs including patisiran typically feature a fully modified nucleotide sequence including a combination of phosphorothioates, 2′-O-Me, 2′-MOE and 2′-deoxy-2′-fluoro (2′-F)[243].

The therapeutic potential of other small non-coding RNAs such as tRNAs has long been recognized but has not yet reached clinical approval. Between 10% and 15% of all inherited diseases are caused by premature stop codons; nonsense mutations leading to premature stop codons can, in principle, be corrected through the use of artificial tRNAs that bypass the stop signal and instead incorporate the desired amino acid[244,245]. Anticodon-edited tRNAs (ACE-tRNAs) have been used to rescue premature stop codons in the cystic fibrosis transmembrane regulator (*CFTR*) gene and restored CFTR channel function in gene-edited immortalized human bronchial epithelial cells[246]. Also, intravenous and intratracheal administration of engineered suppressor tRNAs (sup-tRNAs) in lipid nanoparticles can restore the production of functional CFTR in mice with nonsense mutations and in patient-derived nasal epithelia, and restore airway volume homeostasis[247].

In an alternative approach, disease-causing premature stop codons were corrected by converting uridine into Ψ in stop codons using engineered snoRNAs[248–250]. Artificial H/ACA box guide RNAs designed to target the mRNA premature stop codon restored translational read-through of the full-length protein in a cystic fibrosis cell line with the $CFTR^{G542X}$ nonsense mutation[249] and in mouse cells carrying a nonsense mutation of *Idua* (encoding α-L-iduronidase)[250], which is analogous to the *W402X* mutation commonly found in patients with Hurler syndrome, a lysosomal storage disorder[251]. However, how to guide the engineered RNAs to selected transcripts to prevent read-through from native stop codons, as well as how to deliver the RNAs to specific tissues, are issues that remain to be resolved.

The ability of tRNA modifications to increase the fidelity, accuracy and speed of translation of transcripts with distinct codon abundances (codon optimality)[252] can also be used to upregulate protein synthesis. Modifications in the tRNA anticodon loop, such as m^1G at position 37

Box 3

Naturally occurring and synthetic RNA modifications for clinical use

RNA hydrolysis proceeds in vitro and in vivo through the nucleophilic attack of a deprotonated 2′-hydroxyl ribose functionality to the phosphodiester moiety bridging two nucleotides in an RNA chain[288]. Consequently, biochemical modifications of the same moiety successfully stabilize RNAs for use in basic and therapeutic applications[289]. Naturally occurring modifications of RNA that have been successfully exploited for this purpose are the use of deoxyribose oligonucleotides (in other words, DNA) and methylation of the 2′-hydroxyl function (abbreviated as Nm in the epitranscriptomics field, or as 2′-O-methylation (2′-O-Me)). A successfully used synthetic modification at this position is the use of 2′-deoxy-2′-fluoro (2′-F) nucleosides, which are present in small interfering RNA (siRNA)-based therapeutics that are approved for clinical use[290,291]. The same RNA degradation pathway can also be affected by phosphothioate modification of RNA, which involves the substitution of an oxygen on the phosphodiester backbone by a sulfur. Phosphothioates were first made synthetically[288,292] and later discovered as naturally occurring modifications in bacterial DNA[293]. Whether phosphothioates occur naturally in RNA is currently debated[294,295]. Another promising group of modifications are alkylations at the 5-position of pyrimidines, which discriminate thymidine (the 5-methylated modification of uridine (m^5U)) in longer-lasting DNA from uridine in shorter-lived RNA. Thus, the modified pyrimidines 5-methylcytidine (m^5C) and m^5U (thymidine) protect RNA from degradation, presumably through biophysical stabilization owing to increased base stacking. In general, RNA polymerases tolerate 5-substitutions in pyrimidines, allowing their incorporation into RNA during in vitro transcription.

Coding RNAs that are stabilized by these modifications have increased translation into proteins in eukaryotes, as well as decreased stimulation of the mammalian innate immune system[230]. Such effects have also been observed for pseudouridine (Ψ) modification and for the N^1-methylpseudouridine ($m^1\psi$) modification used in the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) mRNA vaccines. $m^1\psi$ is, despite the misleading nomenclature, isosteric to m^5U and thus can be viewed as a 5-substituted pyrimidine, which seems to combine the properties of Ψ and m^5U in terms of RNA stabilization, translation efficacy and lack of immune stimulation[227].

Glossary

Anticodon
A sequence of three nucleotides on a tRNA molecule that recognizes and binds to a complementary trinucleotide codon sequence on mRNA during protein synthesis.

Antisense oligonucleotides
(ASOs). Short synthetic strands of nucleic acids that bind to complementary RNA sequences, offering targeted gene regulation and therapeutic potential for various genetic and disease-related applications.

Base stacking
Non-covalent interaction between adjacent aromatic nitrogenous bases in RNA that contributes to the stability of the secondary and tertiary structures of the RNA.

Charged tRNAs
tRNA molecules that carry a specific amino acid and are ready to participate in protein synthesis during translation.

Codon optimality
Refers to the preferential use of certain synonymous codons over others in a given organism or gene owing to differences in their usage frequency or interactions with tRNA molecules and ribosomes.

Elongator complex
A multi-protein complex composed of two subcomplexes, ELP1–ELP2–ELP3 and ELP4–ELP5–ELP6, that modifies tRNAs in their wobble position to regulate protein synthesis and ensure proteome stability.

Fragile X syndrome
A genetic disorder characterized by developmental delays, learning disabilities and social and behavioural problems, caused by a mutation in *FMR1*, which is needed for brain development.

Mitochondrial respiratory chain complex deficiency
A type of mitochondrial disease caused by defects in the enzymes involved in oxidative phosphorylation (OXPHOS), resulting in impaired energy production.

Oxidative phosphorylation
(OXPHOS). A metabolic pathway taking place inside mitochondria, in which cells use enzymes to oxidize nutrients, thereby releasing chemical energy in the form of ATP.

Queuosine
A hypermodified guanosine present in certain tRNAs in bacteria and eukaryotes containing the nucleobase queuine, which has a role in maintaining the proper reading frame during mRNA translation.

Unfolded protein response
A cellular stress response mechanism that is activated by the accumulation of unfolded or misfolded proteins in the endoplasmic reticulum.

Wobble position
The third nucleotide position of the anticodon trinucleotide sequence, which pairs with more than one complementary nucleotide in the mRNA codon.

or N^6-isopentenyladenosine at position 37 (t^6A37) as well as elongator complex-dependent modifications at the wobble position, all have the potential to stabilize codon–anticodon interactions to improve the speed and accuracy of translation[253–255].

tRNA-derived fragments are an alternative approach to modulate the translation of specific mRNAs that might conceivably be developed as therapeutics. Although the precise molecular action of tRNA fragments is unclear, they inhibit protein synthesis in response to stress[256] and their distribution is often cell type-specific[257]. For example, a stem cell-enriched tRNA fragment subtype requires the Ψ modification to selectively inhibit aberrant protein synthesis and to promote the engraftment and differentiation of haematopoietic stem and progenitor cells in patients with myelodysplastic syndrome[84,85].

Conclusions and future perspectives

The development of novel detection methods, state-of-the-art molecular approaches and advanced genetic model systems have all contributed to a better understanding of the functions of RNA modifications in physiology and human disease. Only now are we starting to comprehend the wide breadth and extensive functional roles of RNA modifications in higher organisms, and to appreciate their full potential in drug discovery and drug design. Some key limitations and challenges still need to be overcome to make use of the entire epitranscriptome in clinical applications.

We need to further advance mapping and quantification methods for RNA modifications. The discovery of novel functions of RNA modifications requires their unbiased detection in whole transcriptomes (Box 1). However, reliable and reproducible high-throughput methods to map rare modifications globally and in all types of RNA simultaneously are still lacking. Most nucleotide modifications are not restricted to specific types of RNA, and some RNA-modifying proteins target both coding and non-coding RNAs. Yet the stoichiometry of individual modifications in the different RNA types can vary markedly. Most mRNA modifications also occur in highly abundant tRNAs and, therefore, disentangling RNA type-specific molecular and cellular functions of modifications is challenging. Moreover, with the exception of tRNAs and rRNAs, the functions of other non-coding RNAs are often less well defined. Deciphering the importance of RNA modifications in non-coding RNAs with unknown function is nearly impossible because cellular or molecular read-outs are missing. Future advances in the sensitivity and reproducibility of mapping methods to achieve single-cell resolution of modifications in all types of RNA will provide important information regarding their dynamic deposition and physiological functions.

We need to better understand the regulatory network for RNA modifications. The longevity of an RNA molecule directly depends on its modification profile. Yet once RNA modifications are installed, they can outlive expression of the respective RNA-modifying enzymes. For example, mature mammalian tRNAs are extremely stable with a half-life of approximately 4 days[258], whereas the modifying enzymes are often transiently expressed in a cell type-specific manner. Consequently, the correlation between RNA modification levels and expression of their modifying enzymes can be distorted. Furthermore, the functions of RNA modifications are often mediated by specific reader proteins, which increase or decrease the stability of the respective RNA. Accordingly, the same modification, even in the same sequence context, can have different functions depending on the presence or absence of reader or eraser proteins.

Drug discovery pipelines targeting RNA-modifying proteins and RNA-based therapies need to be optimized. The currently identified drugs that target RNA-modifying enzymes often lack specificity, which is responsible for adverse effects that reduce their clinical relevance. This lack of specificity could be improved by optimizing computational prediction models and in vitro high-throughput enzymatic assays and identifying interlinked cellular pathways.

RNA-based therapies already use RNA modifications to enhance their stability, efficacy and specificity. Their pharmacological properties could further be optimized by using both naturally occurring and synthetic chemical modifications and by determining optimal sequence context and location for these modifications within the RNA.

Ultimately, the combination of comparative analyses at the single-cell level and functional analyses using advanced molecular, biochemical and cellular approaches in healthy and diseased human cells will provide a path forward to further integrate RNA modifications into clinical approaches.

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