The expanding world of tRNA modifications and their disease relevance

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Abstract | Transfer RNA (tRNA) is an adapter molecule that links a specific codon in mRNA with its corresponding amino acid during protein synthesis. tRNAs are enzymatically modified post-transcriptionally. A wide variety of tRNA modifications are found in the tRNA anticodon, which are crucial for precise codon recognition and reading frame maintenance, thereby ensuring accurate and efficient protein synthesis. In addition, tRNA-body regions are also frequently modified and thus stabilized in the cell. Over the past two decades, 16 novel tRNA modifications were discovered in various organisms, and the chemical space of tRNA modification continues to expand. Recent studies have revealed that tRNA modifications can be dynamically altered in response to levels of cellular metabolites and environmental stresses. Importantly, we now understand that deficiencies in tRNA modification can have pathological consequences, which are termed 'RNA modopathies'. Dysregulation of tRNA modification is involved in mitochondrial diseases, neurological disorders and cancer.

Anticodon
A unit of three nucleotides at positions 34–36 in transfer RNA, which corresponds with three nucleotides of an mRNA codon.

Reading frame maintenance
Of the three possible mRNA reading frames, only one reading frame generates the correct amino acid sequence. Transfer RNA modification in the anticodon is crucial for maintaining the correct reading frame.

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https://doi.org/10.1038/s41580-021-00342-0

In 1965, Holley et al.[1] determined the chemical structure of yeast transfer RNA (tRNA), $tRNA^{Ala}$, which included modified nucleosides. This work revealed that tRNA adopts a cloverleaf-like secondary structure (FIG. 1a). The anticodon nucleotide triplet was predicted to be ICG, where inosine (I) at the first position is a modified adenosine, leading Francis Crick to propose the wobble hypothesis[2]. Subsequently, tRNA modifications were analysed in various organisms, revealing that tRNA is a molecule heavily decorated with a wide variety of RNA modifications.

During protein synthesis, tRNAs are crucial for accurately deciphering the genetic code of the mRNA codons and translating them to the corresponding amino acids. tRNA is an adaptor molecule that carries an amino acid at its 3′ end and transfers it to the growing peptide chain. In the cloverleaf-like secondary structure of tRNA (FIG. 1a), the D loop (dihydrouridine loop) and the T loop (TΨC loop) form a kissing interaction to fold into an L-shaped tertiary structure (FIG. 1b). The anticodon, which is located at positions 34, 35 and 36 of the tRNA (FIG. 1a), recognizes a specific codon on the mRNA through hydrogen bonding at the A site of the ribosome (FIG. 1c). The anticodon is about 70 Å from the amino acid moiety at the 3′ end of the tRNA, which corresponds to the distance between the decoding and peptidyltransferase centres of the ribosome. In the codon–anticodon interaction, the first and second letters of the codon respectively pair with the third and second positions (positions 36 and 35, respectively) of the anticodon by strictly obeying Watson–Crick pairing (FIG. 1a). By contrast, the base pairing between the third letter of the codon and the first position (position 34) of the anticodon does not always follow the Watson–Crick pairing, but accommodates various irregular pairings, collectively called 'wobble pairing'[2]. Wobble pairing allows 61 sense codons to be deciphered by a limited number of tRNAs. There is enough room at the A site of the small subunit of the ribosome to accept various types of wobble pairs[3]. A wide variety of modified nucleosides (wobble modifications) are present at position 34 (FIG. 2a; Supplementary Fig. 1) and are important regulators of wobble pairing[4,5].

Other than the wobble modifications, position 37 is another hotspot of tRNA modification. This position is 3′ adjacent to the anticodon (FIGS 1a,2a; Supplementary Fig. 1). The modifications at position 37 contribute to ensuring accurate decoding and reading frame maintenance in mRNA[6,7]. In addition to the anticodon region, many modifications are present in the tRNA body, consisting of the D arm, the T arm and the variable loop (FIGS 1a,b,2), which are involved in maintaining the structural stability of the tRNA[8,9]. Some tRNA modifications control the charging specificity of cognate amino acid by aminoacyl-tRNA synthetases (aa-tRNA synthetase)[10,11] (FIG. 1c). Thus, the various tRNA modifications are intimately associated with the roles of tRNAs as adapters of protein synthesis.

Acceptor stem
A stem structure made of seven base pairs formed by the 5′ and 3′ ends of transfer RNA. The 3′ CCA sequence and the discriminator base protrude from this stem.

Discriminator base
The fourth nucleotide (position 73) from the 3′ end of transfer RNA (tRNA); frequently recognized by aminoacyl-tRNA synthetases and is a determinant of aminoacylation.

Following their aminoacylation, all tRNAs, except the initiator tRNA, are recognized by a common translation elongation factor — eukaryotic elongation factor 1α (eEF1α; EF-Tu in prokaryotes) — and recruited to ribosomes, where they bind the A site (FIG. 1c). Decoding occurs at the A site through codon–anticodon base pairing, which triggers GTP hydrolysis by the elongation factor and its release from the ribosome. Following the formation of the peptide bond and translocation, the tRNA moves from the A site to the P site, and then to the E site of the ribosome[12] (FIG. 1c). To be recognized by the common elongation factor and interact with the same tRNA-binding sites on the ribosome, all tRNAs adopt the same L-shaped tertiary structure (FIG. 1b). However, each tRNA species accepts a corresponding amino acid and strictly recognizes cognate codons. For this reason, each tRNA must have identity elements consisting of distinctive sequences or structures[13–15]. tRNA has determinants that enhance recognition by the corresponding aa-tRNA synthetase, as well as antideterminants that prevent recognition by non-cognate synthetases (FIG. 1c). Although these determinants comprise mainly sequence variations of the anticodon, acceptor stem and discriminator base (position 73), tRNA modifications are important for accurate and efficient aminoacylation[10,11,16–18] (FIG. 1c). Without tRNA modifications, many tRNAs have poor aminoacylation capability[19]. In addition, several tRNA modifications act as antideterminants for the non-cognate synthetases[16,20]. These observations suggest that a wide variety of tRNA modifications evolved to confer chemical exclusivity to tRNA molecules without significantly affecting their common structure and function. Furthermore, each tRNA maintains the optimal rate and accuracy of translation[21]. Codon–anticodon pairing strength on the ribosome and the binding affinity between the amino acid moiety of the aa-tRNA and the elongation factor differ for each tRNA species[22,23]. To maintain optimal translation, tRNA modifications compensate for these differences between tRNA species, thereby standardizing the process of translation[21].

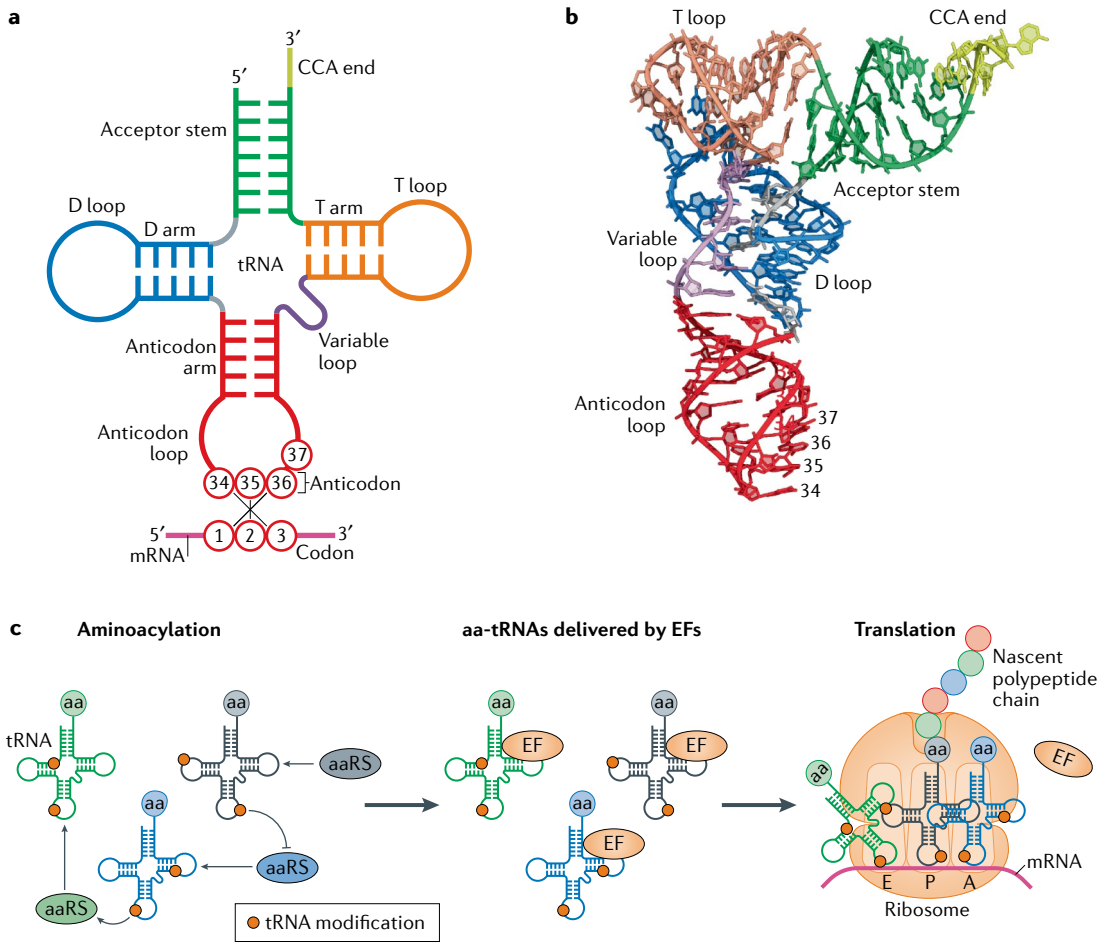


Fig. 1 | **Basic structure and function of tRNA. a** | Cloverleaf structure of transfer RNA (tRNA) with codon–anticodon pairing. A tRNA is composed of five parts: the acceptor stem with a CCA end, the D arm (dihydrouridine arm), the T arm (TΨC arm), the variable loop and the anticodon arm. The D loop and the T loop are kissing to form an L-shaped tertiary structure. The anticodon, which is located at positions 34, 35 and 36 of the tRNA, recognizes a specific codon (positions 1, 2 and 3) on the mRNA through hydrogen bonding at the A site of the ribosome. **b** | Tertiary structure of tRNA. The coordinates are obtained from Protein Data Bank entry 1EHZ. The colour code is the same as in part **a**. **c** | Functional role of tRNA in protein synthesis. tRNA modifications both promote and inhibit aminoacyl-tRNA synthetase (aaRS)-mediated aminoacylation. Elongation factor (EF) recognizes and delivers aminoacylated tRNA (aa-tRNA) to the A site (A) of the ribosome. The codon–anticodon interaction at the A site is facilitated by tRNA modifications.

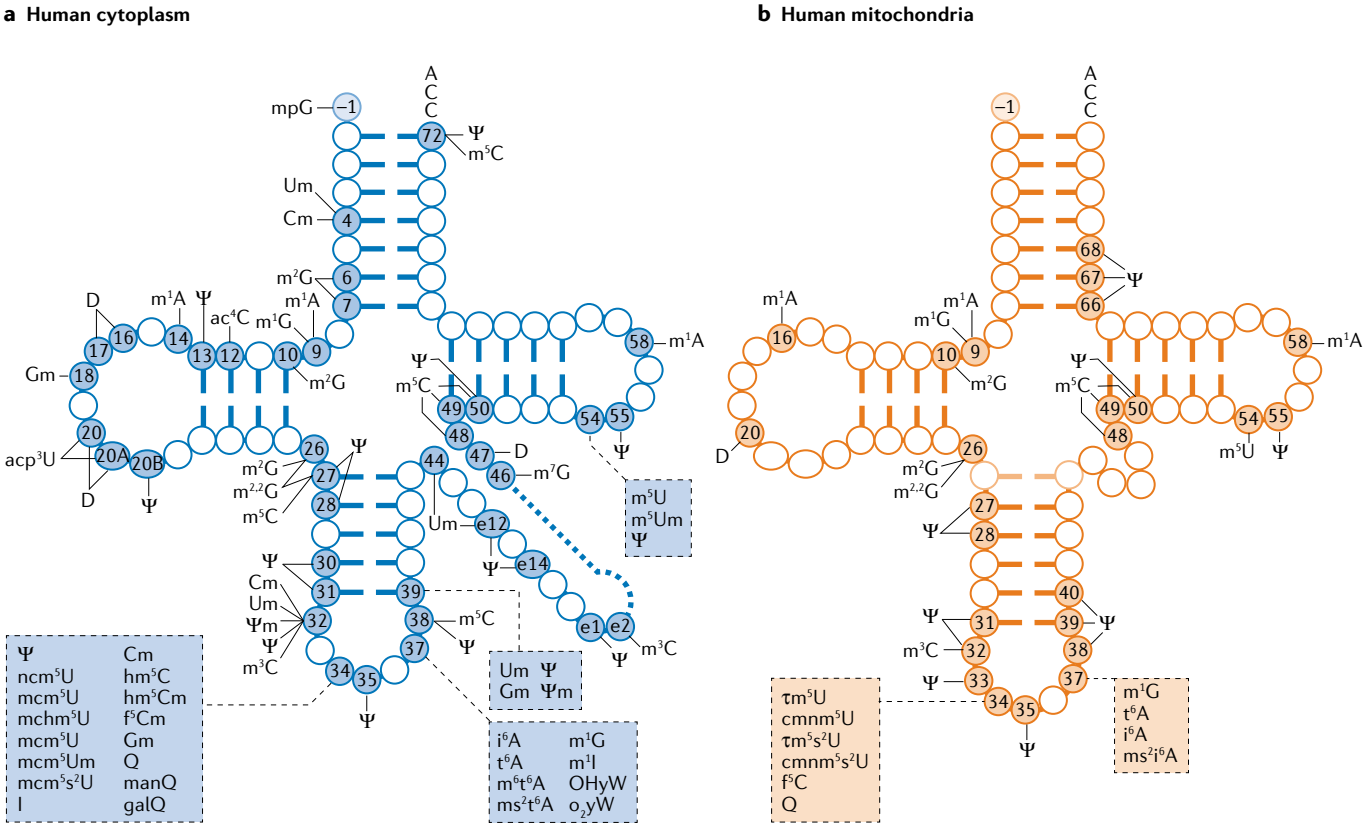


Fig. 2 | **Integrated view of human tRNA modifications. a** | Modifications of human cytoplasmic transfer RNAs (tRNAs)[27,230]. **b** | Modifications of human mitochondrial tRNAs[44]. Nucleotides that undergo modification are numbered, and the relevant modifications are indicated. The nucleotide at position −1 in $tRNA^{His}$ is added post-transcriptionally. The abbreviation of each RNA modification conforms with the RNA modification database MODOMICS[26]. ac⁴C, *N*⁴-acetylcytidine; acp³U, 3-(3-amino-3-carboxypropyl)uridine; Cm, 2′-*O*-methylcytidine; cmnm⁵s²U, 5-carboxymethylaminomethyl-2-thiouridine; cmnm⁵U, 5-carboxymethylaminomethyluridine; D, dihydrouridine; f⁵C, 5-formylcytidine; f⁵Cm, 5-formyl-2′-*O*-methylcytidine; galQ, galactosyl-queuosine; Gm, 2′-*O*-methylguanosine; hm⁵C, 5-hydroxymethylcytidine; hm⁵Cm, 2′-*O*-methyl-5-hydroxymethylcytidine; I, inosine; i⁶A, *N*⁶-isopentenyladenosine; m¹A, 1-methyladenosine; manQ, mannosyl-queuosine; m³C, 3-methylcytidine; m⁵C, 5-methylcytidine; mchm⁵U, 5-(carboxyhydroxymethyl)uridine methyl ester; mcm⁵s²U, 5-methoxycarbonylmethyl-2-thiouridine; mcm⁵U, 5-methoxycarbonylmethyluridine; m¹G, 1-methylguanosine; m²G, *N*²-methylguanosine; m²,²G, *N*²,*N*²-dimethylguanosine; m⁷G, 7-methylguanosine; m¹I, 1-methylinosine; mpG, 5′-methylphosphoguanosine; ms²i⁶A, 2-methylthio-*N*⁶-isopentenyladenosine; ms²t⁶A, 2-methylthio-*N*⁶-threonylcarbamoyladenosine; m⁶t⁶A, *N*⁶-methyl-*N*⁶-threonylcarbamoyladenosine; m⁵U, 5-methyluridine; m⁵Um, 2′-*O*-methyl-5-methyluridine; ncm⁵U, 5-carbamoylmethyluridine; OHyW, hydroxywybutosine; o₂yW, peroxywybutosine; Q, queuosine; t⁶A, *N*⁶-threonylcarbamoyladenosine; Um, 2′-*O*-methyluridine; τm⁵U, 5-taurinomethyluridine; τm⁵s²U, 5-taurinomethyl-2-thiouridine; Ψ, pseudouridine; Ψm, 2′-*O*-methylpseudouridine.

Compared with other non-coding RNAs, tRNAs and ribosomal RNAs (rRNAs) are ubiquitously expressed and are relatively stable in the cell. Although other non-coding RNAs form ribonucleoprotein complexes with partner proteins, tRNA basically functions as a naked molecule without stable partners, and it is the most dynamic molecule involved in translation. From a physicochemical point of view, a tRNA modification confers conformational rigidity to the base or ribose, and provides a hydrophobic or hydrophilic environment to the local structure, thereby stabilizing the higher-order structure of the tRNA[9,24]. On the ribosome, tRNA directly binds to the codon on mRNA and forms many contacts with rRNAs. In addition, tRNA dramatically changes its conformation during the process of accommodation and translocation[12,25]. Thus, tRNA may have fine-tuned its function by acquiring various chemical modifications, which enable it to move dynamically on the ribosome. It is intriguing that many rRNA modifications are clustered near their tRNA contact sites in the ribosome, supporting the idea that RNA modifications modulate direct RNA–RNA interactions.

In this review, I describe newly discovered tRNA modifications and discuss the general features of tRNA modification, their regulation of decoding in protein synthesis, metabolic regulation of tRNA modification and human diseases caused by aberrant tRNA modifications.

The expending space of modifications

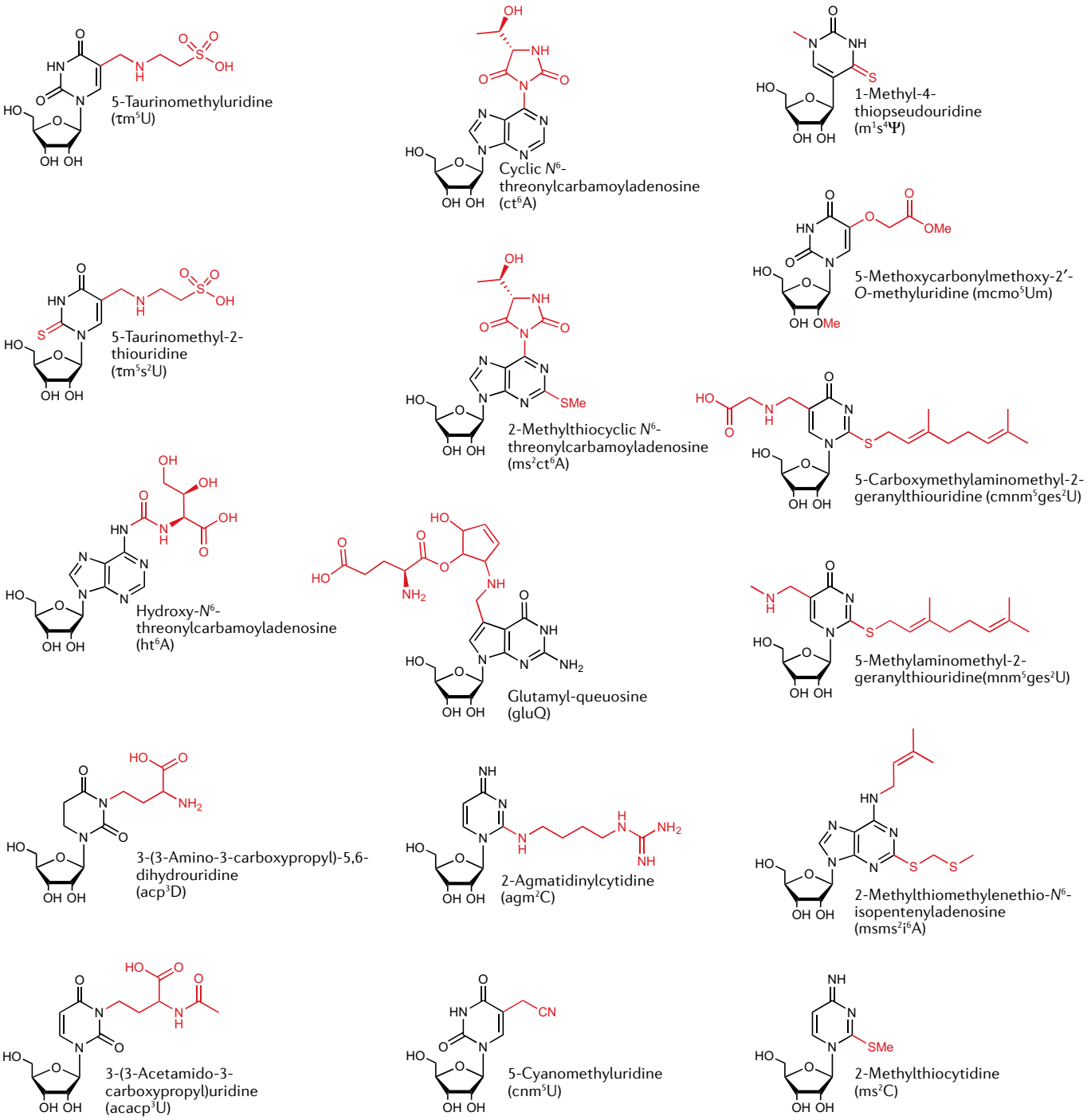
Compared with the relatively small number of different DNA modifications, RNA modifications are much more diverse. In chemical terms, these modifications include methylation, hydroxylation, acetylation, deamination,

isomerization, selenylation, reduction, cyclization and conjugation with amino acids and sugars. To date, about 150 types of RNA modifications have been identified in all domains of life[26]. About 80% of them were identified in tRNAs[26]. Between 10% and 20% of tRNA residues are modified[27]. Historically, RNA modifications in tRNAs, rRNAs and small nuclear RNAs were studied extensively owing to their abundance in the cell. Recent advances in sequencing technology have enabled us to map RNA modifications in mRNAs and various non-coding RNAs. The field that studies RNA modification has been termed 'epitranscriptomics' and has uncovered a new layer of gene regulation at the post-transcriptional level[28].

In the 1970s and 1980s, a large number of tRNA modifications was discovered[8,29,30], and their chemical structures were determined[26]. In this century, 16 tRNA modifications have been discovered (FIG. 3), thereby expanding the chemical space of RNA modification.

The landscape of tRNA modifications

The human genome encodes a set of 429 high-confidence cytoplasmic tRNA genes with distinct sequences[31]. According to the mapping in various mammalian species and tissues of genome-wide occupancy of RNA polymerase III, which is the polymerase that transcribes tRNA genes, about of half of the tRNA



Family box
In the genetic code, a codon box in which four codons are synonymous (encode the same amino acid).

Two-codon sets
In the genetic code, a codon set in which two codons ending in pyrimidines or in purines are synonymous (specifying the same amino acid).

Ribose puckering
A low-strain conformation of the ribose sugar ring in which atoms in the ring are displaced from the plane.

C3′ *endo* form
Major puckering of ribose found in A-form duplex of RNA.

genes are transcribed[32]. About 200 species of tRNAs are detected by comprehensive profiling[33–35]. However, post-transcriptional modifications have been mapped to only 18 different cytoplasmic tRNAs deposited in the tRNA database[27]. Human tRNA modifications are tissue type and cell type dependent[36,37]. Thus, having a complete picture of cytoplasmic tRNA modifications will be helpful to better understand the physiological and pathological processes mediated by human tRNAs. A sequencing method based on the blocking of reverse transcription by RNA modification can provide a more rapid and sensitive assessment of tRNA modification dynamics, at least for some modifications, including 1-methyladenosine (m^1A), 1-methylguanosine (m^1G), 3-methylcytidine (m^3C), 3-(3-amino-3-carboxypropyl) uridine (acp^3U) and pseudouridine (Ψ), which can be detected and quantified by deep sequencing[38–42]. By compiling the published data, we can see that up to 39 types of RNA modifications have been identified in human cytoplasmic tRNAs (FIG. 2a). A current list of genes responsible for cytoplasmic tRNA modifications is provided in Supplementary Table 1.

Mitochondria have their own genomes and gene expression machinery, which is independent of the nuclear system. The genetic code of mammalian mitochondria deviates from the universal code, using AUA for Met, UGA for Trp and AGR for stop codons[43]. Twenty-two species of mitochondrial tRNAs (mt-tRNAs), which are encoded in the mitochondrial DNA (mtDNA), translate essential subunits of the respiratory chain complexes. Very recently, 22 human mt-tRNAs were isolated and analysed by mass spectrometry and biochemical approaches, revealing 18 types of modifications at 137 positions (8.7% of the 1,575 nucleobases) in the 22 mt-tRNAs[44] (FIG. 2b). An up-to-date list of genes responsible for mt-tRNA modifications is provided in Supplementary Table 2. In contrast to higher eukaryotes, the function and biogenesis of tRNA modifications have been extensively studied in *Escherichia coli* and *Saccharomyces cerevisiae*. To date, 31 types of RNA modifications have been found in *E. coli* tRNAs (Supplementary Fig. 1a), and 26 types have been found in *S. cerevisiae* tRNAs (Supplementary Fig. 1b).

tRNA modifications of the anticodon

The anticodon region contains complex tRNA modifications that are involved in codon recognition and reading frame maintenance. A wide variety of modified nucleosides (wobble modifications) are present at the first position (position 34) of the anticodon (FIG. 1a), which forms wobble base pairing with the third position of the codon at the ribosome to ensure accurate decoding of the genetic code[4]. To date, more than 30 types of wobble modifications have been identified[26], which restrict or expand the decoding properties of the tRNA.

Restriction of decoding. According to the classical wobble hypothesis[2], an anticodon with unmodified uridine (U) at the first position (position 34) recognizes codons bearing A or G at the third codon position. However, in the decoding systems of bacteria and organelles (mitochondria and chloroplasts), U34 can recognize all four nucleotides (U, C, A and G) at this position (FIG. 4a), a phenomenon termed 'four-way wobbling'[43]. Thus, in principle, if a tRNA with U34 is assigned to a family box, all four codons in that family box will be decoded by that single tRNA species. By contrast, when modified uridines are present at the wobble position of tRNAs, they specifically decode two-codon sets, recognizing A or G at the third position (FIG. 4a). Derivatives of 5-methyluridine (xm^5U34) represent a major group of modified uridines, found in many organisms. For example, in eukaryotes, 5-methoxycarbonylmethyluridine (mcm^5U) and its derivatives are present in cytoplasmic tRNA[45,46], whereas in the mitochondria, 5-taurinomethyluridine (τm^5U)[47] and 5-carboxyaminomethyluridine ($cmnm^5U$)[44,48] are present.

In some tRNA species, xm^5U34 modifications are further modified into xm^5s^2U34, in which the C2 carbonyl group is thiolated. In other cases, xm^5U34 is subjected to 2′-O methylation to form xm^5Um34 derivatives, which are important for efficient recognition of cognate codons ending in purines (R = A and G), and for eliminating misdecoding of non-cognate codons ending in pyrimidines (Y = U and C)[49]. These modifications stabilize ribose puckering to the C3′ *endo* form, thereby preventing misrecognition of the two-codon set ending in Y. Furthermore, genetic and biochemical analyses have revealed that the C5 substituent of xm^5U modifications enables efficient decoding of G-ending codons[50–52], whereas the 2-thio group of xm^5s^2U contributes to decoding of both A-ending and G-ending codons[52,53]. In yeast, mcm^5s^2U at $tRNA^{Lys}$ and $tRNA^{Glu}$

◀ Fig. 3 | **tRNA modifications discovered in the past two decades.** Breakthroughs in technologies for RNA purification[231,232] have made it possible to isolate individual mitochondrial transfer RNAs (mt-tRNAs), including those of low abundance, from limited amounts of source material. Highly sensitive detection of RNA molecules by mass spectrometry[206] contributed to the discovery of novel RNA modifications. Using these approaches, 5-taurinomethyluridine (τm^5U) and 5-taurinomethyl-2-thiouridine (τm^5s^2U) are found in the anticodon of human mt-tRNAs[47] (FIG. 2b). The two modifications are essential in mammals, and deficiency in these modifications results in human mitochondrial diseases[137]. Hydroxy-N^6-threonylcarbamoyladenosine (ht^6A) was found in sea urchin mt-$tRNA^{Lys}$ (REF.[233]). In *Trypanosoma brucei*, 3-(3-amino-3-carboxypropyl)-5,6-dihydrouridine (acp^3D) — a dihydrouridine derivative of 3-(3-amino-3-carboxypropyl) uridine (acp^3U) — was identified at position 47 in cytoplasmic $tRNA^{Lys}$ (REF.[234]). Very recently, an acetylated form of acp^3U ($acacp^3U$) was identified in *Vibrio cholerae* tRNAs[42]. Cyclic N^6-threonylcarbamoyladenosine (ct^6A)[235,236] and 2-methylthio cyclic N^6-threonylcarbamoyladenosine (ms^2ct^6A)[237] are cyclic forms of N^6-threonylcarbamoyladenosine (t^6A) and 2-methylthio N^6-threonylcarbamoyladenosine (ms^2t^6A), respectively, in tRNAs found in bacteria, yeast, protists and plants. The ct^6A modification supports the decoding efficiency of tRNA and is required for respiratory growth in yeast[224]. Glutamyl-queuosine (gluQ) is a labile modification found in *Escherichia coli* $tRNA^{Asp}$ (REF.[238]). Both 2-agmatidinylcytidine (agm^2C)[239,240] and 5-cyanomethyluridine (cnm^5U)[241] were discovered in the anticodon of archaeal tRNAs, and are required for accurate decoding. 1-Methyl-4-thiopseudouridine ($m^1s^4\Psi$) was recently identified at position 54 of tRNAs from hyperthermophilic archaea[242] and might contribute to thermal stability of tRNA. The modification 5-methoxycarbonylmethoxy-2′-*O*-methyluridine ($mcmo^5Um$) is a minor modification, found in ~4.2% of *E. coli* $tRNA^{Ser1}$ (REF.[243]). Geranylated forms of uridine derivatives — 5-carboxymethylaminomethyl-2-geranylthiouridine ($cmnm^5ges^2U$) and 5-methylaminomethyl-2-geranylthiouridine (mnm^5ges^2U)[244,245] — and the modification 2-methylthiomethylenethio-N^6-isopentenyladenosine ($msms^2i^6A$)[246] were found as highly hydrophobic nucleosides. These modifications are present at very low levels, and seem to be intermediates of other modifications[247,248] and alterations[246]. Finally, 2-methylthiocytidine (ms^2C)[249] might represent an alkylation-damaged nucleoside of 2-thiocytidine (s^2C) in tRNA.

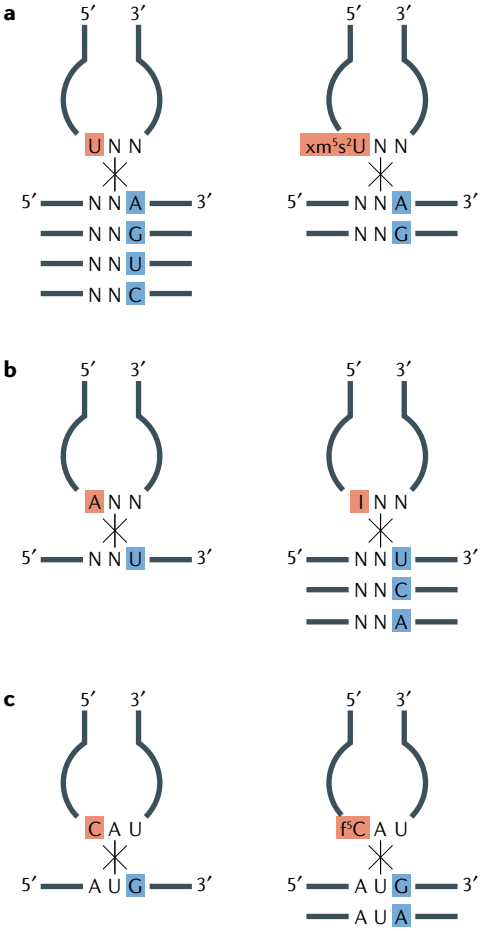


Fig. 4 | **Control of decoding by tRNA wobble modifications. a** | Restriction of codon recognition by 5-methyl-2-thiouridine derivative (xm^5s^2U)-type modifications. **b** | Expansion of codon recognition by inosine (I). **c** | Expansion of codon recognition by 5-formylcytidine (f^5C).

is crucial for maintaining the correct reading frame[54]. Structural analyses of ribosomes in complex with mRNA and tRNA revealed how xm^5U34 contributes to cognate codon–anticodon pairing[50,55]. Ribosome profiling analysis of yeast strains lacking mcm^5s^2U revealed that translation is slow when A-ending codons come in the A site[56,57]. As the 2-thio group of xm^5s^2U promotes translocation[58,59], ribosome pausing at A-ending codons might be explained by slow translocation of hypomodified tRNAs.

Expansion of decoding. An anticodon with unmodified A at position 34 pairs with a U at the third codon position. When A34 is modified to I through hydrolytic deamination, the resulting I34-containing anticodon recognizes codons with U, C or A at the third position; thus, A-to-I editing of the anticodon expands its decoding capacity (FIG. 4b). Crick originally predicted that the I34–A3 purine–purine pairing would involve the *anti* conformation of both bases[2]. This was confirmed by a structural study of I34–A3 pairing within the ribosome[60]. Bacterial tRNA-specific adenosine deaminase (TadA) catalyses A-to-I editing of the anticodon[61], and the Tad2p–Tad3p complex is responsible for the same reaction in budding yeast[62]. In humans, the tRNA-specific adenosine deaminase 2 (ADAT2)–ADAT3 complex catalyses A-to-I editing of the tRNA anticodon[63,64]. These proteins are essential for viability, implying that I34 is a tRNA modification crucial for deciphering the genetic code.

The modification 5-formylcytidine (f^5C) is present at position 34 of mt-$tRNA^{Met}$ (REF.[65]) (FIG. 2b). f^5C34 enables decoding not only of the AUG codon but also of the AUA codon, as Met[66] (FIG. 4c). Thus, f^5C34 facilitates pairing with A at the third position of the AUA codon[67]. My group and others have shown that f^5C34 is biosynthesized through a multireaction process mediated by the methyltransferase NSUN3 (also known as tRNA (cytosine(34)-C(5))-methyltransferase, mitochondrial) and the oxygenase ALKBH1 (REFS[68–71]) (Supplementary Table 2). When the genes encoding NSUN3 and ALKBH1 are disrupted, mitochondrial protein synthesis is markedly reduced, resulting in reduced respiration and mitochondrial activity. Thus, f^5C34 is essential for mitochondrial function.

Metabolic control of tRNA modification

It was once widely believed that tRNA modifications are stable and static, and that their frequencies are rarely regulated, implying that they are structural components that ensure proper functioning of tRNAs in protein synthesis. During tRNA maturation, hypomodified and/or unstructured tRNAs are eliminated by the nuclear surveillance system[72,73]. Specifically, hypomodified tRNAs lacking one or more modifications are degraded by 5′-to-3′ exonucleases through a system called 'rapid tRNA decay' (RTD)[74,75]. However, not all hypomodified tRNAs are eliminated by RTD, and many instances of hypomodified tRNAs are detected in healthy as well as pathogenic cells[43,46,76]. These observations indicate that tRNA modifications undergo dynamic alteration in certain cellular conditions. In this section, I discuss three examples of dynamic regulation of tRNA modification by the cellular metabolic status.

Chemical switching of tRNA modifications by taurine deficiency. As mentioned earlier, τm^5U34 and τm^5s^2U34 in five mt-tRNAs (FIG. 2b) have critical roles in accurate decoding purine-ending two-codon sets[43]. MTO1 and the GTPase GTPBP3 are responsible for the formation of τm^5U34 in mt-tRNAs[48,77] (FIG. 5; Supplementary Table 2). Taurine and 5,10-methylenetetrahydrofolate (CH_2-THF) are the metabolic substrates for the taurine modification of mt-tRNAs[48] (FIG. 5). Taurine is a naturally occurring, sulfur-containing amino acid present at high concentrations in mammalian plasma and tissues[78,79]. In the cytoplasm, taurine accumulates at concentrations of up to 40 mM. Taurine is imported into mitochondria and used for the biogenesis of τm^5U in mt-tRNAs[47] (FIG. 5). Although taurine is biosynthesized from cysteine, it is an essential nutrient for carnivores, including cats and foxes, which have little taurine biogenesis activity[80,81]. In conditions of dietary taurine deficiency, these animals exhibit developmental defects, central retinal degradation, hepatic lipidosis and dilated cardiomyopathy.

Rapid tRNA decay
A pathway of 5′–3′ exonucleolytic degradation of mature transfer RNAs (tRNAs) that lack certain modifications.

These are also major manifestations of human mitochondrial encephalomyopathies[82]. Low capacity for taurine biogenesis is not restricted to carnivores. In humans and possibly other primates, infants have low taurine biogenesis activity, making taurine intake from breast milk essential for normal development[83]. Taurine is also required for aquacultural production of flounder (flatfish), which has little taurine biogenesis activity[84].

To determine whether taurine deficiency affects the τm^{5}U modification, mt-tRNAs were isolated from the liver of cats that developed cardiomyopathy owing to taurine deficiency and subjected to mass spectrometry analyses, which showed that τm^{5}U frequency was slightly but significantly reduced upon taurine depletion and starvation[48] (FIG. 5). A similar observation was made in flounder fed a taurine-depleted diet[48]. In humans, the abundance of τm^{5}U dropped sharply in HeLa cells cultured in taurine-depleted conditions[48], indicating that τm^{5}U is dynamically regulated by extracellular taurine availability even in cells capable of taurine biogenesis. Following taurine depletion, cmnm^{5}U and its 2-thiouridine derivative cmnm^{5}s^{2}U were detected in mt-tRNAs[44,48] (FIG. 5). These derivatives have C-5 substituents in which the taurine moiety of τm^{5}U and τm^{5}s^{2}U is replaced with glycine. Because taurine and glycine have similar chemical structures, it is likely that glycine is incorporated instead of taurine in taurine-depleted conditions. This is a clear example of chemical switching of RNA modifications in physiological conditions. Because the electron density and acidity of the uracil ring are affected by different C-5 substituents[85], the decoding efficiency and fidelity of NNG codons ('N' represents any nucleotide) in mitochondrial translation could be modulated by mt-tRNAs bearing such unusual modifications. These findings might explain, at least in part, why taurine is an essential nutrient for animals. High glycine concentration in blood is a characteristic feature of non-ketotic hyperglycinaemia, which is a mitochondrial disease caused by mutations in the mitochondrial glycine cleavage system[86]. The level of cmnm^{5}s^{2}U34 could be elevated in individuals with this disease; thus, the modification status of mt-tRNAs might be involved in the pathogenesis of non-ketotic hyperglycinaemia.

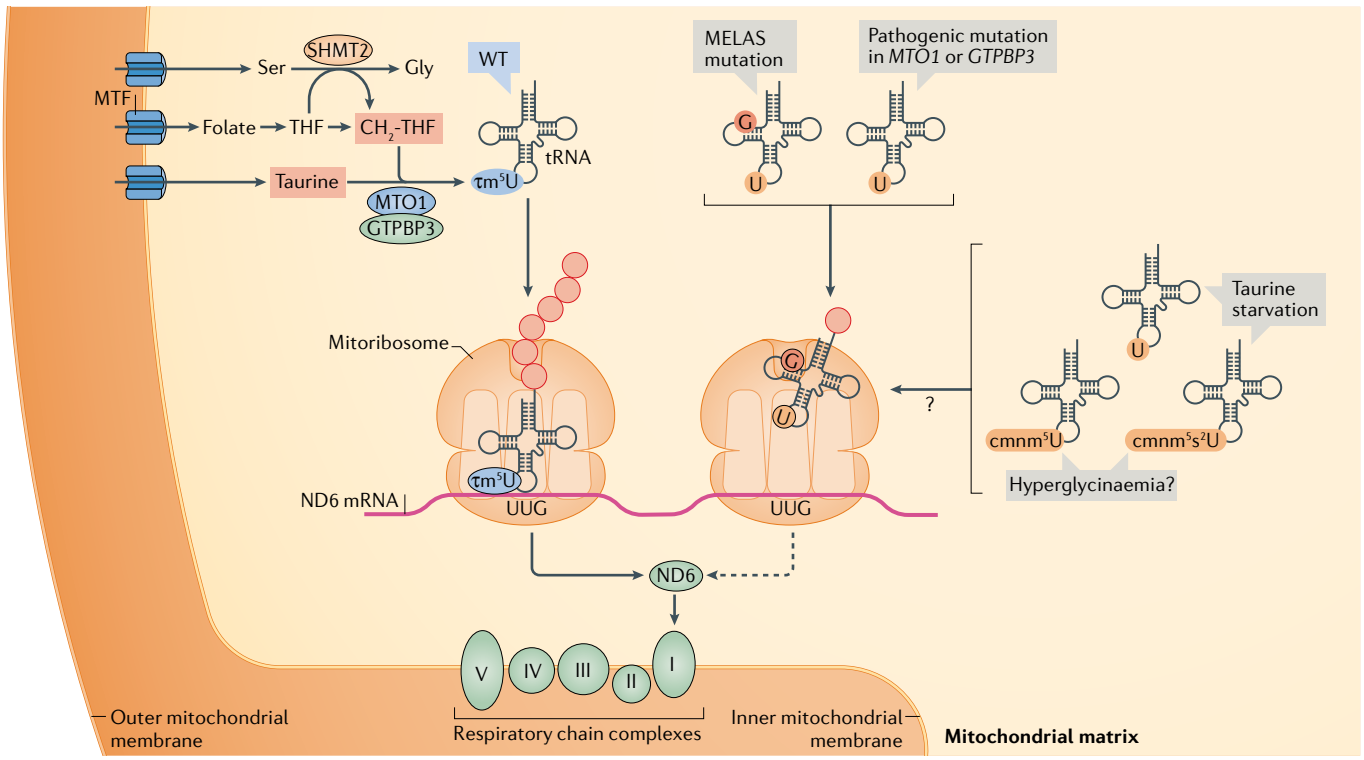


Fig. 5 | **Metabolic and pathological regulation tRNA modifications in human mitochondria.** The transfer RNA (tRNA) modification 5-taurinomethyluridine (τm^{5}U) is biosynthesized from 5,10-methylenetetrahaydrofolate (CH_2-THF) and taurine, which is catalysed by the GTPBP3–MTO1 complex. The modification τm^{5}U in mitochondrial tRNAs (mt-tRNAs) is crucial for efficient translation of mitochondrially encoded proteins of respiratory chain complexes, such as ND6 of complex I, which is essential for oxidative phosphorylation on the mitochondrial inner membrane. Formation of τm^{5}U is impaired by the 3243A>G mutation (G in orange circle) in mt-tRNA$^{Leu(UUR)}$ found in individuals with mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS), as well as by mutations in *MTO1* and *GTPBP3*. The hypomodified tRNA is incapable of decoding the UUG codon efficiently, leading to defective translation of the UUG-rich ND6 mRNA. This is the molecular mechanism of complex I deficiency in such mitochondrial diseases. Taurine starvation also decreases the frequency of τm^{5}U and increases the frequency of unmodified uridine (U). Under this cellular condition, the modifications 5-carboxyaminomethyluridine (cmnm^{5}U) and 5-carboxylmethylaminomethyl-2-thiouridine (cmnm^{5}s^{2}U), in which the taurine moiety of τm^{5}s^{2}U is replaced with glycine, are also introduced into mt-tRNAs. The level of cmnm^5(s^2)U might increase in individuals with hyperglycinaemia. The dynamic regulation of tRNA modification in response to metabolic status implies that translation is regulated in a codon-specific manner in physiological and pathological conditions. MFT, mitochondrial folate transporter; SHMT2, serine hydroxymethyltransferase, mitochondrial; THF, tetrahydrofolate.

In addition to taurine, CH_2-THF is another substrate for τm^5U (REF.[48]). Folic acid derivatives, including CH_2-THF, are metabolites that transfer one carbon atom to various substrates[87]. In mitochondria, serine hydroxymethyltransferase 2 (SHMT2) digests Ser into Gly and transfers the β-carbon from Ser to the methylene group of CH_2-THF (FIG. 5). Consistent with this, τm^5U frequency decreases in cells harbouring mutations in *SHMT2* and in the gene encoding mitochondrial folate transporter (MFT)[48] (FIG. 5). Intriguingly, *SHMT2*-knockout cells exhibit mitochondrial dysfunction, which can be attributed in large part to deficiency of τm^5U in mt-tRNAs[88]. *SHMT2* is highly expressed in various cancers, and suppression of *SHMT2* decreases the tumorigenicity of liver cancer[89,90]. In addition, *SHMT2* expression level decreases with human ageing, thereby decreasing mitochondrial activity[91]. It is therefore not surprising that τm^5U frequency is negatively correlated with ageing. Furthermore, folate deficiency before and after conception causes neural tube defects and spina bifida in the fetus[92]. It is possible that mitochondrial dysfunction owing to reduced levels of τm^5U might be involved in the molecular pathogenesis of this disease.

CO_2-sensitive tRNA modification and the Warburg effect. Another example of metabolic regulation of tRNA modification is N^6-threonylcarbamoyladenosine (t^6A), which is found in all organisms[93]. In t^6A, a Thr residue is bound to the N^6 position of adenine through a carbonyl group[94] (FIG. 6a). t^6A is present at position 37 (t^6A37) (FIG. 2a,b) of tRNAs responsible for decoding ANN codons (position 37 is 3′ adjacent to the anticodon). Structural studies of ribosomes in complex with tRNA and mRNA showed that t^6A stabilizes codon–anticodon pairing by stacking with the adenine base at the first codon[95]. t^6A has crucial roles in various stages of protein synthesis, such as codon recognition, reading frame maintenance, efficient aminoacylation and translocation. In mammals, YRDC and the KEOPS complex are responsible for t^6A37 formation in cytoplasmic tRNAs[96,97], whereas YRDC and OSGEPL1 synthesize t^6A37 in mt-tRNAs[98] (FIG. 6a; Supplementary Tables 1,2). In human mitochondria, t^6A37 is present in four tRNA species[44]. During the biogenesis of t^6A, L-Thr reacts non-enzymatically with CO_2 or HCO_3^- to form a carbamate intermediate (FIG. 6a). YRDC uses ATP to activate this intermediate to form threonylcarbamoyl-AMP (TC-AMP) (FIG. 6a). In cytoplasm, the KEOPS complex transfers the threonylcarbamoyl group of TC-AMP to A37 of the tRNA, thereby forming t^6A37. In mitochondria, OSGEPL1 catalyses the same reaction to form t^6A37 in mt-tRNAs[98] (FIG. 6a). Knockout of *OSGEPL1* eliminates t^6A37 from mt-tRNAs and reduces mitochondrial protein synthesis and respiration[98]. A low level of t^6A37 was found in mt-$tRNA^{Thr}$ carrying the 15923A>G mutation, isolated from cells of an individual with a myoclonic epilepsy with ragged red fibres (MERRF)-like disorder[98], indicating that lack of t^6A37 has pathological consequences.

Kinetic studies have revealed the rate-limiting step of t^6A37 formation in mitochondria. Strikingly, K_m for HCO_3^- is 31 mM, which is extremely high compared with K_m for other substrates[98]. Thus, the cellular concentration of CO_2/HCO_3^- determines the rate of mitochondrial t^6A37 formation. As noted above, in the initial step of t^6A formation, L-Thr reacts with HCO_3^- (CO_2) non-enzymatically to form a carbamate intermediate (FIG. 6a). As this process is in an equilibrium state, high concentration of HCO_3^- (CO_2) is required for efficient carbamate formation, which promotes t^6A formation in the subsequent steps. Thus, the high K_m value for HCO_3^- is a consequence of non-enzymatic carbamate formation. Given that HCO_3^- concentration in mitochondria ranges from 10 to 40 mM (REF.[99]), t^6A37 formation can be dynamically regulated in physiological conditions. When human cells are cultured in CO_2 (HCO_3^-)-depleted conditions, the frequency of t^6A37 in mt-tRNAs is significantly reduced[98]. Given that t^6A37 is essential for protein synthesis, dynamic alteration of t^6A37 frequency would directly affect the decoding ability of tRNAs, indicating that mitochondrial translation is controlled by respiratory conditions as well as by the extracellular environment. Intracellular CO_2 in mitochondria originates largely from the conversion of pyruvate to acetyl-CoA and the tricarboxylic acid cycle during respiration (FIG. 6b). Therefore, in conditions of hypoxia, CO_2 production in mitochondria decreases, and in turn t^6A37 frequency decreases (FIG. 6b). Thus, mitochondrial translation is regulated in a codon-specific manner. In hypoxic conditions, a set of genes related to anaerobic glycolysis are activated through the hypoxia-inducible factor 1α (HIF1α) pathway[100], resulting in massive accumulation of lactate in the cell. In addition, carbonic anhydrase 9 (CA9) is overexpressed on the cell surface as a consequence of HIF1α stabilization, and generates large amounts of bicarbonate and protons[101] (FIG. 6b). The extracellular bicarbonate is used for neutralizing lactate to prevent acidification as well as for supporting t^6A37 biogenesis. However, mitochondrial t^6A37 formation is downregulated in this circumstance, indicating that it serves as a sensor function to monitor hypocarnia in mitochondria. This finding might explain the molecular basis of the Warburg effect[102], in which cells limit mitochondrial function under hypoxia.

Nutritional control of translation by queuosine. Queuosine is a hypermodified guanosine derivative bearing a 7-deazapurine core structure found in bacteria and eukaryotes[103,104]. Queuosine is present at the wobble position of the anticodon in tRNAs responsible for decoding NAY codons. In human cytoplasm, tRNAs for His and Asn have queuosine, while $tRNA^{Asp}$ and $tRNA^{Tyr}$ have mannosyl-queuosine (manQ) and galactosyl-queuosine (galQ), respectively (FIG. 2a). In human mitochondria, queuosine occurs at the wobble positions of four tRNAs — for Tyr, His, Asn and Asp[44]. In bacteria, queuine, a base moiety of queuosine, is biosynthesized in a multistep reaction[105]. As mammals do not have a biosynthetic pathway for queuine, it is obtained as a micronutrient from dietary sources or from the gut microbiota[106]. Loss of queuosine in tRNAs was clearly observed in germ-free mice maintained on a chemically defined diet without queuine[107], indicating that

Warburg effect
Refers to tumours that metabolize glucose anaerobically rather than aerobically even when oxygen is available.

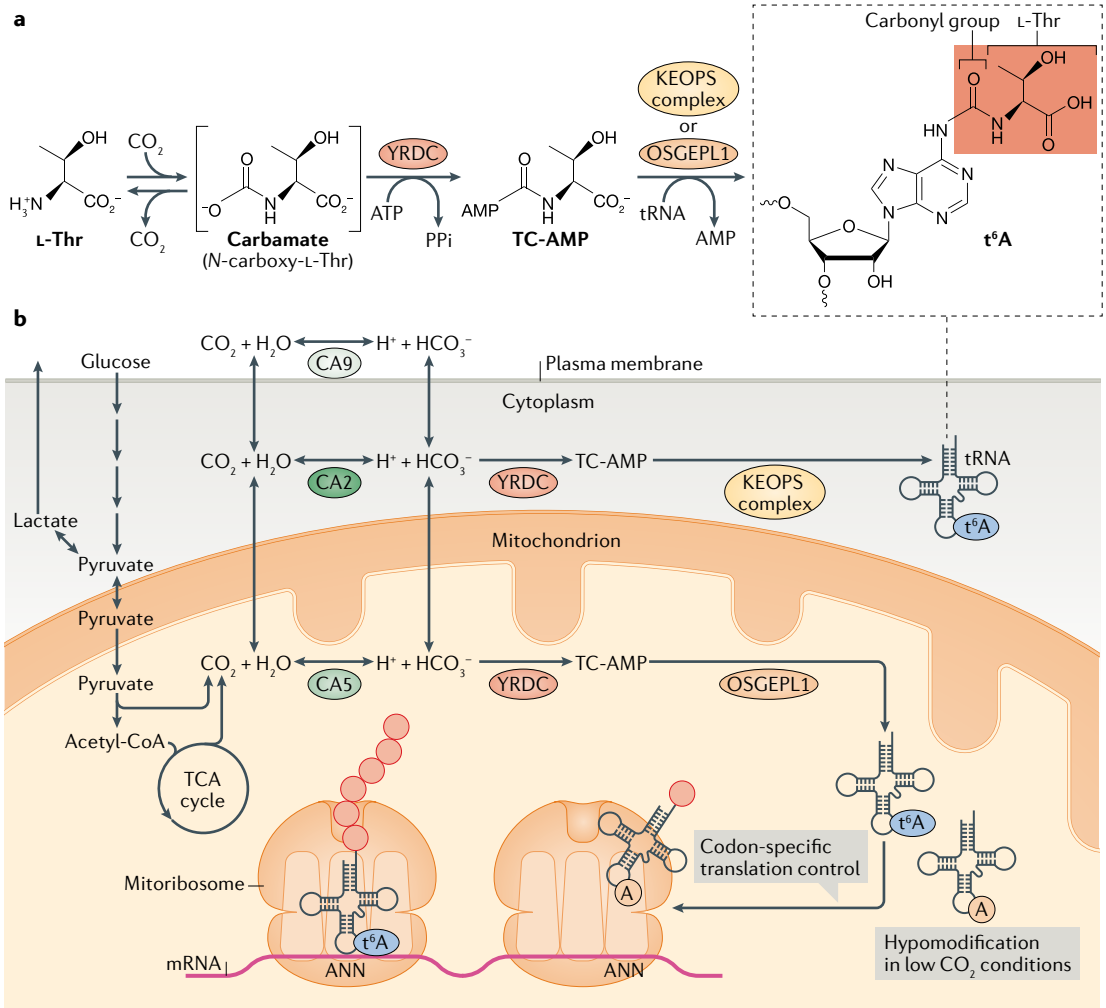


Fig. 6 | **Cellular CO_2 concentration regulates the level of t^6A. a** | Biogenesis of N^6-threonylcarbamoyladenosine (t^6A) in human mitochondria. L-Thr initially reacts with carbon dioxide (CO_2) non-enzymatically to form a carbamate compound. The enzyme YRDC adenylates the carbamate to form a reaction intermediate called 'threonylcarbamoyl-AMP' (TC-AMP). Next, OSGEPL1 or the KEOPS complex use TC-AMP to catalyse the formation of t^6A on tRNA (releasing AMP). **b** | Metabolism of bicarbonate (HCO_3^-) and t^6A formation in the cytoplasm and mitochondria. In mitochondria, CO_2 produced by the tricarboxylic acid (TCA) cycle is hydrated to form bicarbonate by carbonic anhydrase 5 (CA5). Mitochondrial CO_2 is exported to the cytoplasm and hydrated by CA2. YRDC synthesizes TC-AMP from bicarbonate, which in is turn used for the formation of t^6A on transfer RNAs (tRNAs) by the KEOPS complex in the cytoplasm and by OSGEPL1 in mitochondria. In cells cultured without bicarbonate or in hypoxic conditions, t^6A levels in mitochondrial tRNAs decrease, indicating that codon-specific translation regulation is mediated by the sensing of intracellular bicarbonate levels. Under hypoxia, a set of genes related to anaerobic glycolysis, including the gene encoding CA9, are activated by hypoxia-inducible factor 1α (not shown), resulting in massive accumulation of lactate in the cell. The overexpressed CA9 on the cell surface generates large amounts of bicarbonate, which is used to neutralize lactate to prevent acidification and to support t^6A37 biogenesis.

queuosine is dynamically controlled by the nutritional availability of queuine. When queuine and tyrosine were removed from the diet, the germ-free mice died within 2 weeks after birth[106]. Supplementation of queuine rescued the mice. These observations demonstrated that queuosine is a physiologically important tRNA modification; indeed, queuosine has a role in maintaining translation fidelity[108]. Ribosome profiling data revealed that queuosine at position 34 (Q34) enhanced the speed of translation of C-ending codons but reduced that of U-ending codons[109,110]. Loss of queuosine in tRNAs hampered optimal translation and caused protein unfolding, thereby triggering endoplasmic reticulum stress and activation of the unfolded protein response[110].

Q34 is synthesized by tRNA guanine transglycosylase (TGT), which catalyses substitution of guanine with queuine[111]. In eukaryotes, TGT consists of two proteins: the catalytic subunit queuine tRNA-ribosyltransferase catalytic subunit 1 (QTRT1) and the non-catalytic subunit queuine tRNA-ribosyltransferase accessory subunit 2 (QTRT2)[112]. Both QTRT1 and QTRT2 are required for Q34 formation at cytoplasmic tRNAs[113,114] as well as at mt-tRNAs[44]. Mitoribosome profiling of *QTRT2*-knockout human cells revealed that Q34 in mt-$tRNA^{Tyr}$ supports efficient decoding of the UAU codon in mitochondria[44]. Thus, queuosine is a nutritionally controlled tRNA modification that modulates translation elongation in both the cytoplasm and

mitochondria to optimize translation speed, which is necessary for cellular homeostasis.

Diseases of tRNA hypomodification

The physiological importance of tRNA modification has been demonstrated by human diseases caused by aberrant tRNA modification. Given that tRNA-modifying enzymes are ubiquitously expressed in many tissues and cells, it is of great interest to determine why tRNA-modification deficiency frequently causes tissue-specific or organ-specific symptoms. tRNA modifications regulate the activity and steady-state level of tRNAs, thereby optimizing translation speed. Loss of tRNA modification would disrupt optimal translation, leading to mRNA degradation and/or protein misfolding[115]. Characteristic symptoms caused by the loss of specific tRNA modifications imply that different types of tissues and cells have different requirements for fully modified tRNAs.

Mitochondrial diseases. Mitochondrial diseases are disorders caused by mitochondrial dysfunction[116,117]. Because mitochondrial activity is particularly essential in high-energy-consuming tissues and organs, including brain and muscle, mitochondrial diseases are also called 'mitochondrial encephalomyopathies'. Individuals with encephalomyopathies exhibit a wide range of clinical phenotypes, including blindness, deafness, movement disorders and myopathy. Genetic mitochondrial disorders are classified into two groups on the basis of the origin of the mutation: mutations in nuclear genes encoding mitochondrial proteins, and mutations in mtDNA, which encodes 22 tRNAs, 2 rRNAs and 13 mRNAs. Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS)[118] and MERRF[119] are well-characterized classes of mitochondrial encephalomyopathies caused by point mutations in mt-tRNA genes[120,121] (TABLE 1). About 80% of individuals with MELAS have an A-to-G point mutation at position 3243 (3243A>G) in the mt-tRNA^{Leu} gene responsible for the UUR codons[120] (FIG. 5), and another 10% have a 3271T>C mutation in the same tRNA gene[122]. Many individuals with MERRF have an 8344A>G mutation in the mt-tRNA^{Lys} gene[121].

Although such point mutations are strongly associated with specific clinical manifestations, it was not clear how such tRNA mutations result in disease. It is known that some of the mutations in mt-tRNA abolish aminoacylation[123], and the MELAS and MERRF mutant tRNAs showed reduced aminoacylation level to some extent[53,124]. In addition, the steady-state level of the MELAS mutant tRNA decreased, indicating that the mutant tRNA is also destabilized by the mutation[124]. However, the effect of the mutations on aminoacylation and stability is limited. We have isolated these mutant tRNAs from cells of individuals with MELAS and individuals with MERRF, and analysed their post-transcriptional modifications by mass spectrometry. Deficiency in taurine modifications was found in these mutant tRNAs[43,76]. The frequency of τm^{5}U was significantly reduced in mt-tRNA$^{Leu(UUR)}$ harbouring the MELAS mutation[124–126] (FIG. 5); loss of τm^{5}s^{2}U was observed in mt-tRNA^{Lys} harbouring the 8344A>G MERRF mutation;[126,127] and partial modification of m^{1}A at position 58 was also impaired in the same mutant mt-tRNA[128].

The hypomodified mt-tRNA$^{Leu(UUR)}$ lacking τm^{5}U is normally able to decode the UUA codon, but fails to decode the UUG codon efficiently[51] (FIG. 5), indicating that τm^{5}U at position 34 (τm^{5}U34) stabilizes U–G wobble pairing at the A site of the ribosome[50,51]. Ribosome profiling of the cells from individuals with MELAS revealed that mitoribosomes frequently accumulate at the UUG codon[88], demonstrating that τm^{5}U34 is crucial for UUG decoding in mitochondrial translation. As the rate of the MELAS mutation in mtDNA increases, ND6, a mtDNA-encoded subunit of respiratory chain complex I, is synthesized less in mitochondria[129,130] (FIG. 5). Because ND6 has the highest usage of UUG codons among all 13 genes encoded in mtDNA, its translation is severely affected by loss of τm^{5}U in the MELAS mutant tRNA[51]. This molecular mechanism clearly explains why complex I deficiency is the major biochemical symptom of MELAS[118,130]. The MERRF mutant tRNA lacking τm^{5}s^{2}U is unable to efficiently decode either AAA or AAG codons, leading to defective mitochondrial translation and respiratory activity[53]. The tRNA-gene mutations associated with MELAS and MERRF are thought to prevent τm^{5}U-modifying enzymes from recognizing the tRNAs, leading to defects in taurine modification[48]. These findings represent the first example of human disease caused by aberrant RNA modification. Thus, we proposed the term 'RNA modopathy' and suggest it represents a distinct category of human disease[48].

Inspired by reports of τm^{5}U deficiency in individuals with MELAS, the oral administration of a high dose of taurine was investigated in Japan as a potential therapy for MELAS[131]. An expanded clinical trial showed that taurine administration suppressed stroke recurrence in individuals with MELAS[132]. In 2019, taurine administration was officially approved in Japan as a MELAS treatment covered by medical insurance.

Many RNA modopathies have been reported to date[133,134] (TABLE 1). Pathogenic mutations in the gene *MTO1*, which encodes a subunit of the enzymatic complex catalysing the modification of uridine to τm^{5}U, are associated with hypertrophic cardiomyopathy and lactic acidosis[135,136] (TABLE 1; Supplementary Table 2). Individuals with these mutations exhibit mitochondrial dysfunction characterized by reduction in oxygen consumption and respiratory chain complex activity. *Mto1*-knockout mouse embryos die at an early developmental stage, and heart-specific *Mto1*-conditional-knockout newborn mice die in 24 hours[137]. These observations demonstrate that τm^{5}U is essential for animal development. *Mto1*-knockout cells exhibit abnormal mitochondrial protein synthesis[48,137] (FIG. 5) and the accumulation of protein aggregates in the cytoplasm[137]. Mutations in *GTPBP3*, which encodes another subunit of the enzymatic complex that catalyses the τm^{5}U modification, are also associated with hypertrophic cardiomyopathy and lactic acidosis[138] (TABLE 1; Supplementary Table 2). However, unlike individuals with *MTO1* mutations, those with *GTPBP3* mutations develop Leigh syndrome, which is a progressive encephalopathy associated with mitochondrial

Table 1 | **Human diseases caused by aberrant tRNA modifications**

Category	Disease	Gene	RNA modification	tRNA species	Refs
Mitochondrial disease	MELAS	mt-tRNA$^{Leu(UUR)}$ gene	τm^{5}U	mt-tRNA	43,76
	MERRF	mt-tRNA^{Lys} gene	τm^{5}s^{2}U	mt-tRNA	43,76
	Hypertrophic cardiomyopathy and lactic acidosis	*MTO1*	τm^{5}U	mt-tRNA	135,136
	Hypertrophic cardiomyopathy and lactic acidosis and encephalopathy	*GTPBP3*	τm^{5}U	mt-tRNA	138
	RILF	*MTU1*	τm^{5}s^{2}U	mt-tRNA	139,140
	Combined mitochondrial respiratory chain complex deficiency, early-onset mitochondrial encephalomyopathy and seizures	*NSUN3*	f^{5}C	mt-tRNA	69,141
	MLASA	*PUS1*	Ψ	cyto-tRNA, mt-tRNA	142,143
	Encephalopathy and myoclonic epilepsy with multiple OXPHOS deficiencies	*TRIT1*	i^{6}A	cyto-tRNA, mt-tRNA	219
	Multiple mitochondrial respiratory chain complex deficiencies	*TRMT5*	m^{1}G	cyto-tRNA, mt-tRNA	220
Neurological disorder	Familial dysautonomia	*ELP1*	mcm^{5}U and derivatives	cyto-tRNA	46,149,153
	Intellectual disability	*ELP2*	mcm^{5}U and derivatives	cyto-tRNA	155
	Amyotrophic lateral sclerosis	*ELP3*	mcm^{5}U and derivatives	cyto-tRNA	156
	Galloway–Mowat syndrome	KEOPS complex genes (*OSGEP*, *TP53RK*, *TPRKB*, *LAGE3*)	t^{6}A	cyto-tRNA	157,158
	Intellectual disability	*TRMT1*	m^{2,2}G	cyto-tRNA, mt-tRNA	161
	Intellectual disability	*FTSJ1*	2′-O methylation	cyto-tRNA	162
	Intellectual disability, DREAM-PL	*CTU2*	mcm^{5}s^{2}U	cyto-tRNA	163
	Intellectual disability, strabismus, microcephaly, growth delay	*ADAT3*	I	cyto-tRNA	164
	Developmental delay, epileptic encephalopathy	*DALRD3*	m^{3}C	cyto-tRNA	165
	Diabetes, microcephaly and intellectual disability	*TRMT10A*	m^{1}A, m^{1}G	mt-tRNA	221
	Intellectual disability, HSD10 disease	*SDR5C1*	m^{1}A, m^{1}G	mt-tRNA	222
	Severe neurodevelopmental defects	*PUS3*	Ψ	cyto-tRNA, mt-tRNA	223–225
	Intellectual disability, microcephaly, short stature and aggressive behaviour	*PUS7*	Ψ	cyto-tRNA	226
	Galloway–Mowat syndrome, microcephaly	*YRDC*, KEOPS complex genes	t^{6}A	cyto-tRNA	158,227
Cancer	Intellectual disability; skin, breast and colorectal cancer	*NSUN2*	m^{5}C	cyto-tRNA, mt-tRNA	160
	Microcephaly, dwarfism, cancer	*METTL1*, *WDR4*	m^{7}G	cyto-tRNA	171,172
	Colon cancer, breast cancer	*TYW2* (also known as *TRMT12*)	yW	cyto-tRNA	189,228,229
	Bladder cancer, intellectual disability	*ALKBH8*	mchm^{5}U and derivatives	cyto-tRNA	200,201
	Breast cancer	*TRMT2A*	m^{5}U	cyto-tRNA	196
	Colorectal cancer	*NAT10*	ac^{4}C	18S rRNA, cyto-tRNA	191,194
Diabetes	Type 2 diabetes	*CDKAL1*	ms^{2}t^{6}A	cyto-tRNA	202,204

Abbreviations of RNA modifications conform with MODOMICS[26]. ac^{4}C, N^4-acetylcytidine; cyto-tRNA, cytosolic transfer RNA; DREAM-PL, dysmorphic facies, renal agenesis, ambiguous genitalia, microcephaly, polydactyly and lissencephaly; f^{5}C, 5-formylcytidine; I, inosine; i^{6}A, N^6-isopentenyladenosine; KEOPS complex, kinase, putative endopeptidase and other proteins of small size complex; m^{1}A, 1-methyladenosine; m^{3}C, 3-methylcytidine; m^{5}C, 5-methylcytidine; mchm^{5}U, 5-(carboxyhydroxymethyl)uridine methyl ester; mcm^{5}s^{2}U, 5-methoxycarbonylmethyl-2-thiouridine; mcm^{5}U, 5-methoxycarbonylmethyluridine; MELAS, mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes; MERRF, myoclonic epilepsy with ragged red fibres; m^{1}G, 1-methylguanosine; m^{2,2}G, N^2,N^2-dimethylguanosine; m^{7}G, 7-methylguanosine; MLASA, myopathy, lactic acidosis and sideroblastic anaemia; ms^{2}t^{6}A, 2-methylthio-N^6-threonylcarbamoyladenosine; mt-tRNA, mitochondrial transfer RNA; m^{5}U, 5-methyluridine; OXPHOS, oxidative phosphorylation; RILF, reversible infantile liver failure; rRNA ribosomal RNA; t^{6}A, N^6-threonylcarbamoyladenosine; tRNA, transfer RNA; yW, wybutosine; τm^{5}s^{2}U, 5-taurinomethyl-2-thiouridine; τm^{5}U, 5-taurinomethyluridine; Ψ, pseudouridine.

dysfunction[138]. *MTU1* (also known as *TRMU*) encodes mitochondrial tRNA-specific 2-thiouridylase 1, which catalyses the formation of τm^5s^2U (REF.[77]) (Supplementary Table 2). Loss-of-function mutations in *MTU1* reduce the frequency of τm^5s^2U in mt-tRNAs, resulting in impairment of mitochondrial translation through respiratory deficiency, and are associated with reversible infantile liver failure[139] (TABLE 1). *Mtu1*-knockout mice exhibit embryonic lethality and die at embryonic day 7.5–8, demonstrating that 2-thiolation of τm^5s^2U in mt-tRNAs is essential for development[140]. Liver-specific *Mtu1*-conditional-knockout mice exhibit lactic acidosis and indications of liver inflammation and injury[140]. This observation demonstrates that *MTU1* deficiency is a direct cause of reversible infantile liver failure[139].

As mentioned earlier, in human mitochondria, f^5C34 is essential for deciphering the AUA codon as Met[65,66] (FIG. 4c). NSUN3 methylates C34 to form 5-methylcytidine (m^5C34)[68–70], followed by hydroxylation and oxidation to form f^5C34, which is mediated by ALKBH1 (REFS[70,71]). Upon knockout of either of the genes encoding these enzymes, f^5C34 is not formed in mt-$tRNA^{Met}$, and mitochondrial translation is markedly reduced, demonstrating that f^5C34 is essential for mitochondrial function. Consistent with this, loss-of-function mutations in *NSUN3* have been detected in an individual with combined mitochondrial respiratory chain complex deficiency[69] (TABLE 1). Recently, novel biallelic missense mutations were found in an individual with early-onset mitochondrial encephalomyopathy and seizures[141] (TABLE 1).

Missense mutations in the gene encoding tRNA pseudouridine synthase A (*PUS1*) have been reported in individuals with myopathy, lactic acidosis and sideroblastic anaemia (MLASA)[142,143] (TABLE 1). Reduction in Ψ levels was observed in tRNAs extracted from MLASA cells, suggesting that the hypomodified tRNAs decrease the efficiency of protein synthesis. PUS1 introduces Ψ not only into mt-tRNAs but also into cytoplasmic tRNAs and mRNAs[144–146], indicating that MLASA might have multiple causes.

Neurological disorders. Familial dysautonomia is a genetic disease that impairs the sensory and autonomic nervous systems[147,148]. Abnormal splicing of elongator complex protein 1 (ELP1) (FIG. 7a) is a major cause of familial dysautonomia[149] (TABLE 1). ELP1 is an essential factor for the biosynthesis of mcm^5U and its derivatives, which are present at position 34 in the anticodon of cytoplasmic tRNAs[150–152] (FIG. 7a; Supplementary Table 1). Indeed, reduced levels of the mcm^5U derivatives have been observed in tRNAs from the cells of individuals with familial dysautonomia[46,153]. *Elp1*-knockout mice show embryonic lethality at embryonic day 10, likely owing to impaired cardiovascular development and function[154], indicating that mcm^5U34 is essential for animal development. Similarly, mutations in *ELP2*, which encodes another elongator subunit involved in mcm^5U34 biogenesis (FIG. 7a), are associated with intellectual disability[155] (TABLE 1), and *ELP3* mutations are associated with amyotrophic lateral sclerosis[156] (TABLE 1). Because mcm^5U34 and its derivatives are crucial for accurate decoding, loss of mcm^5U34 impairs protein synthesis, leading to abnormal development and degeneration of the nervous system.

A homozygous missense mutation associated with global developmental delay with renal defect was found in the *OSGEP* gene (TABLE 1), which encodes an essential component of the KEOPS complex[157]. The KEOPS complex is responsible for the biogenesis of t^6A at position 37 of cytoplasmic tRNAs[93] (FIG. 6a,b; Supplementary Table 1). In addition, mutations were found in genes encoding four components of the KEOPS complex in 32 families with Galloway–Mowat syndrome[158] (TABLE 1), which is an autosomal recessive disease characterized by the combination of early-onset nephrotic syndrome and microcephaly with brain anomalies[159], indicating that defective biogenesis of t^6A in cytoplasmic tRNAs results in severe inherited disorders. It is likely that t^6A in cytoplasmic tRNAs is essential for cell survival[98].

Other genes responsible for tRNA modifications associated with intellectual disability include the tRNA methyltransferase genes *NSUN2* (REF.[160]), *TRMT1* (REF.[161]) and *FTSJ1* (REF.[162]), the tRNA thiouridylase gene *CTU2* (REF.[163]) and the tRNA adenosine deaminase gene *ADAT3* (REF.[164]) (TABLE 1). Very recently, DALRD3 was identified to be in complex with the tRNA methyltransfeases METTL2A and METTL2B (Supplementary Table 1), each of which introduces the modification m^3C at position 32 of $tRNA^{Arg}$; a nonsense mutation in *DALRD3* was found in an individual exhibiting developmental delay and early-onset epileptic encephalopathy[165] (TABLE 1).

Cancer. Growing evidence suggests that modulated expression of specific tRNAs contributes to cancer progression[166–169]. Recent studies show that dysregulation of translation through aberrant tRNA modification is associated with cell transformation in cancer[170]. It has been reported that expression of tRNA-modifying enzymes is deregulated in various cancers. Overexpression of *NSUN2* and *METTL1* is widely observed in various cancers[171,172] (TABLE 1). Simultaneous deletion of *NSUN2* and *METTL1* in HeLa cells renders them sensitive to the cancer drug 5-fluorouracil (5-FU)[173], indicating that targeting tRNA modification is a promising strategy for cancer chemotherapy. In budding yeast, the cytotoxic effect of 5-FU is strongly associated with deficient tRNA modification, especially at high cultivation temperature[174]. If 5-FU is partially incorporated into a tRNA molecule during transcription, the resulting hypomodified tRNA might be destabilized and eliminated by a tRNA surveillance system called RTD[74].

NSUN2-mediated m^5C formation in tRNAs is required for normal development, and its dysregulation is implicated in cancer[170,175]. Loss of the *NSUN2* gene causes growth retardation and neurodevelopmental defect in humans and mice[160,176–178] (TABLE 1). In mouse skin tumours, tumour-initiating cells exhibit lower translation levels compared with committed progenitor cells; deletion of *Nsun2* enhances the self-renewal potential of tumour-initiating cells and promotes tumorigenesis[175].

In drug-resistant melanomas, the phosphoinositide 3-kinase signalling pathway enhances the expression

Elongator complex
A transfer RNA-modifying enzyme complex catalysing 5-methoxyarbonylmethyluridine (mcm^5U) formation.

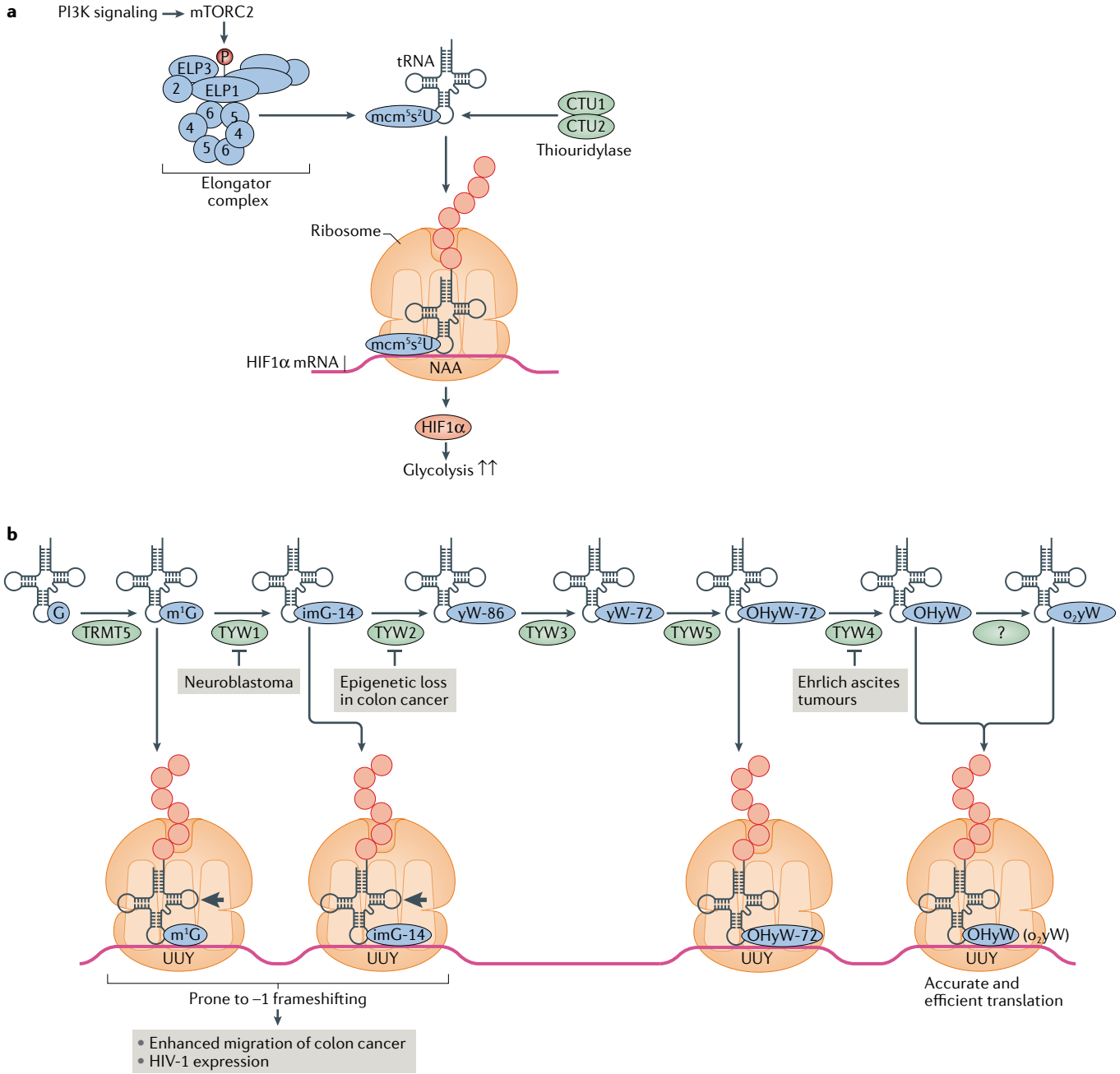


Fig. 7 | **Codon-specific translation control mediated by tRNA modifications in cancer. a** | Codon-specific translation control by 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U). In drug-resistant melanoma, the phosphoinositide 3-kinase (PI3K) signalling pathway activates mechanistic target of rapamycin complex 2 (mTORC2), which phosphorylates Ser1174 of elongator complex protein 1 (ELP1), thereby increasing ELP1 and ELP3 levels in the elongator complex. The elongator complex introduces the 5-methoxycarbonylmethyluridine (mcm^5U) modification derivative mcm^5s^2U into transfer RNAs (tRNAs) for Lys, Glu and Gln, thereby promoting the decoding of NAA codon-rich mRNAs such as the hypoxia-inducible factor 1α (HIF1α) mRNA and thus anaerobic glycolysis and tumour growth. Cytoplasmic tRNA 2-thiolation protein 1 (CTU1) and CTU2 constitute a thiouridylase that is responsible for 2-thiolation of mcm^5s^2U. **b** | Alteration of wybutosine (yW) derivatives in $tRNA^{Phe}$ in cancer. The modification yW and its derivatives, hydroxywybutosine (OHyW) and peroxywybutosine (o_2yW), are found at position 37 of eukaryotic $tRNA^{Phe}$. Derivatives of yW, which are crucial for efficient codon recognition and reading frame maintenance, are biosynthesized by a multistep reaction mediated by tRNA (guanine(37)-N1)-methyltransferase (TRMT5), TYW1, TYW2, TYW3, TYW5 and TYW4. Deficiency of yW derivatives has been found in hepatoma, neuroblastoma, Ehrlich ascites tumours and colorectal cancer. *TYW2* is epigenetically silenced through DNA hypermethylation of its promotor region in colorectal cancers. The hypomodified $tRNA^{Phe}$ induces −1 ribosome frameshifting, thereby enhancing migration and epithelial–mesenchymal transition in colorectal cancer. The hypomodified $tRNA^{Phe}$ augments the replication efficiency of human immunodeficiency virus 1 (HIV-1) RNA, which is mediated through −1 frameshifting in the *gag–pol* open reading frame that is required to produce functional reverse transcriptase. The numerical suffix in each modification represents a different molecular mass from its parental modification. imG-14, 4-demethylwyosine; m^1G, 1-methylguanosine; OHyW-72, 7-aminocarboxypropylhydroxywyosine; yW-72, 7-aminocarboxypropylwyosine; yW-86, 7-aminocarboxypropyldemethylwyosine.

of *ELP1*, which results in efficient introduction of the mcm^5s^2U modification into tRNAs for Lys, Gln and Glu (FIG. 7a; Supplementary Table 1). Those fully modified tRNAs contribute to the efficient translation of the HIF1α mRNA, thereby inducing anaerobic glycolysis that promotes tumour growth[179,180]. Thus, the mcm^5s^2U modification in the tRNA anticodon is a key regulator of translation of NAA codon-rich mRNAs, including HIF1α mRNA (FIG. 7a).

Hypomodification of tRNA is frequently found in cancer. Wybutosine (yW) and its derivatives hydroxywybutosine (OHyW) and peroxywybutosine (o_2yW) are tricyclic nucleosides with large side chains found at position 37 of eukaryotic $tRNA^{Phe}$, and are crucial for efficient codon recognition and reading frame maintenance[181,182]. Derivatives of yW are biosynthesized in a multistep reaction initiated by m^1G formation by tRNA (guanine(37)-N^1)-methyltransferase (TRMT5)[183], and followed by TYW1, TYW2, TYW3, TYW5 and TYW4 (REFS[184–186]) (FIG. 7b; Supplementary Table 1). Deficiency of yW derivatives has been found in hepatoma[187], neuroblastoma, Ehrlich ascites tumours[188] and colorectal cancer[189]. *TYW2* is silenced through DNA hypermethylation of its promotor region in colorectal cancers[189] (FIG. 7b; TABLE 1). The hypomodified $tRNA^{Phe}$ induces −1 ribosome frameshifting (FIG. 7b), thereby dysregulating mRNA abundance through nonsense-mediated RNA decay. Epigenetic loss of TYW2 confers enhanced migration and epithelial–mesenchymal transition in colorectal cancer[189]. The hypomodified $tRNA^{Phe}$ also influences the replication efficiency of human immunodeficiency virus 1 RNA, which is mediated by −1 frameshifting in the *gag–pol* reading frame required to produce functional reverse transcriptase[190]. *N*-Acetyltransferase 10 (NAT10; also known as RNA cytidine acetyltransferase) is highly expressed in malignant tumours[191,192], and is also a promising therapeutic target in laminopathies and premature ageing[193]. NAT10 was found to be an ATP-dependent RNA acetyltransferase responsible for the formation of N^4-acetylcytidine (ac^4C) at position 1842 in helix 45 (REF.[194]) and at position 1337 in helix 34 (REF.[195]) of human 18S rRNA. In addition, NAT10 uses an adaptor protein, THUMPD1, to form ac^4C at position 12 of cytoplasmic tRNAs[195] (Supplementary Table 1). Inactivation of NAT10 induced apoptosis and growth retardation in human culture cells, and this was accompanied by high-level accumulation of the 18S rRNA precursor[194,195], suggesting that NAT10-mediated ac^4C formation is crucial for rRNA processing and ribosome biogenesis. The ac^4C12 modification might also contribute to tRNA stability and function, and additional studies are necessary to fully reveal the biological significance of NAT10–THUMPD1 function.

TRMT2A is a tRNA methyltransferase that catalyses the formation of 5-methyluridine (m^5U) at position 54 of cytoplasmic tRNAs (Supplementary Table 1). TRMT2A is highly expressed in $HER2^+$ breast cancer cells and could be a biomarker of increased risk of recurrence in patients[196].

ALKBH8 is a dioxygenase and a tRNA methyltransferase responsible for biogenesis of 5-methoxycarbonylmethyluridine ($mchm^5U$) and its derivatives in the anticodon of cytoplasmic tRNAs[197–199] (Supplementary Table 1). ALKBH8 is highly expressed in human bladder cancers[200]. Silencing of ALKBH8 significantly suppressed invasion, angiogenesis and growth of an in vivo model of human urothelial carcinoma cells through induction of apoptosis[200]. Recessive truncating mutations in *ALKBH8* cause intellectual disability with severe impairment of tRNA wobble modifications[201].

Diabetes. The *CDKAL1* gene is associated with type 2 diabetes across ethnic groups[202] (TABLE 1). CDKAL1 (also known as threonylcarbamoyladenosine tRNA methylthiotransferase) is a tRNA-modifying enzyme that introduces the 2-methylthio group of the modification 2-methylthio-N^6-threonylcarbamoyladenosine (ms^2t^6A), which is present at position 37 of cytoplasmic $tRNA^{Lys}$ (REFS[203,204]) (Supplementary Table 1). The level of ms^2t^6A at position 37 is greatly reduced in the pancreas of β-cell-specific *Cdkal1*-knockout mice[204]; these mice lose control of blood glucose levels and have lower rates of insulin secretion. The low expression of proinsulin in β-cells might be due to the low translational capacity of the hypomodified $tRNA^{Lys}$. A recent study showed that mice lacking iron-regulatory protein 2 (IRP2) develop diabetes[205]. Lack of IRP2 dysregulates cellular iron homeostasis mediated by transferrin receptor 1 and ferritin, which causes iron deficiency in β-cells. As CDKAL1 requires an iron–sulfur cluster for its enzymatic activity, iron loss strongly impairs CDKAL1 activity and reduces ms^2t^6A levels at $tRNA^{Lys}$, leading to inefficient translation of proinsulin.

Conclusions and future perspective

A number of tRNA modifications were discovered in the 1970s and the 1980s. Following these discoveries, study of tRNA modifications has been sluggish for a long time. Recent advancement in epitranscriptomics renewed the interest in tRNA modification. Structural and functional roles of tRNA modifications have been extensively studied in bacteria and yeast. Research on tRNA modifications in human and mammals, however, has lagged behind. To understand the functional and physiological roles of tRNA modifications in human, comprehensive analysis of each tRNA modification is necessary. It is also essential to determine all the human proteins responsible for forming tRNA modifications, and the spatio-temporal regulation of their genes in various biological contexts, to enable us to understand the global dynamics of tRNA modifications.

Why do tRNAs have such complex and diverse modifications? Why does lack of a tRNA-modifying enzyme cause characteristic symptoms? Do tRNA modifications change their frequency during development and cell differentiation, or in various environmental conditions? To answer these questions, it is indispensable to develop an innovative technology for measuring tRNA modification status more easily and accurately. Even if the expression level of a tRNA-modifying enzyme changes in a certain context, the tRNA modification status does not always correlate with the expression level of the cognate tRNA-modifying enzyme because most tRNA modifications are not reversible and tRNA has quite a long

lifetime in the cell. Many studies of tRNA modification do not actually analyse the tRNA modification status in their various experimental conditions. Nucleoside analysis by liquid chromatography–mass spectrometry of total tRNA fractions is a conventional and general method to quantify tRNA modifications[206–211]. However, it is impossible to grasp the tRNA modification status at a specific site, especially when tRNA abundance changes. Thus, we need a highly sensitive analytical method to accurately and comprehensively analyse the status of individual tRNA modifications in a limited quantity of tissues or clinical specimens. RNA mass spectrometry is capable of mapping and quantifying RNA modifications at any given site accurately and reliably because it can detect both modified and unmodified RNA fragments digested by residue-specific RNases[44,98,208,212]. However, RNA mass spectrometry requires considerable amounts of material and is not suitable for high-throughput analyses of RNA modifications in multiple analytes. Sequencing-based methods are highly sensitive and suitable for detecting several tRNA modifications from small numbers of cells and clinical specimens, although this method is applicable only for specific tRNA modifications[38,39,42]. Nanopore-based sequencing is a unique method capable of directly analysing RNA molecules without converting them into cDNA[213]. When an RNA molecule passes through the nanopore, a characteristic ion-current signal originating from a modified residue is observed. If the base call accuracy for RNA modification is increased in the future, nanopore-based sequencing will become the most promising method for detecting and quantifying tRNA modifications at a single-molecule level[214–217].

Finally, I would like to discuss potential reader proteins that could recognize and bind tRNA modifications. To date, a reader of tRNA modification has not been reported. However, as tRNA modifications function as determinants of aminoacylation, in a certain sense, aa-tRNA synthetases might function as readers of tRNA modifications. In addition, because lack of tRNA modification destabilizes the tRNA tertiary structure, hypomodified tRNAs are degraded by RTD. Some factors involved in RTD might have a role in quality control of tRNA by sensing the tRNA modification status. Recently, tRNA modifications in tRNA-derived fragments (tRFs; degradation intermediates of tRNAs) have been attracting attention. Pseudouridine synthase 7-mediated pseudouridylation at position 8 (Ψ8) of tRFs has an important role in controlling protein synthesis in stem cells[218]. In the translation initiation complex, tRFs bearing Ψ8 sequester polyadenylate-binding protein 1 (PABPC1) and downregulate translation initiation, whereas tRFs lacking Ψ8 do not, indicating that PABPC1 is a reader of Ψ8. As tRFs are abundant molecules produced in various stress conditions, many researchers are searching for their physiological roles, and so diverse tRF modifications might be revealed in the future.

Published online 3 March 2021

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Acknowledgements
The author is grateful to members of his laboratory, in particular K. Miyauchi, T. Ohira and K. Minowa, for preparing tables and figures and for providing valuable suggestions. This work was supported by Grants-in-Aid for Scientific Research (18H05272) from JSPS, and Exploratory Research for Advanced Technology (ERATO; JPMJER2002) from JST. The author apologizes to those of his colleagues whose many contributions related to tRNA modifications could not be cited in this Review owing to space limitations.

Competing interests
The author declares no competing interests.

Peer review information
Nature Reviews Molecular Cell Biology thanks Juan Alfonzo, Tao Pan and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

Supplementary information
The online version contains supplementary material available at https://doi.org/10.1038/s41580-021-00342-0.