BBA - Gene Regulatory Mechanisms 1861 (2018) 419–432

Contents lists available at ScienceDirect

BBA - Gene Regulatory Mechanisms

journal homepage: www.elsevier.com/locate/bbagrm

Transfer RNA modification and infection – Implications for pathogenicity and host responses

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ARTICLE INFO

Keywords:
Transfer RNA
Post-translational modification
Infection
Stress
Host-pathogen interaction

ABSTRACT

Transfer RNA (tRNA) molecules are sumptuously decorated with evolutionary conserved post-transcriptional nucleoside modifications that are essential for structural stability and ensure efficient protein translation. The tRNA modification levels change significantly in response to physiological stresses, altering translation in a number of ways. For instance, tRNA hypomodification leads to translational slowdown, disrupting protein homeostasis and reducing cellular fitness. This highlights the importance of proper tRNA modification as a determinant for maintaining cellular function and viability during stress. Furthermore, the expression of several microbial virulence factors is induced by changes in environmental conditions; a process where tRNA 2-thiolation is unequivocal for pathogenicity. In this review, we discuss the multifaceted implications of tRNA modification for infection by examining the roles of nucleoside modification in tRNA biology. Future development of novel methods and combinatory utilization of existing technologies will bring tRNA modification-mediated regulation of cellular immunity and pathogenicity to the limelight.

1. Introduction

Infection is defined as the establishment of a pathogen in its host after invasion. Achieving a successful infection is everything but straightforward, as the pathogen has to encounter a suitable host, attach to and penetrate various mechanical barriers while competing with the commensal normal flora, adapt to a hostile environment that is often scarce on nutrients and laced with toxic metabolites and enzymes, as well as to avoid detection by the host immune system, be it specialized immune cells or rapidly switching signaling pathways and regulatory mechanisms [1]. To achieve this tall feat, pathogens need to rapidly adapt to continuously changing environmental conditions. Factors that affect microbial virulence include: (i) accurate sensing of the environment, i.e. so-called quorum sensing; (ii) secretion of chemicals into the surroundings, often via dedicated secretion systems; (iii) adhering to and colonization of vast areas via biofilm formation; (iv) directed motility; and (v) growth at elevated temperatures [2,3]. These factors are triggered by external signals that indicate poor growth conditions or hostile environments, including high temperature, pH changes, the presence or absence of certain nutrients, cell density, and many more. Continuous adaptation to such physiological stresses necessitates a rapid regulation of transcription, translation, and protein modification. Furthermore, many microbial pathogens have a higher-than-average degree of genomic instability with frequent mutations in specific hot spots, such as the repetitive sequences of minisatellites, telomere regions, and, somewhat surprisingly, genes encoding for transfer RNAs [4].

During recent years, ever more complex regulatory functions have been assigned to various non-coding RNAs, such as microRNAs, long non-coding RNAs, small nuclear/nucleolar RNAs, and other small regulatory RNAs. These RNAs have been reported to facilitate the expression of virulence-associated functions in numerous microbes [4,5] and to regulate host-pathogen interactions, ranging from viral [6] to parasitic infections [7]. Despite intense focus on the link between microbial pathogenicity and in particular microRNAs, far less attention has been paid to the various cellular functions mediated by the most prevalent group of all non-coding RNAs, the transfer RNAs.

Transfer RNAs (tRNAs) are essential adapter molecules in translation that carry specific amino acids and, by complimentary codon-anticodon base pairing, ensure the incorporation of the correct amino acid sequence in the nascent polypeptide [8]. Although tRNAs perform such a vital function, we are only at the verge of uncovering the exact mechanisms by which tRNA interacts with messenger RNA transcripts and the ribosome, as well as the intricate regulatory functions mediated by post-transcriptional RNA modification. A mature tRNA molecule is richly decorated with numerous evolutionary conserved nucleoside

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https://doi.org/10.1016/j.bbagrm.2018.01.015
Received 29 November 2017; Received in revised form 4 January 2018; Accepted 19 January 2018
Available online 31 January 2018

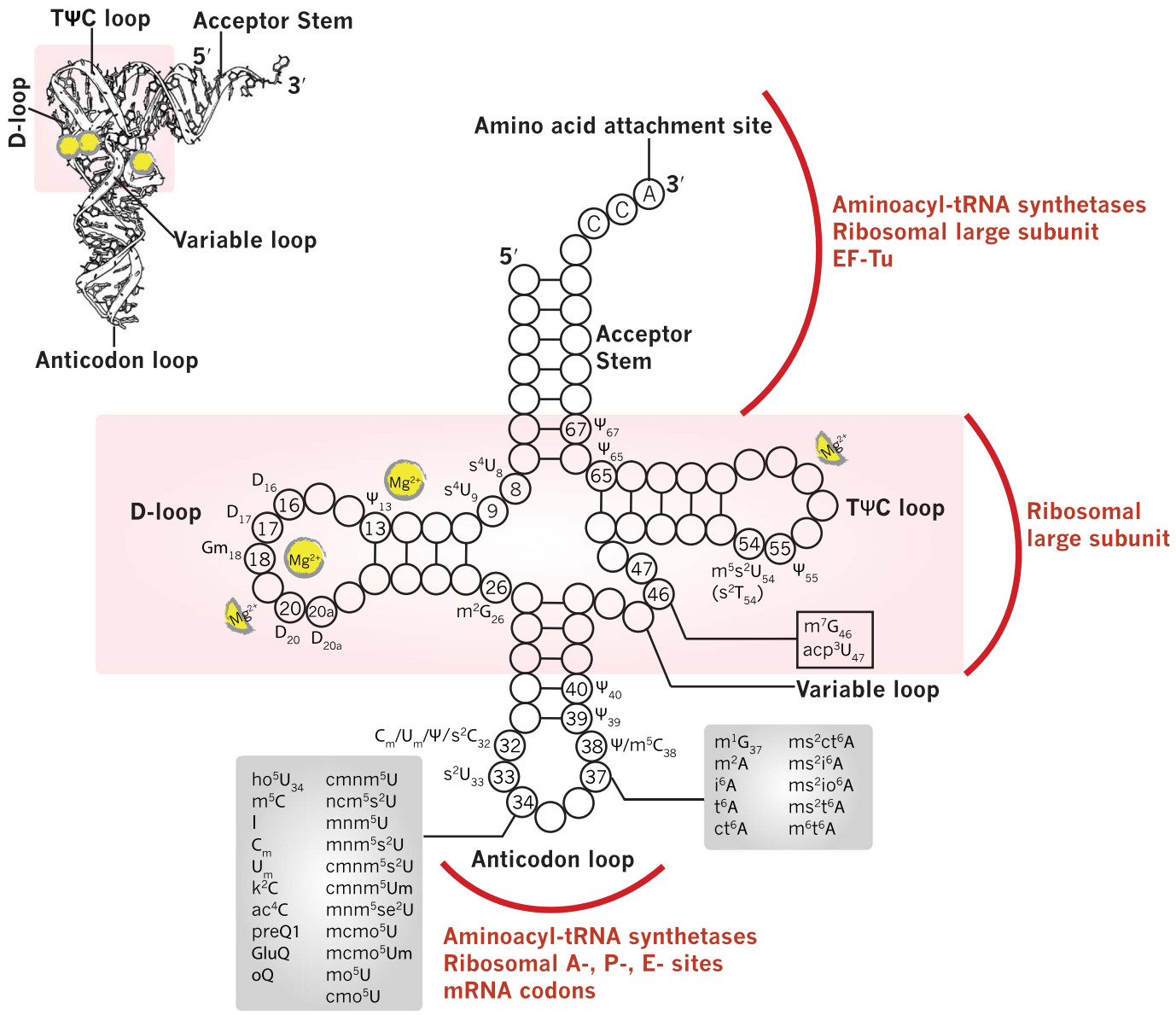

Fig. 1. Transfer RNA molecules are post-transcriptionally modified with functional groups that confer structural stability and modulate codon-anticodon interactions. Schematic two-dimensional cloverleaf representation of a tRNA molecule, and its characteristic three-dimensional L-shaped conformation (top left panel) following TΨC- and D-loop interaction. Structural domains are denoted in black, aminoacyl-tRNA synthetase and ribosomal subunit interaction sites are indicated in red. The putative magnesium ions are positioned based on the crystal structure of *S. cerevisiae* phenylalanine tRNA (PDB ID 1TN2). Frequently occurring (prokaryotic) nucleoside modifications are highlighted. Note the abundance of modifications at or near the anticodon loop, in particular at wobble position 34. Nucleoside modification nomenclature and abbreviations according to the Modomics convention [11].

modifications (Fig. 1) that occur throughout all domains of life – and even beyond, as some viruses carry tRNA-like molecules and nucleoside modifying enzymes [9]. Many modifications are simple additions or substitutions of functional groups, such as methyl (CH_3), amine (NH_2), and thiol (S) groups, whereas others have complex structures, whose biosynthesis require the interplay of numerous enzymatic steps and pathways [10]. Despite their apparent simplicity, > 150 modifications have been reported so far, of which > 90 are found in tRNA [11]. These tRNA modifications are highly dynamic and their prevalence varies according to the metabolic state of the cell [12]. Intriguingly, some modifications are redundant during normal growth and their function is uncovered only once the cell is exposed to physiological stress, such as sudden changes in the environment or a pathogenic infection. This suggests that tRNA modifications are important for modulating general stress responses in the cell, and possibly also for host responses to infection, such as pathogen-associated molecular pattern (PAMP)-triggered immunity in plants [13] or cytokine-induced immunity in metazoans [14,15]. Furthermore, perturbation of tRNA modification has been associated with a number of diseases and disabilities in humans, including developmental and neurological dysfunctions, cancers of lymphoma, leukemia and carcinoma, as well as type II diabetes (reviewed in [16,17]).

To date, the role of tRNA modification following physiological stress, such as oxidation, temperature, pH, salinity, nutrient starvation, etc. is rather well established for several prokaryotic and eukaryotic organisms. However, the implications of tRNA modification for infection – be it in terms of pathogenicity or host cell responses – remain to be elucidated. Nonetheless, several studies on bacterial and fungal pathogens have highlighted tRNA modifications as important regulators of virulence factor expression. This regulation occurs at the post-transcriptional level, as mRNA transcript levels remain unaltered. Indeed, it is thought that changes in tRNA modification constitute a rapid response to sudden external stimuli. In this review, we present aspects of tRNA biology that are of particular importance for adaptation to

cellular and environmental stress, and we discuss the implications of tRNA modification for infection.

2. Impact on structures and folding

Nucleoside modifications have a profound impact on RNA structure and folding (Fig. 1) [18,19]. In the case of tRNAs, post-transcriptional modifications are critical for folding, stability and decoding activity [20–22]. The half-life for tRNAs ranges from a few hours to several days [23–26]. Several studies have shown that tRNAs lacking nonessential modifications have a higher turnover rate, curtailing the half-live from hours to minutes [27]. Nucleoside modifications ubiquitously present on the body of tRNAs are important for establishing tertiary contacts between the D-loop and TΨC-loop, as well as for maintaining the stability of the folded L-shape of many tRNAs (Fig. 1). Moreover, cells undergo post-transcriptional modification and de-modification dynamics of their tRNAs in response to environmental changes and adaptation for cell survival. For example, in archaea and bacteria, tRNA modifications are necessary for maintaining structural rigidity across a wide temperature range [28]. Unlike naked tRNA transcripts, fully modified tRNA molecules are densely folded and thermostable (reviewed in [20]).

2.1. tRNA stability

Rapid tRNA decay following nuclease attack can result from a lack of certain tRNA modifications [27,29]. In eukaryotes, nucleoside modifications protect tRNA transcripts from degradation [30] and provide structural stability [31,32]. Without proper modification, these transcripts would otherwise contribute to the formation of tRNA-derived fragments upon physiological stress [31–34]. Indeed, translation rates are dependent on tRNA availability, and the appearance of stress-induced tRNA fragments leads to protein synthesis inhibition and reduced cell viability. Furthermore, tRNA-targeted RNase activities are tightly controlled and it is thought that tRNA modifications are the *custodians* of tRNA fragmentation [33]. Loss of non-essential tRNA modification may also trigger rapid decay of cytoplasmic and nuclear tRNAs, whereby modifications act as sensors for rapid clearance of non-functional tRNAs or possible implicit immune-stimulatory tRNAs.

Nucleoside modifications also affect codon-anticodon interactions. Sulfur-containing 2-thio (s^2) modifications are commonly found on uridine at wobble position 34 (U_{34}) in tRNA isoacceptors coding for lysine, glutamate, and glutamine, whereas 4-thio (s^4) modifications are found at position 8 (Fig. 1). The s^2U modification is ubiquitous in all domains of life, whereas s^4U is only found in eubacteria [35]. Except for *Mycoplasma*, tRNAs from all organisms with NAA codons have a thiolated uridine derivative as the wobble nucleoside. Replacement of the uridine C2-oxygen atom for a sulfur atom improves the s^2U stacking interactions and RNA duplex stability. Therefore, s^2U_{34} improves stacking interactions between anticodon loop uridines, and stabilizes codon-anticodon interaction. In the presence of thiolation, the equilibrium shifts towards C3′-*endo* sugar puckering conformation [36–39]. Ribonucleosides in RNA typically favor a C3′-*endo* sugar pucker whereas 2′-deoxynucleotides in DNA adopt a C2′-*endo* pucker. Indeed, the five atoms in the pentose sugar are not coplanar, which allows for high flexibility. Sugar puckering happens when there is a displacement of the 2′ and 3′ carbons above the plane of the C1′-O4′-C4′ atoms. The sugar structure (puckering) and base pair configuration (nucleotide incorporation) can influence the sugar conformation in nucleic acids [40]. Furthermore, sugar puckering also influences the polymerase extension fidelity; for example, 2′-fluororibonucleosides that are constrained to a C3′-*endo* conformation are preferentially incorporated by RNA polymerases [41].

2.2. Interactions with and recognition by translational factors

During the life span of a tRNA, which stems from tRNA biogenesis to the tRNA turnover pathway, the tRNA molecule encounters numerous interaction partners. The main translation factors that frequently interact with tRNAs are aminoacyl-tRNA-synthetases (aaRS), elongation factors, ribosomes, mRNAs, as well as some stress response-triggered proteins.

Aminoacyl-tRNA synthetases (aaRS) are key enzymes responsible for charging the tRNA with the cognate amino acid, which constitutes the first step of protein synthesis following the tRNA maturation process. Class I and II aaRS differ by the ATP triphosphate group binding conformation, as well as the tRNA acceptor arm binding mode [42]. The tRNA is recognized based on its acceptor arm and the identity of the nucleosides at position 34–36 in the anticodon loop for some aaRS (Fig. 1) (reviewed in [42,43]). Structurally, no significant differences between fully modified and unmodified tRNA variants binding to aaRS have been found [44]. Furthermore, tRNA modifications play an important role in facilitating recognition by the respective aaRS [42,43,45–48] and preventing frameshifting during translocation [49,50]. In addition, tRNA-aaRS interactions have been shown to protect the anticodon region against potential nuclease cleavage and modification in pathogenic bacteria and yeast [51–53], and to prevent mischarging of amino acids on tRNAs [54,55]. Notably, impaired enzyme function of aaRS has been associated with a variety of diseases and cellular disorders in humans [56].

Efficient translation relies on the productive interaction and coordination between the ribosome, tRNA and mRNA. Recent high-resolution structures of human [57], bacterial [58,59], and eukaryotic [60] ribosomes reveal that ribosomal RNA (rRNA) modifications participate in the molecular recognition of tRNAs in the A-, P-, and E-sites. These rRNA modifications localize close to the 3′-CCA end of A- and P-site tRNAs, which during translation could influence peptide formation, as well as close to the tRNA acceptor stem of the E-site. In *Saccharomyces cerevisiae*, unmodified tRNA has been shown to have a two order-of-magnitude lower binding affinity to the small ribosomal subunit than the fully modified native tRNA [61]. Two observations in support of tRNA modifications facilitating efficient ribosome binding are: (i) the ability of chemically synthesized yeast tRNA anticodon domains to inhibit the binding of native yeast tRNA to the ribosomal subunits, but not to the unmodified anticodon stem loop; and (ii) the disposition of specifically modified DNA analogs of yeast tRNA anticodon domain to bind to the ribosome and effectively inhibit native tRNA from binding, while unmodified DNA analogs fail to perform the task [62]. The authors concluded that the following anticodon characteristics are preferred for a strong yeast tRNA-ribosome binding: (i) a 5-methylcytidine (m^5C) modification-dependent Mg^{2+}-stabilized anticodon domain structure, and (ii) a 1-methylguanosine (m^1G)-aided open loop conformation. Conjointly, biochemical and structural data show that anticodon domain modifications provide stability to the tRNA for ribosome-mediated codon binding [63–65]. For example, 5-hydroxy (xo^5U)-type modifications found at U_{34} and the N6-methyladenosine (m^6A) modification at position 37 confer order and stability to the pre-structured anticodon loop prior to A-site codon binding [63].

In addition to proper ribosome binding, tRNA anticodon loop modifications are also important to ensure mRNA decoding fidelity [66]. Elucidating from X-ray structural data, Rozov and co-workers define a powerful role of tRNA modifications in translation [67]. They demonstrated that bacterial tRNA with a hypermodified 5-metylaminomethyl-2-thiouridine ($mnm^5s^2U_{34}$) wobble position is able to discriminate between cognate and near-cognate stop codons or any unusual base-paring codon. On the other hand, a human unicellular endosymbiont *Candidatus Riesia pediculicola* seems to have lost all post-transcriptional modifications, except those located in the anticodon stem loop, which strongly supports the obligatory nature of these modifications in mRNA decoding [68]. Likewise, for harmful parasites

including *Trypanosoma brucei* [69], *Toxoplasma gondii* [70], *Theileria* [71] and *Plasmodium falciparum* [72], anticodon stem loop modifications could be part of the strategy by which pathogens persist during infection.

Initiator tRNAs ($tRNA_i^{Met}$) are proxy for protein synthesis initiation and strictly discriminated from the elongation process. To prevent $tRNA_i^{Met}$ from the tRNA selection step in translation elongation, two adjacent base-pairs (50:64 and 51:63) in the TΨC stem of the $tRNA_i^{Met}$ are responsible for the introduction of a sequence-dependent RNA helix perturbation to the TΨC stem (Fig. 1) [73]. The resulting disorder is thought to prevent $tRNA_i^{Met}$ from binding to elongation factor eEF1. Usually, a pyrimidine at position 64, together with other initiator tRNA discriminants, is sufficient to constrain its use specifically for initiation. However, when nucleoside 64 of a $tRNA_i^{Met}$ is a purine, 2′-O-phosphoribosylation modification is required. This is the case for most yeast [74,75] and wheat germ [75] initiator tRNAs. Using a heterologous yeast system, Förster and co-workers showed that this single modification of $tRNA_i^{Met}$ can act as a negative discriminant for elongation factor eEF1-α [76]. The 5′-phosphoribosyl residue hinders the binding of $tRNA_i^{Met}$ to eEF1-α. Interestingly, demodification of nucleotide 64 in plant and fungi allows $tRNA_i^{Met}$ to participate in the elongation cycle, but not in the initiation step [75]. Remarkably, removal of this modification in yeast $tRNA_i^{Met}$ actually improves the affinity of eukaryotic $tRNA_i^{Met}$ to bacterial elongation factor EF-Tu·GTP, achieving a comparable affinity as bacterial elongator tRNAs [75].

3. Genome recoding

Messenger RNAs (mRNAs) carry specific genomic information to the protein translational machinery, accompanied by distinct mRNA-tRNA codon-anticodon interactions, to attune protein production. tRNAs close the protein synthesis feedback loop by modulating the output of protein translation via post-transcriptional modifications that are populated at the tRNA anticodon stem loops. This section highlights how tRNA modifications are able to expand the initial coding capacity of a tRNA molecule, triggering a complex network of coding events relating to host-pathogen responses.

Genome recoding by tRNA modifications represent an important mechanism for adapting cells and organisms to changing environmental conditions. In certain budding yeasts, such as *Saccharomyces* and pathogenic *Nakaseomyces*, CUN codons are reassigned from leucine to threonine in mitochondria, suggesting that the genetic code has been evolving and expanding [77,78]. On the other hand, CUN codons code for leucine in other pathogenic yeasts, including *Candida albicans* and *C. parapsilosis* [77].

Wyosine (imG), along with its derivatives wybutosine (yW) and hydroxywybutosine (OHyW), is another prevalent modification found in archaeal [79] and cytoplasmic eukaryotic tRNA [80] at position 37 of the anticodon loop. It is essential for translational fidelity, yet exquisitely reported for the single $tRNA_{Phe}^{GAA}$. Interestingly, imG and yW are absent in bacteria [81]. Only recently, yW and OHyW are found uniquely in cytosolic and mitochondrial tRNA of kinetoplastids, while imG is a strictly mitochondrial modification. These modifications are thought to increase the coding diversity in trypanosomatids [81].

Contrary to the canonical perception that mistranslation often leads to a detrimental outcome to cells, some mistranslation events are actually beneficial. For example, hyperthermophiles adapt their protein translation to lower temperatures by performing temperature dependent mistranslation. At low growth temperatures, the hyperthermophilic archaeon *Aeropyrum pernix* misacylates $tRNA_{Met}$ to $tRNA_{Leu}$ [82].

3.1. tRNA editing expands the coding capacity

Adenosine to inosine (A-to-I) tRNA editing is omnipresent in eukaryotes but occurs only in limited bacterial phyla [83]. Contrary to its eukaryotic counterparts (7–8 tRNAs), only $tRNA_{Arg}^{ACG}$ undergoes A-to-I editing in *E. coli* [84]. Inosine (I), a common wobble position modification, is interpreted as guanosine (G) and allows base pairing with C, 5-methyluridine (m^5U or T), and A during protein synthesis [85]. Without this modification, cognate tRNA selection is affected. In *Salmonella typhimurium*, unmodified A_{34} in $tRNA_{Pro}^{GGG}$ is unable to read the usual CCC codon on mRNA [86]. Despite this, the cells still grow normally. As it turns out, $tRNA_{Pro}^{AGG}$ with a protonated A_{34} is able to wobble base pair with C, hence rescuing the cells from stalling at CCC codons.

Interestingly, the conversion of A-to-I at the wobble position is influenced by cytidine to uridine (C-to-U) tRNA editing. Indeed, the detailed mechanism of early discoveries of C-to-U tRNA editing in eukaryotes [87,88] has only recently been elucidated [89,90]. A majority of the C-to-U editing events are found in the anticodon region, suggesting that they influence codon recognition. For example, the marsupial mitochondrial $tRNA_{Asp}^{GCC}$ anticodon is, upon editing to GUC, capable of recognizing aspartate codons instead of glycine codons [87]. It is noteworthy that this editing event only takes place for a small portion of the tRNAs. Another coding capacity expanding C-to-U editing event has been reported for the non-pathogenic eukaryotic protozoan *Leishmania tarentolae* [88]. In its mitochondria, a single C-to-U nucleotide modification at position C_{34} of the imported, nucleus-encoded $tRNA_{Trp}^{CCA}$ anticodon, remarkably enables decoding of the UGA stop codon as tryptophan. Moreover, C-to-U deamination editing has been reported for several other organisms, such as plant mitochondria, chloroplasts, mammalian apoB mRNA, *T. brucei* and the hyperthermophilic archeon *Methanopyrus kandleri* [90–94]. The ability for both eukaryotes and bacteria, including opportunistic pathogens, to change the information content of their tRNA can be beneficial as base-pairing properties are expanded beyond faithful mirroring of the genetic blueprint when performing specialized cellular functions.

3.2. Preventing reading frame slippage or programmed frameshifting

Reading frame maintenance is another aspect where tRNA modifications play an important role. Together with the ribosome, tRNAs and their modifications act as *gatekeepers* to ensure that mRNA is faithfully translated in-frame, particularly during the elongation step. Loss of the reading frame due to a spontaneous +1 or −1 frameshifting (+1FS or −1FS) error may jeopardize gene expression. However, for some genes it has been shown that moderate frameshifting during expression does not cause deleterious effects in *E. coli* [95]. Although rare, this poses an issue especially for slippery mRNA sequences, such as CC[C/U]-[C/U] read by the GGG and UGG isoacceptors of $tRNA_{Pro}$, where the 0 and +1 frame are indistinguishable [96,97]. Slippery sites are estimated to occur ~2300 times among total *E. coli* sense codons [97], and ~3700 times among > 30,000 transcripts analyzed for protozoa *Euplotes octocarinatus* [98]. Modification at position 37 of several tRNAs is indeed important for maintaining the reading frame. While m^1G modification at position 37 of tRNA is known to prevent +1FS [99], the mechanism was only recently resolved [97]. Gamper and coworkers found that m^1G_{37} in $tRNA_{Pro}$ is the major determinant of +1FS error suppression. However, while it is dominant in $tRNA_{Pro}^{UGG}$, it requires the assistance of the translation factor EF-P to suppress $tRNA_{Pro}^{GGG}$, which is prone to frameshift [97]. Likewise, hypomodification of the 2-methylthio (ms^2) or the 2-methylthio-N6-(*cis*-hydroxyisopentenyl) (ms^2io^6) groups in other tRNA isoacceptors (e.g. $tRNA_{Phe}$, $tRNA_{Tyr}$, etc.) also induces +1FS [50], including the pathogenic bacteria *S. typhimurium* [100,101]. Further examples of FS-inducing hypomodifications at position 37 in tRNA are reviewed in [12]. Furthermore, *S. cerevisiae* $tRNA_{Phe}$ with hypermodified G_{37} (yW, imG) is involved in the stabilization of codon-anticodon interactions [102] and preventing ribosomal −1FS [103].

On the other side of the coin, programmed FS has been reported to regulate gene expression in bacteria, fungi, archaea, mammals, and ciliated protozoa [104–111]. In programmed FS, the ribosome redirects the mRNA reading frame from 0 to +1 or −1 reading frame at a

specific location, without compromising peptidyl transfer, so that translation proceeds until the stop codon. To this end, obligate pathogens such as viruses manipulate the properties of tRNA modification to hijack the host translation machinery by performing programmed FS. Altered tRNA modification and programmed FS propensity have been shown to inhibit viral replication [112]. In Tobacco Mosaic Virus (TMV), the stop codon is read through by wild-type $tRNA_{Tyr}$ carrying a modification at position 10, but not by the hypomodified $tRNA_{Tyr}$ species, to produce viral proteins [113–115]. Moreover, HIV-1 uses programmed −1FS to translate structural and enzymatic domains essential for its replication [116]. However, the exact mechanism by which tRNA modifications regulate translation of viral proteins remains an enigma.

3.3. Codon usage

More than one codon synonymously decodes an amino acid. Among different organisms, the prevalence of preferred codons used to translate genes differ, creating species-specific codon usage bias. Novoa and co-workers have shown that tRNA modifications shape the genome structure and define the codon usage in all kingdoms [117]. The authors bring forward two specific tRNA modifications – I_{34} and xo^5U_{34} – that significantly fine-tune the correlation between genomic codon usage and tRNA gene frequencies in gene expression profile analyses. I_{34} modification is positively-selected among eukaryotes and present in two bacterial phyla [83] while 5-hydroxyuridine derivatives modification at position 5 of the uracil base (xo^5U_{34}-derivatives) is present primarily in bacterial tRNAs [36]. Conversion of A to I allows I_{34} to wobble with A, C, and U [118]. On the other hand, xo^5U_{34} modification expands the wobble capacity of U to read 3 to 4 codons in a family codon box [119,120]. Unlike bacteria and eukaryotes, archaea presents the simplest decoding scenario with a minimal set of tRNA isoacceptors and uniform abundance of tRNA genes [117].

In mycobacteria, hypoxia reprograms tRNA modifications and selectively translates mRNAs from families of codon-biased persistence genes [121] (see also Section 5.2). Consistent with the translation of transcripts enriched in its cognate codon, ACG, which includes the master regulator of hypoxic bacteriostasis, uridine 5-oxyacetic acid (cmo^5U) modification in $tRNA^{UGU}_{Thr}$ also increases survival during early hypoxia. Interestingly, a systematic analysis of the tRNA transcriptome of *Lactococcus lactis* reveals that tRNA abundance upon protein overexpression does not correlate with the codon usage of the overexpressed gene. The changes *in lieu* suggest that tRNA concentrations are adjusted to the codon usage of the housekeeping genes to counteract stress [122].

tRNAs specific for lysine, glutamate, and glutamine from all organisms, except Mycoplasma, and organelles have a 2-thiouridine derivative (xm^5s^2U) as the wobble nucleoside. In eukaryotic cytoplasmic tRNAs, the conserved modification (xm^5-) in position 5 of uridine is 5-methoxycarbonylmethyl (mcm^5). Loss of the conserved mcm^5s^2U modification in lysyl-tRNA impairs growth and protein homeostasis in *S. cerevisiae* [123–126]. While the primary function of $mcm^5s^2U_{34}$ modification is to modulate the translation of cognate codons in yeast [123], bacteria seem to have positively selected for this modification to prevent missense error in decoding [119]. Hence, codon usage, tRNA availability, and tRNA modification all synergistically affect translation efficiency.

3.4. Reassigning codons

The ability of tRNA to wobble at the third nucleotide allows more than one codon to be decoded. Taking this into account, less tRNAs are needed to translate the standard genetic code set of 61 codons into 20 amino acids [127]. Not only that, nucleoside modifications embellishing particularly the anticodon loop of a tRNA molecule further authorize sense codon reassignment. For example, addition of 5-formylcytidine (f^5C) at the first position of the anticodon in mitochondrial $tRNA_{Met}$ in species ranging from fruit flies to humans, concedes the tRNA to translate both AUG and nonconventional AUA codons as methionine. Using an *in vitro* translation system from bovine liver mitochondria, Takemoto and co-workers have shown that $mt-tRNA^{CAU}_{Met}$ lacking f^5C can only recognize the AUG codon [128]. Mitochondrial translation can be initiated with the universal isoleucine codons (AUU and AUC), attributed to the unique modification of f^5C_{34} [129]. Strikingly, f^5C modification is also found in the $mt-tRNA_{Met}$ of the parasitic nematode *Ascaris suum* [130], which lacks the T-stem but still folds into a reasonable L-shaped tRNA structure.

Bacterial and archaeal tRNAs are able to convert AUG to AUA with lysidine-containing cytidine modified nucleoside 34 at $tRNA_{Ile}$ anticodon [131,132]. In bacteria like *Lactobacillus plantarum*, which is commonly found in fermented food products, lysidine modification at position 34 of $tRNA^{LAU}_{Ile2}$, but not the unmodified $tRNA^{UAU}_{Ile2}$, enables efficient charging with isoleucine [133]. The enzyme responsible for this modification is **t**RNA^{Ile}-**l**ysidine **s**ynthetase (*TilS*) (reviewed in [134]). Loss of TilS enzyme function is thought to be detrimental to bacterial protein synthesis, as the ribosomes are likely to stall at AUA codons [131]. While no mammalian nuclear and mitochondrial counterpart of TilS has been reported, homologs are almost uniformly present in bacteria [135], such as *E. coli* and *Pseudomonas aeruginosa*. Chemically similar to lysidine, agmatidine (C+ or 2-agmatinylcytidine) is incorporated at the first (wobble) position of some tRNAs in archaea. Similar to the role of lysidine, C+ of the archaeal $tRNA_{Ile2}$ anticodon is essential for AUA decoding of Ile-CAU and not Met-AUG [136,137]. Hence, bacteria and archaea have developed very similar isoleucine AUA codon decoding strategies that strongly disfavor methionine codon AUG selection.

3.5. tRNA priming

Specific tRNAs are often used to prime reverse transcriptases to initiate DNA synthesis. Retroelements found in retroviruses, plant pararetroviruses, and retrotransposons – i.e. transposon containing long terminal repeats – contain primer-binding sites complementary to the primer tRNA [138]. The selection of tRNAs for priming is affected by modified nucleosides. In avian myeloblastosis virus, only the non-methylated species of $tRNA_{Trp}$ at position 7, instead of N2-methylated guanosine (m^2G_7) $tRNA_{Trp}$, is encapsidated and used as a primer for reverse transcription [139].

Lentiviruses of the *Retroviridae* family are human and animal pathogens that cause persistent infections [140]. Lentiviruses are compelling agents for gene therapy, as reviewed in [141–143]. For example, HIV-1 has been used as a gene delivery vehicle because of its specificity of integration at localized hotspots within the human genome. As compared to murine leukemia virus, HIV-1 lentiviral vectors are less likely to insert their cargo genes randomly or to interfere with critical host genes [144]. All known mammalian lentiviruses, including HIV-1, use host $tRNA_{Lys3}$ as a primer for the reverse transcription replication step [138,145,146]. Yarian and co-workers have shown that the N6-threonylcarbamoyladenosine (t^6A_{37}) modification on $tRNA^{UUU}_{Lys3}$ anticodon stem-loop enables the binding of tRNA to the ribosomes [147]. This suggests that tRNA modification is crucial for priming of host $tRNA^{UUU}_{Lys3}$ to initiate the reverse transcription of the lentivirus genomic RNA. Concomitantly, a NMR-derived structure of human $ASL-tRNA^{UUU}_{Lys3}$—t^6A_{37} also supports the importance of t^6A_{37} and other position 37 modifications, such as in human $tRNA^{UUU}_{Lys3}$ where A_{37} is modified to 2-methylthio-N6-threonylcarbamoyladenosine ($ms^2t^6A_{37}$), for optimal ribosome binding through an open, structured loop of $ASL-tRNA^{UUU}_{Lys3}$—t^6A_{37} [148].

4. Metabolism regulation

The nucleoside modification content of any given tRNA is a dynamic

representation of the metabolic state of the cell. The availability of cellular metabolites governs the extent of nucleoside modification, e.g. methionine, cysteine, or threonine is required for the formation of methylated, thiolated, or threonylated tRNA species, respectively. However, although amino acid starvation or protein synthesis inhibition yield hypomodified tRNAs, metabolic disturbances are not the main cause of hypomodification. Indeed, studies conducted on *E. coli* show that modifications such as $mnm^5s^2U_{34}$ are invariant of the growth phase [149], whereas queuosine (Q_{34}) and 2-methylthio-N6-isopentenyladenosine ($ms^2i^6A_{37}$) formation is clearly growth phase dependent [150,151], and others, like s^4U_8, decrease in some tRNA isoacceptors while remaining stable in others [149]. Remarkably, two *Saccharomyces cerevisiae* strains were reported to have a temperature-dependent 2-thiolation deficit, preventing synthesis of $mcm^5s^2U_{34}$, the eukaryotic equivalent of mnm^5s^2U, at elevated temperatures. Interestingly, this deficit was not found in other closely related yeasts, including the opportunistic pathogen *C. glabrata* [152]. Since tRNA modification synthesis is dependent on the growth rate and phase of the organism, as well as on physiological stress factors, it is thereby highly unlikely that all tRNAs are constantly modified. Instead, the ratio of hypomodified vs. modified tRNAs dynamically changes in response to the prevailing environmental conditions and translational needs of the cell.

The dependence of tRNA modifications on metabolites has interesting implications for pathogenicity. For example, the iron uptake mechanism in gram-negative bacteria requires the presence of enterochelin, a high affinity siderophore that is synthesized from the aromatic amino acids precursor chorismic acid [153]. Enterochelin is also required for the synthesis of the 2-methylthio (ms^2) group in a number of modifications, including ms^2io^6A, ms^2i^6A, and 2-methylthio-N6-threonylcarbamoyladenosine (ms^2t^6A) [154,155], as well as for the conversion of G to Q [156]. Hence, the presence of enterochelin is determinant for the level of these modified nucleosides in tRNA. Interestingly, the lack of 2-methylthiolation seems to initiate a feedback loop by stimulating the transport of aromatic amino acids, including enterochelin's precursor, chorismic acid. Moreover, loss of function in the isopentenyl-pyrophosphate transferase MiaA, which is responsible for catalysis of the N6-isopentenyl (i^6) group in gram-negative bacteria, increases the frequency of GC to TA transversions under specific stress conditions [150,157,158]. Thus, hypomodification of ms^2i^6A (by MiaB) and ms^2io^6A (hydroxylation by MiaE) facilitates adaptation to iron-limited environments by activating genes involved in iron accumulation. This constitutes an important pathogenicity trait that furthers virulence during invasive infection [159], as hosts strive to limit bacterial growth by reducing the iron available through iron-chelating proteins (Fig. 2).

Chorismic acid is also required as an intermediate in the synthesis of tyrosine and phenylalanine, and the tRNAs coding for these amino acids frequently contain cmo^5U, uridine 5-oxyacetic acid methyl ester ($mcmo^5U$), and Q modifications [160]. These modifications are all located at or near the tRNA anticodon, with cmo^5U and $mcmo^5U$ being essential for accurate decoding of G-ending codons [120], whereas ms^2i^6A and ms^2io^6A stabilize codon-anticodon interaction by increased stacking [161]. Indeed, ribosomal P-site slippage leading to +1FS was found to increase three to nine-fold in tRNAs with ms^2-hypomodification at position 37, whereas tRNAs with m^1G at this position were far less sensitive to +1FS [50]. Furthermore, lack of i^6A modification in the diarrhea-causing enterobacteria *Shigella flexneri* was shown to significantly decrease the expression of virulence factors required for invasion and motility. Interestingly, this reduction was accounted solely to post-transcriptional regulation, as mRNA levels remained constant [162]. Therefore, an insufficient supply of intermediary metabolic products may have far-reaching consequences for translation, giving rise to pleiotropic effects on cell physiology that reduce the fitness of the bacteria.

The effect of metabolism on tRNA modification manifests differently in eukaryotes than in bacteria (Fig. 2). Loss of the MiaA homolog in *S. cerevisiae*, Mod5, has almost no effect on growth rate in various media and temperatures, with failure to sporulate being the only noticeable effect in homozygous mutants [163]. However, nuclear Mod5 is directly involved in tRNA gene-mediated silencing and it has the ability to bind to tRNA-like substrates, which facilitates aggregation of Mod5 [164,165]. Contrary to wild type *Schizosaccharomyces pombe*, *tit1* mutants are sensitive to rapamycin, displaying phenotypic changes including reduced growth rate and protein translation. It is thought that the lack of i^6A modification might confer rapamycin sensitivity by altering the amino acid sequence of TORC1, since isopentenylation is known to promote $tRNA^{G\Psi A}_{Tyr}$ misreading of near cognate $tRNA^{UGC}_{Cys}$ codon [166,167]. Metazoan isopentenylation occurs mainly in the mitochondria, where aberrant ms^2i^6A modification is associated with respiratory chain defects [168]. Only two cytoplasmic tRNAs, both serine-encoding, are i^6A modified by TRIT1 in humans. Selenoprotein expression is entirely dependent on selenocysteine $tRNA^{Sec}_{Ser}$, whereby a single nucleoside exchange in this tRNA can abolish this activity, resulting in complex symptoms [169,170]. Furthermore, knockout mutants of the tRNA modifying enzymes AtTRM11 and AtELP1 in *Arabidopsis thaliana*, which synthesize m^2G and 5-carbamoylmethyluridine (ncm^5U), respectively, were found to have an early flowering phenotype as well as reduce organ growth, although it was unclear whether this was directly caused by loss of the tRNA modification or due to disruption of other cellular pathways [171]. These examples highlight the complexity of tRNA modification and translational control, as hypomodification may lead to a slight reduction of fitness in fungi, whereas the outcome may be far more diverse and severe in plants and metazoans.

4.1. Sulfur relay

Sulfur is an essential element that is present in all major classes of biomolecules, such as iron/sulfur (Fe/S) clusters, enzyme cofactors, and thionucleosides. The synthesis of thionucleosides requires complex sulfur-transfer systems consisting of multiple mediators, which have been described in detail for bacterial, archaeal, and eukaryotic pathways (recently reviewed in [12,172–174]). Thionucleosides are in most cases created by replacing the keto-oxygen on the base with sulfur [125,175–177], although a methylthio-group from *S*-adenosyl-L-methionine (SAM) and a currently unknown sulfur source provides the basis for thiolated adenines [178,179]. Thionucleosides are ubiquitous and their function follows the canonical division, i.e. anticodon modifications modulate translational accuracy and efficiency whereas acceptor stem, TΨC-loop, and D-loop modifications provide structural stability, or act as recognition elements for appropriate tRNA aminoacyl synthetases or modification enzymes [21]. Recently, much focus has been given to the interconnected nature of sulfur relay pathways and their relation to other cellular processes, such as stress response, regulatory functions, and other metabolic pathways, especially those related to cofactor metabolism. Indeed, thiomodified tRNAs are essential mediators in a large number of stress responses, including oxidative stress [126,180–182], temperature stress [152,183–185], nutrient starvation [186,187], etc. (Fig. 2). These functions and their implications for infection will be discussed in Section 5. For additional information on thiomodification and disease, the following review articles are recommended: [16,17].

5. Infection and host responses – adaptation to stress

As we have discussed above, tRNA modifications are crucial for a number of structural and functional features. They stabilize the folded L-shape of the tRNA molecule, limit tRNA turnover rates, as well as affect genome recoding and cellular metabolism, which is an essential determinant for the expression of e.g. virulence factors or immune responses. To better understand the concerted action of these functions, it is important to determine the dynamics of tRNA modification. As

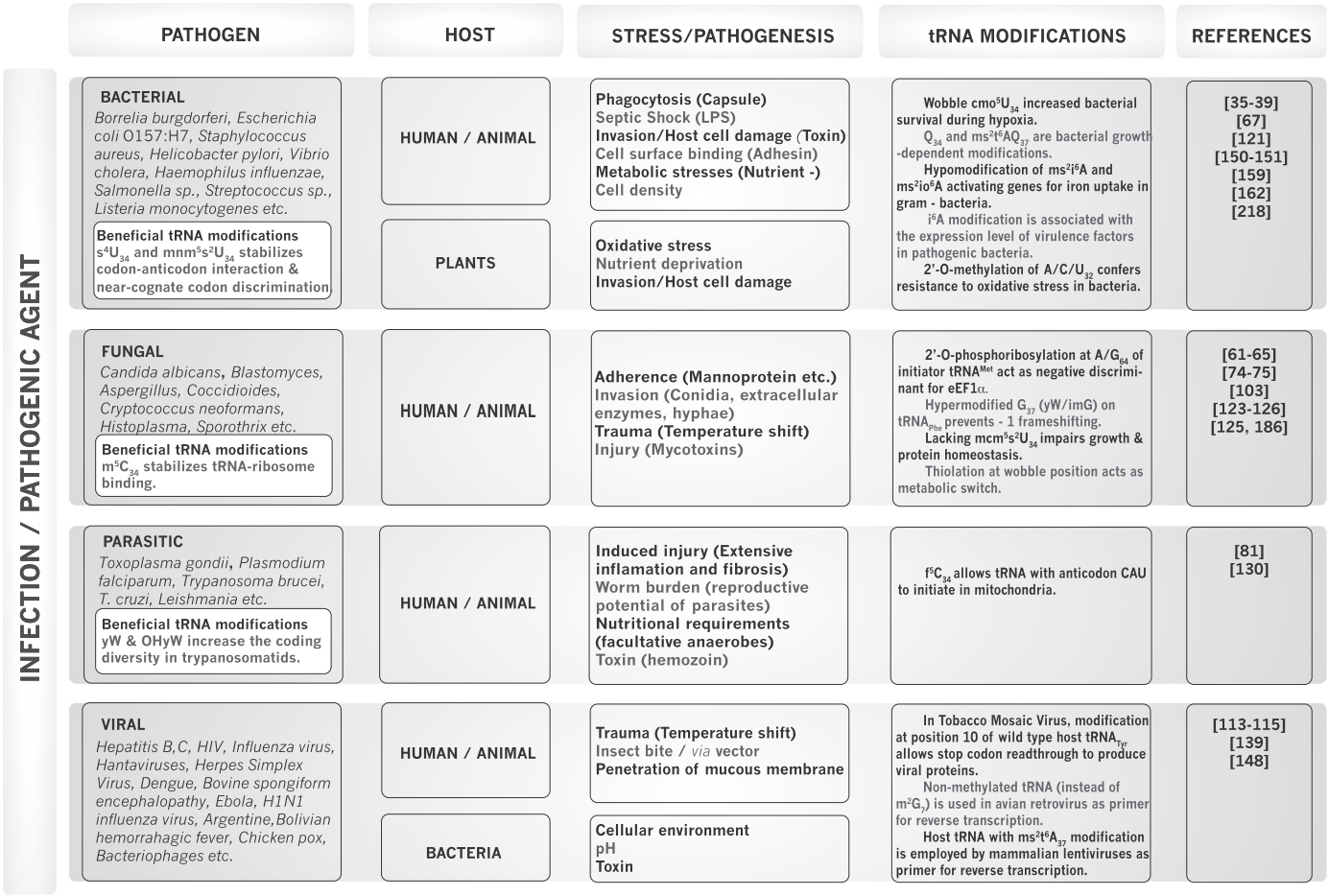

Fig. 2. Transfer RNA modifications in pathogens associated with metabolism and physiological stress responses. Summary of tRNA modifications in bacteria, fungi, parasites, and viruses that have been reported to be important for pathogenicity and/or host-pathogen interactions. Nucleoside modification nomenclature abbreviations according to the Modomics convention [11].

quantitative liquid chromatography mass spectrometry has advanced and become more accessible, we have gained the first insights into tRNA modification dynamics [188–190]. These studies provide a detailed account of tRNA modification following various stresses [121,191–193], not just for one or two modified nucleosides, but for the whole tRNA modification landscape of an organism and sometimes even for multiple, evolutionarily related species [152]. Furthermore, the advent of ribosome profiling and RNA sequencing has enabled detailed insights into post-transcriptional events that have cemented the importance of tRNA modifications as translational modulators [126,181]. Although tRNA transcription and transcript processing can be regulated by physiological stress (reviewed by [182,194]), the focus of this chapter will be on post-transcriptional modifications and their role in stress response mechanisms – with special attention on how this is relevant for pathogenicity and infection (Figs. 2 and 3).

5.1. Temperature stress

As discussed in Section 2, nucleoside modifications maintain the structural stability and function of tRNA molecules, allowing for widespread adaptation to various environments. Indeed, tRNA modification patterns have been observed to differ markedly depending on the growth temperature of the organism, and extremophiles feature more tRNA modifications than their mesophilic counterparts [28,152]. For instance, 7-methylguanosine (m^7G) at position 46 is critical for cell viability at high growth temperatures in *Thermus thermophilus* [195], whereas lack of pseudouridine (Ψ) at position 55 in the same bacteria gives rise to an abnormal increase of other nucleoside modifications and subsequent growth retardation at low temperatures [196]. Moreover, thionucleosides located outside the anticodon stem loop also increase the structural stability of tRNAs in extremophiles [20,197,198].

In contrast to extremophiles, mammals maintain a constant body temperature that ranges from 36.5 °C for humans [199] and elephants [200] up to 40.1 °C for rabbits [201]. However, an infection may increase the normal body temperature by as much as 4 °C. Thus, successful pathogens must be able to withstand growth temperatures in excess of 40–42 °C to proliferate in mammals. A rapid loss of viability was seen for *Escherichia coli* TruB deletion mutants deficient in Ψ_{55} tRNA modification when grown above 37 °C [202]. In *S. cerevisiae*, strains deficient in TRM8, the m^7G methyltransferase, are temperature-sensitive in synthetic media containing glycerol [203]. Thus, the structural rigidity conferred by m^7G_{46} and Ψ_{55} modification is important for viability at elevated temperatures.

The anticodon stem-loop modifications have also been linked to high-temperature growth and pathogenicity (Fig. 3) [152,204–206]. Particular focus has been given to 2-thiolation of U_{34}, which occurs as $mcm^5s^2U_{34}$ in eukaryotes, $mnm^5s^2U_{34}$ in bacteria, and 5-taurinomethyl-2-thiouridine (τm^5s^2U) in mitochondria, and is synthesized by two separate pathways responsible for the addition of the xm^5 and s^2U moiety, respectively (reviewed in [174]). Notably, $mcm^5s^2U_{34}$ modification is an important translational modulator that prevents the occurrence of FS and alters the rate and accuracy of translation [126,181,207,208], with similar observations being reported for the corresponding bacterial and archaeal modifications [209,210]. Loss of $mcm^5s^2U_{34}$ modification causes cellular dysfunction due to disruption of protein homeostasis in yeast and nematodes [126], and severe neurological defects in humans

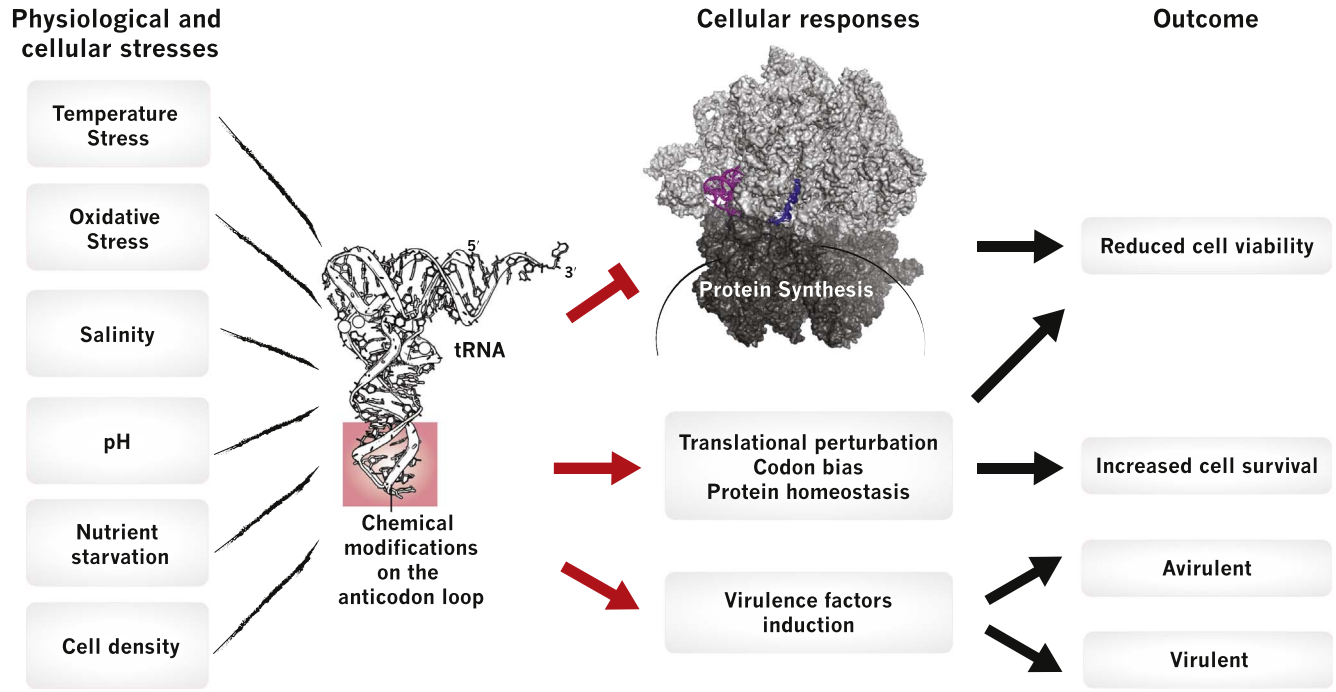

Fig. 3. Physiological stress elicits complex cellular responses that are mediated by anticodon loop tRNA modifications. Hypomodification of the anticodon loop has been associated with numerous translational perturbations that affect stress responses, disrupt protein homeostasis, or reduce the expression of virulence factors, thereby limiting the organism's fitness and viability during physiological stress [121,126,181,207,212]. Conversely, reinstating anticodon modification clears cellular stress and enhances viability.

(reviewed in [16,17]). Interestingly, the stringent translational regulation mediated by 2-thiolation also seems to promote high-temperature growth [152,184]. A recent study comparing the tRNA modification profile from evolutionary related yeasts following temperature stress revealed that *S. cerevisiae* BY4741 and W303 have a transient temperature-dependent 2-thiolation deficit that limits growth to 39 °C. However, 2-thiolation levels remained unaltered for the opportunistic pathogen *C. glabrata*, which grows well at temperatures up to 43 °C [152]. Furthermore, quantitative trait loci mapping for pathogenic isolates of *S. cerevisiae*, which grow at 41 °C, identified the Ncs2 (a.k.a. Tuc2) enzyme as the causative agent for the high-temperature growth phenotype [211]. Interestingly, Ncs2 catalyzes s^2U_{34} modification [177] and is also associated with invasive and pseudohyphal growth [204]. Sequence analysis of Ncs2 reveals that pathogenic yeasts have acquired a single histidine to leucine mutation that does not occur in non-pathogenic strains. Consequently, it is possible that this mutation stabilizes the Ncs2 enzyme at elevated temperatures whereby 2-thiolation is maintained. A back-to-back publication also uncovered that a loss of Ψ_{38} modification at the anticodon stem-loop in *S. cerevisiae* W303 abolishes growth at elevated temperatures, which is also linked to an observed deficit in 2-thiolation [185].

5.2. Oxidative stress

Reactive oxygen species (ROS) act as cellular messengers in redox signaling, which is an important signal pathway that regulates cellular growth, hypoxia, immune cell recruitment, and mobility (Figs. 2, 3). Despite their important function, oxidative stress may emerge if the antioxidant enzyme system is unable to balance the ROS levels in the cell. This might occur following exposure to environmental toxins, which can cause toxic effects through the release of peroxides and free radicals that damage the cell. Oxidative stress has a profound effect on tRNA modification and translation. Exposing *S. cerevisiae* to hydrogen peroxide yields a specific increase in 2′-O-methylcytidine (Cm), m^5C, and N2,N2-dimethylguanosine (m_2^2G) modification levels, whereas strains deficient in methyltransferases TRM4 (m^5C) and TRM1 (m_2^2G) are non-viable following hydrogen peroxide exposure [191]. Other mechanistically different toxicants, including various oxidizing and alkylating agents, did not elicit similar m^5C responses although changes were observed for other modifications [191,193], suggesting that tRNA modification patterns can be used to distinguish between chemically similar stressors. However, the increased modification level of m^5C at wobble base position 34 of $tRNA^{CAA}_{Leu}$ is thought to enhance selective translation of stress-related genes with over-represented Leu-UUG codons [212]. Many pathogenic bacteria, such as *Streptococcus pneumoniae*, utilize a similar strategy for several genes regulating oxidative stress responses and virulence factors, which feature specific codons indicative of codon-biased translational regulation (Fig. 3) [213]. A recent study shows that human cells that lack TRMT1, and therefore do not have cytoplasmic or mitochondrial m_2^2G modification, show perturbations in redox homeostasis with increased endogenous ROS levels and hypersensitivity to oxidizing agents [214]. Furthermore, Q modification has been found to promote the antioxidant defense system by increasing catalase activity and thereby relieving ROS stress [215].

Codon-biased translation has also been associated with cellular stress responses following alkylation damage (Fig. 3). In *S. cerevisiae*, the loss of mcm^5U_{34} modification, which is exclusively found on $tRNA^{UCU}_{Arg}$ and $tRNA^{UUC}_{Glu}$, has been linked to this stress. Addition of the final methyl group onto mcm^5 is catalyzed by tRNA methyltransferase TRM9. The expression of three damage response proteins, YEF3, RNR1, and RNR3, is substantially reduced in TRM9-deficient strains due to perturbed translation of transcripts with an over-representation of AGA and GAA codons, rendering TRM9-deficient cells susceptible to DNA alkylation [216]. Alkbh8, the mammalian TRM9 homolog, is also induced following oxidative stress and, similar to its yeast counterpart, it too is required for the efficient translation of selenocysteine-containing damage response proteins. Furthermore, loss of mcm^5U modification also abolishes mcm^5s^2U and mcm^5Um modifications, which has profound effects on cell viability and stress clearance [217].

Moreover, single-gene-deletion mutants of *E. coli* lacking thiolation pathway enzymes were found to have numerous perturbations in cellular respiration, the ATP/ADP ratio, nucleoside triphosphate levels, and DnaA activity caused by an aberrant intracellular redox state [180].

It was suggested that these perturbations might have been caused by the loss of 2-thiolation on tRNA and subsequent translational alterations. Indeed, induction of oxidative stress in *S. cerevisiae* and *C. elegans* revealed that strains deficient in 2-thiolation, which affects $mcm^5s^2U_{34}$ modification of $tRNA^{UUU}_{Lys}$, $tRNA^{UUG}_{Gln}$, and $tRNA^{UUC}_{Glu}$, resulted in translational slowdown at the ribosomal A-site [126,181], causing severe protein aggregation and imbalanced protein homeostasis. This reduction in decoding speed stems from perturbed codon-anticodon interactions and might increase the probability of rare coding events, such as misreading or FS, or induce co-translational misfolding (Fig. 3) [126].

A recent study suggests that codon-biased translation is integrally involved in regulating the way that microbial pathogens respond to infection-related stress. Hypoxia-induction and subsequent anaerobic resuscitation was shown to reprogram all 40 tRNA modifications detected [121], which has also been observed for other stresses and organisms and highlights the dynamics of tRNA modification in stress response [152]. The onset of hypoxia was found to increase the amount of cmo^5U_{34} found in $tRNA^{UGU}_{Thr}$, correlating to increased translation of transcripts rich in the cognate codon ACG, which includes DosR, the master regulator of hypoxic bacteriostasis [121].

In addition to wobble position 34 modifications, 2′-O-methylation of A, C, and U at position 32 by a methyltransferase homologous to *E. coli* TrmJ confers resistance to oxidative stress in *P. aeruginosa* [218]. Hypomethylation of the 2′-O-ribose moiety at position 32 is linked to reduced catalase activity, similar to m^6A_{37} modification in *E. coli* [219], which is thought to be due to perturbed codon-anticodon interaction and the resulting translation insufficiency and transcript misreading [220,221].

5.3. Salinity, pH, and nutrient starvation

Certain tRNA modifications are important both for maintaining structural stability and ensuring correct transcript decoding. Perturbation of these specific tRNA modifications may have important consequences for a number of different stresses (Figs. 2, 3). In particular, the expression of virulence factors is often linked to environmental sensing and stress response. This is also the case for the plant pathogenic fungus *Colletotrichum lagenarium*, where expression of APH1, a tRNA methyltransferase homolog of *S. cerevisiae* Trm8 that catalyzes the addition of m^7G_{46}, is required for pathogenicity and tolerance to salinity and oxidative stress [222]. Methylation of G_{46} is also associated with reduced temperature tolerance (Section 5.1) and heat shock treatment yields penetration hyphae and forms lesions, suggesting that m^7G_{46} is required for virulence in *C. lagenarium*. Furthermore, APH1-deficient strains showed a reduced invasive growth and higher sensitivity to antimicrobial compounds [222].

Numerous studies on the bacterial GidA/MnmE heterodimeric complex have highlighted the connection between tRNA modification, environmental stress responses, and pathogenicity. GidA catalyzes the addition of the 5-carboxymethylaminomethyl ($cmnm^5$) group on U_{34}, whereas MnmE is required for 2-thiolation in $mnm^5s^2U_{34}$ [223,224]. Deletion of GidA gives rise to a filamentous morphology and significantly reduced motility in *S. enterica* sv. *Typhimurium* [225], which suggests that $cmnm^5U_{34}$ hypomodification might be responsible for the reduced expression of motility genes. Various pleiotropic phenotypes have been reported for GidA-deficient *Agrobacterium tumefaciens* and *P. syringae* [226,227]. GidA deletion also reduces cytotoxicity induction in macrophages through an unknown mechanism that might involve 2-thiolation [183]. Interestingly, growth in an acidic or divalent cation-depleted environment induces GidA expression [187], whereas GidA and MnmE-deficient *Streptococcus pyogenes* mutants are highly attenuated in soft tissue infection models [228]. This suggests that U_{34} modification is critical for microbial virulence during infection.

As discussed in Section 4, nutrient availability influences tRNA modification and gene expression is adjusted accordingly. The intracellular amount of methionine and cysteine directly controls 2-thiolation in *S. cerevisiae* [125,186]. During sulfur starvation, $mcm^5s^2U_{34}$ modification is downregulated, whereby sulfur consumption and growth is reduced, and the expression of enzymes involved in methionine, cysteine, and lysine biosynthesis is increased [186]. Lack of essential metabolites, as experienced by cells grown in minimal synthetic defined media, yields a global rearrangement of tRNA modification [229], which can have considerable implications for transcript decoding [50]. Indeed, invasive pathogens often encounter similar hostile changes to their environment (Fig. 2). Reduced expression of virulence genes is often the cause of poor translation efficiency of essential transcription factors, such as VirF in *S. pyogenes* [230]. Consequently, careful regulation of tRNA modification ensures efficient translation and swift adaptation to various physiological stresses.

6. Discussion

The omnipresent nature of post-transcriptional tRNA modifications is indicative of their immense importance in translation. We have presented numerous studies that assign several critical functions to the correct and timely modification of tRNA nucleosides. These nucleoside modifications confer structural stability to the three-dimensional shape of the tRNA, protecting it against physical, chemical, or biological degradation, as well as regulating base pairing affinity of tRNA anticodons to mRNA codons and affecting recognition by ribosomes (Fig. 1) [231]. Studies on bacteria, yeast, and nematodes have unequivocally shown that perturbations in tRNA modification severely affect the translation efficiency for a number of critical codons, limiting the cells ability to cope with physiological stresses and leading to a reduction in fitness and viability [126,181,207,208].

These stresses are relevant to microbial pathogenicity, as the translation of virulence factors is triggered by external stimuli, including changes in temperature, pH, oxidation state, nutrient availability, metabolites, etc. (Fig. 2). Therefore, conclusions and assumptions as to how tRNA modification levels in a pathogen and a host react to infection can to a certain extent be drawn from studies on stress. Indeed, loss of the s^2U_{34} modification has been directly linked to significant attenuation of gram-negative pathogenic bacteria in the *Escherichia*, *Salmonella*, and *Shigella* genera [162,183,228]. The exact molecular mechanism of attenuation has not been fully determined, but it is thought that reduced translation of the key virulence factor, VirF, following loss of s^2U_{34} is the main contributing factor.

Although such conclusions are logical and rather straightforward to verify, it is far more challenging to dissect the intricate network of interaction partners and stress response/signaling pathways triggered by infection. For example, an opportunistic microbial pathogen that infects its host has to simultaneously adapt to a completely different environment, encountering multiple stresses, while reprogramming the entire transcriptional and translational apparatus (Fig. 3). Pathogens also have to fight off a large number of commensal microbes – a recent study found 47 bacterial genera and numerous fungi and archaea associated with plant roots [232] – and evade toxic metabolites produced by these. With such an overwhelming amount of interaction partners, simplified infection models are needed in order to track the effect of individual tRNA modifications, as well as to distinguishing between host and pathogen modifications and their effect on translation.

Assigning function to tRNA modification in plant and metazoan hosts is further complicated by strong compensatory mechanisms. Although these mechanisms tend to counteract deficits in modification, even subtle changes in the tRNA modification pattern may be sufficient to ultimately affect downstream processes, including host immunity responses. The effect on general and specific host immunity responses needs to be established, as a screen on > 1000 natural accessions of *A. thaliana* found diverse mechanisms of resistance to the same pathogen, *P. syringae* [233]. Furthermore, it must be assessed whether any observed phenotype actually stems from perturbed tRNA modification, or if it can be traced back to *moonlighting* activities of the modification

enzyme or pathway. A recent study showed that Tum1, which is part of the 2-thiolation pathway in *S. cerevisiae*, is involved in sterol ester regulation in a way that is entirely 2-thiolation independent [234]. Indeed, such diverging functions of tRNA modifying enzymes need to be considered when evaluating cellular functions.

Quantitative high-resolution LC-MS studies have given us a detailed account of tRNA modification patterns in numerous organisms following stress [121,152,191]. While this has highlighted the sensitivity of tRNA modification to external stimuli, it is still not clear whether changes in tRNA modification levels constitute an active form of adaptation or a simple passive response to altered translational requirements. In the active adaptation scenario, tRNA modifications need to be constantly monitored by modification/demodification enzymes so that mature tRNAs and nascent tRNA transcripts can be efficiently modified according to cellular demands. Though this might be energetically feasible in specific nutrient rich environments, it is less likely to occur in most other situations. However, the importance of tRNA structural stability and translational efficiency upon stress response does merit the notion of tight control for key nucleosides modifications [121,212]. In the passive adaptation scenario, tRNA modifications are not actively altered but changes occur following tRNA turnover, whereby only nascent tRNA transcripts are modified according. This energetically favorable model is supported by work in *S. cerevisiae*, where loss of 2-thiolation occurs gradually over > 7 h when cells are shifted to non-permissive temperatures, only to recover fully in the same extent of time when temperature stress is removed [152]. Nonetheless, such a delayed response is counterintuitive to studies that show the importance of 2-thiolation in cellular functions that require rapid responses, such as translation of virulence factors. Hence, tRNA modification regulation is likely to be far more complicated than either scenario suggests, and it is conceivable that elements from both the active and the passive model contribute towards tRNA modification homeostasis.

7. Perspectives

Post-transcriptional nucleoside modifications are universal on RNA species and they have been assigned numerous cellular functions ranging from translational control to epigenetic regulation. This insight has required methods that enable accurate identification and quantification of RNA modification, as well as unbiased analysis of translation. For example, bisulfite sequencing has been extensively used to map m^5C methylation sites on individual RNAs and for genome-wide methylation analysis [235], whereas LC-MS analysis is often the preferred choice for quantitative analysis of RNA modification patterns [189,190]. Many qualitative and quantitative approaches initially developed for mapping modifications in rRNA have been revisited for their applications in tRNA modifications analysis. One recent breakthrough in method development is the adaption and application of the next-generation sequencing-based RiboMethSeq protocol for analysis of tRNA 2′-O-methylation [236]. On the other hand, ribosome profiling provides vast insights into post-transcriptional regulation the cell, revealing which transcripts are translated into proteins at any given time [237].

Although each method yields valuable data, we are now at a crossroad where less and less knowledge can be gained using only one specific methodological approach. Indeed, to comprehensively understand how and why tRNA modification responds to infection, systematic and unbiased studies that temporally dissect tRNA modification pattern changes – and correlate those to translational alterations – are needed to unravel complex host-pathogen interactions in a step-by-step-fashion. To this end, new analytical methods that require far less input material will greatly expand the range of samples to be analyzed [238], and user-friendly software pipelines will facilitate the analysis of LC-MS [239] and high-throughput sequencing data [240], thereby greatly furthering our understanding of tRNA modification-mediated regulation in cellular immunity and pathogenicity mechanisms.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgements

The authors thank the Guest Editor, Prof. Magdalena Boguta, for inviting us to contribute to this Special Issue. The authors also thank Dr. Tamara Bar-Magen Numhauser and M.Sc. Pavlína Gregorová for scientific discussions and valuable suggestions on this review.

Funding

This work is supported by the Academy of Finland [grant numbers 294917 and 307215; to L.P.S.] and the Sigrid Jusélius Foundation [Three-year grant 2017–2020; to L.P.S.]. Open access charges are covered by the Helsinki University Library.

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