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tRNA modifications regulate translation during cellular stress

Chen Gu[1], **Thomas J. Begley**[2,3,*], and **Peter C. Dedon**[1,4,*]

[1]Department of Biological Engineering and Center for Environmental Health Science, Massachusetts Institute of Technology, Cambridge, MA

[2]State University of New York - College of Nanoscale Science and Engineering, Albany, NY

[3]The RNA Institute at the University at Albany, Albany, NY

[4]Singapore-MIT Alliance for Research and Technology, Singapore

Abstract

The regulation of gene expression in response to stress is an essential cellular protection mechanism. Recent advances in tRNA modification analysis and genome-based codon bias analytics have facilitated studies that lead to a novel model for translational control, with translation elongation dynamically regulated during stress responses. Stress-induced increases in specific anticodon wobble bases are required for the optimal translation of stress response transcripts that are significantly biased in the use of degenerate codons keyed to these modified tRNA bases. These findings led us to introduce the notion of tRNA modification tunable transcripts (MoTTs – transcripts whose translation is regulated by tRNA modifications), which are identifiable using genome-wide codon counting algorithms. In support of this general model of translational control of stress response, studies making use of detailed measures of translation, tRNA methyltransferase mutants, and computational and mass spectrometry approaches reveal that stress reprograms tRNA modifications to translationally regulate MoTTs linked to arginine and leucine codons, which helps cells survive insults by damaging agents. These studies highlight how tRNA methyltransferase activities and MoTTs are key components of the cellular stress response.

Keywords

modified ribonucleoside; transfer RNA; stress response; translation; codon usage

*Correspondence should be addressed to: Thomas Begley, SUNY College of Nanoscale Science and Engineering, Albany, NY, tbegley@sunycnse.com; and Peter Dedon, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, pcdedon@mit.edu.

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INTRODUCTION

Cellular responses to stress and damage

Damage, stress and disease—The chemical nature of DNA, protein and lipids makes these vital cellular macromolecules susceptible to damage from endogenous and exogenous agents. Normal metabolic processes can produce alkylating agents (*i.e.,* formaldehyde and nitrosamines) and a wide array of reactive oxygen and reactive nitrogen species (*i.e.,* OH, H_2O_2 and NO) [1]. Similarly, environmental exposures to pesticides, consumer products and radiation sources can also promote cellular alkylation, oxidation and nitrosation damage. Alkylating and oxidizing agents have the potential to disrupt the cellular redox balance by depleting cellular glutathione levels and can damage DNA, proteins and lipids [2–4]. DNA damage can drive mutagenesis and the resulting DNA sequence changes can drive carcinogenesis and cancer progression. Protein damage can deplete the cell of vital activities, cause protein aggregation and result in the formation of reactive carbonyls (*i.e.*, advanced glycation end products), with the latter two mechanisms implicated in neurodegenerative diseases [5]. Lipid damage in the form of peroxidation can generate DNA- and protein-damaging agents, as well as disrupt cellular membranes. Lipid damage is linked to both cancer and neurodegenerative disease [5]. Lastly, disruption of cellular redox balance can promote inflammation, with this being linked to a host of chronic diseases [6–8]. Cells respond to all of these stresses by controlling expression of a variety of response genes, including the DNA damage response.

DNA damage response—The DNA base and sugar moieties are subject to damage by oxidation and alkylation that generate adducts and cause single- and double-strand breaks [1]. All types of cells, ranging from simple prokaryotes and eukaryotes to mammals, have built in defense mechanisms to respond to chemical and physical changes to the genome in the form of the DNA damage response. For example, DNA double-strand breaks are recognized by sensor proteins, which in turn recruit transducer proteins to activate a cascade of signals. The activated DNA damage response will regulate the cell cycle, increase the level of DNA repair proteins and in some cases signal for cell death [9, 10]. In humans, the sensor, transducer and DNA repair proteins can include MRN complex (Mre11-Rad51-Nbs1), ATM, p53 and Brca1, to name a few, with disruptions leading to genome instability syndromes and increased cancer incidence [11–13]. Single-strand breaks, specific mismatches and bulky DNA adducts will also activate the DNA damage response. The signalling cascade linked to the DNA damage response will optimize the cell for DNA repair and in most cases maintaining the integrity of the DNA and health of the cell and organism [14].

Heat shock and unfolded protein responses—In addition to DNA damage, alkylating and oxidizing agents can promote protein damage to activate protein-stress response pathways [15–17]. Translation errors and compounds that disrupt post-translational processing of proteins can also promote folding problems. The *Streptomyces*-produced nucleoside antibiotic mixture Tunicamycin is a compound that prevents N-linked glycosylation [18]. Misfolded and unfolded proteins are readily recognized by cellular machinery and activate the cytoplasmic heat shock response (HSR) and endoplasmic

reticulum (ER)-associated unfolded protein response (UPR) [19]. Both HSR and UPR activate chaperone and signal transduction systems to refold proteins and in some cases reprogram the cell. For example, the UPR will recognize misfolded proteins in the ER lumen using a chaperone protein. The activated UPR turns on an elegant signal transduction pathway that includes splicing of a specific mRNA (*i.e., HAC1* in budding yeast) to promote the production of an active transcription factor and up-regulation of systems to promote folding and, if prolonged, promote cell death [20, 21]. Tunicamycin components are classic activators of the UPR [22]. An overactive UPR is implicated in the pathogenesis of cancer and amyotrophic lateral sclerosis, Parkinson's, Huntington's and Alzheimer's diseases [23].

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) response and detoxification—ROS can include $O_2^{\bullet-}$ and H_2O_2 from the mitochondria and $OH^{\bullet}$ from Fenton reduction of H_2O_2 and breakdown of reactive nitrogen species such as peroxynitrite ($ONOO^-$) [1]. ROS and RNS can damage all types of biomolecules, including RNA, lipids, proteins and carbohydrates, with damage and response best characterized for DNA. Increased $O_2^{\bullet-}$ and H_2O_2 levels inside the cell will promote the oxidation of protein-based cysteine amino acids. The AP-1 like transcription factors have harnessed oxidized cysteine to sense fluxes in cellular ROS levels and activate detoxification systems [3, 24]. Superoxide dismutase can detoxify $O_2^{\bullet-}$ to produce H_2O_2, which is further detoxified by catalase and glutathione peroxidase (Gpx) enzymes to make H_2O [3, 25]. Gpx proteins require reduced glutathione as a cofactor to detoxify H_2O_2 and have also been shown to work on peroxidized lipids [26, 27], with many of the Gpx enzymes possessing the amino acid selenocysteine (Sec) as a key catalytic residue.

Targeted changes in gene expression are the key to an appropriate and efficient response to DNA or protein damage or increased ROS and RNS levels. Gene expression can be regulated at many different levels in eukaryotic systems, including transcription, translation and post-translational mechanisms. In this review, we will focus on translational control mechanisms. Specifically we will focus on how tRNA modification enzymes regulate the translation of key stress response proteins. In addition we will discuss how tRNA modification defects lead to protein errors, with the latter phenotypes potentially exploitable for disease treatments.

tRNA modifications

tRNA structure and function—With their 3′-linked amino acids, tRNA molecules are composed of 70–90 nucleotides of linear sequence that folds into a cloverleaf-shaped secondary structure and L-shaped tertiary structure, which fits into the tRNA binding sites (P and A) in the ribosome. They are initially transcribed with canonical U, A, C and G bases, but the nucleobases and ribose sugars are chemical modified by a large system of enzymes to form one of >120 different known chemical structures. There are ~25–30 types of modified ribonucleosides in an organism and an average of 11 and 13 modifications spread throughout each tRNA in yeast and humans, respectively [28–32]. As shown in Figure 1 for budding yeast, the structures of these modifications on tRNA are highly diverse, ranging in complexity from simple methylation to amino acid conjugation to multi-step biosynthetic reactions leading to complex ring structures [33, 34]. To a certain extent there

are conserved locations for many of these modifications, such as the presence of dihydrouridine (D) and pseudouridine (ψ) in the D stem and loop and T stem and loop of many tRNAs, respectively. There are also a large number of chemically distinct modifications found in the anticodon stem and loop [35]. Interestingly, although these non-canonical nucleosides can be located throughout the structure of tRNA, the significance of their functions remains elusive. It was initially believed that these modified ribonucleosides played mainly structural roles by stabilizing the unique secondary and tertiary structures of tRNA. For example, the highly conserved D imparts flexibility [36], whereas ψ and 2′-*O*-methylation stabilize base stacking [33, 34, 37]. More importantly, the large diversity of chemical structures in the anticodon loop, and especially at the wobble position, has been shown to be critical for translational fidelity, frame-shift prevention and translation efficiency [33, 34, 38–40]. We recently expanded these roles for wobble modifications to include fine-tuning of the efficiency of translation of codon-biased mRNAs from classes of stress response genes [41–45]. The clear regulatory function of tRNA modifications thus raises the issue of pathology and disease caused by defects in tRNA function.

Chemistry of modification in anticodon stem and loop and its link to translational control—The diversity of tRNA modification structures, the fact that some wobble base modifications are only found on a subset of tRNAs that interact with select codons and the known role of wobble modifications in modulating anticodon-codon interactions, all suggest a role for anticodon stem and loop tRNA modifications in regulating translation by virtue of their ability to control the rate of translational elongation [33, 34]. Indeed, if they play a regulatory role, tRNA modifications must change in response to specific alterations in cell state. In addition the changes must alter the codon-reading properties of the associated tRNA. The variety of chemical structures at wobble positions supports this model. In budding yeast, 9 of the 24 modified ribonucleosides (Figure 1) are found at the wobble position 34 in tRNA [46]: ψ, hypoxanthine (I), 2′-*O*-methylguanosine (Gm), 2′-*O*-methylcytidine (Cm), 5-methoxycarbonylmethyluridine (mcm^5U), 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U), 5-carbamoylmethyluridine (ncm^5U) and 5-carbamoylmethyl-2′-*O*-methyluridine (ncm^5Um). It is important to note that the majority of these wobble modifications occur at U, which gives this pyrimidine significant regulatory flexibility in reading a codon. For example, yeast tRNA methyltransferase 9 (Trm9) participates in the biosynthesis of mcm^5s^2U and mcm^5U by adding the final methyl group at wobble positions in five tRNA species ($tRNA^{Arg(\underline{U}CU)}$, $tRNA^{Gly(\underline{U}CC)}$, $tRNA^{Lys(\underline{U}UU)}$, $tRNA^{Gln(\underline{U}UG)}$ and $tRNA^{Glu(\underline{U}UC)}$) [47, 48]. It has been shown that $tRNA^{Arg(\underline{U}CU)}$ plays a central role in the response to alkylating agents, with enhanced translation of mRNAs containing its cognate AGA codon [45]. Another feature of wobble modifications is their frequent tRNA specificity. For example, tRNA methyltransferase 4 (Trm4) catalyzes the formation of m^5C in over 34 species of tRNA, most frequently at position 48 between the variable arm and T stem loop [32]. However, $tRNA^{Leu(\underline{C}AA)}$ is the only tRNA with m^5C at the wobble position [32] and we have shown that this uniqueness plays a role in the regulation of translation during the oxidative stress response [42]. The role of wobble base methylation of pyrimidine nucleobase structures thus emerges as a central feature of a tRNA-based translation regulatory system.

tRNA methyltransferases

General reaction mechanism—tRNA methyltransferases (Trm) transfer the methyl group from *S*-adenosyl methionine (SAM) to the 2′-OH of the ribose sugar, to the carbon and nitrogen atoms of the nucleobase, or to nucleophilic sites in modification intermediates (some examples are shown in Figure 2). There are 18 known Trm enzymes in *S. cerevisiae*, with genomic analyses predicting 36 human Trms [49]. In many cases, and for both Trm4 and Trm9, there are 2 or more human homologs for each yeast Trm, which suggests diversification or specialization of Trm activity to new modifications in humans, modification of different tRNAs or RNA substrates, or functions other than tRNA modification. Such is the case for the human Trm9 homologs, ALKBH8 and hTRM9L. There is also a diversity of function among Trms, with ALKBH8 homologs in mammalian, bacterial and protozoan cells showing DNA dealkylation repair activity and RNA oxidation activity, both derived from the Fe(II) and 2-oxoglutarate-dependent oxygenase domain of the protein [50]. Mammalian and plant ALKBH8 homologs are tRNA hydroxylases that convert mcm^5U to (*S*)-5-methoxycarbonylhydroxymethyluridine ($mchm^5U$) at the wobble position of $tRNA^{Gly(\underline{U}CC)}$ [51, 52]. At the same time, ALKBH8 also catalyzes methylation of wobble U derivatives to form mcm^5U and mcm^5s^2U in certain tRNA species, such as the tRNA for Sec [51, 53–55]. Regardless of enzyme identity or regulation, modified ribonucleosides can promote tRNA structural stability and folding, translational fidelity, frame-shift prevention and translation efficiency, with evidence for roles in tRNA quality control, cellular stress responses and cell growth [34, 38–40, 46, 56, 57].

Human tRNA modification systems and disease—Akin to the emerging recognition of defects in tRNA aminoacyl synthases in a variety of human diseases [58], several clinical observations point to critical roles for tRNA modifications in human diseases such as cancer. This point is illustrated with ALKBH8 and hTRM9L human homologs of yeast Trm9 [51, 53, 54]. ALKBH8 has been shown to be over-expressed in human bladder cancers and thought to be anti-apoptotic, as silencing its expression down-regulated NOX-1 activity and caused activation of the JNK and p38 pathway, leading to increased apoptosis [53]. Its homologous partner, hTRM9L, on the other hand, appears to be epigenetically silenced in breast, testicular, bladder and colon cancers [59]. Indeed, re-expression of hTRM9L in SW620 and HCT116 colorectal cancer cell lines, in which hTRM9L is silenced, suppresses tumor growth and promotes senescence [59]. We also note that hTRM9L maps to the short arm of chromosome 8, a region commonly lost or silenced in many cancers, including colorectal and breast [60–64]. Available data suggest a model in which ALKBH8 and hTRM9L have opposing roles in managing cell survival and cell death. For example, ALKBH8 has been shown to be vital for cell viability in late stage tumors, with knockdown leading to cell death [53]. Similarly, turning off hTRM9L in late stage tumors is required for them to grow, [59, 64, 65] as re-expression of TRM9L in late-stage models drives these cells into senescence [59].

Yeast tRNA modification systems and associated phenotypes—Biochemical characterization of tRNA modification systems in *E. coli* and *S. cerevisiae* has led to the identification of many of the proteins and synthetic steps needed to generate specific tRNA modifications. The creation and systematic use of a library of *S. cerevisiae* gene deletion

mutants have also allowed researchers to observe the association between tRNA modification deficient cells (*i.e.*, *trm*Δ) and stress phenotypes (examples in Figure 2). Deletion of a specific tRNA modification system can lead to global or specific hypo-modification of tRNA. Hypo-modification of tRNA is linked to disease pathology in humans, with specific under-modification of yeast tRNA leading to sensitivity to agents that promote increased ROS, DNA damage and protein errors. For example, *trm4*Δ cells are sensitive to killing by H_2O_2, suggesting that they have a compromised response to ROS-inducing agents, which could be due to decreased translation of a critical detoxification protein (Figure 2) [42]. Sensitivity to agents that promote DNA double-strand breaks and S-phase damage (IR, MMS and HU) have been demonstrated for *trm9*Δ cells, which could be due to a defect in the translation of critical DNA replication activity [44, 66]. In support of this translation defect idea, *trm9*Δ cells demonstrate sensitivity to aminoglycoside antibiotics that promote protein synthesis errors, with the cells revealing increases in arginine for serine misincorporation events and frame-shifting [66]. The aminoglycoside-induced sensitivity and increased translational errors in *trm9*Δ cells promote protein errors and misfolding, with the absence of Trm9 leading to activation of the UPR and HSR (Figure 2). Notably tRNA modification deficient strains have reported phenotypes that include slow growth and sensitivity to 5-fluorouracil, ultraviolet radiation, cycloheximide and heat, with each phenotype potentially due to defects in stress signalling and/or protein synthesis [67, 68, 69 70].

The connection between stress phenotypes, hypo-modification of tRNA, and protein synthesis defects in *trm* mutants supports the idea that there may be a distinct translational response to stress. To help decipher the translational responses, new technologies and analytic approaches have been developed. Coming in the form of mass spectrometry-based ribonucleoside analysis and genome-wide codon bias analytics, these approaches have helped link tRNA modifications to the regulation of critical stress response proteins. Systems-based approaches reveal that groups of codon-biased transcripts over-use codons that can be linked to specific tRNA modifications, with subsequently translated proteins also functioning in stress response pathways. Together, these results support the idea that many stress response proteins are translated from tRNA modification tunable transcripts (MoTTs), a concept discussed in detail below.

Methyl-based modifications regulate gene expression

Transcriptional regulation by m^5C—We have observed a critical role for tRNA modifications, and tRNA methylation in particular, in the control of translation following stress. A common theme associated with the control of gene expression, and in some cases epigenetics, is the use of enzyme-catalyzed methylation to regulate transcription, with the corresponding epigenetic marks regulated or dramatically altered in response to environmental stimuli or in different cancers. For example, enzyme-catalyzed methylation by DNA methyltransferases (DNMT's) to form m^5C is a well-established regulator of gene transcription [71–74], with methylation patterns in some promoter regions reprogrammed by stress or altered in some cancers [75, 76]. Promoter CpG methylation can silence the transcription of tumor suppressor activities leading to decreased DNA damage signaling and DNA repair, thus contributing to the etiology of different cancers [76]. Histone methylation

by protein methyltransferases (PMT's) functions in a similar manner as a well-recognized regulator of gene expression, with the "epigenetic" methylation marks occurring on histone tails [77]. As part of an integrated system with DNA methylation, histone methylation is theorized to be part of a complicated "histone code" that is altered by environmental signals and disease pathologies to control gene expression. Lysine N^7-methylation (H3K4, H3K36) in histone H3 and the subsequent demethylation are considered to be dueling signals that regulate transcription. At their simplest, both promoter and histone methylation affect gene expression by regulating how much of a transcript is made, with these epigenetic signals altered in cancer to drive pathogenesis and reprogrammed after environmental exposures. However, the simplicity of methylation as the sole chemistry of epigenetic marks has now been complicated by the emergence of 5-hydroxylmethylcytidine, 5-formylcytidine and 5-carboxycytidine as putative epigenetic marks in DNA [78].

tRNA methylation and translational regulation—In parallel with DNA and histone protein methylation, we introduce the concept of RNA modifications, including RNA methylation, as marks that reprogram in response to environmental changes and control gene expression at the level of translation. We and others have demonstrated that tRNA modification enzymes and their homologs are tumor growth suppressors and down-regulated in some cancers [59] [64, 79]. We have also observed that tRNA methylation affects gene expression by regulating how well a transcript is translated [41–45, 66]. The DNA and RNA modification activities represented by DNMTs, PMTs and Trms share a common theme of regulating gene expression by enzyme-catalyzed methylation, with altered regulation/patterns linked to environmental exposure and cancer. The concept of tRNA wobble methylation expands methylation signals to regulators of translation and links tRNA modifications and Trms to the synthesis of proteins vital to stress responses [41–45, 66]. A major problem with studying RNA modification signals is the availability of technology to analyze and quantify them, which we have solved by developing a novel bioanalytical and bioinformatic platform.

Stress-induced changes in tRNA modification levels are linked to MoTTs

Quantifying changes in tRNA modification levels: A mass spectrometry and bioinformatic platform for identifying and quantifying modified ribonucleosides—

The model we have developed posits tRNA modifications as regulatory elements, which requires that they be coordinately regulated and dynamically altered in response to a stimulus. Following on our initial observations linking tRNA wobble mcm^5U and selective translation of codon-biased mRNAs in response to alkylation stress [45], we undertook an assessment of stress-induced changes in the full set of 24 tRNA modifications in budding yeast, with the goal of identifying patterns and behaviors for different stresses. To facilitate this systems-level analysis, we developed a chromatography-coupled mass spectrometry (LC-MS) platform [80] that entails (1) RNA isolation and HPLC purification of tRNA [81], (2) enzymatic hydrolysis to ribonucleosides for reversed-phase HPLC resolution, (3) mass spectrometry-based identification and quantification of individual ribonucleosides and (4) multivariate statistical analysis of the resulting fold-change data comparing controls to treatment conditions (Figure 1) [41, 42, 80]. We then used this platform to analyze changes in the levels of tRNA modifications after exposing

yeast to equitoxic doses of four mechanistically distinct toxicants: H_2O_2, MMS, sodium arsenite ($NaAsO_2$) and sodium hypochlorite (NaOCl). As we reported [41], the levels of 23 tRNA modifications uniquely changed in response to each toxicant, with hierarchical clustering of fold-change data distinguishing both agent and dose as signature patterns of increase and decrease. These stress-specific patterns of tRNA modification changes were then linked to selective translation of codon-biased mRNAs for stress response proteins [42], which raises the concept of MoTTs.

MoTTs: Codon specific regulation of translation—Changes in wobble base tRNA modification levels have the potential to work in concert with codon usage patterns in specific transcripts to regulate translation of response proteins. These can be designated as modification tunable transcripts (MoTTs), with Figure 3 detailing the methodology used to identify MoTTs in any organism [43]. The concept of MoTTs is similar to the idea in mammals that the transcription of some but not all genes can be regulated by promoter methylation (m^5C) and is analogous to transcripts specific to enzymes containing Sec, a non-standard amino acid with a tRNA that reads internal stop codons, as discussed in the next section. The idea is that the MoTTs preferentially use one of several degenerate codons for an amino acid. In order to identify MoTTs and evaluate the use of specific codons and codon combinations in genes and gene networks, we developed a gene-specific codon counting (GSCC) algorithm as a codon usage and statistical analysis tool. Further we employed the GSCC algorithm to analyze the 5,780 genes in *S. cerevisiae* [82]. Visualization approaches were then used to identify distinct codon usage patterns in specific genes and groups of genes. A computational analysis of *S. cerevisiae* cDNAs revealed 425 open reading frames that possess statistically significant deviations in the usage of 29 codons compared to other transcripts [45, 82]. This over-usage includes the presence of many non-preferred (*i.e.*, non-optimal) codons, relative to genome averages. The 425 codon-biased transcripts represent potential MoTTs and they over- or under-use specific mono-codons throughout their open reading frames. Interestingly, several quad-codon patterns (*i.e.*, 4 repeats of a codon) are well represented in the 425 identified transcripts. Many of the MoTTs-associated codons are found in mixed codon boxes in which wobble base tRNA modifications enhance interactions with one codon (*i.e.*, AGA for Arg) while restricting interactions with others (*i.e.*, AGC for Ser). Functional analysis of the 425 potential MoTTs found that their corresponding proteins are over-represented in activities associated with protein synthesis, metabolism and stress responses, with four prominent members of the DNA damage response (*RNR1-4*) identified. As described in detail shortly, several published studies support the idea that translation of specific codon-biased transcripts can be regulated by tRNA modifications, with stress response genes well represented in our list of candidate MoTTs. This concept of codon usage, tRNA modification reprogramming and selective translation is illustrated by stop-codon recoding for mRNAs of Sec-containing proteins.

Alkylation damage and mcm^5U—One example of the connection between the modification status at a tRNA wobble position and cellular stress response is showcased in our report that the presence of mcm^5U at the wobble position of certain tRNA was crucial for cell survival following DNA alkylation damage [45]. In *S. cerevisiae*, TRM9 catalyzes the addition of the final methyl group on the modifications mcm^5U and mcm^5s^2U, which are

found at the uridine wobble base of $tRNA^{ARG(\underline{U}CU)}$ and $tRNA^{GLU(\underline{U}UC)}$. The mRNAs for yeast translation elongation factor 3 (*YEF3*) and ribonucleotide reductases 1 (*RNR1*) and 3 (*RNR3*) are over-represented with AGA and GAA codons. *YEF3*, *RNR1* and *RNR3* fit the criteria for MoTTs because they over-use specific degenerate codons and the last two correspond to established stress response activities. The basal translation of *YEF3*, *RNR1* and *RNR3* mRNA was found to be dramatically decreased in *trm9*Δ cells lacking mcm^5U and mcm^5s^2U in the corresponding tRNA anticodons [45]. The decrease in the tRNA modifications led to reduced expression of these key damage response proteins even though the transcription of these genes remained unperturbed, and ultimately caused enhanced susceptibility of *trm9*Δ cells to DNA alkylation agents [45].

Oxidative damage and m^5C—Previously, we showed that the *S. cerevisiae* Trm4-catalyzed modification of C to m^5C at the wobble position of $tRNA^{Leu(\underline{C}AA)}$ increased in response H_2O_2 exposure, which stimulated translation of mRNAs (MoTTs) derived from the 38 genes in yeast in which 90% or more of the leucines are encoded by UUG [42]. Among these UUG-enriched MoTTs is that for the ribosomal protein Rpl22a, one of two alternative proteins for Rpl22, which, in terms of mRNA sequence, stands in sharp contrast with the mRNA for its paralog Rpl22b that lacks significant enrichment of UUG, despite apparent homology at the amino acid level. As expected, H_2O_2 exposure did not increase the rate of translation of Rpl22b, a non-MoTT, and only deletion of the gene for Rpl22a, a MoTT, rendered the cells sensitive to killing by H_2O_2-induced oxidative stress [42]. These results provide a direct link between stress-induced increases in a specific wobble tRNA modification and enhanced translation of codon-biased mRNAs for critical stress response genes. This again illustrates the concept of MoTTs. Notably, mRNA levels for *RPL22A*, *YEF3*, *RNR1* and *RNR3* are identical in wild-type, *trm4*Δ (for *RPL22A*) and *trm9*Δ (for *YEF3*, *RNR1*, and *RNR3*) cells [42, 45], which further demonstrates that the tRNA modification-dependent gene regulation program operates at the level of translation.

Stop-codon recoding as a well-studied example of MoTTs—The connection between tRNA modifications and stress response enzymes has previously been described during the process of translational recoding. Sec is considered the 21^{st} amino acid and a dedicated codon for this amino acid is not found in the genetic code. To accommodate incorporation of this non-standard amino acid, some organisms, including mice and humans, use an internal stop codon (UGA) and specific sequences in the 3′ untranslated region (UTR) of the mRNA to signal for Sec incorporation. Sec is the key active site amino acid in some Gpxs and thioredoxin reductases (TrxRs). Many selenoproteins are regulated by selenium levels and have stress response roles specific to the detoxification of ROS [83–84]. Sec is charged on specific tRNAs that contain anticodons that pair with the UGA stop codon, $tRNA^{Sec(\underline{U}CA)}$, and the enzyme-catalyzed tRNA modifications mcm^5U and mcm^5Um are found at the wobble position. The modifications 1-methyladenosine (m^1A) at position 58, ψ at position 55, and isopentenyladenosine (i^6A) at position 37 are also found on $tRNA^{Sec(\underline{U}CA)}$ [85]. The presence of m^1A, ψ, i^6A and mcm^5U is required for the formation of mcm^5Um, with the levels of this modification being sensitive to selenium concentration inside the cell and promoting a distinct tertiary structure [86]. The two different states for $tRNA^{Sec(\underline{U}GA)}$ suggest a highly regulated modification pattern and support the translation of

distinct subclasses of selenoproteins. Elegant studies in mice have demonstrated that mcm^5U and mcm^5Um are required for efficient incorporation of Sec into specific selenoproteins [54, 87]. There are 25 selenoproteins in humans, as identified by computational approaches that identify internal stop codons and regulatory sequences [88]. From the perspective of codon usage, transcripts corresponding to selenoproteins are severely biased as they contain more than one stop codon, which is significant compared to the thousands of transcripts in humans that use standard amino acids and contain only a single stop codon. The concept of codon bias being used to regulate the translation of stress response proteins is an exciting prospect, and we put forth the Sec transcripts are MoTTs.

Regulation of translation elongation

Model for increased translation by anticodon loop modifications—Studies on mcm^5U and m^5C support a general model (Figure 4) in which translation elongation is regulated to promote cellular stress responses. The ability to change the kinetic and thermodynamic properties of specific anticodon-codon interactions is linked to wobble base tRNA modifications, making them ideal regulatory points. Stress-induced increases in mcm^5U and m^5C (or any wobble modification) allow for increased decoding of specific codons, which can be regulatory in MoTTs that over-use the codon. The increased modification of tRNA selectively increases the translation of MoTTs, which can, in effect, accelerate the translation of specific transcripts and lead to increased levels of critical response proteins. The increased tRNA modification also has the potential to increase translational fidelity, which should correspond to more active proteins. Such decreased protein errors would allow the cell to repurpose protein stress response systems during times of increased external stimuli. Our model is supported by (1) codon reporter systems and studies that support idea that specific codons need specific tRNA modifications to be efficiently translated; (2) the observation of increased wobble base modifications under specific stressors; (3) polysome profiles that demonstrate altered distribution of codon-biased transcripts in *trm* mutants; (4) matched mRNA and protein studies that show decreased levels of critical stress response proteins in *trm* mutants and (5) the observation that specific tRNA modifications promote translational fidelity [41–45, 66, 84, 89, 90]

Potential for tRNA modifications to restrict translation—The other side of translational regulation is the potential to down-regulate translation of specific proteins in response to environmental changes. This idea is illustrated in our codon reporter assays in budding yeast, which demonstrate that GAG-GAG-GAG-GAG is translated at higher levels in *trm9*Δ cells relative to wild-type cells [44]. This suggests that mcm^5s^2U tRNA modifications, which are found in $tRNA^{Glu}$ that decodes GAG, can repress translation of mRNAs that contain specific codon sequences. We have used our Gene-Specific Codon Counting database to identify 8 genes containing at ≥1 GAG-GAG-GAG-GAG sequence [82]. Functional analysis of these proteins indicates that many are involved in ribosomal RNA regulation and vesicle function.

Exploiting tRNA modifications for disease treatment

Cancer therapeutics—Carcinogenesis and cancer progression can be attributed to many different endogenous and environmental agents that promote damage, cell stress or alter

physiological conditions that promote cell growth. As described above, tRNA modification systems specific to the anticodon loop of key tRNAs regulate cellular stress responses and can promote the detoxification of damaging agents and efficient DNA repair to prevent cell death. Regulating responses to stress is a classic role for tumor suppressor proteins, with p53 as the archetype. Defects in p53 can corrupt cellular responses to DNA damaging agents, by preventing activation of downstream components and cell cycle checkpoints. p53-inactivating mutations are reported for many cancers and can allow for increased proliferation of cancer cells [91–93]. Decreased expression of known and potential tRNA modification enzymes have also been reported in lung and colorectal cancers. The tRNA-isopentenyltransferase (tRNA-IPT) TRIT1 is responsible for the formation of i^6A and the modification is found at position 37 of many tRNAs, most notably $tRNA^{Sec}$. TRIT1 levels are decreased in lung adenocarcinomas [79], which could compromise stress responses and give TRIT1 a growth suppressive role in some lung cancers. The human hTRM9L homolog of yeast Trm9 has been identified as a tumor growth suppressor in colorectal cancers, with deficiencies in hTRM9L found in specific cell models (SW620, HCT116, LoVo) and colorectal tumors from the clinic. The tumor growth suppressor role of hTRM9L suggests that it could be involved in stress response regulation, with this under investigation. Based on data generated in bacterial, yeast and mammalian cell culture models, deficiencies in specific tRNA modification enzymes and tRNA modifications should sensitize cancer cells to particular therapeutics. For example, he tRNA-IPT activity is required for modification of $tRNA^{Sec}$. Knockdown of tRNA-IPT or failure to modify A37 leads to decreased selenoprotein levels [94], with ROS detoxifying enzymes as notable members. These data suggest that TRIT1-deficient lung tumors would be sensitized to therapeutics that promote increased ROS. Similarly, a deficiency in hTRM9L has been shown to sensitize colorectal cancer cells to killing by aminoglycoside antibiotics [59]. The US Food and Drug Administration has already approved aminoglycoside antibiotics for Gram-negative, select Gram-positive and protozoal infections. Together with the cell-based killing data, this existing clinical use makes aminoglycoside drugs like paromomycin and gentamicin attractive personalized medical therapeutics for colorectal cancer.

Antibiotics—tRNA modification enzymes may also serve as potential targets for anti-fungal and antibiotic development, as they are already used by competing organisms to kill other species. For example, the mcm^5s^2U modification of *S. cerevisiae* is targeted by killer toxin system of *K. lactus*, with the associated endoribonuclease cleaving the ASL of specific tRNAs to shut down translation in *S. cerevisiae* [95, 96]. A potential drug-able example is found for TrmD, which is a tRNA methyltransferase that methylates a guanine at position 37 of various bacterial tRNAs [97–100]. The gene for TrmD is essential in many types of bacteria [98, 99], which points to its potential as an antibiotic target. In addition humans use a different family of Mtase enzymes to methylate guanine at position 37, which supports the idea that any drugs that target TrmD would be specific to bacteria. Several groups have explored the development of inhibitors of SAM binding to TrmD, with identification of several SAM analogs that bind with relatively high affinity [101, 102]. In one case, fused thieno-pyrimidones were identified as competitive inhibitors of SAM binding, with nanomolar binding affinity and a lack of activity against human homologs of TrmD [102].

Conclusions and Perspectives

In conclusion, we have described the technology and studies that support the idea that dynamic tRNA modifications regulate the translation of codon-biased transcripts. The unique reprogramming of tRNA modifications observed after cells were treated with DNA-damaging or ROS-inducing agents will most likely be a conserved theme for responses to other distinct stressors. For example, nutritional stress is predicted to promote reprogramming of tRNA modifications to drive a translational response program, which could also be coupled to tRNA degradation and altered aminoacylation programs. We have made the case that tRNA modification reprogramming is tightly linked to MoTTs, with these distinct transcripts serving as blue prints for translation. While we describe MoTTs as having codon biases that signal for “on” or more translation, there exists a strong possibility that specific tRNA modification patterns can be used to signal for “off” and slow down translation of specific transcripts. Turning off stress responses is an important and understudied area, but from the perspective of the cell, efficient down-regulation of cellular programs (*i.e.*, DNA replication) can help protect against offending agents. There are established and exploitable connections between tRNA modification systems and disease. We envision personalized cancer therapeutics that target specific modification programs, with the tRNA modification signature giving cancers cells a growth advantage but making them susceptible to a specific stressor. Aminoglycoside antibiotics are potential route towards realizing this potential, but further study and pharmacological optimizations are needed. Similarly the targeting of organism specific tRNA modification systems also has great potential for treating infectious agents, as pathogen specific Trms and their unique chemically modified tRNAs are attractive targets.

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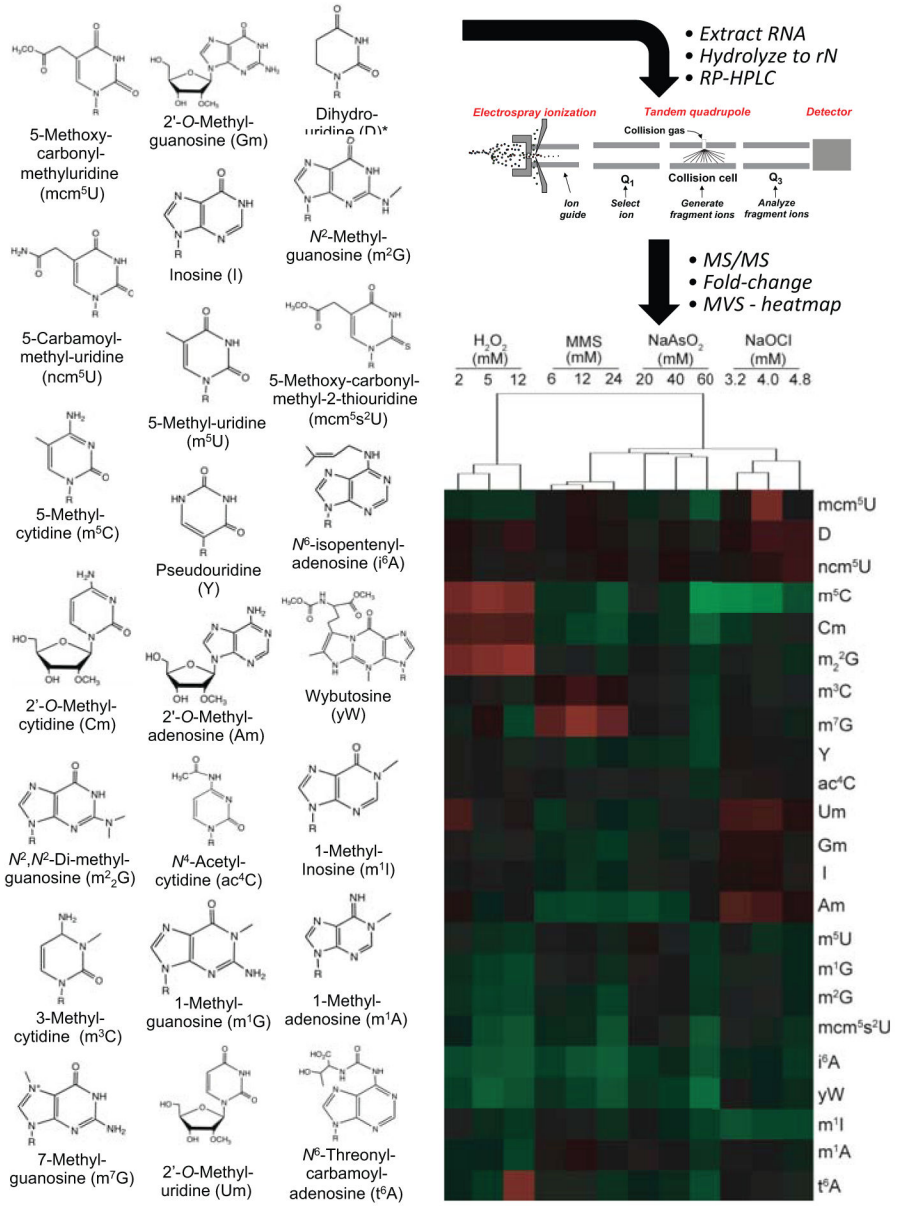

Figure 1. Stress-induced changes in tRNA modification as measured by LC-MS/MS

Budding yeast contain 25 modified ribonucleosides, of which 23 can be measured by chromatography-coupled tandem mass spectrometry (LC-MS/MS). Following tRNA isolation and hydrolysis, individual modified ribonucleosides are resolved by reversed-phase HPLC and quantified by tandem mass spectrometry. The data are used to calculate fold-change values comparing control cells to stressed cells, with the fold-change values analyzed by multivariate statistics to identify patterns of stress-induced changes. The heat map represents fold-change data for four mechanistically distinct toxicants and shows both agent- and dose-specific signatures. The heat map image was reproduced from Chan *et al.* (2010) *PLoS Genetics* **6**: e1001247.

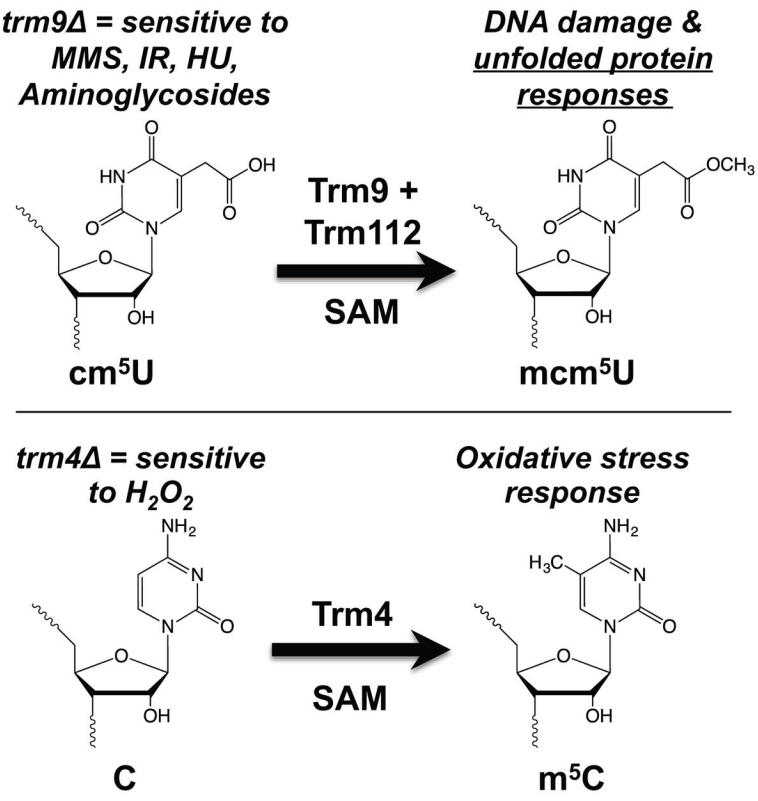

Figure 2. tRNA modifications and their relation to stress signaling pathways

Description of mutant phenotypes for cells deficient in Trm9 and Trm4, structure of substrates and products for each enzyme catalyzed tRNA modification, the pathways regulated by each tRNA methyltransferases, and pathways whose activation is prevented (underlined) by proper tRNA modification, via the prevention of amino acid misincorporation errors.

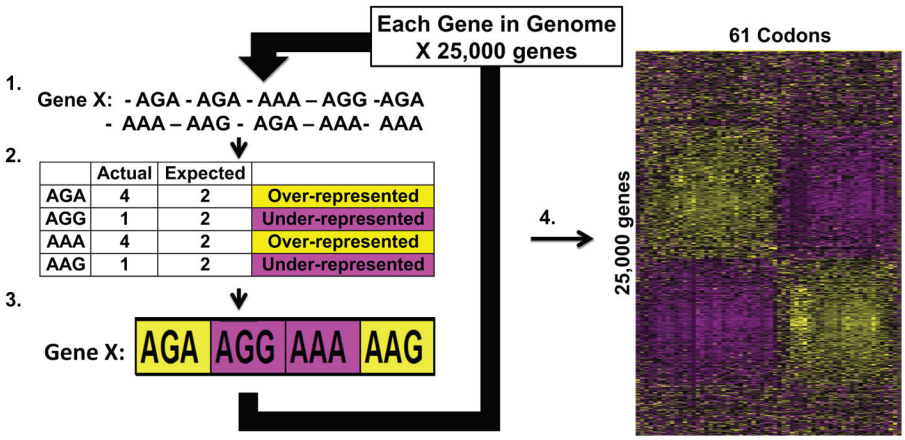

Figure 3. Genome-based identification of MoTTs

Iterative analysis of each open reading frame is used to count the number of codons in each gene and determine the frequency of use of synonymous codons for each amino acid (Steps 1 – 3). After analysis of all genes in a genome, the average value for all genes is then used to identify specific genes that are over- (yellow) or under- (purple) using a codon, with groups of genes that have similar codon over- and under-usage patterns identified by clustering and heat map visualization (Step 4).

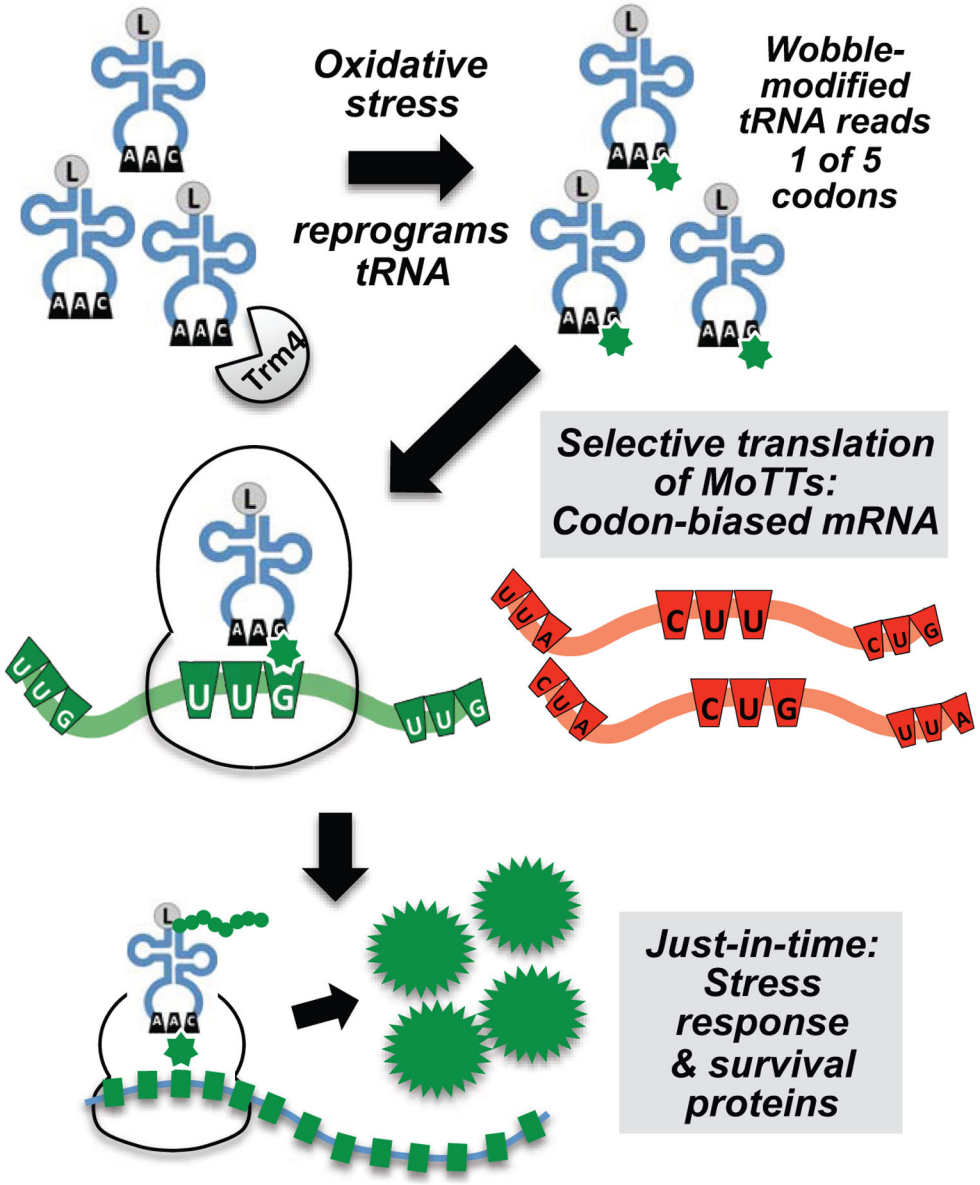

Figure 4. Changes in tRNA modification regulate the translation of MoTTs

The scheme depicts the concept of stress-induced tRNA reprogramming and selective translation of codon-biased mRNAs (MoTTs) for oxidative stress in budding yeast.