NAIL-MS Elucidates Crucial tRNA U34 Modifications in Response to Heat Stress across Eukaryotes and Prokaryotes ☆

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Abstract

Global warming leads to rising temperatures, necessitating organismal adaptation at the cellular level. One potential mechanism for maintaining proteome integrity during stress is the adaptation of tRNA modifications. While tRNA modification reprogramming has been well-studied under chemical stressors, its role in heat stress remains unclear. To address this, we performed a comparative analysis of tRNA modifications in *Arabidopsis thaliana*, *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Dictyostelium discoideum*, and *Escherichia coli* under heat stress. We assessed the abundance of 30 modified nucleosides using isotope dilution mass spectrometry under control conditions. *A. thaliana* showed a similar diversity and abundance of tRNA modifications compared to other eukaryotes, suggesting conservation across species. Under heat stress, overall tRNA modification levels were largely stable, with no significant changes in modifications such as dihydrouridine and N4-acetylcytidine. However, one to four modifications per organism were altered, with uridine modifications at position 34 (U34) being the most prominent change. Here, pulse-chase NAIL-MS (nucleic acid isotope labeling coupled mass spectrometry) experiments in *E. coli* and *S. cerevisiae* revealed that changes in U34 modifications occurred not only in pre-existing tRNAs but also in newly transcribed tRNAs. These results suggest that existing tRNAs adapt as an early response to heat stress, while newly transcribed tRNAs are reprogrammed to ensure long-term survival under prolonged heat. Our findings highlight the potential role of tRNA modification reprogramming in heat stress adaptation.

Introduction

Prokaryotes and Eukaryotes are continuously exposed to hostile environments, and survival requires maintaining a precise cellular response and functional proteome. To cope with stress conditions, gene expression on the transcriptional level is commonly regulated as a primary response to fine-tune cellular adaptation [1–4]. Additionally, studies on the post-transcriptional level of gene regulation have shown that RNA modifications are present in all living organisms [5] and play important roles in RNA metabolism, such as

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configuring RNA stability, translation efficiency, RNA structure, and alternative splicing [6].

The major function of tRNAs as adaptors of amino acids during protein synthesis is very well known, and, essentially, studies have demonstrated that amino acid charging, tRNA abundance and chemical modifications are essential for this process [7]. Although small in size, tRNAs are the most extensively modified RNAs in cells, both in abundance and chemical diversity. tRNA modifications are dynamic and contribute to the translation of mRNAs in different environmental settings, cellular types and ages [8,9]. The tRNA anticodon at positions 34, 35 and 36 binds to the codon (triplets) within the mRNAs. This binding occurs in the third position of the mRNA codon using a non-Watson-Crick interaction with tRNA position 34 (known as the wobble position), which is highly modified and, therefore, influences translation in many ways. In addition, these tRNA modifications at the wobble position are reprogrammed to adjust translation in hostile environments [10,11]. RNA modification reprogramming is most commonly detected using highly sensitive mass spectrometry analysis (LC-MS/MS), which is especially required for low-abundant RNA modifications. Such modifications are located in the anticodon (positions 34–36) of tRNA or its surrounding stem-loop region (positions 31–33 and 37–39), and they are crucial regulators of translation thus, these modifications often adjust due to environmental changes. In all organisms, uridine modifications at position 34 are chemically complex and require a multi-enzyme cascade for their incorporation. The shared molecular features among all organisms are the 2-thiolation and the C5 substitution of U34. Yet, *E. coli* adds a glycine to C5 (followed by deacetylation and methylation), while eukaryotes add acetic acid (followed by either methylation or condensation with ammonia). In particular, these U34 modifications enable proper base-pairing with both A- and G-ending codons and ensure the translocation of U34 into the minor groove, which leads to a correct and ideally timed read-out by the ribosome and thus maintains proteome integrity [12].

As a significant indicator of global warming, high temperature is a concern that broadly impacts human needs, activities and wildlife. As sessile organisms, plant growth is commonly compromised under heat. Besides, different (micro)organisms are constantly exposed to variations of temperatures that go beyond their optimal growth. Physiologically, high temperatures alter membrane fluidity, accumulation of reactive oxygen species (ROS) and protein denaturation [13]. Selective autophagy is a consequence to detoxify the cell and retain precious biomolecules and their building blocks. Especially plants depend on autophagy as a key player in survival and recovery from heat stress [14]. Moreover, vital cellular processes such as photosynthesis in plants and cyanobacteria, fermentation in yeast cells and the regulation of virulent genes in pathogenic bacteria are commonly affected by temperature [15,16]. Cells, in response, induce a manifold of stress-related factors for proper adaptation. Under heat, the well-described heat shock transcription factors and the heat shock proteins [17] are crucial for cellular survival at high temperatures. In addition, RNA modification reprogramming under heat stress (continuous exposure to mildly elevated temperatures) and heat shock (spontaneous exposure to highly elevated temperatures) has been recently reported as an important regulatory step in bacterial stress response. Under these conditions, *E. coli* presented an increase of s^4U, Gm and m^7G, whereas a decrease in modifications at position 32 (Cm, Um or s^2C) was observed [18]. In fact, a recent direct RNA sequencing study in conjunction with LC-MS analysis confirmed these findings [19]. In *S. cerevisiae*, a systematic analysis of various strains revealed a differential adaptation of tRNA modifications under heat stress. *E.g.* the commonly used strain Sc288, from which the strain BY4741 is derived, showed a decrease in 5-methoxycarbonylmethyluridine (mcm^5U), at the wobble position 34, while the strain W303 showed an increase in mcm^5U abundance under high temperatures. The 2-thiolated derivative mcm^5s^2U, in turn, was decreased in both strains [20], indicating that the tRNA reprogramming in yeast potentially works in a strain-dependent manner. In *A. thaliana*, the impact of stress on mRNA 6-methyladenosinylation is commonly reported [21]. In rice, crop adaptation and development were improved by the introduction of 5-methylcytidine (m^5C) in mRNAs under heat stress. This particular RNA modification is incorporated by Nsun2, an enzyme known to introduce m^5C also in tRNAs [22]. In reality, the impact of RNA modifications on plant tRNAs is still insufficiently addressed. For the social amoeba *D. discoideum*, which, upon starvation, progresses from a single-cell state to form a multicellular fruiting body, the tRNA reprogramming during this cellular transition was also reported [23], but it lacks further studies under stress conditions. In *C. elegans*, no tRNA modification profiling under heat has been reported so far, but one study found that loss of m^5C leads to increased ribosome occupancy at leucine and proline codons. Upon heat shock, Leu-UUG codons showed the highest ribosome density observed in a m^5C-depleted strain, suggesting that the translation of this codon is slowed during heat stress in the absence of m^5C in *C. elegans* [24]. Although some reports on heat-induced tRNA modification reprogramming exist for *E. coli* and *S. cerevisiae*, a comparative study examining changes in tRNA modifications across Prokaryotes and Eukaryotes in response to heat is still missing. Furthermore, the molecular mechanism driving changes in tRNA modification abundances is yet to be identified.

To address this knowledge gap, we performed a comparative tRNA modification analysis using different organisms (*E. coli, A. thaliana*, *C. elegans, S. cerevisiae and D. discoideum*), a common stressor (heat) and isotope dilution mass spectrometry for absolute quantification. Initially, the absolute abundance of 30 modified nucleosides was assessed for all organisms under optimal growth conditions. As previously reported, we verified that abundance and chemical diversity depend on the studied organism, with *E. coli* being the least modified organism in both aspects [25]. Similarly, we found a dependence of tRNA modification abundance on the growth medium and mostly a lower abundance in the minimal medium for both *E. coli* and *S. cerevisiae*. After establishing the tRNA modification density under control conditions, all organisms were subjected to heat stress. In general, we found tRNA modification abundance to be stable, including modifications such as dihydrouridine (D) and N4-acetylcytidine (ac^4C). Yet, within each organism's tRNAs, 1–4 modifications changed, and, most importantly, at least one uridine modification found at position 34 was altered due to heat stress. Remarkably, for *E. coli* and *S. cerevisiae*, we performed pulse-chase NAIL-MS experiments, and we revealed that changes in U34 modifications are not only due to adaptation in the tRNAs originated from the beginning of the heat exposure (original tRNAs) but also in newly transcribed tRNAs during the heat stress. Moreover, tRNA isoacceptors are purified and modification changes are not in all cases connected to adaptations in the molar abundance of a modification but potentially due to changes in tRNA isoacceptor abundance.

Results

Steady-state abundance of tRNA modifications across different species

To compare changes in tRNA modifications under stress, it is important to determine the absolute abundance of tRNA modifications in the studied organisms. For this purpose, we cultivated *Escherichia coli* (*E. coli* or Ec), *Dictyostelium discoideum* (*D. discoideum* or Dd), *Arabidopsis thaliana* (*A. thaliana* or At), *Saccharomyces cerevisiae* (*S. cerevisiae* or Sc) and *Caenorhabditis elegans* (*C. elegans* or Ce) using their respective optimal (control) growth conditions and harvested total RNA for purification of tRNAs. Next, we applied different periods of heat stress to *E. coli* (1 h), *A. thaliana* (4 days), *C. elegans* (4 h), *S. cerevisiae* (3 h) and *D. discoideum* (Figure S1). To estimate the influence of growth and development of each organism and to acquire heat-exclusive effects, organisms were cultivated under control and heat stress conditions in parallel. Except for *D. discoideum,* which was cultivated for 36 h either under control or under heat, all the other organisms were grown first under control until a certain growth phase (OD_{600} 0.8 for Yeast, OD_{600} 0.6 for *E. coli*, 2 weeks for *A. thaliana* and L4/ early young adult stage for *C. elegans*) and then heat stress was applied in half of the samples. The other half was kept under control for the final comparison of both heat and untreated samples. A phylogenetic tree depicting the relationship among these 5 organisms and the phenotype of each organism under control and heat is also shown (Figure S1b and S7). After isolation of total RNA, tRNA was isolated by size exclusion chromatography (Figures S2–S6) and tRNA purity was ensured by either chip or agarose gel electrophoreses (Figures S2–S6). Absolute quantification of RNA modifications was achieved after enzymatic hydrolysis of tRNA using a cocktail of nucleases and phosphatase to yield nucleosides. Through this process all sequence information is lost. Yet, as the position within a tRNA is well known for many tRNA modifications, and in case of hypermodified U34 modifications even limited to a single position, we are confident in our subsequent assignments. Samples were split into two aliquots to allow quantitative analysis of dihydrouridine (D) and cytidine modifications by isotope dilution mass spectrometry (LC-MS/MS) [26] (Tables S1–S4).

Figure 1 shows the absolute abundance of tRNA modifications in all 5 organisms under control conditions. The absolute abundance was calculated by dividing the abundance of a modification in fmol by the sum of all 4 canonical nucleosides (in fmol). Subsequently, the value was multiplied by 100 to receive the modification per 100 nucleotides. (Given the length of tRNA from 76 to 93 nts, normalization to 100 nts leads to a near estimate of modification abundance within an average tRNA molecule). Dihydrouridine (D) and pseudouridine (Ψ) were the most abundant modifications in all five organisms, followed by 5-methyluridine (m^5U) and other structure-stabilizing tRNA modifications (Figure 1a). As previously observed [25], *E. coli* RNA modifications are less chemically diverse, and their abundance is lower than that of other organisms. *E. coli* presents four unique modifications, namely the thiolated nucleosides 2-thiocytidine (s^2C) and 4-thiouridine (s^4U), the N6-methylated variant of N6-threonyladenosine (m^6t^6A) (Figure 1c) and the hypermodified uridine modification 5-methylaminomethyluridine (mnm^5U). mnm^5U is found at position 34 of bacterial tRNAs, and it facilitates the correct binding of the anticodon to the codon [12]. Similar modifications are found in eukaryotic tRNAs with 5-methoxycarbonylmethyl(−2-thio)uridine (mcm^5U and mcm^5s^2U). These modifications are incorporated by a complex modification pathway, and mcm^5U can be considered a precursor of mcm^5s^2U [27]. The same mod-

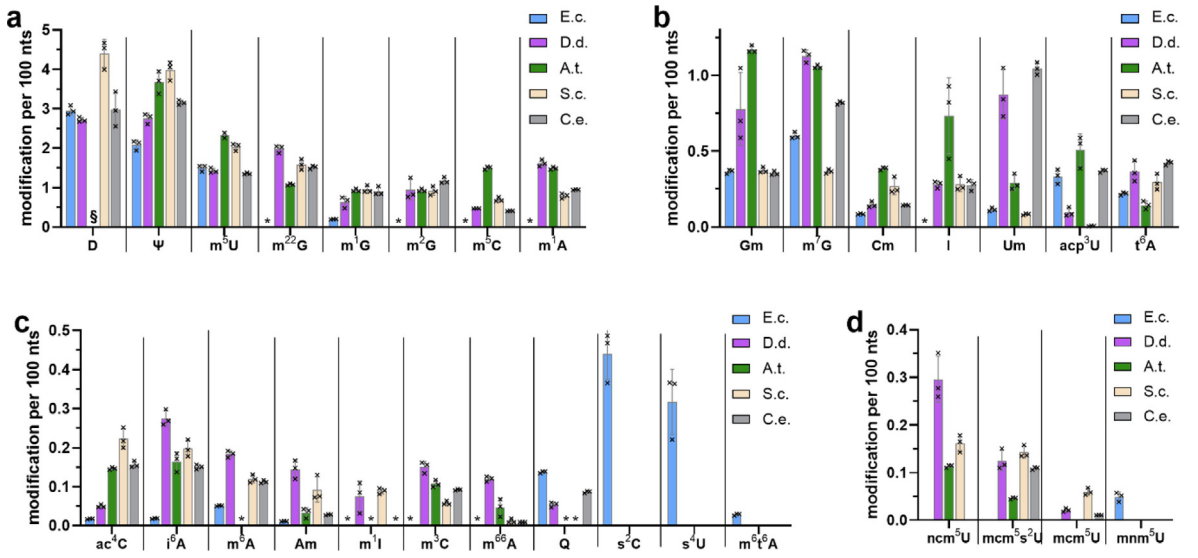

Figure 1. Absolute abundance of tRNA modifications in five organisms as modification per 100 nucleotides (nts). a High abundant modifications (1–5 modifications/100 nts = 1–5 %). b Medium abundant modifications (0.5–1 modifications/100 nts = 0.5–1 %). c Low abundant modifications (<0.5 modifications/100 nts = <0.5 %). d U34 modifications. *E. coli* (blue), *D. discoideum* (purple), *A. thaliana* (green), *S. cerevisiae* (orange) and *C. elegans* (grey). From 3 biological replicates. Error bars reflect standard deviation. * below limit of detection § not analyzed.

ification machinery can also produce 5-carbamoylmethyluridine (ncm^{5}U), which we found in high abundance in *D. discoideum* but is mostly absent in *C. elegans* [25,28].

E. coli and S. cerevisiae tRNA modification abundance is influenced by the medium composition

Before the intended analysis of tRNA modifications under stress, we also defined the abundance of these modifications in *E. coli and S. cerevisiae* using minimal media, M9 and YNB, respectively (Figure 2). As already reported, in both *E. coli* and *S. cerevisiae*, the abundance of tRNA modifications depends on the used growth media and nutrient availability [29–31]. For bacteria and yeast, LB and YPD are rich growth media, while M9 and YNB are considered minimal media for bacteria and yeast, respectively. For example, less 2′-O-methylguanosine, 2′-O-methyluridine and queuosine (Gm, Um and Q, respectively) are observed in *E. coli* grown in minimal M9 medium compared to rich LB-grown *E. coli*. The low abundance of Q in M9-cultured *E. coli* is probably caused by the absence of vitamin B12, which is a crucial cofactor for Q *de novo* synthesis in *E. coli* [32]. M9 lacks cobalt ions (LB medium usually contains trace amounts of cobalt ions) which *E. coli* requires for biosynthesis of vitamin B12. Thus, bacteria cannot synthesize Q efficiently in minimal medium. In *S. cerevisiae*, m^{5}U and mcm^{5}s^{2}U are less abundant in YNB-grown cells, while mcm^{5}U and Um are more abundant. This observation supports previous studies that determine tRNA modification adaptation as a means to overcome nutrient depletion [29] and supports the hypothesis of tRNA modifications as metabolism and nutrient sensors [31,33].

tRNA modification abundances change upon heat stress in all studied organisms

In the next step, we split the organisms into two cohorts; one cohort was left at the ideal temperature, and the second was exposed to elevated temperatures. The exposure time was chosen in accordance with the organism's estimated speed of growth. *E.g. E. coli* was exposed for 1 h, *S. cerevisiae* for 3 h, *C. elegans* for 4 h, *D. discoideum* for 36 h and *A. thaliana* for 4 days. The elevated temperature did not change the phenotype of the microorganisms (Figures S7). Again, total RNA was isolated, followed by tRNA purification and LC-MS/MS analysis. The abundance of each modification/100 nts found under heat was normalized to the abundance found under control conditions which resulted in a fold-change. Most changes were not statistically significant (student *t*-test), and therefore, we decided to use a bubble-heatmap where the size of the data point reflects the statistical significance and the colour and its intensity reflects the fold-change (Figure 3a). Modifications such as D (dihydrouridine), ac^{4}C or m^{7}G are plotted as absolute values, as these modifications were reported to play a role in heat stress adaptation of thermophiles [34]. For these modifications, we only observed a small but statistically significant

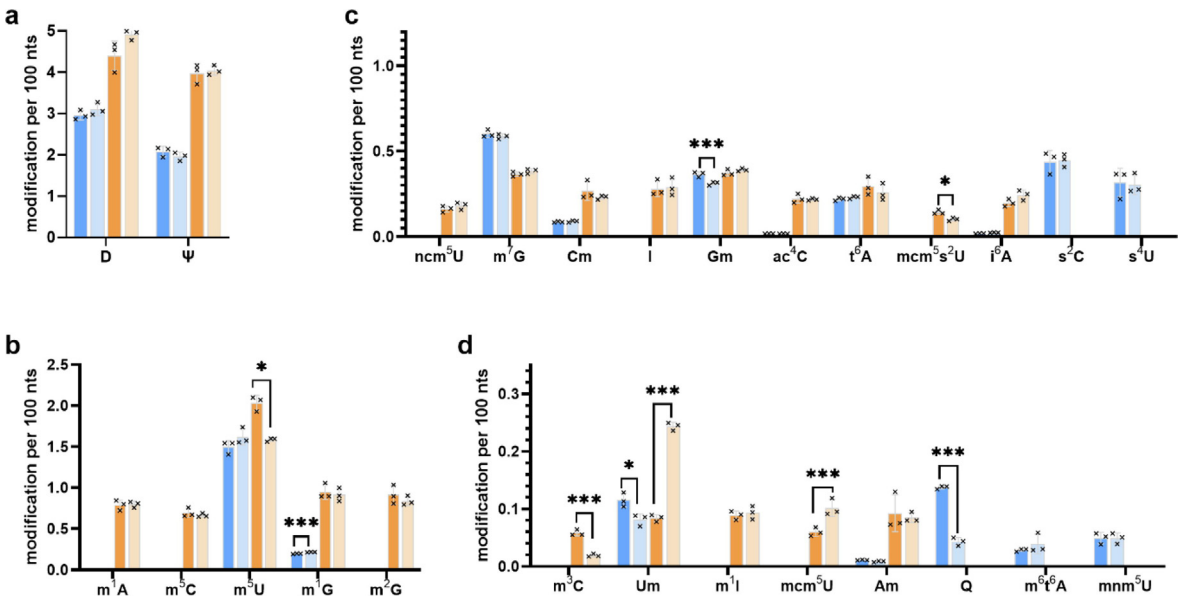

Figure 2. Absolute abundance of tRNA modifications in *E. coli* and *S. cerevisiae* grown in rich (LB and YPD) and minimal medium (M9 and YNB) a high abundant modifications dihydrouridine (D) and pseudouridine (Ψ). b modifications of ~1 % abundance. c modifications ranging from 0.5-1 % abundance. d modifications of abundance below 0.5 %. *E. coli* (LB medium – dark blue; M9 medium – light blue) and *S. cerevisiae* (YPD medium – dark orange; YNB medium – light orange). From 3 biological replicates. Error bars reflect standard deviation. Statistics performed with student *t*-test with p-values < 0.05 indicated by * and p < 0.01 indicated by ***.

increase in ac^{4}C in *E. coli* tRNA (LB-grown, p-value: 0.01) (Figure 3b–d). Furthermore, Um decreased in heat-exposed *E. coli*, while mnm^{5}U increased.

A decrease in Am and m^{6,6}A is also observed in *E. coli*, most likely reflecting a lower degree of rRNA fragment contamination in the tRNA preparation [35], which cannot be excluded even though the size of purified RNA matches the expected size of tRNAs. In *A. thaliana*, we see a higher abundance of m^{5}C and ncm^{5}U (p-value: <0.1) which suggests that plants may also experience tRNA modification adaptation. In *S. cerevisiae* grown in the minimal medium an increase in m^{5}U is observed (p-value: 0.01). For *C. elegans*, we observed a minor 0.9-fold reduction in acp^{3}U (p-value: 0.03) and a 1.14-fold increase in mcm^{5}U (p-value: 0.04). All in all, the changes in tRNA modification abundance are very mild under heat stress. However, one pattern that is noteworthy is the change in U34 modifications, which appears to be altered in most organisms examined in this study. For example, in *E. coli*, mnm^{5}U is elevated after heat stress (in both M9 and LB, Figure 3e), albeit not reaching a p-value below 0.05. In *S. cerevisiae*, mcm^{5}s^{2}U is decreased, and its precursor mcm^{5}U is increased (Figure 3f), as previously reported [20]. For *A. thaliana*, only two biological replicates had signals above the lower limit of quantification for hypermodified uridines and thus statistical testing was not possible. In the remaining two replicates we observed a 0.67-fold lower abundance of mcm^{5}U, a higher abundance of ncm^{5}U and a constant abundance of mcm^{5}s^{2}U (Figure 3g). Further testing, potentially with harvesting at multiple intervals in between, are needed to study the relevance of this observation. Finally, for *D. discoideum*, no statistically relevant changes in U34 modifications were noticed, while in *C. elegans,* a higher abundance of mcm^{5}U was found. Altogether, these results indicate that U34 modifications may adapt under heat stress and that the adaptation is observable across prokaryotes and eukaryotes (here represented by 5 model organisms). Thus, we next focused on revealing the molecular mechanisms that lead to U34 modification abundance changes using NAIL-MS.

NAIL-MS in E. coli reveals tRNA modification reprogramming on pre-existing and new tRNAs

tRNA modification abundances can be modulated by various molecular mechanisms. Namely, the abundance of a modification may increase by additional modification of already existing tRNAs (original tRNAs), loss of hypomodified tRNAs or by faster modification of new tRNAs. A decrease in modification abundance is possible by slowed modification of new tRNAs but also loss of highly modified existing tRNAs. A first step towards deciphering the mechanism that causes tRNA modification reprogramming is a discriminant analysis of tRNAs that existed prior to the heat stress and tRNAs transcribed during heat stress.

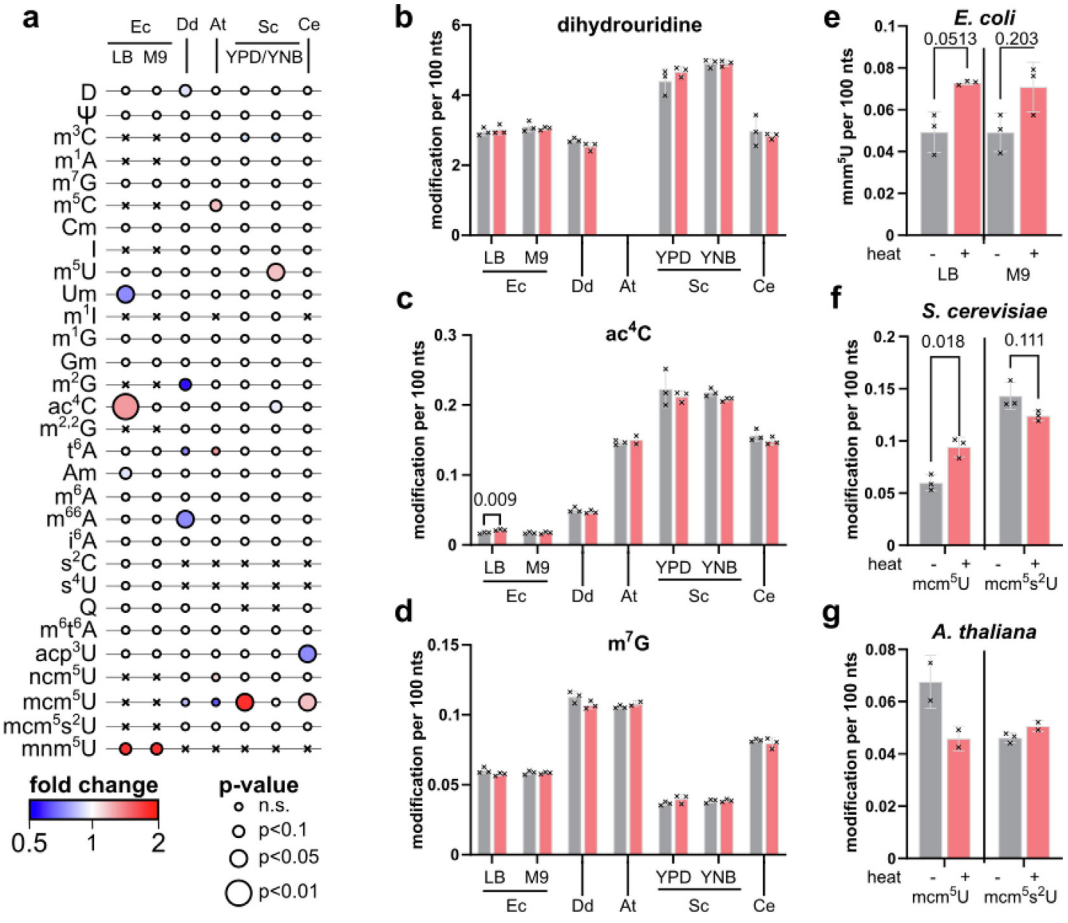

Figure 3. Trna modification abundance changes under heat stress. a heatmap of trna modification fold-changes under heat stress where the colour and its intensity indicate the fold-change and the size of the circle the statistical significance. the x symbol indicates not detected RNA modifications. b Absolute abundance of dihydrouridine. (Note: D abundance was not quantified in A. thaliana due to the presence of THU in the hydrolysis [26]) c Absolute abundance of N4-acetylcytidine (ac^4C). d Absolute abundance of N7-methylguanosine (m^7G). e Absolute abundance of mnm^5U in E. coli grown in LB or M9 medium under heat stress. f Absolute abundance of mcm^5U and mcm^5s^2U in S. cerevisiae grown in YPD medium. g Absolute abundance of mcm^5U and mcm^5s^2U in A. thaliana (n = 2). Legend b-f: Except for A. thaliana, all measurements are from 3 biological replicates. Gray and red bars indicate control and heat stress, respectively. Error bars reflect standard deviation. Statistics were performed with student *t*-test.

For this purpose, we performed a NAIL-MS experiment. *E. coli* was grown in unlabelled M9, and upon exposure to heat, the medium was exchanged to a fully nitrogen-15-containing medium spiked with $[CD_3]$-S-methionine. A concept sketch is shown in Figure 4a. This experiment allows the discrimination of pre-existing tRNAs containing unlabelled nucleosides and unlabelled methylation marks ($m/z \pm 0$) from pre-existing tRNAs that receive an additional $[CD_3]$-methylation mark *e.g.* due to heat ($m/z + 3$, "post-methylation"). Furthermore, new tRNAs have $[^{15}N]$-labelled nucleosides and $[CD_3]$-methylation marks and can be easily distinguished from pre-existing tRNAs by mass spectrometry (cytidine $m/z + 3$, uridine $m/z + 2$, purines $m/z + 5$, methylation marks an additional $m/z + 3$) [36] (Table S3).

After isolation of tRNAs extracted from the NAIL-MS experiment, the abundance of new canonical nucleosides over the sum of all canonical nucleosides in tRNA was plotted (new transcript ratio, NTR). As expected, the abundance of new transcripts rises over time for both temperatures (Figure 4b). During heat stress, the NTR is higher compared to the untreated control. This indicates that either transcription is increased or that rapid tRNA decay (RTD) is faster in heat-stressed cells. To assess which scenario is more plausible, an inspection of modification abundance in the different tRNA species is needed.

For this, we plotted the abundance of pre-existing tRNA modifications normalized to the abundance of pre-existing canonical nucleosides. For s^2C, s^4U, Ψ, Um, acp^3U, ac^4C, Gm and t^6A, we observed identical abundances in heat-exposed and control

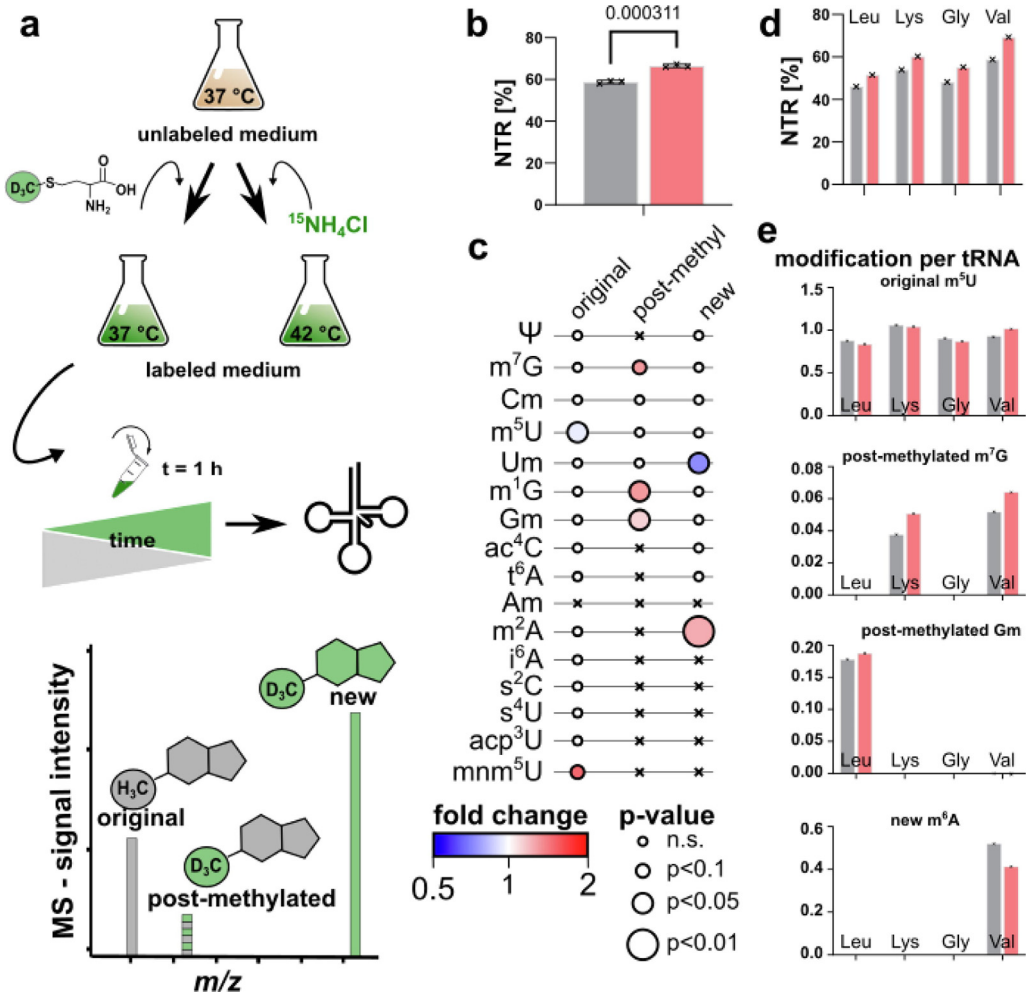

Figure 4. Pulse-chase NAIL-MS analysis of *E. coli* grown in minimal medium under heat exposure. a Concept sketch of the performed NAIL-MS experiment. Bacteria were grown in unlabelled M9 medium to OD 0.6, and the medium was exchanged to ^{15}N-labelled M9 supplemented with [CD_3]-S-methionine. After 1 h, tRNA was isolated and analysed by MS to distinguish original, post-methylated and new nucleosides. b New transcript ratio (NTR) from NAIL-MS experiment. Calculated by dividing the sum of new canonical nucleosides by the sum of (new + original) canonical nucleosides. control in grey and heat in red. c Heatmap of tRNA modification fold-changes where the colour indicates the fold-change and the size of the circle the statistical significance. The results shown are from 3 biological replicates. Statistics were performed with student *t*-test. X indicates not detected. d New transcript ratio (NTR) from tRNA isoacceptors under NAIL-MS conditions. Legend d and e: tRNAs Leu-UAA, Lys-UUU, Gly-UCC and Val-UAC; control in grey and heat in red, from 3 pooled biological replicates. e Absolute abundance of modified nucleosides in tRNA isoacceptors during heat stress in a pulse-chase NAIL-MS set-up matching c. Y-axis: modification per tRNA.

cells, which argues against global RTD (Figure 4c, column "original"). In organisms such as *V. cholerae* [37] and yeast [38], hypomodified tRNAs are targets of RTD, which would result in an increased modification density in pre-existing tRNAs in our NAIL-MS set-up. As we do not observe increased tRNA modification abundances, it is most likely that *E. coli* increases tRNA transcription as a response to heat stress. Furthermore, RTD is mainly reported in amino acid-depleted *E. coli* [39] and not in heat stress. For original mnm^{5}U, we observed a 1.3-fold increase (p-value 0.1) which aligns well with our findings from the unlabelled cultures, where we observed a similar increase. For m^{5}U, where we observed no effect in the unlabelled cultures, we found a decreased abundance during heat stress for original m^{5}U (1.08-fold decrease, p-value: 0.02). For Um, where we found a decrease in unlabelled culture, but no change in pre-existing tRNAs, we noticed a statistically significant reduction in the new tRNAs (1.3-fold decrease p-value: 0.01). In addition, m^{2}A increases 1.15-fold in new tRNAs (p-value: 0.0002), while m^{6}A (found exclusively at A37 in *E. coli* $tRNA^{Val}_{UAC}$ [5]) remains unaltered. Other modifications, such as Ψ, Gm and ac^{4}C showed comparable abundance

in new tRNAs from control and heat-exposed cells. Finally, we evaluated the degree of post-methylation in pre-existing tRNAs. Post-methylation species occur if additional methyl marks are deposited on pre-existing tRNAs. Here, Gm showed a 1.13-fold increase, m^1G a 1.3-fold increase and m^7G a 1.3-fold increase under heat stress (p-values: 0.02, 0.04 and 0.06, respectively) while other modifications remained unaltered (Figure 4c). Correspondingly, the increase in Gm aligns well with previous reports that described an increase in Gm in heat-stressed and heat-shocked *E. coli* [18,19,40].

With the goal to further deconvolute the data, we pooled total tRNA of the 3 biological replicates which yielded enough total tRNA for purification of 4 tRNA isoacceptors. We performed the isoacceptor purification using reverse complementary-biotinylated DNA probes (Table S5) and streptavidin coated magnetic beads. For *E. coli*, tRNAs Leu-UAA, Lys-UUU, Gly-UCC and Val-UAC were purified and the stoichiometry of modifications per tRNA is given in Figure S8. Although the pooling resulted in a single data point and statistical significance cannot be determined, we found the data to be informative. While we found most expected modifications in the expected stoichiometry for each tRNA isoacceptor, some expected modifications were below the limit of quantification and could not be assessed (*e.g.* mnm^5U, Um and m^1G). The new transcript ratio (NTR) is higher for all 4 tRNAs under heat compared to control, which can be either caused by increased transcription or by increased degradation of pre-existing tRNAs (Figure 4d). On the isoacceptor level, we did not find a decrease in the stoichiometry of m^5U per tRNA (Figure 4e). This observation in conjunction with the higher NTR under heat argues towards an increased degradation of mature, m^5U-modified tRNAs. Next, we assessed the degree of post-methylation for m^7G and Gm. 2 out of 2 tRNA isoacceptors harbor m^7G and both tRNAs showed a stoichiometric increase in m^7G upon heat exposure. Under control conditions, 3.7 % of lysyl-tRNA have a post-methylated m^7G while under heat the abundance increases to 5.1 % and for valyl-tRNA the increase is from 5.2 % to 6.4 %. Post-methylation of Gm, which is contained in leucyl-tRNA, increases slightly (1.05-fold) under heat stress. Interestingly, in new tRNA isoacceptors, no stoichiometric changes are observed, except for m^6A in valyl-tRNA, where we see a 1.25-fold decrease in m^6A abundance (Figure 4e).

In a next step, we analysed how *E. coli* cells lacking tRNA modification enzymes performed under short (1 h) and prolonged heat stress using both minimal and rich growth medium. We chose knockout lines that lack the writers for the following tRNA modifications: Gm(trmH), s^2U (trmU), m^6A (yfiC), cmnm (trmE), m^5U (trmA) and mnm^5U(gidA). As can be seen in Figure S9, the absence of certain tRNA writers does not impact bacterial growth under heat within the first hour of analysis. Yet, after prolonged heat exposure, we noticed a delay in growth in LB medium under heat for the knockout lines gidA, trmE and trmU. This indicates that these writers, and consequently, the respective tRNA modifications can play a role under heat stress. In M9 medium, we surprisingly see a better performance of growth for all the knockout lines under prolonged heat stress. In summary, we deciphered the molecular origin of previously observed changes in tRNA modification abundance in *E. coli* under heat stress. We demonstrated that pre-existing tRNAs receive additional methylations, especially Gm and m^7G, under heat stress and that mnm^5U is more abundant in total tRNA.

tRNA modifications in S. cerevisiae change in original and new tRNAs

We have previously observed an increase of the U34 modification mcm^5U in *S. cerevisiae* under heat using an unlabelled YPD-rich medium (Figure 3f). To decipher the molecular mechanism, we needed to perform a NAIL-MS experiment in a rich medium. For this purpose, we decided to use a ^{15}N-YPD medium (Silantes, Munich, Germany) supplemented with unlabelled glucose. This medium leads to a full exchange of all nitrogen atoms with nitrogen-15. Uridine and its modifications receive a m/z + 2, cytidine and its modifications a m/z + 3 and purines and their modifications a m/z + 5 [41] (Table S4). As we were mainly interested in understanding the molecular mechanisms of the mcm^5U increase, we omitted additional labelling with $[CD_3]$-S-methionine, which would have led to the formation of isotopologues of identical mass but different origin (*e.g.* 5-$[CH_3]$-$^{15}N_3$-cytidine and 5-$[CD_3]$-$^{14}N_3$-cytidine). The concept of the assay is shown in Figure 5a.

As with *E. coli*, we first judged the new transcript ratio of *S. cerevisiae*. We found that transcription is substantially slowed in the heat-exposed yeast (Figure 5b). Thus, we conclude that RTD plays a minor role in our NAIL-MS context as RTD would lead to an elevated NTR. Next, we assessed the abundance of mcm^5U and mcm^5s^2U in original and new tRNAs. As expected, we observe a 1.2-fold increase in mcm^5U (p-value: 0.03) in the original tRNAs and a 1.3-fold decrease in mcm^5s^2U (p-value 0.001) (Figure 5c). In new tRNAs, we found no changes in abundance for mcm^5U (1.08-fold increase p-value: 0.18) and a slight 1.08-fold increase in mcm^5s^2U (p-value: 0.02). In addition to the U34 modifications, we monitored the abundance of modifications in original tRNAs. The most interesting effect was seen for acp^3U, which decreased 1.2-fold (p-value: 0.02) amidst a minor decrease for m^3C and

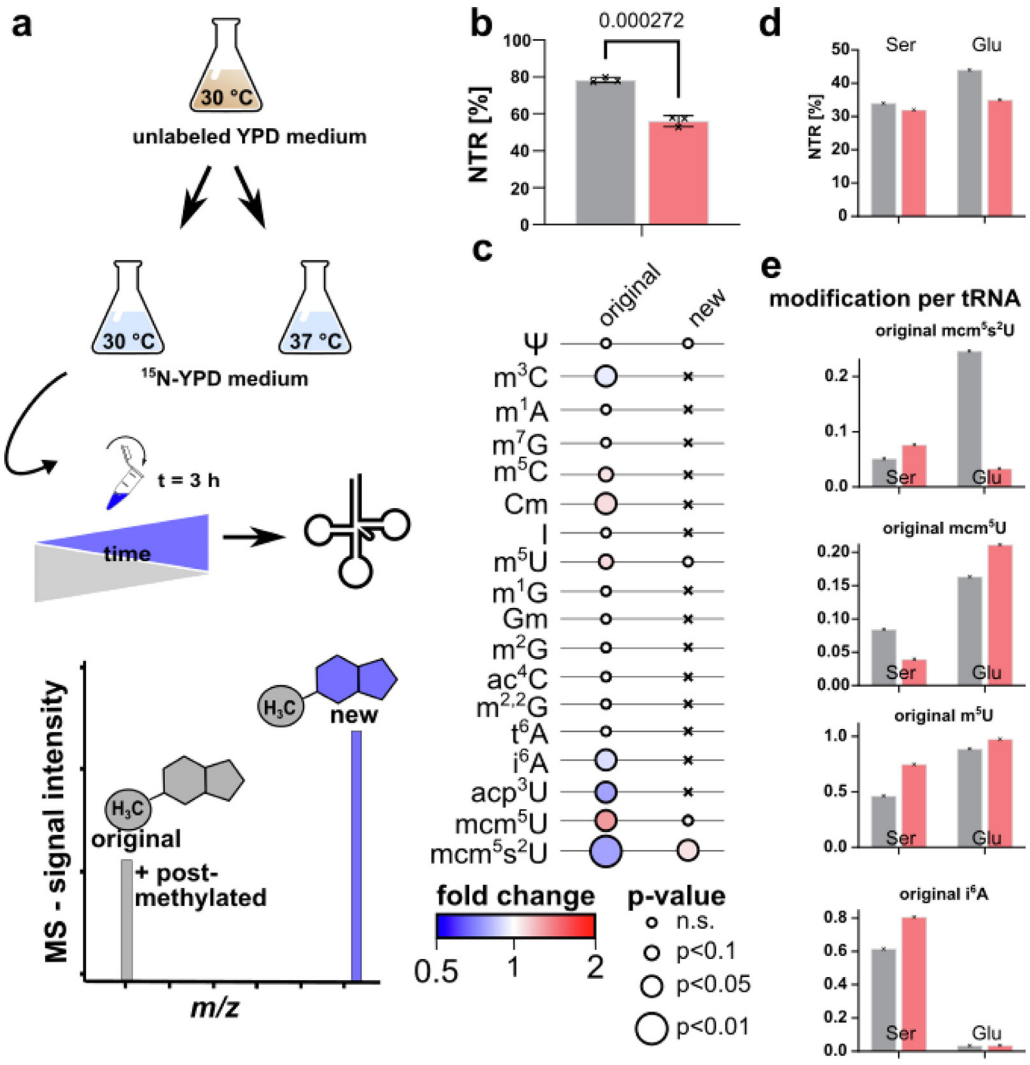

Figure 5. Pulse-chase NAIL MS analysis of *S. cerevisiae* grown in rich medium (YPD) under heat exposure. a Concept sketch of the performed NAIL-MS experiment. Yeast was grown in unlabelled YPD medium to OD 0.8, and the medium was exchanged for ^{15}N-labelled YPD medium. After 3 h, tRNA was isolated and analysed by MS to distinguish original and new nucleosides. b New transcript ratio (NTR) from NAIL-MS experiment. Calculated by dividing the sum of new canonical nucleosides by the sum of (new + original) canonical nucleosides. control in grey and heat in red. c Heatmap of tRNA modification fold-changes where the colour indicates the fold-change and the size of the circle the statistical significance. The results shown are from 3 biological replicates. Statistics were performed with student *t*-test. X indicates not detected. d New transcript ratio (NTR) from tRNA isoacceptors under NAIL-MS conditions. Legend d and e: tRNAs Ser-UGA and Glu-UUC; control in grey and heat in red, from 3 pooled biological replicates. e Absolute abundance of modified nucleosides in tRNA isoacceptors during heat stress in a pulse-chase NAIL-MS set-up matching c. Y-axis: modification per tRNA.

i^{6}A and a small increase for m^{5}C, Cm and m^{5}U (Figure 5c).

As done for *E. coli*, the remaining total tRNA was pooled which allowed purification of 2 tRNA isoacceptors, namely, Ser-UGA and Glu-UUC. Although the pooling resulted in a single data point and statistical significance cannot be determined, we found the data to be informative. First, we calculated the NTR, and interestingly the NTR is substantially lower for heat-treated glutamyl-tRNA (1.28-fold) than for seryl-tRNA (1.06-fold) (Figure 5d). Two scenarios may explain these results. The first involves RTD being more pronounced for seryl-tRNA than glutamyl-tRNA leading to a higher NTR for seryl-tRNA and the second involves lower transcription of glutamyl-tRNA under heat. Which of the scenarios is correct, cannot be deciphered from our data. Next, we quantified the abundance of modified nucleosides per tRNA under control and heat conditions. The modification profile was in good agreement with the expected modification profile

for each tRNA which indicates a reasonable degree of enrichment (Figure S8). As shown in Figure 5e, ~ 23 % pre-existing (=original) glutamyl-tRNAs contain mcm^5s^2U under control conditions but during heat, this number drops to below 5 %. Inversely, the abundance of mcm^5U increases under heat stress. We want to emphasize that this is a single datapoint from the pooled tRNA of 3 biological replicates. Therefore, we do not wish to over-interpret the data.

Discussion

In this study, we determined the absolute abundance of 30 modified nucleosides in tRNA taken from five organisms under control and heat stress conditions. Our initial analysis of modification abundances aligns well with our 2019 reported findings for *E. coli*, *S. cerevisiae* and *D. discoideum* [25]. For *A. thaliana*, this is the first report on absolute tRNA modification abundances. In comparison to the other organisms here studied, *A. thaliana* has a high abundance of Gm and m^7G, but a low abundance of the U34 modifications included in our study. Future studies will show whether this is a general trend for plant-derived tRNAs and what the functional consequences are.

Regarding the heat stress, we only observed a limited number of tRNA modifications that change within each organism (Figure 3a). This agrees well with previous stress studies using nutrient depletion [29] or chemical stressors [10,11,36] that identified a subset of tRNA modifications as reprogrammed. Interestingly in all stress studies, including this current study, U34 modifications appear to be most prone to reprogramming. In *C. elegans*, Bystrom and colleagues studied *elpc-1* and *elpc-3*, which are required for mcm^5U and ncm^5U formation, and *tuc-1* required for mcm^5s^2U under heat stress. At 33 °C heat shock, all mutants showed less translation of a reporter gene construct under a heat-shock promoter, suggesting these modifications are required for translation efficiency upon heat shock [42]. As we observe a higher abundance of mcm^5U in heat stress, we suggest that U34 modifications may play a role in translation of heat-shock proteins in *C. elegans*.

Here, we deciphered how the reprogramming of U34 modifications is achieved in *E. coli* (BW25113) and *S. cerevisiae* (BY4741). In *E. coli*, we discovered that mnm^5U likely increases in pre-existing tRNAs, which can be explained by two scenarios of its biosynthetic pathway [43]. (i) MnmC, the enzyme responsible for mnm^5U formation, is more active under heat stress. (ii) MnmA, the 2-thiouridine synthase which converts mnm^5U to mnm^5s^2U in tRNAs glutamine, glutamate and lysine, is less active and thus more mnm^5U occurs. Due to the low abundance of mnm^5s^2U in tRNA in combination with its low MS detection efficiency (caused by the 2-thiolation), we could not quantify mnm^5s^2U in our NAIL-MS experiment. Thus, we cannot clearly define which pathway is causative for the increase in mnm^5U. For this more sensitive MS instrumentation and more efficient tRNA isoacceptor isolation protocols for parallel isolation are needed. Interestingly, *E. coli* with a deficiency for certain tRNA modifications perform better under prolonged heat stress in minimal medium compared to the respective wild type (Figure S9). Further studies will show whether the tRNA modification adaptations described here and by others [19] are indeed wanted and genetically programmed or are unwanted biochemical responses to the stress exposure.

In yeast, we observed a similar increase in the C5-modified U34 modification in both labelled and NAIL-MS-derived tRNA. Yet, we could also detect and quantify the 2-thiolated derivative allowing a clearer discrimination of the underlying mechanisms in yeast. Here, mcm^5s^2U is decreased and its precursor mcm^5U increased (Figure 3f), as previously reported by the Leidel lab [20]. Interestingly, the increase in mcm^5U is only the case using YPD medium and not YNB medium, which might be explained by the already high abundance of mcm^5U in tRNA from YNB-grown cells (Figure 2c,d).

In the NAIL-MS context, we also observed a decreased abundance of mcm^5s^2U and higher abundance of mcm^5U in original total tRNAs. Purification of a mcm^5s^2U-modified tRNA (Glu-UUC) revealed that mcm^5s^2U is nearly lost from original tRNA-Glu while mcm^5U rises. We can only speculate how the cells achieve this loss, but the effect might be connected to tRNA transcription as previously described [20]. According to our NAIL-MS data, slowed RNA modification is not involved in the underlying mechanisms as we find a higher abundance of mcm^5s^2U and mcm^5U in the new tRNAs (1.08-fold increase, p-value: 0.02 and 0.18, respectively). The higher abundance of mcm^5U and mcm^5s^2U might be attributed to the lower number of new tRNAs available under heat stress, and thus, the ratio of enzyme:substrate and co-factor:substrate increases the modification kinetics (Figure 5b). Our previous analysis of mcm^5s^2U under chemical stress argues against the enzyme:substrate ratio hypothesis as we observed a slowed incorporation of mcm^5s^2U in new tRNAs, even though fewer new tRNAs were available under chemical stress [11]. However, our previous study was performed in a YNB-based NAIL-MS context, whereas we work with a nutrient-rich YPD medium in this study. As U34 modifications have been proposed as sensors of nutrition availability [33] and depend on various co-factors such as S-adenosylmethionine (SAM), glycine and folate, which are derived from different metabolic pathways, the difference in growth medium most likely

impact the results. Thus, we suggest that the ratio of co-factor:substrate might cause the elevation in $mcm^5(s^2)U$ in new tRNAs.

For multicellular organisms such as *A. thaliana* and *C. elegans,* nucleic acid isotope labeling is possible, but the temporal resolution for discrimination of tRNA modification processes by a pulse-chase experiment is too low. Therefore, we cannot study the molecular mechanisms which led to the adaptation of U34 modifications (Figure 3a). But judging from the observations in *E. coli* and *S. cerevisiae* we hypothesize that existing tRNAs adapt due to heat stress as an early response to overcome it. Given the continuous rise of global temperatures, more research, especially in plants, is needed to understand and exploit the power tRNA modification reprogramming might have on organismal survival.

Material and Methods

Cultivation of Escherichia coli

A single colony of *E. coli* BW25113 strain was picked from a fresh streaked LB-agar plate and inoculated in a 5–10 mL LB medium (Roth, Karlsruhe, Germany). The bacterial cells were grown overnight at 37 °C, shaking at 150 rpm. The next day, the cell density was assessed by OD_{600} measurement (DiluPhotometer™, Implen, Los Angeles, USA) and the cells diluted to OD 0.05. After that, the cells were grown under the same conditions to reach the mid-log phase, more specifically to OD 0.6. At this moment, the control samples (time point 0) were taken from 6 biological replicates. For heat stress, 3 of these samples were then transferred to 42 °C and 3 of them remained at 37 °C. After 1 h of heat exposure at 42 °C, the heat-treated as well as the control samples were taken for comparison. The medium was discarded after centrifugation (30 min, 4000×*g*, 24 °C), and the resulting cell pellet was stored at −80 °C for RNA extraction using TRI Reagent (Sigma Aldrich, St. Louis, MO, USA) on the next day (see section on RNA extraction). The same procedure was performed using M9 minimal medium (prepared by mixing a 10× M9 stock solution – Na_2HPO_4 (68 g/L), KH_2PO_4 (30 g/L), NaCl (2.5 g/L), and NH_4Cl (10 g/L) – which was diluted to 1× and supplemented with $MgCl_2$ (0.002 M), $CaCl_2$ (0.0001 M), Na_2SO_4 (0.002 M), and glucose (0.4 %) at final concentrations).

Cultivation of Saccharomyces cerevisiae

A single colony of *S. cerevisiae* BY4741 strain was picked from a fresh streaked YPD-agar plate and inoculated in a 12 mL YPD medium (Roth, Karlsruhe, Germany). The yeast cells were grown overnight at 30 °C, shaking at 200 rpm. On the next day, the cell density was assessed by OD_{600} measurement (DiluPhotometer™, Implen, Los Angeles, USA) and the cells diluted to OD 0.2. After that, the cells were grown until OD 0.8. At this moment, the control samples (time point 0) were taken from 6 biological replicates. For heat stress, 3 of these samples were then transferred to 37 °C and 3 of them remained at 30 °C. After 3 h of heat exposure at 37 °C, the heat-treated and the control samples were taken for comparison. The medium was discarded after centrifugation (30 min, 4000 × g, 24 ○C), and the resulting cell pellet was used for RNA extraction using hot phenol (see below). The same procedure was performed using YNB (Yeast-nitrogen-base) minimal medium (prepared by mixing a 10× YNB stock solution − Carl Roth, Karlsruhe, Germany – which was diluted to 1× and supplemented with glucose (0.01 g/L), uracil (0.02 g/L) and methionine (0.02 g/L) and a mix of unlabelled amino acids at final concentration: 0.02 g/L arginine, 0.02 g/L histidine, 0.06 g/L leucine, 0.03 g/L lysine, 0.05 g/L, phenylalanine, 0.4 g/L serine, 0.2 g/L threonine, 0.04 g/L tryptophane, 0.03 g/L tyrosine and 0.15 g/L valine, glutamine 0.1 g/L and aspartate 0.1 g/L).

Cultivation of Arabidopsis thaliana

We used the *A. thaliana* accession Col-0 as the wild-type control. Plants were cultivated on half-strength Murashige and Skoog (MS) medium supplemented with 1.5 % sucrose or on soil under long-day control conditions (16 h at 22 °C and 80 mmol photons m^{-2} s^{-1} /8h at 18 °C in the dark) using light-emitting diode cabinets (LED-41 HIL2, Percival Scientific, Perry, IA, USA). For Trizol RNA extraction (Section 2.6), surface-sterilized seeds were stratified at 4 °C for 2 days and then cultivated for 14 days on soil under control conditions. For cold and heat treatments, part of the plants was further exposed to temperatures of 4 °C and 32 °C, respectively, for more than 4 days. The other part of the plants was kept under control conditions the entire time for comparison.

Cultivation of Caenorhabditis elegans

C. elegans wild-type N2 Bristol strain was maintained on the Nematode Growth Medium (NGM) agar plates using *Escherichia coli* HB101 as diet. The strain was obtained from the *Caenorhabditis* Genetics Centre (CGC), University of Minnesota, USA. *C. elegans* larvae were synchronised in the L1 larval stage by bleaching using hypochlorite treatment (2:2:1 solution of 4.5 % sodium hypochlorite, H_2O and 10 M NaOH) as previously described (Stiernagle, 2006). Synchronised L1 larvae were split into four populations and maintained at 20 ℃ until heat shock. L4/ early young adult stage animals were

either collected as pre-treatment controls or were heat shocked for 4 h at 37 ℃. Untreated controls were maintained for 4 h at 20 ℃. Worms were washed thrice in the M9 buffer (42 mM Na_2HPO_4, 22 mM KH_2PO_4, 86 mM NaCl, 1 mM $MgSO_4$). After the final wash, the worm pellet was mixed with Invitrogen TRIzol Reagent (Cat. No. 15596018) and snap frozen.

Cultivation of Dictyostelium discoideum

The cultivation of the Dictyostelium discoideum AX2 wild-type strain was carried out at 21 °C or 27 °C (heat stress) in constant light. 2×10^7 cells (biological triplicates) were grown axenically in HL5 + medium (Formedium, Norfolk, UK) for 36 h in shaking culture. Cells were collected via centrifugation at room temperature and washed twice with 1 × Soerensen buffer (2 mM Na_2HPO_4, 15 mM KH_2PO_4, adjusted with H_3PO_4 to pH 6.0). Cells were lysed with 1–2 mL TRI Reagent (Sigma Aldrich, St. Louis, MO, USA) with 20 mM EDTA (ethylenediaminetetraacetic acid) and incubated for 5 min at room temperature. Isolation of total RNA was performed according to the manufacturer's protocol.

NAIL-MS using e. Coli and S. Cerevisiae

A single colony of *E. coli* BW25113 strain was picked from a fresh streaked LB-agar plate and inoculated in a 5–10 mL M9 minimal medium. For sterility check, the same medium was grown without inoculation. The bacterial cells were grown overnight at 37 °C, shaking at 150 rpm. Next day, cells were re-inoculated in 30 ml M9 minimal medium and cells were grown overnight using the same conditions. The cell density was assessed by OD_{600} measurement (DiluPhotometer™, Implen, Los Angeles, USA) and the cells diluted to OD 0.1 in 50 ml unlabelled M9 medium. For t0 (time point zero), cells were grown until OD 0.6 and part of the samples (7 ml) were once again collected. Next, the culture was centrifuged (10 min, 4000 × g) and cells were washed with 1 ml full labelled medium (prepared by mixing a 10 × $^{15}NH_4Cl$-labelled M9 stock solution with glucose, $MgCl_2$, Na_2SO_4, $CaCl_2$ and CD_3-methionine) [36]. Cells were then resuspended in the same fully labelled medium and samples were either incubated at 37 °C (control) or at 42 °C (heat stress). After 1 h of growth, both samples were taken for comparison. The medium was discarded after centrifugation (30 min, 4000 × g, 24 °C) and the resulting cell pellet was stored at −80 °C for RNA extraction using TRI Reagent (Sigma Aldrich, St. Louis, MO, USA).

For *S. cerevisiae*, a single colony of the BY4741 strain was picked from a fresh streaked YPD-agar plate and inoculated in a 12 mL YPD medium (Roth, Karlsruhe, Germany). The yeast cells were grown overnight at 30 °C, shaking at 150 rpm. On the next day, the cell density was assessed by OD_{600} measurement (DiluPhotometer™, Implen, Los Angeles, USA) and the cells diluted to OD 0.2. After that, the cells were grown until OD 0.8. At this moment, the control samples (time point zero) were taken and medium exchange was performed using a labelled medium (prepared by mixing ^{15}N Silantes rich growth medium – Silantes, Munich, Germany – supplemented with 1 % (w/w) glucose). Cells were then grown either at 30 °C (control) or at 37 °C (heat stress). After 3 h of growth, both samples were taken for comparison. The medium was discarded after centrifugation (30 min, 4000 × g, 24 °C) and the resulting cell pellet was used for RNA extraction using Hot Phenol.

Isolation of total RNA – Hot phenol

Total RNA was extracted according to the protocol from Collart *et al.* [44], followed by ice-cold ethanol precipitation. In summary, 0.1× V 5 M NH_4OAc and 2.5× V 100 % ethanol were added to the resulting aqueous phase and samples were directly stored at − 20 °C. The next day, samples were centrifuged (40 min, 12,000×*g*, 4 °C), the supernatant was discarded and the remaining pellet was washed twice using 200 μL of 70 % ethanol. Finally, the supernatant was carefully discarded, the pellet air-dried for 10 min and the total RNA suspended in 30 μL of H_2O.

Isolation of total RNA – Trizol

C. elegans cells were harvested using 500 μL of the TRI reagent. The total RNA was isolated following the supplier's manual (Roth, Karlsruhe, Germany) using 100 μL of chloroform and subsequent cold isopropanol precipitation. The next day, samples were centrifuged (30 min, 12,000×*g*, 4 °C), the supernatant was discarded, and the remaining pellet was washed twice using 200 μL of 70 % ethanol. Finally, the supernatant was carefully discarded, the pellet air-dried for 10 min, and the total RNA suspended in 30 μL of H_2O.

Isolation of tRNAs using SEC (Size exclusion Chromatography)

tRNAs were isolated exclusively by SEC (AdvanceBio SEC 300 Å, 2.7 μm, 7.8 × 300 mm for tRNA, Agilent Technologies) according to Heiss et al., 2021. After isolation, the RNA was precipitated overnight using 0.1× V 3 M NH_4OAc and 2.5× V 100 % ice-cold ethanol. The next day, samples were centrifuged (1 h, 12,000×*g*, 4 °C), the supernatant was discarded and the remaining pellet was washed once using 200 μL of 70 % ethanol. Finally, the supernatant was carefully discarded, the pellet air dried for 10 min and the total tRNAs were suspended in 20 μL of H2O.

Isoacceptor purification

For isoacceptor purification 1 μg of total tRNA was mixed with 100 μM biotinylated antisense oligonucleotides in 5× SSC buffer (0.75 M NaCl, 75 mM trisodium citrate at pH 7.0) in a total volume of 100 μL. This mixture was denatured at 90 °C for 3 min followed by an annealing step at 65 °C for 10 min. For purification 25 μL of Magnetic Dynabeads® MyoneTM Streptavidin T1 (Thermo Fisher Scientific, Waltham, MA, USA) was used for each sample. Beads for multiple samples were prepared simultaneously. During each washing step the volume of buffer used was equal to 25 μL times the number of samples. The magnetic beads were washed thrice using Bind and Wash buffer (5 mM Tris-HCl (pH 7.5), 0.5 M EDTA, 1 M NaCl) and once using 5× SSC buffer before resuspending in 5× SSC buffer. During each washing step, samples were placed in a magnetic rack to allow for separation of supernatant and beads. To each sample 25 μL of the prepared magnetic beads were added and samples were shaken at 25 °C and 600 rpm for 30 min. After immobilisation samples were washed once using 50 μL of 1× SSC buffer followed by three washing steps using 25 μL 0.1× SSC buffer. For RNA elution the beads were resuspended in 25 μL of deionized water and heated to 75 °C for 3 min. The supernatant was transferred to a fresh Eppendorf tube and directly digested for mass spectrometry.

RNA digestion for mass spectrometry

Total tRNAs were digested into single nucleosides by using 0.2 U phosphodiesterase I (VWR, Radnor, Pennsylvania, USA), 2 U alkaline phosphatase (Merck, Darmstadt, Germany) and 2 U benzonase (Merck, Darmstadt, Germany) in Tris (pH 8, 5 mM, Carl Roth, Karlsruhe, Germany) and $MgCl_2$ (1 mM, Carl Roth, Karlsruhe, Germany) containing buffer. In addition, 10 μM butylated hydroxytoluene (Merck, Darmstadt, Germany), 5 μg tetrahydrouridine (Merck, Darmstadt, Germany) and 1 μg pentostatin (Merck, Darmstadt, Germany) were added to avoid oxidation and deamination of the nucleosides. This digestion mixture (20 μL) was added to the samples (150 ng of tRNAs in 15 μL) and incubated for 2 h at 37 °C. tRNA isoacceptors were hydrolysed using the same protocol with the exception that benzonase was exchanged for nuclease P1 (Merck, Darmstadt, Germany) and phosphodiesterase was omitted due to prolonged market unavailability. For the modifications analysed in this manuscript, this exchange does not result in significant changes in nucleoside release [26].

QQQ mass spectrometry

For the mass spectrometry analysis, we used an Agilent 1290 Infinity II system equipped with a diode-array detector (DAD), in conjunction with an Agilent Technologies G6470A Triple Quadrupole system and electrospray ionization (ESI-MS, Agilent Jetstream). The operating conditions were set to positive-ion mode, with a skimmer voltage of 15 V, a cell accelerator voltage of 5 V, and a nitrogen gas temperature of 230 °C at a flow rate of 6 L/min. The sheath gas (N2) was maintained at a temperature of 400 °C with a flow rate of 12 L/min. Additional parameters included a capillary voltage of 2500 V, a nozzle voltage of 0 V, and a nebulizer pressure of 40 psi. The instrument operated in dynamic MRM (Multiple Reaction Monitoring) mode. For separation, a Core-Shell Technology column (Synergi, 2.5 μm Fusion-RP, 100 Å, 100 × 2 mm, Phenomenex, Torrance, CA, USA) at a temperature of 35 °C was used and a flow rate of 0.35 mL/min. The mobile phase consisted of a binary mixture: buffer A was a 5 mM NH_4OAc aqueous solution adjusted to pH 5.6 with 65 μL of glacial acetic acid, while buffer B was pure acetonitrile (Roth, LC-MS grade, purity ≥99.95). The gradient program began with 100 % solvent A for 1 min, followed by a gradual increase to 10 % solvent B over 3 min. From 4 to 7 min, solvent B was increased to 40 % and maintained for 1 min before returning to 100 % solvent A, followed by a 3-minute re-equilibration period.

Quantification

For absolute quantification, synthetic standards of all modified nucleosides used in this study were weighed, dissolved and mixed into a single calibration mix. The calibration mix was serially diluted to receive 12 calibration levels with 100 – 0.025 pmol of canonical nucleosides, 20 – 0.005 pmol of dihydrouridine and pseudouridine and 5 – 0.00125 pmol of other modified nucleosides per 10 μL injection. To each sample and calibration 1 μL of SILIS (stable isotope-labelled internal standard, gen[2]) [45] was added during injection. The peak areas of all biological samples were within the calibrated range.

Data analysis

The acquired data was analysed using Agilent's MassHunter Quantitative Analysis (for QQQ) (Version B.09.00 / Build 9.0.647.0) software. Here, the dMRM (dynamic multiple reaction monitoring) signal of the respective nucleoside and the SILIS were integrated. Followingly, the nucleoside isotope factor (NIF) was calculated (Eq. (1)).

$$NIF = \frac{signalarea(Nucleoside)}{signalarea(SILIS)} \quad (1)$$

A linear regression was fitted through the calculated NIFs for all levels of the calibration. The slope of the resulting graph was the relative response factor for the respective nucleosides (rRFN). This allowed for absolute quantification of the respective nucleosides (Eq. (2)).

$$n_{samplenucleoside} = \frac{signalarea_{samplenucleoside}}{rRFN_{nucleoside} \times signalarea_{SILIS}} \quad (2)$$

Those calculations were automatically performed by the MassHunter software. For further analysis the data was exported to Microsoft Excel (Version 2108, Build 14332.21007). Here, the absolute quantities of nucleosides [fmol] were normalised to the amount of canonicals [fmol] present in each sample. We opted to calculate the amount of modifications in modification per 100 nucleotides (Eq. (3)).

$$modifications_{per100canonicals} = 100 * \frac{n_{modification}}{(n_C + n_U + n_G + n_A)} \quad (3)$$

For NAIL-MS experiments the labelled modifications were referenced to the respective labelled canonicals.

Plotting of the data and statistical analysis was done using Graphpad Prism (Version 10.2.3). To test for statistical significance student t-tests with varying p-values (see respective experiment) were carried out. Dot plots were manually created using Excel data and Inkscape (Version 1.2).

Microscopy

For *S. cerevisiae* and *E. coli*, samples were collected and observed on slides before and after 1 h of heat treatment. Microscopy was performed with a Zeiss Axiostar plus microscope equipped with 10×, 40× and 100× Zeiss objectives and assembled using Fiji and Inkscape software. For *C. elegans*, Images were taken using a Zeiss Stemi 305 Stereo microscope with an integrated camera and assembled using Fiji and Affinity Designer 2 software. Original images were adjusted using the brightness tool in Fiji.

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CRediT authorship contribution statement

Alexandre Magno Vicente: Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Katarzyna Hencel:** Writing – original draft, Investigation. **Jannick Schicktanz:** Investigation. **Christian Hammann:** Project administration, Methodology, Investigation, Funding acquisition, Formal analysis. **Alper Akay:** Writing – original draft, Supervision, Investigation, Funding acquisition. **Stefanie Kaiser:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization.

DATA AVAILABILITY

Data is available from the corresponding author upon request.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmb.2025.169228.

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