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Independent suppression of ribosomal +1 frameshifts by different tRNA anticodon loop modifications

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ABSTRACT

Recently, a role for the anticodon wobble uridine modification 5-methoxycarbonylmethyl-2-thiouridine (mcm⁵s²U) has been revealed in the suppression of translational +1 frameshifts in *Saccharomyces cerevisiae*. Loss of either the mcm⁵U or s²U parts of the modification elevated +1 frameshift rates and results obtained with reporters involving a tRNA$^{Lys}_{UUU}$ dependent frameshift site suggested these effects are caused by reduced ribosomal A-site binding of the hypomodified tRNA. Combined loss of mcm⁵U and s²U leads to increased ribosome pausing at tRNA$^{Lys}_{UUU}$ dependent codons and synergistic growth defects but effects on +1 frameshift rates remained undefined to this end. We show in here that simultaneous removal of mcm⁵U and s²U results in synergistically increased +1 frameshift rates that are suppressible by extra copies of tRNA$^{Lys}_{UUU}$. Thus, two distinct chemical modifications of the same wobble base independently contribute to reading frame maintenance, loss of which may cause or contribute to observed growth defects. Since the thiolation pathway is sensitive to moderately elevated temperatures in yeast, we observe a heat-induced increase of +1 frameshift rates in wild type cells that depends on the sulfur transfer protein Urm1. Furthermore, we find that temperature-induced frameshifting is kept in check by the dehydration of N6-threonylcarbamoyladenosine (t⁶A) to its cyclic derivative (ct⁶A) at the anticodon adjacent position 37. Since loss of ct⁶A in *elp3* or *urm1* mutant cells is detrimental for temperature stress resistance we assume that conversion of t⁶A to ct⁶A serves to limit deleterious effects on translational fidelity caused by hypomodified states of wobble uridine bases.

Introduction

tRNA molecules from all three domains of life undergo extensive post-transcriptional modification, some of which are capable of modulating certain tRNA functions during the translation process.[1] One of these is the conserved wobble base modification 5-methoxy-carbonyl-methyl-2-thio-uridine (mcm⁵s²U), which is found in the anticodons of tRNA$^{Gln}_{UUG}$, tRNA$^{Glu}_{UUC}$ and tRNA$^{Lys}_{UUU}$.[2-4] Three distinct enzyme sets are required to convert the unmodified wobble uridine to mcm⁵s²U.[5] The six subunit Elongator complex (Elp1-Elp6) along with accessory proteins is required for the addition of a chemical side chain (likely a 5-carboxymethyl-chain, cm⁵) that is subsequently converted to mcm⁵ by the action of the Trm9-Trm112 methyltransferase complex.,[2,6–19] The thiolation (s²U) involves members of the *URM1* pathway, a sulfur transfer system consisting of Nfs1, Tum1, Urm1, Uba4 and the thiolase complex Ncs2-Ncs6.[7,20–22] In this pathway, sulfur is mobilized from cysteine and transferred to the thiolase complex via the sulfur transfer protein Urm1, which also acts as an ubiquitin-like protein modifier.[23–26] In budding yeast, the modifications of the uracil base at C2 (s²U) and C5 (mcm⁵U) occur independently of each other and inactivation of either alone results in shared pleiotropic phenotypes including sensitivities to various stressors and growth defects at elevated temperature.[5]

Simultaneous inactivation of both s²U and mcm⁵U modifications results in synergistic growth defects that are partly suppressible by elevated copy numbers of the three tRNAs normally carrying mcm⁵s²U.[27,28] Suppression efficiency is maximal when copy numbers of all three tRNAs are jointly elevated but detectable phenotypic rescue became already apparent when tRNA$^{Lys}_{UUU}$ was individually overexpressed, suggesting a major part of the growth defects does result from tRNA$^{Lys}_{UUU}$ malfunction in the absence of the wobble uridine modification.[27] Recently, the mcm⁵U and s²U parts of the mcm⁵s²U modification were further implicated in the maintenance of translational accuracy.[29] Absence of either increased ribosomal +1 frameshifting at tRNA$^{Glu}_{UUC}$ and tRNA$^{Lys}_{UUU}$ dependent test codons in dual luciferase based reporter constructs. To clarify whether induced frameshifting in the absence of mcm⁵U is due to weakened A-site or P-site binding, a series of modified Ty1 frameshift sites fused to a *lacZ* reporter gene were employed.[29] In the original Ty1 sequence, frameshifting is induced by a "hungry" AGG (Arg) codon following the CUU (Leu) codon.[30] The hungry Arg codon was replaced by the tRNA$^{Lys}_{UUU}$ dependent AAA codon and strongly increased frameshift rates were recorded when the mcm⁵ part of the mcm⁵s²U modification was lacking in tRNA$^{Lys}_{UUU}$ of an Elongator (*elp3*) mutant.[29] Furthermore,

elevated copy numbers of tRNA$^{Lys}_{UUU}$ suppressed the increase in frameshifting in the elp3 mutant, suggesting that loss of mcm^{5}U in tRNA$^{Lys}_{UUU}$ induces $+1$ frameshifts mainly by reduced A-site binding of the hypomodified tRNA rather than causing effects by affecting efficiency of P-site binding.[29] This conclusion is further supported by in vitro assays demonstrating a reduced A-site binding capacity of tRNA$^{Lys}_{UUU}$ in the absence of either s^{2}U or mcm^{5}U.[31]

Recently, the combination of mcm^{5}U and s^{2}U modification defects was shown to induce an increased ribosomal stalling at mcm^{5}s^{2}U dependent AAA (Lys) and CAA (Gln) codons. This has a negative impact on co-translational folding of nascent polypeptide chains and triggers a widespread protein aggregation phenomenon.[28] Ribosomal stalling and protein aggregation were significantly suppressed by elevated levels of the three tRNAs normally carrying the mcm^{5}s^{2}U modification.[28] Since growth phenotypes and protein aggregation are suppressible by elevated tRNA copy numbers, it seems likely that phenotypes in the mcm^{5}s^{2}U minus mutant are caused by the protein aggregation effect.[28] However, in light of the contribution of mcm^{5}U and s^{2}U to the suppression of translational frameshifting,[29] it seemed possible that combined loss of mcm^{5}U and s^{2}U not only leads to increased protein aggregation but also impairs translational accuracy, which was investigated in this study. Further, wobble uridine thiolation was recently found in several independent studies to decrease in budding yeast under conditions of mild heat stress,[32–35] suggesting that elevated temperature may already cause measurable effects on $+1$ translational frameshifting. Since tRNA$^{Lys}_{UUU}$, additionally harbors a modified adenosine at position 37 (cyclic N6-threonyl-carbamoyl-adenosine, ct6A), which is already implicated in suppression of ribosomal frameshifts[36,37] we monitored how this modification impacts on the translational effects of heat induced suppression of wobble uridine thiolation.

Results and discussion

Independent suppression of $+1$ frameshifts by s^{2}U and mcm^{5}U

To further check for a role of the mcm^{5}U or s^{2}U parts of the wobble uridine modification mcm^{5}s^{2}U in translational accuracy, we utilized $+1$ frameshift reporters containing a modified Ty1 frameshift site (Fig. 1).[29] Different yeast strains of the BY4741 background were transformed with an in-frame control construct (F12) and the test construct (W12). Both constructs contain the modified Ty1 frameshift site CTT-AAA-C decoded by tRNA$^{Leu}_{UAG}$ (CTT) and tRNA$^{Lys}_{UUU}$ (AAA) in a HIS4::lacZ reporter cassette (Fig. 1B). F12 and W12 constructs differ in the position of the lacZ gene, which is in the 0 frame in F12 and in the $+1$ frame in W12. Previous work established that in the W303 strain background, loss of elp3, which removed the mcm^{5}U side chain from tRNA$^{Lys}_{UUU}$ decoding the AAA codon in the frameshift site, strongly induced normalized frameshift rates in comparison to the wild type.[28] We also detected a significant induction of $+1$ frameshift rates (4.5-fold) in the BY4741 elp3 mutant (Tab. 1; Fig. 2A). Deletion of the gene for the sulfur carrier Urm1, which is essential for wobble uridine thiolation,

similarly increased frameshift rates (5.6-fold) (Tab. 1; Fig. 2A). To test for a potential contribution of the protein modifier function of Urm1, we compared frameshift rates between urm1 and ncs2. Loss of URM1 disables both protein modification and tRNA thiolation, whereas loss of the thiolase subunit Ncs2 disables tRNA thiolation alone.[7,22,23,25] We detected no significant difference between frameshift rates of both mutants (Tab. 1), suggesting the effects of URM1 deletion on translational fidelity exclusively result from loss of s^{2}U formation. Since double deletion of elp3 and urm1, which concomitantly removes both mcm^{5}U and s^{2}U from wobble uridines is permitted in BY4741 (but not in the W303 strain used in the previous study)[27,38] we also analyzed frameshift rates in the double mutant and found a synergistic $\sim$16-fold increase compared with the wild type. Frameshift rates in the double mutant were significantly increased ($p < 0.05$) in comparison to either single mutant (Tab. 1; Fig. 2A), indicating the sulfur at position 2 and the mcm side chain at position 5 of the wobble base (U34) do contribute to translational accuracy independently of each other. To test whether loss of s^{2}U and combined loss of mcm^{5}U and s^{2}U increase frameshift rates through an A site effect (Fig. 1C-E), we analyzed whether the increase in the frameshift rates is suppressible by an extra tRNA$^{Lys}_{UUU}$ gene copy, tK(UUU), as was previously described for rescue of elp3 phenotypes by higher-than-normal levels of tRNA$^{Lys}_{UUU}$.[29] Indeed, the extra tK(UUU) copy present in the reporter constructs significantly reduced frameshift levels in urm1 and elp3 urm1 (Table. 1; Fig. 2A) mutants, consistent with the interpretation that a delayed A-site entry of hypomodified tRNA$^{Lys}_{UUU}$ induces a reading frame shift of the tRNA$^{Leu}_{UAG}$ occupying the ribosomal P-site (Fig. 1D). Since combination of mcm^{5}U and s^{2}U modification defects has deleterious effects on growth and stress resistance and these effects are suppressible by elevated levels of those three tRNAs that normally harbor the modification,[27] we conclude that the observed strong increase in frameshift rates may contribute to growth defects, in addition to the described effects on protein homeostasis.[28] Complete loss of another anticodon loop modification (ct6A) similarly causes growth defects, stress sensitivity, protein aggregation and ribosomal frameshift induction, which may be due to changes in the efficiency of codon occupancy.[39] Hence it seems likely that tRNA modifications other than mcm^{5}s^{2}U independently add to the efficiency of A-site binding and thereby explain synergistic phenotypes observed upon combined removal of different modifications from bases in tRNA anticodon loops.[40]

Elevated temperature impacts on translational accuracy via the Urm1 pathway

Recent work revealed an unanticipated sensitivity of several of the thiolation pathway components to elevated temperature.[32–35] This in turn leads to significantly reduced wobble uridine thiolation levels which are already detectable in BY4741 wild type cells after modest increase of temperatures to 33°C.[33] Hence, it can be expected that mild heat stress increases translational $+1$ frameshift levels due to its destabilizing effects on the tRNA thiolation pathway. To
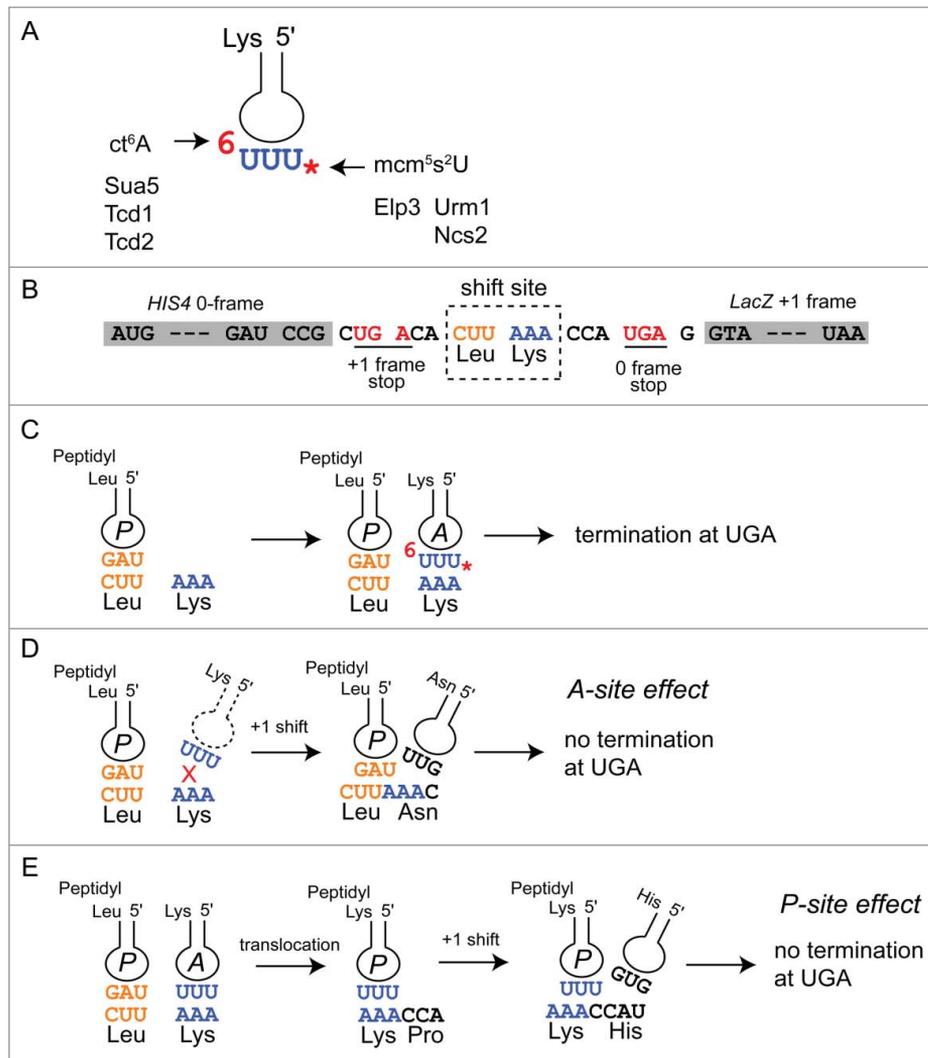
Figure 1. Anticodon loop modifications of tRNA$^{Lys}_{UUU}$ and translational +1 frameshift models. (A) Schematic representation of tRNA$^{Lys}_{UUU}$ anticodon loop with ct^{6}A (6) and mcm^{5}s^{2}U (*) modifications. Gene-products relevant for synthesis of the modifications and addressed in this study are indicated. (B) Schematic representation of the modified Ty1 frameshift construct sensitive to tRNA$^{Lys}_{UUU}$ modification defects.[28] Shown is the W12 construct where *lacZ* is in the +1 frame with respect to *HIS4A*.[28] The shift site as well as amino acids specified and +1 and 0 frame UGA stop codons are indicated. (C) With fully modified tRNA$^{Lys}_{UUU}$ frameshift rates are low and termination at the UGA downstream of the Lys codon occurs efficiently. (D) Frameshift induction by tRNA$^{Lys}_{UUU}$ modification defects via an A-site effect. Hypomodified tRNA$^{Lys}_{UUU}$ (absence of * and 6, stippled) inefficiently binds to the AAA codon while peptidyl-tRNA$^{Leu}_{UAG}$ occupies the P-site. This pausing allows the peptidy-tRNA$^{Leu}_{UAG}$ to shift to the +1 frame and the appearance of an AAC codon in the A site (read by tRNA$^{Asn}_{GUU}$). This effect would be suppressible by extra copy of tRNA$^{Lys}_{UUU}$.[28] (E) Frameshift induction by tRNA$^{Lys}_{UUU}$ modification defects via a P-site effect. Hyopmodified tRNA$^{Lys}_{UUU}$ (absence of * and 6) binds to the AAA codon and translocation occurs. Once present in the P-site as peptidyl tRNA$^{Lys}_{UUU}$, binding is weakened (loosing P-site grip) and +1 frameshift occurs. This effect would not be suppressible by extra copy of tRNA$^{Lys}_{UUU}$.

test this assumption, we grew wild type cells harboring the W12 or F12 constructs at mild heat stress conditions (37°C), measured +1 frameshift rates and compared them to rates observed under non-stress condition. We found that such elevated growth temperature increases +1 frame-shift levels ~5-fold (Tab 1; Fig. 2B), a value similar to that seen in *urm1* cells grown under non-stressed conditions. Furthermore, extra copies of tRNA$^{Lys}_{UUU}$ significantly reduce the frameshift inducing effect of temperature stress (Tab. 1; Fig. 2B). Since both elevated temperature and loss of *URM1* have comparable effects on +1 frameshift levels and similarly respond to increased tRNA$^{Lys}_{UUU}$ doses, it seemed likely that the temperature effect is largely due to inactivation of the Urm1 dependent tRNA thiolation pathway. We took advantage of the fact that *urm1* mutants are hardly growth affected under such mild heat stress

conditions [25-27] and tested whether temperature effects on +1 frameshift levels depend on a functional *Urm1* pathway. *urm1* mutants harboring the W12 and F12 construct were grown at 37°C, +1 frameshift rates were measured and compared with levels measured under non-stress conditions. As shown in Table 1 and Fig. 2B, there is no significant dif-ference in +1 frameshift levels between *urm1* cells grown at 30°C or at 37°C. These results suggest that moderate heat stress causes a significant increase in translational inaccu-racy via the destabilization of the Urm1 pathway compo-nents. To test whether moderate heat stress (37°C) indeed reduces sulfur flow through the Urm1 pathway, we moni-tored the relative efficiency of Ahp1 urmlyation, a process that is dependent on the thiocarboxylate formation at the Urm1 C-terminus. We have recently shown that more harsh heat stress (39°C) reduces both sulfur dependent Ahp1
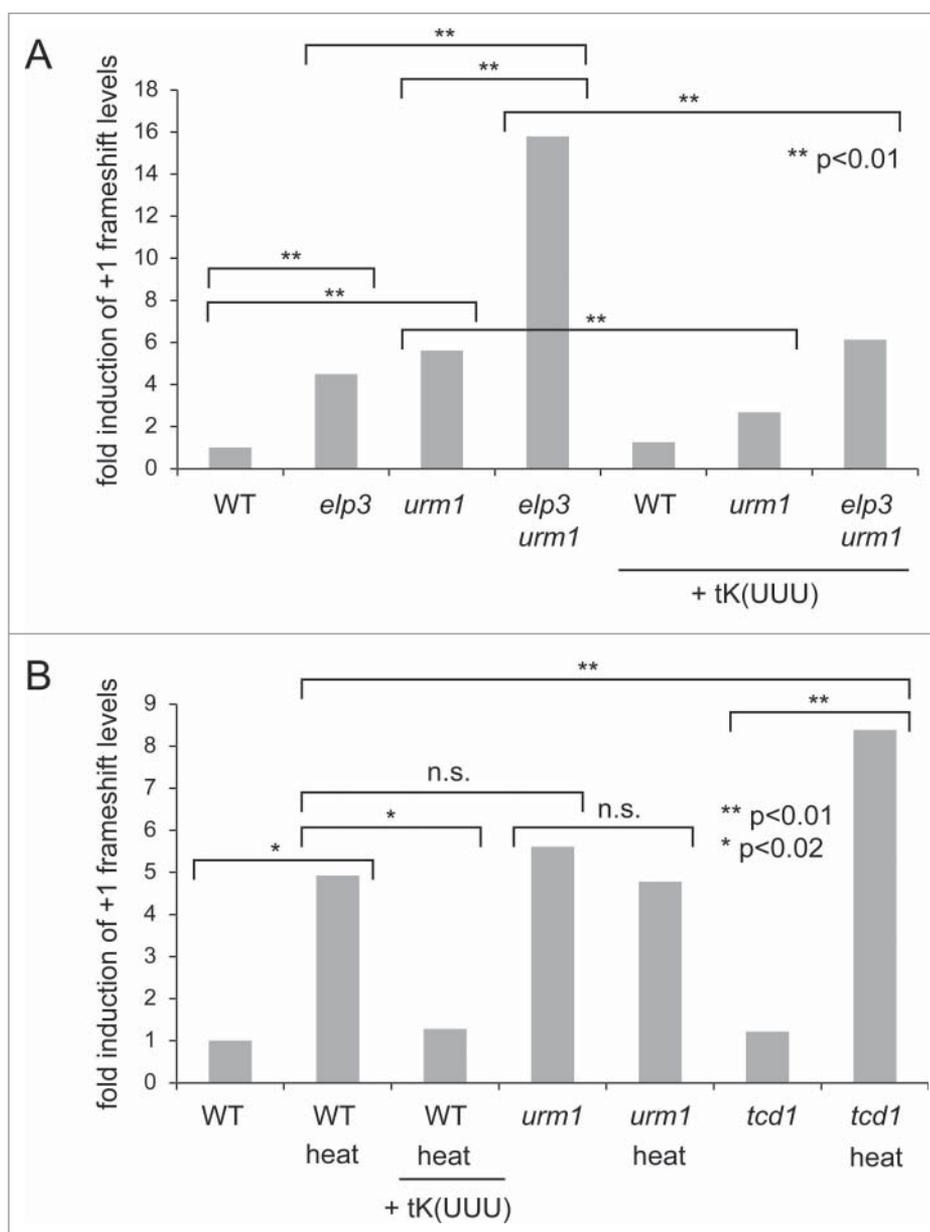
Figure 2. Induction of +1 translational frameshift rates relative to the wild type. Fold induction rates were calculated based on normalized frameshift values [%] shown in Table 1 and significance of differences determined using two-tail t-test. n.s.: not significantly different. Heat indicates moderate thermal stress condition during growth (37°C) and + tK(UUU) refers to frameshift levels measured with the WA-2 and FA-1 constructs[28] that carry an additional copy of the *tK(UUU)* gene.

urmylation and Urm1 stability without affecting Ahp1 levels.[26] Thus, in order to check whether temperature shift to 37°C already affects both the tRNA thiolation and urmylation branches of the Urm1 pathway, we analyzed levels of Ahp1-Urm1 conjugates and free Urm1 after temperature shift from 30°C to 37°C by Western analysis (Fig. 3). We detected a clear reduction of Ahp1 urmylation already after 2.5h at 37°C and a reduction of detectable free Urm1 after 7.5 h. This result suggests that mild heat stress which increases +1 frameshift rates (Tab. 1, Fig. 2) has a direct effect on the Urm1 pathway, which might result in part from a destabilization of the sulfur carrier and a reduction of sulfur flow to Urm1. The latter notion is supported by a more rapid decline of Ahp1-Urm1 conjugates compared with Urm1 itself (Fig. 3) and a previously reported instability of Tum1 at 37°C, which operates upstream of Urm1 in

the sulfur transfer pathway.[34] Since neither wild type nor the *urm1* mutant show a significant growth defect under mild heat stress conditions that induce +1 frameshift rates via downregulation of the Urm1 pathway, we assume that cells are well capable of handling the consequences of such disturbance of the translation machinery. Most likely, this occurs by proteasomal degradation of non-functional polypeptides generated due to translational frameshift events. In support, proteins induced in *urm1* and *elp3* cells are strongly enriched in proteasomal subunits and regulators,[31] suggesting an increased need for protein degradation and turnover in the absence of s²U modification. We show here that upon combination of mcm⁵U and s²U defects, frameshift rates synergistically increase to much higher levels compared with those seen in heat stressed cells; at the same time, a general growth defect becomes apparent,[27]

Table 1. Normalized frameshift rates in different yeast strains and growth conditions.

Strain	Modification missing	Temperature	Normalized frameshift rate[% (SD)]
WT	—	30	0.26 (0.07)
WT + tK	—	30	0.33 (0.07)[ns]
WT	$(s^2U)^a$	37	**1.30 (0.20)**[*]
WT + tK	$(s^2U)^a$	37	0.34 (0.04)[ns]
urm1	s^2U	30	**1.48 (0.16)**[**]
urm1 + tK	s^2U	30	**0.71 (0.02)**[**]
urm1	s^2U	37	**1.26 (0.22)**[**]
ncs2	s^2U	30	**1.41 (0.41)**[**]
elp3	n/mcm^5U	30	**1.19 (0.27)**[**]
elp3 urm1	$n/mcm^5U + s^2U$	30	**4.17 (1.44)**[**]
elp3 urm1 + tK	$n/mcm^5U + s^2U$	30	**1.62 (0.39)**[**]
tcd1	ct^6A	30	0.32 (0.08)[ns]
tcd1 urm1	$ct^6A + s^2U$	30	**2.22 (0.77)**[*]
tcd1	$ct^6A + (s^2U)^a$	37	**2.21 (0.22)**[**]

+tK refers to frameshift levels measured with the WA-2 and FA-1 constructs [28] that carry an additional copy of the *tK(UUU)* gene.
[a] temperature induced loss of s^2U [32]
[ns] not significantly different from wild type (30°C) levels as determined by *t*-test (p > 0.05).
[*] significantly different from wild type (30°C) levels as determined by *t*-test (p < 0.05).
[**] significantly different from wild type (30°C) levels as determined by *t*-test (p < 0.01).

suggesting that growth defects may correlate with an overflow of the cell's capacity to handle non-functional polypeptides. In support of this notion, it was demonstrated that cells lacking mcm^5U and s^2U display a severely reduced ability to respond to acute proteotoxic stress,[28] a condition that puts an additional burden on the cellular resources for handling damaged proteins.
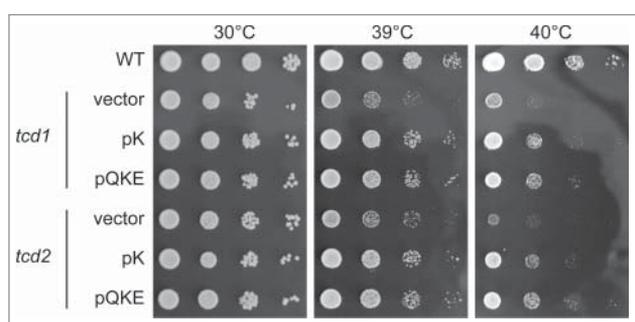
Role of the anticodon adjacent ct⁶A modification in the prevention of heat induced +1 frameshifts

Since the frameshift events recorded in this study are caused by a wobble uridine modification defect in $tRNA^{Lys}_{UUU}$, we investigated the relevance of cyclic N6-threonyl-carbamoyl-adenosine which is found in the anticodon loop of this tRNA at
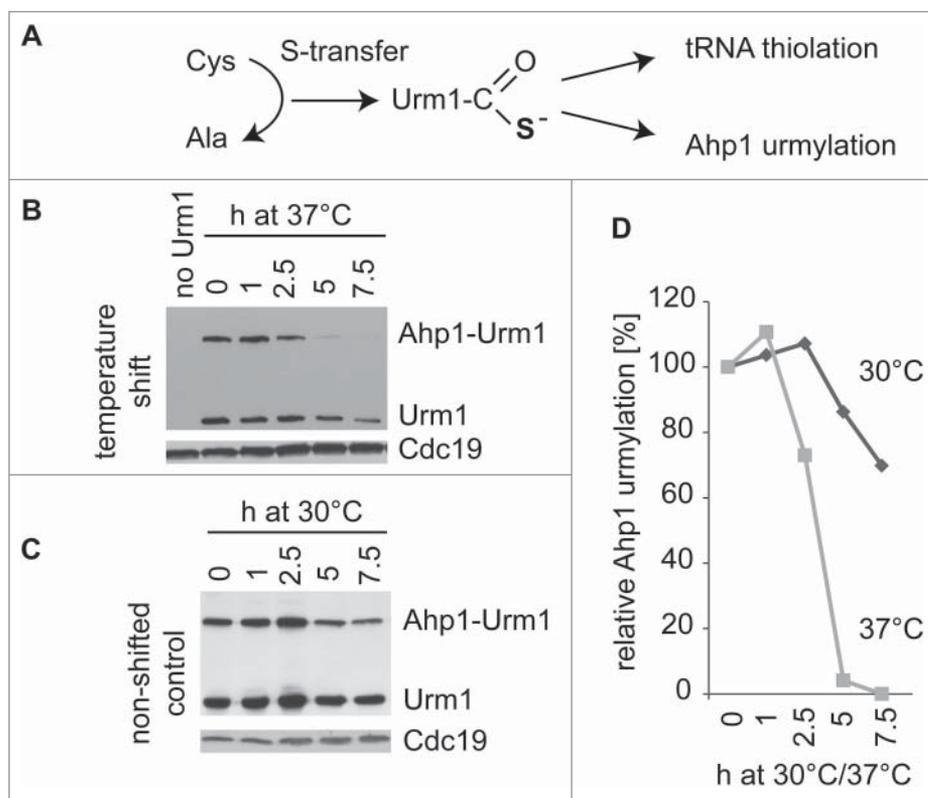
Figure 3. Effect of temperature shift on Ahp1 urmylation and Urm1 levels. (A) Sulfur transfer from cysteine to Urm1 results in formation of Urm1 thiocarboxylate, which is required for covalent attachment of Urm1 to Ahp1 (urmylation) and tRNA thiolation. (B) Temperature shift assay. *urm1* mutants expressing TAP-tagged Urm1[25] were cultivated at 30°C in YNB media to $OD_{600nm} = 1$ after which one half of the culture was shifted to 37°C. At indicated time points, total protein extracts were prepared and subjected to anti-TAP Western analysis to detect free Urm1 as well as the slower migrating Ahp1-Urm1 conjugate. Previous studies with *ahp1*mutants confirmed the the slower migrating band to result from Ahp1 urmylation by the tagged Urm1 variant.[25,26] To verify equal loading, anti-Cdc19 antibodies were utilized. (C) As in (B) but with the culture part that remained at 30°C. (D) Signal intensities of slower migrating band in (B) and (C) were quantified and relative levels of Ahp1 urmylation at 30°C or 37°C were calculated by dividing signal intensity at time points 2.5h, 5h or 7.5h by the one of timepoint 0.

position 37 (modomics.genesilico.pl). Complete loss of this modification is known to induce translational frameshift events in yeast,[36] however, the role of the dehydration of t^6A to its cyclic form ct^6A in yeast is unknown.[37] Conversion of t^6A to ct^6A is abolished in *tcd1* and *tcd2* mutants and both show negative genetic interaction with Urm1 pathway and Elongator genes.[37,40,41] In addition, combination of *tcd1* and Elongator mutations was shown to increase the formation of protein aggregates, which is likely due to increased ribosomal pausing.[40] Hence, ct^6A might play a functionally overlapping role with mcm^5U and s^2U in the suppression of translational frameshifts by independently contributing to the efficiency of A-site binding. To investigate this possibility, we introduced the W12 and F12 frameshift reporter constructs into a *tcd1* mutant strain and measured frameshift rates at non-stress and mild heat stress conditions (37°C). While loss of the modification has no effect on frameshift levels in non-stressed cells, there is a significant increase in +1 frameshift events at 37°C when ct^6A is absent compared with wild type cells at the same condition (Tab. 1; Fig. 2B). At further elevated temperatures, both *tcd1* and *tcd2* single mutants display a growth defect that can be rescued specifically by overexpression of $tRNA^{Lys}_{UUU}$ (Fig. 4), suggesting that in the absence of ct^6A (presence of t^6A) and elevated temperature, malfunction of $tRNA^{Lys}_{UUU}$ is responsible for the observed growth defect. Among the three tRNAs that are subject to heat induced shut-off of wobble uridine thiolation, $tRNA^{Lys}_{UUU}$ is the single species that also carries ct^6A (modomics.genesilico.pl). Since elevated temperature induces a loss of s^2U, it appears likely that temperature sensitivity of *tcd1* or *tcd2* cells occurs due to further increased translational frameshifting by $tRNA^{Lys}_{UUU}$ when both ct^6A and s^2U are lacking. To verify this assumption, we measured frameshift rates in *tcd1 urm1* double mutants at non-stress conditions. Indeed, frameshift levels are elevated to similar levels in *tcd1* single mutants grown under heat stress and *tcd1 urm1* double mutants grown under non-stress conditions (Tab. 1). We conclude that the dehydration of t^6A to ct^6A counteracts the frameshift inducing effects of mild temperature stress in budding yeast. Since ct^6A is the natural configuration of t^6A in several, but not all cell types and temperature effects on the thiolation pathway occur only in some yeast species,[31] it would be interesting to see if this form of t^6A has co-evolved with a

Table 2. Strains used in this study.

Strain	Genotype	Reference/source
S. cerevisiae BY4741	MAT**a**, his3Δ, leu2Δ, met15Δ, ura3Δ	Euroscarf, Frankfurt
S. cerevisiae elp3	BY4741 elp3Δ::KanMX4	Euroscarf, Frankfurt
S. cerevisiae urm1	BY4741 urm1Δ::KanMX4	Euroscarf, Frankfurt
S. cerevisiae tcd1	BY4741 tcd1Δ::KanMX4	Euroscarf, Frankfurt
S. cerevisiae tcd2	BY4741 tcd2Δ::KanMX4	Euroscarf, Frankfurt
S. cerevisiae ncs1	BY4741 ncs1Δ::KanMX4	Euroscarf, Frankfurt
S. cerevisiae elp3 urm1	BY4741 elp3Δ::KanMX4 urm1Δ:: HIS3	Ref. ([39])
S. cerevisiae tcd1 urm1	BY4741 tcd1Δ::KanMX4 urm1Δ:: SpHIS5	Ref. ([39])

temperature responsive thiolation pathway to limit its negative influence on translational accuracy. The finding that loss of ct^6A does not influence +1 frameshift rates when tRNA thiolation is unaffected but increases rates of *urm1* mutants or wild type cells grown under mild heat stress identifies ct^6A as a modification that becomes crucial for translational efficiency particularly when s^2U is missing or downregulated.

Methods

Strains, plasmids and growth assay

Yeast strains used in this study are listed in Table 2. Constructs containing the *HIS4A::lacZ* reporter cassette and modified Ty1 frameshift sites are described in[29] and are based on pMB38–9merWT or pMB38–9merFF, respectively.[30] Construct F12 contains the frameshift site CUU-AAA-C and the *lacZ* gene in reading frame 0 (in frame control). Construct W12 contains the same frameshift site and the *lacZ* gene in frame +1 with respect to HIS4A. Suppression analysis of frameshift rates involved constructs WA-2 and FA-1, which are similar to W12 and F12 but carry an additional insert with the tK(UUU) gene.[29] Plasmids were transformed into yeast strains using the lithium acetate method[42] and transformants selected on yeast nitrogen based media (Foremedium, Norfolk, UK) lacking uracil. Selection plates were incubated at 30°C until colonies were formed. Independent colonies from the selection plates were used to inoculate cultures for β galactosidase assays. Growth phenotypes of *tcd1* and *tcd2* single mutants at different temperatures and suppression by tRNA overexpression were determined by serial dilution and spot assay as described.[27] Overexpression plasmids for $tRNA^{Lys}_{UUU}$ (pK) and for combined overexpression of $tRNA^{Lys}_{UUU}$, $tRNA^{Gln}_{UUG}$ and $tRNA^{Glu}_{UUC}$[10,43] were used along with pRS425[44] as the empty vector control. Assays were performed on solid yeast peptone dextrose medium (2% glucose, 1% yeast extract, 2% peptone) and plasmid carrying cells were precultured on yeast nitrogen based minimal medium (Foremedium, Norfolk, UK) lacking leucine.

Determination of frameshift levels

Freshly transformed cells were grown at 30°C or 37°C in liquid yeast nitrogen based media containing 2% glucose and lacking uracil to $OD_{600 nm}$ of 2–3 and harvested by centrifugation. Cells were washed with water and resuspended in Z-buffer (60 mM

Figure 4. Heat induced growth defects in *tcd1* and *tcd2* single mutants are suppressible by elevated levels of $tRNA^{Lys}_{UUU}$. *tcd1* and *tcd2* mutants were transformed with either pRS425 (vector), pK (multi copy $tRNA^{Lys}_{UUU}$) or pQKE (multi copy $tRNA^{Lys}_{UUU}$, $tRNA^{Gln}_{UUG}$ and $tRNA^{Glu}_{UUC}$) and serial dilutions of cultures transferred to YPD plates that were incubated at the indicated temperatures for two days.

Na$_2$HPO$_4$, 40 mM NaH$_2$PO$_4$, 10 mM KCl, 50 mM 2-mercaptoethanol, pH 7). Dilutions of this suspension were used to determine cell density by measuring the absorbance at 600 nm. 500 μl aliquots of undiluted suspension were removed to new tubes and 2 drops of 0.01% SDS solution and chloroform added followed by vortex-aided mixing of samples for 30 sec each. Samples were then incubated at 37°C for 5 min and 100 μl of 4 mg ml^{-1} ortho-nitrophenyl-β-galactoside dissolved in Z-buffer was added. Upon color development during incubation at 37°C reactions were stopped by the addition of 250μl 1M Na$_2$CO$_3$ and centrifuged at 10.000 g for 5 min. Upper phases were transferred to optical cuvettes and absorbance at 420nm determined using a photometer. β galactosidase activity units were calculated using absorbance data at 600 and 420nm as well as reaction times by employing Miller's formula.[44] Frameshift rates [%] were calculated by dividing the β galactosidase activity measured with the W12 (or WA-2) construct by the one measured with the F12 (or FA-1) construct. For each strain at least three independent cultures were run with both the test constructs and the in-frame control constructs and measured. Normalized frameshift rates of the biological replicates were averaged and standard deviations calculated. Statistical significance was analyzed using two-tail t-test.

Ahp1 urmylation assay

TAP-tagged *Urm1* was expressed in BY4741 urm1 mutants[26] and cells were grown to OD$_{600nm}$ = 1 after which the culture was split. One half was further incubated at 30°, whereas the other half was shifted to 37°C incubation temperature. At time points 0, 1h, 2.5h 5h and 7.5h, aliquots were removed, total protein extracts generated and Ahp1 urmylation as well as free Urm1 levels determined as described previously.[25,26] In parallel, equal loading was verified by anti Cdc19 Western analysis as described.[25,26]

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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