Functional importance of $\Psi_{38}$ and $\Psi_{39}$ in distinct tRNAs, amplified for $\text{tRNA}^{\text{Gln(UUG)}}$ by unexpected temperature sensitivity of the $s^2\text{U}$ modification in yeast

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ABSTRACT

The numerous modifications of tRNA play central roles in controlling tRNA structure and translation. Modifications in and around the anticodon loop often have critical roles in decoding mRNA and in maintaining its reading frame. Residues U$_{38}$ and U$_{39}$ in the anticodon stem–loop are frequently modified to pseudouridine ($\Psi$) by members of the widely conserved TruA/Pus3 family of pseudouridylases. We investigate here the cause of the temperature sensitivity of $\text{pus3}^{\Delta}$ mutants of the yeast $\text{Saccharomyces cerevisiae}$ and find that, although $\Psi_{38}$ or $\Psi_{39}$ is found on at least 19 characterized cytoplasmic tRNA species, the temperature sensitivity is primarily due to poor function of $\text{tRNA}^{\text{Gln(UUG)}}$, which normally has $\Psi_{38}$. Further investigation reveals that at elevated temperatures there are substantially reduced levels of the $s^2\text{U}$ moiety of mcm$^5$s$^2\text{U}_{34}$ of $\text{tRNA}^{\text{Gln(UUG)}}$ and the other two cytoplasmic species with mcm$^5$s$^2\text{U}_{34}$, that the reduced $s^2\text{U}$ levels occur in the parent strain BY4741 and in the widely used strain W303, and that reduced levels of the $s^2\text{U}$ moiety are detectable in BY4741 at temperatures as low as 33°C. Additional examination of the role of $\Psi_{38,39}$ provides evidence that $\Psi_{38}$ is important for function of $\text{tRNA}^{\text{Gln(UUG)}}$ at permissive temperature, and indicates that $\Psi_{39}$ is important for the function of $\text{tRNA}^{\text{Ile(CAA)}}$ in $\text{trm10}^{\Delta}$ $\text{pus3}^{\Delta}$ mutants and of $\text{tRNA}^{\text{Leu(CAA)}}$ as a UAG nonsense suppressor. These results provide evidence for important roles of both $\Psi_{38}$ and $\Psi_{39}$ in specific tRNAs, and establish that modification of the wobble position is subject to change under relatively mild growth conditions.

Keywords: PUS3; UBA4; KTI12; $\text{Saccharomyces cerevisiae}$; pseudouridine

INTRODUCTION

Modifications are universally found in tRNA molecules from all environmental niches examined, including the simplest organisms with the most streamlined genomes. These modifications are known to play important roles in ensuring the folding and stability of the tRNA (Helm et al. 1999; Kadaba et al. 2004; Alexandrov et al. 2006), high accuracy of charging (Muramatsu et al. 1988), maintenance of the correct reading frame (Urbonavicius et al. 2001), and accurate decoding of mRNAs (Murphy et al. 2004; Weixlbaumer et al. 2007). Modifications in and around the anticodon loop have profound roles in the cell. Several modifications found at the wobble residue N$_{34}$ in the yeast $\text{Saccharomyces cerevisiae}$ likely exert their biological effects through decoding. Mutants lacking t$^6$A$_{37}$ (N$^6$-threonlycarbamoyladenosine) (El Yacoubi et al. 2011; Srinivasan et al. 2011) or m$^1$G$_{37}$ grow very poorly (Björk et al. 2001), and lack of i$^6$A$_{37}$ (N$^6$-isopen
tenyladenosine) results in reduced nonsense suppression (Dihanich et al. 1987) and tRNA gene-mediated silencing (Pratt-Hyatt et al. 2013), as well as increased resistance to certain antifungal drugs (Suzuki et al. 2012). Similarly, lack of $\text{oxycarbonylmethyluridyne;} \text{mcm}^3\text{s}^2\text{U}, \text{5-methoxycarbonylmethyl-2-thiouridyline}; \text{mcm}^3\text{U}, \text{5-carbamoylmethyluridyline}$ and $\text{mcm}^3\text{Um}, \text{5-carbamoylmethyl-2′-O-methyluridyline}$ are temperature-sensitive (Jablonski et al. 2001; Krogan and Greenblatt 2001) and have defects in transcription, exocytosis, silencing, and DNA damage response (Otero et al. 1999; Rahl et al. 2005; Li et al. 2009), all owing to two of the 11 tRNAs with these modifications (Esberg et al. 2006; Chen et al. 2011). Modifications at other positions of the anticodon stem–loop also affect function in $\text{S. cerevisiae}$. At residue 37, mutants lacking t$^6$A$_{37}$ (N$^6$-threonlycarbamoyladenosine) (El Yacoubi et al. 2011; Srinivasan et al. 2011) or m$^1$G$_{37}$ grow very poorly (Björk et al. 2001), and lack of i$^6$A$_{37}$ (N$^6$-isopen
tenyladenosine) results in reduced nonsense suppression (Dihanich et al. 1987) and tRNA gene-mediated silencing (Pratt-Hyatt et al. 2013), as well as increased resistance to certain antifungal drugs (Suzuki et al. 2012). Similarly, lack of...
Pseudouridine is the most common modification found in tRNA from all domains of life, and \( \Psi_{38} \) and \( \Psi_{39} \) are two of the four most-conserved pseudouridine modifications (Charette and Gray 2000). Among characterized tRNA species in archaea, eukaryota, eubacteria, and viruses, \( \Psi \) is found in 37 of 56 tRNAs with \( U_{38} \) and in 163 of 178 tRNAs with \( U_{39} \) (Table 1; Juuling et al. 2009). Furthermore, \( \Psi_{39} \) is found in several tRNA species of the molliculite Mycoplasma capricolum, a bacterial species in the phylum firmicutes with a streamlined genome and only 13 different tRNA modifications (Andachi et al. 1989). The family of genes encoding the pseudouridine synthase responsible for \( \Psi_{38} \) and \( \Psi_{39} \) modifications is also highly conserved (Koonin 1996; Mueller and Ferre-D’Amare 2009). In Escherichia coli and Salmonella typhimurium, TruA catalyzes formation of \( \Psi_{38}, \Psi_{39}, \) and \( \Psi_{40} \) (Singer et al. 1972; Hur and Stroud 2007), whereas in S. cerevisiae, the related Pus3 (Deg1) catalyzes \( \Psi_{38} \) and \( \Psi_{39} \) modification (Lecointe et al. 1998) and in Haloflexx volcanii, HVO_1852 catalyzes \( \Psi_{39} \) and likely \( \Psi_{38} \) modification (Blaby et al. 2011). Furthermore, the TruA/Pus3 family has a homolog in the streamlined bacterial genomes of endosymbionts, derived from a \( \Psi \) and \( \gamma \) proteobacter, and in molliculites (de Crécy-Lagard et al. 2012).

The TruA/Pus3 pseudouridylases are important, but not essential. Mutation of \( \text{hisT} \) (TruA), the pseudouridylase catalyzing formation of \( \Psi_{38} \) in E. coli and Salmonella typhimurium results in derepression of the histidine operon, and a modest-to-severe reduction in growth rate (Chang et al. 1971; Tsui et al. 1991), while S. cerevisiae pus3Δ mutants are distinctly slow growing (Carbone et al. 1991) and temperature-sensitive (Lecointe et al. 2002), and have reduced –1 frameshifting owing to lack of \( \Psi_{39} \) (Bekaert and Rousset 2005). Biochemical and structural analysis shows that \( \Psi \) stabilizes both duplex and single-stranded RNA in part owing to coordination of a water molecule through the N\(_3\)H group of \( \Psi \) and the adjacent 5′ phosphates, and from enhanced stacking in both single-stranded and duplex helices owing to its favoring of 3′ endo conformation (Arnez and Steitz 1994; Davis 1995; Durant and Davis 1999; Charette and Gray 2000).

We investigated here the cause of the temperature sensitivity of pus3Δ mutants of S. cerevisiae. Our results demonstrate that although \( \Psi_{38} \) or \( \Psi_{39} \) are found on at least 19 tRNA species in yeast (all of the characterized species with \( U_{38} \) or \( U_{39} \)), the primary defect at high temperature is due to poor function of tRNA\(^{\text{Gln(UUG)}}\). Surprisingly, we find that the defect in tRNA\(^{\text{Gln(UUG)}}\) is due to loss of both \( \Psi_{38} \) and s\(^2\)U at high temperature, and that the loss of s\(^2\)U occurs at high temperature in commonly used wild-type S. cerevisiae strains. Moreover, we provide evidence that \( \Psi_{39} \) has a role in tRNA\(^{\text{Gln(UUG)}}\) at low temperature, and that \( \Psi_{39} \) has a role in the function of tRNA\(^{\text{Trp(CCA)}}\) and in suppression by tRNA\(^{\text{Leu(CAA)}}\). Our results emphasize that Pus3 has distinct effects on specific tRNA species and demonstrate that relatively mild temperature changes can alter the modification spectrum of cellular tRNAs.

### RESULTS

The temperature sensitivity of pus3Δ mutants is primarily due to the defect of tRNA\(^{\text{Gln(UUG)}}\)

To begin elucidating the important role of PUS3, we performed a screen for high copy suppressors of the temperature sensitivity of pus3Δ mutants. A reconstructed pus3Δ mutant strain was temperature-sensitive on rich (YPD) plates at 38°C and grew more poorly than wild type in liquid YPD media at 30°C and 37°C (Fig. 1A,B), essentially as previously reported (Carbone et al. 1991; Lecointe et al. 1998). Based on the temperature sensitivity on plates, we transformed 17 pools of plasmids from an arrayed library of \( \sim 1700 \) high copy (2μ) plasmids with inserts spanning nearly the entire yeast genome (Jones et al. 2008), and plated pools of transformants at 38°C and 39°C in YPD. We identified two pools containing potential suppressors that did not contain PUS3, each of which after deconvolution had in common a tQ(UUG) gene (encoding tRNA\(^{\text{Gln(UUG)}}\)), which proved to be responsible for suppression of the temperature sensitivity when expressed on a 2μ plasmid containing no other genes (Fig. 1A). As the modifications of tRNA\(^{\text{Gln(UUG)}}\) are uncharacterized and there is an encoded uridine at residue 38, this uridine was a potential Pus3 substrate. Indeed, we found that after growth at 30°C, tRNA\(^{\text{Gln(UUG)}}\) purified from pus3Δ mutants had one less mol of \( \Psi \) than in wild-type cells (2.88 mol/mol compared with 3.73 mol/mol), whereas the levels of m\(^1\)A and mcm\(^5\)s\(^2\)U were virtually unchanged in both strains (Fig. 1C).

To define the tRNA specificity for suppression of the temperature sensitivity of pus3Δ mutants, we tested each of the 25 tRNA species bearing a \( \Psi \) or uncharacterized uridine at residue 38 or 39 in yeast, after overexpression of a representative tRNA gene on a 2μ plasmid containing no other genes. We found that only the plasmid expressing tRNA\(^{\text{Gln(UUG)}}\) substantially improved the growth of the pus3Δ strain at 38°C.

| TABLE 1. The occurrence of \( \Psi_{38} \) and \( \Psi_{39} \) is conserved in all domains of life |
|-----------------|----------------|----------------|
| Origin          | No. of available tRNA sequences\(^a\) | Residue 38         | Residue 39         |
|                 | \( U^b \) \( \Psi \) Total | \( U^b \) \( \Psi \) Total |
| Archaea         | 76             | 11 1 12 | 3 7 10 |
| Eukaryota       | 242            | 2 26 28 | 8 108 116 |
| Eubacteria      | 137            | 5 8 13 | 4 41 45 |
| Virus           | 17             | 1 2 3 | 0 7 7 |
| Total           | 472            | 19 37 56 | 15 163 178 |

\(^a\) published by Juuling et al. (2009). \(^b\) Unmodified uridine.
and 39°C, whereas overproduction of the other tRNA species had no discernable effect on growth (Fig. 1D). As the pus3Δ strain overexpressing tRNA$^{\text{Gln(UUG)}}$ grew nearly as well as the pus3Δ strain expressing PUS3, these results suggest strongly that tRNA$^{\text{Gln(UUG)}}$ is the major biologically important target of Pus3 at high temperature.

It was possible that the temperature sensitivity of the pus3Δ mutant was due to reduced levels of tRNA$^{\text{Gln(UUG)}}$ at high temperature caused by tRNA degradation by the rapid tRNA decay pathway (Chernyakov et al. 2008; Dewe et al. 2012) or the nuclear surveillance pathway (Kadaba et al. 2004), both of which are known to target specific tRNA species in certain hypomodified strains. However, we found that tRNA$^{\text{Gln(UUG)}}$ levels were unaffected in the pus3Δ mutant after growth at 30°C or after shift to 38°C for three or four generations, relative to the levels of the control tRNA$^{\text{Val(AAC)}}$, which is not a substrate for Pus3 and does not have U39 or U39 (Fig. 1E).

Furthermore, analysis of tRNAs isolated under acidic conditions to preserve charging showed that charging was unaffected after growth at 37°C or 38°C (Fig. 1F). Thus, the defect in the pus3Δ strain that impairs tRNA$^{\text{Gln(UUG)}}$ function must be due to some other property of the hypomodified tRNA$^{\text{Gln(UUG)}}$.
strain, and tested the mutants for growth after introduction of a low copy \([\text{CEN LEU2}]\) complementing plasmid, a high copy \(tQ(UUG)\) plasmid, control high copy plasmids bearing other tRNAs, or an empty vector, followed by selection against the \([\text{CEN URA3 PUS3}]\) plasmid on media containing 5-fluoroorotic acid (5-FOA). We found that \(kti12\Delta pus3\Delta\) mutants were inviable, as the \(kti12\Delta pus3\Delta\) \([\text{CEN URA3 PUS3}]\) strain did not grow on media containing 5-FOA when it harbored a \([\text{CEN LEU2}]\) empty vector (vec), but behaved like the corresponding single mutant when it harbored a complementing \([\text{CEN LEU2 KTI12}]\) or \([\text{CEN LEU2 PUS3}]\) plasmid (Fig. 2A).

Remarkably, overproduction of tRNA\(^{\text{Gln(UUG)}}\) suppressed the lethality of \(kti12\Delta pus3\Delta\) mutants, whereas no suppression was observed by introduction of high copy plasmids overproducing tRNA\(^{\text{Lys(UUU)}}\), which has the same mcm\(^5\)s\(^2\)U modification as tRNA\(^{\text{Gln(UUG)}}\); tRNA\(^{\text{Gln(CUG)}}\), the other isoacceptor of this tRNA family; or tRNA\(^{\text{Pro(UGG)}}\), another tRNA bearing \(\Psi_{38}\) (Fig. 2A). Overproduction of tRNA\(^{\text{Gln(UUG)}}\) resulted in modest growth at temperatures up to 35°C, and temperature sensitivity at 37°C and higher temperatures (Fig. 2B). We also found that suppression of \(kti12\Delta pus3\Delta\) mutants was further enhanced by overproduction of both tRNA\(^{\text{Gln(UUG)}}\) and tRNA\(^{\text{Pro(UGG)}}\) (which has mcm\(^5\)\(^5\)U), but was not further enhanced by overproduction of tRNA\(^{\text{Glu(UUC)}}\) in combination with either of the other two tRNAs with mcm\(^5\)s\(^2\)U (tRNA\(^{\text{Lys(UUU)}}\) or tRNA\(^{\text{Gln(UUG)}}\)); or any of the other tRNAs containing both xcm\(^5\)U and encoded U38 or U39 (Fig. 2B).

\(s^2\)U levels are reduced at 33°C to 39°C in both \(pus3\Delta\) mutants and wild-type strains

We speculated that the temperature sensitivity of the \(pus3\Delta\) strain might be due to loss of both \(\Psi_{38}\) and mcm\(^5\)s\(^2\)U of

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**FIGURE 2.** PUS3 has negative synthetic genetic interactions with genes required for mcm\(^5\)s\(^2\)U modification, which are partially suppressed by tRNA\(^{\text{Gln(UUG)}}\). (A) Overproduction of tRNA\(^{\text{Gln(UUG)}}\) suppresses the synthetic lethality of \(pus3\Delta kti12\Delta\) cells. A \(pus3\Delta kti12\Delta\) \([\text{CEN URA3 PUS3}]\) strain was transformed with \([\text{LEU2}]\) plasmids as indicated, and cells were grown overnight in SD-Leu medium, diluted, and spotted onto SD-Leu medium containing 5-FOA to select against the \(URA3\) plasmid, and plates were incubated at 30°C for 5 d. (lc) \(CEN\) plasmid and (hc) 2 \(\mu\) plasmid. (B) Overproduction of tRNA\(^{\text{Pro(UGG)}}\) further improves the growth of the \(kti12\Delta pus3\Delta\) \([2\mu HIS3 tQ(UUG)]\) mutant. Strains containing plasmids as indicated were grown in SD-His-Leu medium, diluted, and spotted as in A, and incubated at the indicated temperature for 2 d. (C) The synthetic growth defect of \(pus3\Delta uba4\Delta\) cells is suppressed by overproduction of tRNA\(^{\text{Gln(UUG)}}\). A \(uba4\Delta pus3\Delta\) \([\text{CEN URA3 PUS3}]\) strain was transformed with \([\text{LEU2}]\) plasmids as indicated, and cells were grown and analyzed as in A. (D) Overexpression of other known Uba4 substrate tRNAs does not further improve the growth of the \(uba4\Delta pus3\Delta\) \([2\mu HIS3 tQ(UUG)]\) mutant. Strains with plasmids as indicated were grown and analyzed as in B.
tRNA Glu(UUG) because of the suppression of the synthetic growth defects of the kit12Δ pus3Δ and the ubn4Δ pus3Δ strains by tRNA Glu(UUG) overexpression. To test this hypothesis, we purified tRNA Glu(UUG) from the pus3Δ strain and the wild-type parent strain BY4741 after growth at 30°C and 37°C, and examined modifications. As expected, the levels of Ψ and m1A were virtually unchanged in both strains at the two different temperatures (Table 2). However, consistent with our hypothesis, we found reduced levels of mcm5s2U in tRNA Glu(UUG) from the pus3Δ mutant after growth at 37°C, compared with that at 30°C (0.19 compared with 0.87 mol/mol), accompanied by a commensurate increase in mcm5U levels (from undetectable levels to 0.62 mol/mol) (Table 2; Fig. 3A). This result implies that the temperature-sensitive phenotype of the pus3Δ mutant was due to the lack of Ψ38 in combination with the partial loss of s2U from tRNA Glu(UUG) at 37°C.

To our surprise, we also found that tRNA Glu(UUG) from the wild-type strain had comparably reduced levels of mcm5s2U at 37°C (from 0.85 to 0.20 mol/mol), accompanied by an increase in mcm5U from undetectable levels to 0.69 mol/mol. Furthermore, both wild-type and pus3Δ strains grown at 37°C had comparably reduced levels of s2U in their tRNA Glu(UUG) and tRNA Lys(UUU) (Fig. 3B), the other two cytoplasmic tRNA species known to have mcm5s2U. For tRNA Glu(UUG) purified from the wild-type strain at 37°C, mcm5s2U levels were reduced from 0.75 to 0.17 mol/mol, while mcm5U increased from undetectable levels to 0.60 mol/mol, whereas other modifications remained at constant levels (Table 2). A similar reduction of mcm5s2U levels and commensurate increase in mcm5U levels was found in tRNA Glu(UUG) from the pus3Δ mutant, and in tRNA Lys(UUU) from both the wild-type and the pus3Δ strain at 37°C (Table 2). We infer that at 37°C the s2U modification is not efficiently made in the BY4741 wild-type strain and derivative strains. As might be expected, there was no change in the modification levels of representative tRNAs bearing mcm5s2U (tRNA Glu(UUG)), mcm5s2U34 (tRNA Pro(UGG)), or mcm5s34 (tRNA Ser(UAA)) after growth of either the wild-type or the pus3Δ strain at 37°C (Table 3).

Further investigation showed that the reduced levels of the s2U moiety of tRNAs were increasingly obvious as temperatures increased above 30°C. In YPD medium, reduced levels of the s2U moiety of tRNA Glu(UUG) were significant after growth for three generations at 33°C (mcm5s2U reduced from 0.92 to 0.78 mol/mol, with a commensurate increase in mcm5U), and were more extreme after growth for three generations at 35°C,

### TABLE 2. Quantification of nucleosides of tRNA Glu(UUG), tRNA Lys(UUU), and tRNA Glu(UUG) from wild-type and pus3Δ cells after three generations at 30°C and 37°C

| tRNA | Modification | Moles expected | wt 30°C | wt 37°C | pus3Δ 30°C | pus3Δ 37°C |
|------|--------------|----------------|---------|---------|-----------|-----------|
| tRNA Glu(UUG) | Ψ | 4 | 3.73 ± 0.14 | 2.88 ± 0.07 | 3.91 ± 0.04 | 2.97 ± 0.13 |
| | mcm5s2U | 1 | 0.85 ± 0.10 | 0.87 ± 0.04 | 0.20 ± 0.03 | 0.19 ± 0.01 |
| | mcm5U | 0 | n/d | n/d | 0.69 ± 0.02 | 0.62 ± 0.09 |
| | m1A | 1 | 0.64 ± 0.04 | 0.75 ± 0.08 | 0.74 ± 0.13 | 0.76 ± 0.14 |
| tRNA Lys(UUU) | Ψ | 5 | 4.70 ± 0.16 | 4.78 ± 0.15 | 4.79 ± 0.13 | 4.83 ± 0.08 |
| | mcm5s2U | 1 | 0.83 ± 0.04 | 0.84 ± 0.01 | 0.20 ± 0.02 | 0.33 ± 0.07 |
| | mcm5U | 0 | 0.13 ± 0.03 | 0.17 ± 0.08 | 0.72 ± 0.10 | 0.76 ± 0.04 |
| | m7G | 1 | 1.03 ± 0.04 | 1.01 ± 0.03 | 1.03 ± 0.02 | 1.03 ± 0.02 |
| | m7A | 1 | 0.86 ± 0.04 | 0.88 ± 0.04 | 0.88 ± 0.05 | 0.88 ± 0.03 |
| tRNA Glu(UUG) | Ψ | 3 | 2.90 ± 0.04 | 3.02 ± 0.03 | 3.02 ± 0.04 | 3.06 ± 0.09 |
| | mcm5s2U | 1 | 0.75 ± 0.04 | 0.75 ± 0.05 | 0.17 ± 0.01 | 0.27 ± 0.09 |
| | mcm5U | 0 | n/d | n/d | 0.60 ± 0.02 | 0.48 ± 0.09 |
| | m1C | 1 | 0.91 ± 0.01 | 0.89 ± 0.04 | 0.90 ± 0.03 | 0.89 ± 0.04 |

*Mean and standard deviation based on three individual growths and RNA preparations.

### FIGURE 3. tRNA Glu(UUG) has reduced mcm5s2U levels at 37°C, accompanied by an increase in mcm5U. (A) Loss of the s2U moiety of mcm5s2U from tRNA Glu(UUG) occurs at 37°C in the wild-type and the pus3Δ strain. A wild-type strain and a pus3 mutant at log phase at 30°C were harvested three generations after a shift to 37°C, tRNAs were purified, and nucleosides were analyzed as described in Materials and Methods. The HPLC-UV chromatograph of modified nucleosides from tRNAs was shown at 265 nm, and the region containing mcm5s2U and mcm5U modification was shown, all of which bear the mcm5s2U34 modification.
37°C, and 39°C (mcm$s^2$U levels reduced to 0.64, 0.44, and 0.36 mol/mol, respectively) (Table 4; Fig. 4A). This graded temperature-dependent loss of the s$^2$U from tRNA$^{Gln}$($UUG$) suggests increasingly reduced capacity for synthesis of the s$^2$U moiety at elevated temperatures. Consistent with this interpretation, we also found that loss of the s$^2$U moiety occurred gradually as a function of the number of generations after the temperature was increased to 37°C (Table 5; Fig. 4B). The levels of mcm$s^5$U decreased steadily in each generation, accompanied by an increase in mcm$s^2$U, as expected for progressive loss of synthetic capacity for the s$^2$U modification at high temperature.

The reduced s$^2$U levels extended to minimal media and another commonly used strain. We found a similar loss of the s$^2$U moiety of mcm$s^5$U in tRNA$^{Gln}$($UUG$) in the BY4741 strain after growth at 37°C in synthetic complete medium or after growth of the S. cerevisiae W303 strain in YPD medium for three generations at 37°C (Fig. 4C). Thus, the reduced levels of s$^2$U occur in two widely used S. cerevisiae wild-type strains grown at 37°C in conventional media. However, we note that there was a distinctly more modest loss of s$^2$U modification after growth of the BY4741 strain for three generations in YM medium containing glycerol (Fig. 4C).

Interestingly, a shift back to 30°C does not completely correct the defect in s$^2$U modification of tRNA within two generations (Table 6; Fig. 4D). Although there was an increase in mcm$s^5$U modification in the two generations after the cells were shifted from 37°C back to 30°C, there was only a partial increase. It is striking that the amounts of mcm$s^5$U that remain at each generation after the shift back to 30°C are very similar to those predicted if previously matured tRNA$^{Gln}$($UUG$) containing mcm$s^2$U was not efficiently subsequently modified to mcm$s^5$U.

### TABLE 3. Quantification of nucleosides of tRNA$^{Gly}$($UCC$), tRNA$^{Pro}$($UGG$), and tRNA$^{Leu}$($UAA$) from wild-type and pus3Δ cells after three generations at 30°C and 37°C

| tRNA$^a$ | Modification | Moles expected | 30°C | 37°C |
|---------|--------------|----------------|------|------|
| tRNA$^{Gly}$($UCC$) | Ψ | 2 | 1.98 ± 0.02 | 2.00 ± 0.03 | 2.00 ± 0.02 |
| | mcm$s^2$U | 1 | 0.92 ± 0.03 | 0.93 ± 0.00 | 0.90 ± 0.03 |
| | Gm | 1 | 0.87 ± 0.08 | 0.93 ± 0.00 | 0.90 ± 0.02 |
| tRNA$^{Pro}$($UGG$) | Ψ | 4 | 3.87 ± 0.08 | 3.80 ± 0.03 | 2.93 ± 0.03 |
| | mcm$s^2$U | 1 | 0.97 ± 0.02 | 0.93 ± 0.00 | 0.94 ± 0.02 |
| | m$^2$A | 1 | 0.73 ± 0.07 | 0.75 ± 0.11 | 0.81 ± 0.04 |
| | Cr | 1 | 0.88 ± 0.03 | 0.90 ± 0.01 | 0.89 ± 0.02 |
| tRNA$^{Leu}$($UAA$) | Ψ | 2 | 1.96 ± 0.07 | 2.19 ± 0.04 | 2.37 ± 0.03 |
| | mcm$s^2$U | 1 | 1.68 ± 0.01 | 1.63 ± 0.05 | 1.82 ± 0.02 |
| | U$m^m$ | 0 | 0.19 ± 0.02 | 0.21 ± 0.03 | 0.10 ± 0.02 |
| | m$^2$G | 1 | 0.96 ± 0.00 | 1.02 ± 0.07 | 1.04 ± 0.03 |
| | Cm | 1 | 0.83 ± 0.03 | 0.81 ± 0.11 | 0.76 ± 0.06 |
| | Gm/m$m^1$G | 2 | 1.92 ± 0.04 | 1.96 ± 0.01 | 1.89 ± 0.02 |
| | ac$^1$G | 1 | 0.86 ± 0.04 | 0.83 ± 0.05 | 0.80 ± 0.01 |
| | m$^2$G | 1 | 1.03 ± 0.02 | 1.04 ± 0.01 | 1.00 ± 0.02 |
| | m$^2$G | 1 | 0.89 ± 0.03 | 0.90 ± 0.00 | 0.87 ± 0.01 |

*Mean and standard deviation based on three individual growths and RNA preparations.

### TABLE 4. Quantification of nucleosides of tRNA$^{Gln}$($UUG$) in BY4741 wild-type cells after three generations at various temperatures

| Modification$^a$ | Moles expected | 30°C | 33°C | 35°C | 37°C | 39°C |
|------------------|----------------|------|------|------|------|------|
| Ψ | 4 | 3.84 ± 0.04 | 3.87 ± 0.02 | 3.86 ± 0.10 | 3.94 ± 0.10 | 4.02 ± 0.01 |
| mcm$s^2$U | 1 | 0.92 ± 0.05 | 0.78 ± 0.01 | 0.64 ± 0.01 | 0.44 ± 0.00 | 0.36 ± 0.02 |
| m$^2$U | 0 | n/d$^b$ | 0.11 ± 0.03 | 0.25 ± 0.03 | 0.44 ± 0.02 | 0.53 ± 0.01 |
| m$^2$A | 1 | 0.55 ± 0.19 | 0.63 ± 0.04 | 0.64 ± 0.08 | 0.60 ± 0.09 | 0.70 ± 0.07 |

*Mean and standard deviation based on three individual growths and RNA preparations.

$^b$Not detected.

**ψ$_{38}$ affects the function of tRNA$^{Gln}$($UUG$) at 30°C**

We also found evidence that tRNA$^{Gln}$($UUG$) function is compromised by lack of ψ$_{38}$ at lower temperatures, under conditions where mcm$s^5$U is intact. Previous experiments established that the normally lethal deletion of the single copy $tQ(CUG)$ gene could be suppressed by overexpression of tRNA$^{Gln}$($UUG$), implying that the overexpressed tRNA could decode CAG codons (Johansson et al. 2008). However, we found that this suppression was strongly $pus3$-dependent, as the $tQ(CUG)Δ$ [cen $ura3$ $tQ(CUG)$] [2μ LEU2 $tQ(UUG)$] strain grew well on media containing 5-FOA, but the corresponding $tQ(CUG)Δ pus3Δ$ [cen $ura3$ $tQ(CUG)$] [2μ LEU2 $tQ(UUG)$] strain did not (Fig. 5).

### At standard temperatures ψ$_{39}$ affects the function of tRNA$^{Trm}$($CCA$) and of tRNA$^{Lan}$($CAA$)

We examined the synthetic phenotype reported for mutation of $PUS3$ and $TRM10$, which encodes the m$^1$G$_9$ methyltransferase (Jackman et al. 2003), because the $pus3Δ trm10Δ$ interaction was the most severe of those reported (Costanzo et al. 2010). We found that $pus3Δ trm10Δ$ [cen $ura3$ $PUS3$] strains were not viable on standard FOA media, but were weakly viable on media containing lower amounts of FOA (Fig. 6A), consistent with previous results demonstrating a
growth defect of trm10Δ strains on media containing 5-fluorouracil (Gustavsson and Ronne 2008). This growth defect was fully complemented by a [CEN LEU2 PUS3] plasmid (Fig. 6A) or a [CEN LEU2 TRM10] plasmid (data not shown). We determined which, if any, of the tRNAs that have m1G9 (Swinehart et al. 2013) and Ψ38 or Ψ39 (or an uncharacterized U38 or U39) could improve this growth defect on media containing FOA. We found that tRNATrp(CCA) overexpression suppressed the severe growth defect of the pus3Δ trm10Δ strain, whereas overexpression of the other substrate tRNAs of Trm10 and Pus3 did not; however, the pus3Δ trm10Δ [2μ LEU2 tw(CCA)] strain still grew poorly compared with the control pus3Δ trm10Δ [CEN LEU2 PUS3] strain, particularly at higher temperatures (Fig. 6B). As tRNATrp(CCA) has a relatively unstable anticodon helix by Ψ90, as Ψ has been shown to increase the stability of duplex RNAs (Durant and Davis 1999).

To further investigate the function of Ψ39 in stabilization of the anticodon helix, we compared the function of three tRNAs with Ψ39 and different predicted stabilities of the anticodon stem, by evaluation of nonsense suppression. We examined tRNATrp(GUA), tRNAVal(CGA), and tRNALeu(CAA), with predicted anticodon stem stabilities of −2.4, −3.4, and −4.9 kcal/mol, respectively (Reuter and Mathews 2010), after changing the anticodon to CUA to read the UAG (am-

![Graphs and tables from the text]

**FIGURE 4.** The reduced levels of s2U in tRNAAsn(UUG) are dependent on temperature and growth medium. (A) Reduced levels of the s2U moiety of tRNAAsn(UUG) are detectable after growth for three generations at 33°C and are more extreme at higher temperatures. BY4741 was grown to log phase at 30°C, shifted to different temperatures, and grown for three generations. Then tRNAAsn(UUG) was purified and analyzed from harvested cells. (Dark gray bars) mcm5U; (light gray bars) mcm5s2U. (B) Reduced levels of the s2U moiety occur gradually as a function of time after temperature shift to 37°C. BY4741 cells were grown as in (A) and harvested one to five generations after shifting the temperature to 37°C, and nucleosides in tRNAAsn(UUG) were analyzed as in A. (C) Reduced levels of the s2U moiety of tRNAAsn(UUG) occur in two widely used wild-type strains in glucose-containing media. Strains were grown in rich medium (YPD), synthetic complete medium (SDC), or YP medium with glycerol (YPG) to log phase and shifted to 37°C as indicated for three generations, followed by purification of tRNAAsn(UUG) from harvested cells and nucleoside analysis. (D) Reduced levels of the s2U modification of tRNA are not fully restored within two generations after a shift back to 30°C. BY4741 cells were grown in YPD media for four generations at 37°C as in A, followed by dilution in media prewarmed to 30°C, and growth for two more generations, and tRNAAsn(UUG) was purified from cells harvested as indicated and analyzed for nucleosides.

**TABLE 5.** Quantification of nucleosides of tRNAAsn(UUG) in BY4741 wild-type cells after growth at 30°C and 37°C for various generations

| Temperature | Modification | Moles expected | Generation |
|-------------|--------------|----------------|------------|
|             | Ψ            | 1              | 2          | 3          | 4          | 5          |
| 30°C        | m1A          | 0.65 ± 0.13    | 0.58 ± 0.12| 0.55 ± 0.19| 0.59 ± 0.12| 0.59 ± 0.15|
|             | mcm5U        | 0/n/d         | n/d        | n/d        | n/d        | n/d        |
|             | mcm5s2U      | 0.86 ± 0.02   | 0.86 ± 0.01| 0.92 ± 0.05| 0.92 ± 0.07| 0.91 ± 0.05|
| 37°C        | Ψ            | 4              | 3.81 ± 0.04| 3.77 ± 0.05| 3.84 ± 0.04| 3.81 ± 0.03| 3.84 ± 0.04|
|             | m1A          | 1              | 0.65 ± 0.13| 0.58 ± 0.12| 0.55 ± 0.19| 0.59 ± 0.12| 0.59 ± 0.15|
|             | mcm5U        | 0/n/d         | n/d        | n/d        | n/d        | n/d        |
|             | mcm5s2U      | 0.86 ± 0.02   | 0.86 ± 0.01| 0.92 ± 0.05| 0.92 ± 0.07| 0.91 ± 0.05|

| Temperature | Modification | Moles expected | Generation |
|-------------|--------------|----------------|------------|
| 37°C        | Ψ            | 4              | 3.81 ± 0.04| 3.77 ± 0.05| 3.84 ± 0.04| 3.81 ± 0.03| 3.84 ± 0.04|
|             | m1A          | 1              | 0.65 ± 0.13| 0.58 ± 0.12| 0.55 ± 0.19| 0.59 ± 0.12| 0.59 ± 0.15|
|             | mcm5U        | 0/n/d         | n/d        | n/d        | n/d        | n/d        |
|             | mcm5s2U      | 0.86 ± 0.02   | 0.86 ± 0.01| 0.92 ± 0.05| 0.92 ± 0.07| 0.91 ± 0.05|

a Mean and standard deviation based on three individual growths and RNA preparations.
b Not detected.
Remarkably, we found that the reduced levels of s2U occur in and that reduced s2U is detectable at temperatures as low as strains that are in widespread use in the yeast community, both the BY4741 strain and the W303 strain at 37°C, two (Table 8; Fig. 6C), strongly indicating that tant role in the function of tRNALeu(CAA). In contrast, the reduction of s2U in YP media containing glycerol may be due more, there was little effect of the Δpus3 mutation relative to that in the corresponding tRNALeu(CAA). In contrast, the Δpus3 mutant has an important role in the function of tRNALeu(CAA). As shown in Figure 5, the Δpus3 mutation had no effect on suppression in the tY (GUA)am strain relative to that in a PUS3+ strain at all three temperatures (from 0.871 to 0.886 at 28°C, from 0.624 to 0.691 at 33°C, and from 0.351 to 0.380 at 37°C). Furthermore, there was little effect of the Δpus3 mutation on suppression in the tsL(CAA)am strains at 28°C (from 0.721 to 0.663), and only a modest reduction of suppression at 33°C and 37°C (from 0.455 to 0.130 and from 0.099 to 0.014, respectively). These results suggest that the role of PUS3 extends to Ψ39, but not in a manner that can be explained by increased stability of the anticodon stem–loop.

**DISCUSSION**

We have provided evidence here that yeast Δpus3 mutants are temperature-sensitive owing to reduced function of tRNA\(^{\text{Gln(UUG)}}\), because of the absence of Ψ38 in combination with reduced levels of the s\(^2\)U moiety of mcm\(^5\)s\(^3\)U of tRNA\(^{\text{Gln(UUG)}}\). Remarkably, we found that the reduced levels of s\(^2\)U occur in both the BY4741 strain and the W303 strain at 37°C, two strains that are in widespread use in the yeast community, and that reduced s\(^2\)U is detectable at temperatures as low as 33°C, and occurs in both YPD and synthetic media. As the loss of s\(^2\)U is associated with slower decoding of VAA codons and up-regulation of GCN4 by a GCN2-independent mechanism (Zinshteyn and Gilbert 2013), numerous biological effects reported in the literature at even mildly elevated temperatures may need to be reinterpreted because of the accompanying partial loss of the s\(^2\)U moiety. The more minor reduction of s\(^2\)U in YP media containing glycerol may be due to the reduced growth rate, which would allow increased time for mcm\(^5\)s\(^3\)U biosynthesis, or might be due to different transcription and proteome content in a carbon source requiring respiration (Gasch et al. 2000).

The reduction in the s\(^2\)U moiety of the mcm\(^5\)s\(^3\)U modification at modestly elevated temperatures is part of an emerging theme of altered modification programs observed under different stress conditions. It is intriguing that at 37°C the s\(^2\)U modification was also previously found to be reduced in yeast mitochondrial tRNA\(^{\text{Lys(UUU)}}\), which normally has cmnm\(^5\)s\(^3\)U \(^{34}\), whereas the known s\(^2\)U of mitochondrial tRNA\(^{\text{Gln(UUG)}}\) and tRNA\(^{\text{Glu(UUC)}}\) was not (Kamenski et al. 2007). It thus seems possible that s\(^2\)U formation of mitochondrial tRNA\(^{\text{Lys(UUU)}}\) has components in common with the cytoplasmic s\(^2\)U modification that are not shared by the other two mitochondrial tRNA species. Other examples of altered modifications during growth of yeast include increased m\(^5\)C in the anticodon of tRNA\(^{\text{Leu(CAA)}}\) and loss of m\(^2\)G and cm during oxidative stress (Chan et al. 2010, 2012), and the increased m\(^6\)C at C\(_{48}\) and C\(_{50}\) of tRNA\(^{\text{His}}\) during the onset of stationary phase, amino acid starvation, and rapamycin treatment (Preston et al. 2013). It thus seems plausible that these alterations in modifications are an integral part of a stress response.

Remarkably, our data suggest that once mature tRNA\(^{\text{Gln(UUG)}}\) is synthesized with mcm\(^5\)U, the s\(^2\)U moiety is not easily added to form mcm\(^5\)s\(^3\)U, following a return to 30°C and log phase growth. Although this result might imply that there is a biochemical requirement for s\(^2\)U to be made before the mcm\(^5\)U moiety is added, available evidence shows instead that several mutants required for mcm\(^5\)U biosynthesis are also partially lacking the s\(^2\)U modification (Nakai et al. 2008; Noma et al. 2009). Two other explanations might explain the slow recovery of tRNAs that are fully modified with mcm\(^5\)s\(^3\)U. First, once tRNA biogenesis is finished and tRNA with the mcm\(^5\)U modification enters into the translation cycle, there may be a reduced likelihood for that tRNA to be subsequently modified with the s\(^2\)U modification. This could occur if the tRNA is being sequestered by the translation machinery and is not able to effectively bind the s\(^2\)U modification enzymes, or if there is a specific time during tRNA biogenesis when the s\(^2\)U modification should be made; for example, tRNA\(^{\text{Phe}}\) maturation requires retrograde transport of spliced tRNA to the nucleus (Murthi et al. 2010), in part to ensure modification of G\(_{37}\) to m\(_{1}G_{37}\) prior to yW formation after reexport (Ohira and Suzuki 2011). Second,

**TABLE 6.** Quantification of nucleosides of tRNAGln(UUG) in BY4741 wild-type cells after growth at 37°C, followed by shift to 30°C

| Modificationa | Moles expected | Gen.4 | Gen.1 | Gen.2 |
|---------------|----------------|-------|-------|-------|
| Ψ             | 4              | 3.93 ± 0.10 | 3.88 ± 0.03 | 3.91 ± 0.06 |
| m\(^1\)A       | 1              | 0.58 ± 0.05 | 0.64 ± 0.07 | 0.68 ± 0.08 |
| mcm\(^5\)U     | 0              | 0.52 ± 0.02 | 0.28 ± 0.00 | 0.14 ± 0.02 |
| mcm\(^5\)s\(^2\)U | 1              | 0.31 ± 0.01 | 0.50 ± 0.03 | 0.64 ± 0.02 |

Superscript aMean and standard deviation based on three individual growths and RNA preparations.
there might be limiting biosynthetic capacity for making the $s^2U$ modification, thereby requiring a significant amount of time to generate $mcm5s2U$ on all tRNA species after the return to 30°C.

The suppression of the pus3Δ temperature sensitivity by increased dosage of tRNA$^{Gln(UUG)}$ emphasizes that, as with other modifications mutants, there is a primary target tRNA for which the modification appears to have the major biological effect (Esberg et al. 2006; Björk et al. 2007; Phizicky and Alfonzo 2010; Dewe et al. 2012; Guy et al. 2012). Two previous reports also emphasize a central role of $mcm5s2U$ of tRNA$^{Gln(UUG)}$, the high copy suppression by tRNA$^{Gln(UUG)}$ and tRNA$^{Lys(UUU)}$ of the multiple phenotypes of $elp$ mutants, which lack the $cm5U$ moiety (Esberg et al. 2006); and the enhanced high copy suppression by tRNA$^{Gln(UUG)}$ (in addition to tRNA$^{Lys(UUU)}$) of the lethality of $elp3Δ tue1Δ$ mutants, which lack both the $cm5U$ and $s^2U$ moieties of $mcm5s2U$ (Björk et al. 2007).

Our results also indicate a distinct role of $Ψ_{38}$ of tRNA$^{Gln(UUG)}$ separate from $mcm5s2U$, as suppression of the lethality of a $tQ(CUG)Δ$ strain by high copy expression of tRNA$^{Gln(UUG)}$ (Johansson et al. 2008) is much more efficient when Pus3 is present than in a pus3Δ mutant; this Pus3 dependence of the tRNA$^{Gln(UUG)}$ suppression occurs at 30°C, conditions in which $mcm5s2U$ levels are normal. Consistent with the observation that suppression of the $tQ(CUG)Δ$ lethality by overexpression of tRNA$^{Gln(UUG)}$ in a PUS3Δ strain requires $mcm5s2U$ (Johansson et al. 2008), we found that suppression was reduced at higher temperatures, when $s^2U$ levels are reduced (Fig. 5). The effect of $Ψ_{38}$ on tRNA$^{Gln(UUG)}$ function might be due to reduced ability to decode CAG codons (Johansson et al. 2008), or to an overall

| tRNA   | $ΔG^{ab}$ (kcal/mol) | $Ψ_{38}$ | $Ψ_{39}$ |
|--------|----------------------|----------|----------|
| Lys(UU) | -1.2                 | $Ψ_{39}$ |
| Met(CAU)| -2.0                 | $Ψ_{39}$ |
| Tyr(GUA)| -2.4                 | $Ψ_{39}$ |
| Trp(CCA)| -2.4                 | $Ψ_{39}$ |
| Ser(GA)| -2.7                 | $Ψ_{39}$ |
| Thr(UGU)| -2.9                 | $U_{39}$ |
| Ser(UG)| -3.2                 | $Ψ_{39}$ |
| Arg(UC)| -3.4                 | $Ψ_{39}$ |
| Ser(GA)| -3.4                 | $U_{39}$ |
| Leu(UG)| -3.4                 | $Ψ_{39}$ |
| Leu(UA)| -3.5                 | $Ψ_{39}$ |
| Glu(UCC)| -3.7               | $Ψ_{39}$ |
| His(UCC)| -3.9               | $Ψ_{39}$ |
| Glu(CUC)| -4.0                | $Ψ_{39}$ |
| Ile(UAU)| -4.1                | $Ψ_{39}$ |
| Gly(CGC)| -4.2                | $Ψ_{39}$ |
| Thr(GT)| -4.4                | $Ψ_{39}$ |
| Lys(UU)| -4.4                | $Ψ_{39}$ |
| Ala(UC)| -4.4                | $Ψ_{39}$ |
| Gly(CGC)| -4.6                | $Ψ_{39}$ |
| Gln(UUG)| -4.7               | $Ψ_{39}$ |
| Thr(GT)| -4.7                | $Ψ_{39}$ |
| Phe(GAA)| -4.9               | $Ψ_{39}$ |
| Leu(CAA)| -4.9               | $Ψ_{39}$ |
| Asn(GUU)| -5.0               | $Ψ_{39}$ |
| Gln(UUG)| -5.0               | $U_{39}$ |
| Arg(GCG)| -5.4               | $Ψ_{39}$ |
| Val(CAC)| -5.5                | $Ψ_{39}$ |
| Ile(AAU)| -5.6               | $Ψ_{39}$ |
| Arg(CCU)| -5.6               | $Ψ_{39}$ |
| Cys(GCA)| -5.7                | $Ψ_{39}$ |
| Val(AC)| -5.9               | $Ψ_{39}$ |
| Leu(GAG)| -6.0               | $Ψ_{39}$ |
| Ser(GU)| -6.0               | $Ψ_{39}$ |
| Gly(CGC)| -6.4               | $Ψ_{39}$ |
| Pro(AGG)| -6.8               | $Ψ_{39}$ |
| Gly(UCC)| -6.9               | $Ψ_{39}$ |
| iMet(CAU)| -6.9              | $Ψ_{39}$ |
| Asp(GUC)| -7.0               | $Ψ_{39}$ |
| Val(UAC)| -7.4               | $Ψ_{39}$ |
| Pro(UGG)| -7.7               | $Ψ_{39}$ |
| Ala(AGC)| -8.4               | $Ψ_{39}$ |

$^a$Reuter and Mathews (2010).
$^b$tRNA$^{Arg}^{CCG}$ did not form a stable anticodon stem–loop with this program.
reduced functional level of tRNA$_{\text{Gln(UUG)}}^{\text{am}}$. Although the precise role of Ψ$_{39}$ in tRNA$_{\text{Gln(UUG)}}^{\text{am}}$ is not clear, Ψ$_{39}$ is involved in noncanonical interactions with N$_32$ (Auffinger and Westhof 1999), and Ψ stabilizes single-stranded regions (Davis 1995).

Although it is well established that Ψ stabilizes stacking in both single-stranded regions and duplexes of helices by promoting the C3’ endo conformation and coordination of a water molecule (Arnez and Steitz 1994; Davis 1995; Durant and Davis 1999; Charette and Gray 2000), it is not clear that this stabilization is the only significant biological effect of Ψ$_{39}$. In favor of stabilization is the apparently biased distribution of Ψ$_{39}$ or an uncharacterized U$_{39}$ on those yeast cytoplasmic tRNAs with less stable anticodon stems (Table 7), and the observed suppression of the trm10Δ pus3Δ growth defect by the tRNA species with m1G$_{39}$ and the least stable anticodon stem (tRNA$_{\text{Trp(CCA)}}^{\text{am}}$). However, the significant role for Ψ$_{39}$ on tRNA$_{\text{Leu(CAA)}}^{\text{am}}$ function and the more modest role for Ψ$_{39}$ on function of tRNA$_{\text{Gln(GUG)}}^{\text{am}}$ and tRNA$_{\text{Ser(CGA)}}^{\text{am}}$, argues for an additional role of Ψ$_{39}$ in yeast as tRNA$_{\text{Leu(CAA)}}^{\text{am}}$ is predicted to have a much more stable anticodon stem than the two other species. We note that the identity and pairing ability of the 31–39 pair at the bottom of the anticodon stem have both been implicated in first base decoding accuracy of tRNA$_{\text{Gln(CUG)}}^{\text{am}}$ (Murray et al. 1998; Kemp et al. 2013), and that Ψ$_{39}$ has been shown to increase –1 frameshifting in certain constructs (Bekaert and Rousset 2005). Thus, in this case Ψ$_{39}$ may increase decoding efficiency of tRNA$_{\text{Leu(CAA)}}^{\text{am}}$. However, its role remains to be defined.

It is intriguing that tRNA$_{\text{Gln(UUG)}}^{\text{am}}$ appears to be balanced on a knife edge of function. As reported here, tRNA$_{\text{Gln(UUG)}}^{\text{am}}$ function is critically dependent on Pus3 function at all temperatures, and as previously reported, tRNA$_{\text{Gln(UUG)}}^{\text{am}}$ and tRNA$_{\text{Gln(UUG)}}^{\text{am}}$ function in several phenotypes depends on both moieties of mcm3$^s$U (Esberg et al. 2006; Björk et al. 2007). Thus, tRNA$_{\text{Gln(UUG)}}^{\text{am}}$ may have an important regulatory role in the cell. Indeed, it is known that tRNA$_{\text{Gln(CUG)}}^{\text{am}}$ function is important for nitrogen sensing (Murray et al. 1998) and it is known that increased tRNA$_{\text{Gln(UUG)}}^{\text{am}}$ levels can suppress the nitrogen sensing defect (Kemp et al. 2013) and the lethal phenotype of tQ(CUG)Δ strains (Johanson et al. 2008). As s$^u$U levels appear regulated by temperature in wild-type cells, and yeast mutants lacking either s$^u$U or xcm$^u$U constitutively activate the GCN4 stress response (Zinshteyn and Gilbert 2013), it seems plausible that the stress response at high temperature is in part mediated by the loss of s$^u$U and reduced function of tRNA$_{\text{Gln(UUG)}}^{\text{am}}$.

**MATERIALS AND METHODS**

**Yeast strains**

Strains used for this study are derived from BY4741 and are listed in Table 9. Because PUS3 (YFL001W) is very near the centromere of chromosome VI, we replaced the first 379 nt of the gene with a bleR marker, similar to the approach previously reported (Carbone et al. 1991; Lecointe et al. 2002), using the pUG66 ble cassette with forward primer 5’-CCACATGCAATCTTTACTGCCCTACTAT.

| Strain name | Parent strain | Genotype Source |
|-------------|---------------|----------------|
| BY4741      | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 | This study |
| W303        | MATa leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,15 | This study |
| YK428-1     | BY4741 pus3Δ:ble$^R$ | This study |
| YK435-1     | BY4741 pus3Δ:ble$^R$ can1-100 ade2-1 | This study |
| YHL028-1    | YK435-1 pus3Δ:ble$^R$ his3-11,15 | This study |
| YHL029-3    | YK435-1 pus3Δ:ble$^R$ kti12Δ:kanMX can1-100 ade2-1 | This study |
| YHL030-1    | YK435-1 pus3Δ:ble$^R$ kti12Δ:kanMX his3-11,15 | This study |
| YK613-1     | BY4741 can1:PCAL1 GFPam PUS3 can1:PCAL1 GFPam | This study |
| YHL567-1    | YK613-1 can1:PCAL1 GFPam PUS3 can1:PCAL1 GFPam PUS3 | This study |
| YHL649-1    | YK613-1 can1:PCAL1 GFPam PUS3 can1:PCAL1 GFPam PUS3 ade2:2Y(GUA)am | This study |
| YHL650      | YHL567-1 can1:PCAL1 GFPam PUS3 can1:PCAL1 GFPam PUS3 ade2:2Y(GUA)am | This study |
| YHL644-1    | YK613-1 can1:PCAL1 GFPam PUS3 can1:PCAL1 GFPam PUS3 ade2:2Y(GUA)am | This study |
| YHL642-1    | YHL567-1 can1:PCAL1 GFPam PUS3 can1:PCAL1 GFPam PUS3 ade2:2Y(GUA)am | This study |
| YHL645-1    | YK613-1 can1:PCAL1 GFPam PUS3 can1:PCAL1 GFPam PUS3 ade2:2Y(GUA)am | This study |
| YHL643-1    | YHL567-1 can1:PCAL1 GFPam PUS3 can1:PCAL1 GFPam PUS3 ade2:2Y(GUA)am | This study |
| YHL686-1    | YBY4741 can1:PCAL1 GFPam PUS3 can1:PCAL1 GFPam PUS3 ade2:2Y(GUA)am | This study |
| YHL690      | YK428-1 pus3Δ:ble$^R$ can1:PCAL1 GFPam PUS3 can1:PCAL1 GFPam PUS3 ade2:2Y(GUA)am | This study |
AACCTCCCTTGACAGCTGAAGCTTGCTCTGACGC-3′ and reverse primer 5′-CAGATCCCACTAGTGGCCTATGCCCATGAAAGA GTGTAACTTGTTCTCCCTGAGGTTTAT-3′. We tested eight independent isolates for growth phenotype on different media, all of which were identical, and then transformed this strain with the [CEN URA3 PUS3] plasmid (pFEN011), which contains PUS3 DNA from −452 to +35, ending 16 bp before the outer edge of CEN VI.

All double-mutant pus3Δ strains were generated by PCR amplification of DNA from the corresponding KanMX strain in the YKO collection (Open Biosystems), followed by linear transformation of the fragment into a pus3Δ::ble R [CEN URA3 PUS3] strain.

The tQ(CUG)MΔ(CEN URA3 tQ(CUG)) strain was constructed by transformation of BY4741 with the [CEN URA3 tQ(CUG)] plasmid containing the tQ(CUG) in a cassette with the 5′ flanking DNA of tH(GUG)G2, followed by PCR amplification of the hygR marker and linear transformation to delete the tQ(CUG) gene.

The GFPam flow cytometry reporter strain YK613-1 (relevant genotype: BY4741 can1::P GAL1 GFPam α, P GAL10 RFP) was made by PCR amplification of the P GAL1 GFPam α, P GAL10 RFP DNA and its adjacent MET15 marker from pEKD1294 (Dean and Grayhack 2012) with CEN1 primers, followed by linear transformation, selection on SD-Met, screening on SD-Arg, and PCR amplification and sequencing. Then we made a pus3Δ derivative strain of the YK613-1 GFPam-reporter strain (YHL567-1) by PCR amplification of the pus3Δ::ble R cassette from strain YK428-1 and linear transformation. Then tY(GUA)am H1(CAA)am and tS(CGA)am derivatives of YK613-1 and YHL567-1 were constructed by linear transformation to integrate the Stu I fragments of plasmids (derived from pAB230-1) containing the corresponding tRNA genes and the HIS3 marker, at the ADE2 locus, resulting in the corresponding ade2::5′tH(GUG)G2::tRNAam::HIS3 strain. Three independent transformants were then tested for each tRNA gene inserted. In addition, three independent versions of strain YHL567-1 were transformed and tested with integrated tY(GUA)am, each yielding the same GFP expression within 1.9%.

### Plasmids

Plasmids used in this study are listed in Table 10. Plasmids expressing tRNAs were made either by ligation-independent cloning (LIC) of a tRNA with its own flanking sequence into the 2μ LEU2 plasmid pAVA577 or by insertion of a tRNA sequence into the Bgl II, Xho I site of a tRNA expression plasmid (pMAB813A or pW097) as previously described (Whipple et al. 2011). The same LIC cloning method was also used to construct plasmids bearing KTI12, UBA4, TRM10, and PUS3. The integrating plasmids for the wild-type tRNAs and tRNA variants were constructed by replacement of Fluc DNA with the corresponding tRNA sequence in the Bgl II, Xho I site of plasmid pAB230-1, essentially the same as described previously.

| Table 10. Plasmids used in this study |
|--------------------------------------|
| **Plasmid** | **Parent** | **Description** | **Source** |
| pMP881 | 2μ URA3 | Laboratory strain |
| pMP822 | CEN LEU2 | Laboratory strain |
| pMP824 | 2μ LEU2 | Laboratory strain |
| pEMP1744-2 | 2μ HIS3 | Laboratory strain |
| pAVA577 | 2μ LEU2 LIC vector | Alexandrov et al. (2006) |
| pMAB813A | 2μ LEU2 th(CUG) | Guy et al. (2012) |
| pAVA581 | CEN LEU2 LIC vector | Quarterly et al. (2009) |
| pAVA579 | CEN URA3 LIC vector | Quarterly et al. (2009) |
| pW097 | CEN URA3 tH(GUG)G2::tS(CGA) | Whipple et al. (2011) |
| pW132 | ade2::5′tH(GUG)G2::tS(CGA) | Whipple et al. (2011) |
| pAB230-1 pW132 | ade2::5′tH(GUG)G2::Fluc | Guy et al. (2014) |
| pMG16A pMAB813A | 2μ LEU2 tF(GAA) | Guy et al. (2012) |
| pW038 | 2μ LEU2 tS(CGA) | Whipple et al. (2011) |
| pEH101-1 | 2μ LEU2 tA(AGC) | This study |
| pEH102-1 | 2μ LEU2 t(UCC) | This study |
| pEH103-1 | 2μ LEU2 t(UCU) | This study |
| pEH104-1 | 2μ LEU2 t(CCU) | This study |
| pEH105-1 | 2μ LEU2 t(AGA) | This study |
| pEH106-1 | 2μ LEU2 t(AGU) | This study |
| pEH107-1 | 2μ LEU2 t(GUA) | This study |
| pEH112-1 | 2μ LEU2 t(CCA) | This study |
| pDL871a | 2μ LEU2 t(RCC) | This study |
| pKD9073 | 2μ LEU2 t(AGG) | This study |
| pKD9088 | 2μ LEU2 t(UGG) | This study |
| pKD9089 | 2μ LEU2 t(UAG) | This study |
| pKD9072 | 2μ LEU2 t(UUC) | This study |
| pKD9080 | 2μ LEU2 t(CC) | This study |
| pMG24A pMAB813A | 2μ LEU2 t(UAA) | Guy et al. (2012) |
| pMG18B pMAB813A | 2μ LEU2 t(CC) | Guy et al. (2012) |
| pKD9092 | 2μ LEU2 t(CGU) | This study |
| pKD9094 | 2μ LEU2 t(CUC) | This study |
| pJE1007B | 2μ LEU2 t(CC) | This study |
| pJE1008A | 2μ LEU2 t(UC) | This study |
| pJE1009B | 2μ LEU2 t(UU) | This study |
| pKD9073 | 2μ LEU2 t(UC) | This study |
| pKD9080 | 2μ LEU2 t(UU) | This study |
| pDL866a | 2μ LEU2 t(UCC) | Letzring et al. (2010) |
| pKD9080 | 2μ LEU2 t(UUU) | This study |
| pMG18B pMAB813A | 2μ LEU2 t(UAA) | Guy et al. (2012) |
| pKD9092 | 2μ LEU2 t(CU) | This study |
| pKD9094 | 2μ LEU2 t(CC) | This study |
| pJE1007B | 2μ LEU2 t(CC) | This study |
| pJE1008A | 2μ LEU2 t(UU) | This study |
| pJE1009B | 2μ LEU2 t(UU) | This study |
| pKD9073 | 2μ LEU2 t(UU) | This study |
| pKD9080 | 2μ LEU2 t(UU) | This study |
| pMG24A pMAB813A | 2μ LEU2 t(UAA) | Guy et al. (2012) |
| pMG18B pMAB813A | 2μ LEU2 t(CC) | Guy et al. (2012) |
| pKD9092 | 2μ LEU2 t(CGU) | This study |
| pKD9094 | 2μ LEU2 t(CUC) | This study |
| pJE1007B | 2μ LEU2 t(CC) | This study |
| pJE1008A | 2μ LEU2 t(UC) | This study |
| pJE1009B | 2μ LEU2 t(UU) | This study |
| pKD9073 | 2μ LEU2 t(UU) | This study |
| pKD9080 | 2μ LEU2 t(UU) | This study |
previously (Guy et al. 2014). The integrating GFP–RFP reporter plasmid pEKD1294 was derived from pEKD1024 (Dean and Grayhack 2012) by LIC cloning to insert a UAG amber stop codon at amino acid 7 of GFP, using the forward oligo 5′-AATTCCCAT CAACCTGAATTTGCTACTGGAAGTTCATAGCAGAAACGC ATCCACCA-3′ and reverse oligo 5′-CTTCCACACCTGGTGGA TGCGGTTTTGCTATTGAACTTCAGTAGACATT-3′.

**Growth of yeast strains**

Wild-type and pas3Δ strains were grown overnight in YPD or other medium at 30°C, inoculated into fresh medium for one generation, and then diluted as necessary into prewarmed media at the desired temperature, and grown at that temperature in a shaking water bath for subsequent generations as noted, prior to cell harvest and quick freezing of pellets.

**Northern blot analysis**

Bulk RNA was prepared from ~3 OD pellets using glass beads, and ~1 μg RNA was resolved by PAGE and analyzed as previously described (Alexandrov et al. 2006). For analysis of charging, RNA was prepared under acidic conditions (pH 4.5) and resolved on 6.5% acrylamide gels at pH 5 for 15 h at 4°C as described (Alexandrov et al. 2006).

**Extraction of bulk low molecular-weight RNA from yeast and purification of tRNA**

Bulk RNA was extracted from ~300 OD pellets using hot phenol, and tRNA was purified from 1.25 mg bulk RNA using 5-biotinylated oligonucleotides (Integrated DNA Technologies) complementary to the corresponding tRNA sequences, as described (Jackman et al. 2003).

**HPLC analysis of nucleosides from tRNA**

Purified tRNA (1.25 μg) was digested with 0.5 μg P1 nuclease, followed by 1 unit of calf intestinal alkaline phosphatase, and nucleosides were subjected to HPLC and quantified based on extinction coefficients, as described (Jackman et al. 2003), using parameters for mcm3U (Gray 1976) and mcm5s2U (Baczynskyj et al. 1969).

**Flow cytometry**

Strains were grown for 24 h at 28°C in S-His dropout medium containing 2% raffinose, followed by overnight growth in YP medium containing 2% galactose and 2% raffinose supplemented with 80 mg/L adenine, dilution and growth to OD ~1, followed by flow cytometry as described (Guy et al. 2014), and data analysis using FlowJo software. Cells that passed the RFP cutoff of 5 × 10^5 were used to determine a median GFP and median RFP for that sample and a calculated GFP/RFP, and biological triplicates were used to obtain an overall median GFP/RFP and a standard deviation.

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