tRNA^{His} 5-methylcytidine levels increase in response to several growth arrest conditions in *Saccharomyces cerevisiae*

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ABSTRACT

tRNAs are highly modified, each with a unique set of modifications. Several reports suggest that tRNAs are hypomodified or, in some cases, hypermodified under different growth conditions and in certain cancers. We previously demonstrated that yeast strains depleted of tRNA^{His} guanylyltransferase accumulate uncharged tRNA^{His} lacking the G₋₁ residue and subsequently accumulate additional 5-methylcytidine (m⁵C) at residues C₄₈ and C₅₀ of tRNA^{His}, due to the activity of the m⁵C-methyltransferase Trm4. We show here that the increase in tRNA^{His} m⁵C levels does not require loss of Thg1, loss of G₋₁ of tRNA^{His}, or cell death but is associated with growth arrest following different stress conditions. We find substantially increased tRNA^{His} m⁵C levels after temperature-sensitive strains are grown at nonpermissive temperature, and after wild-type strains are grown to stationary phase, starved for required amino acids, or treated with rapamycin. We observe more modest accumulations of m⁵C in tRNA^{His} after starvation for glucose and after starvation for uracil. In virtually all cases examined, the additional m⁵C on tRNA^{His} occurs while cells are fully viable, and the increase is neither due to the GCN4 pathway, nor to increased Trm4 levels. Moreover, the increased m⁵C appears specific to tRNA^{His}, as tRNA^{Val}(AAC) and tRNA^{Gly}(GCC) have much reduced additional m⁵C during these growth arrest conditions, although they also have C₄₈ and C₅₀ and are capable of having increased m⁵C levels. Thus, tRNA^{His} m⁵C levels are unusually responsive to yeast growth conditions, although the significance of this additional m⁵C remains unclear.

Keywords: tRNA(His); tRNA modifications; 5-methylcytidine; Trm4; cellular stress

INTRODUCTION

During maturation, tRNA molecules are extensively processed and highly modified with a unique set of post-transcriptional modifications. In *Saccharomyces cerevisiae*, ~13 nt are modified in each tRNA, and there are 25 unique tRNA modifications that have been found at 36 positions on the tRNA (Phizicky and Hopper 2010). These post-transcriptional modifications are highly conserved in different organisms, underscoring their importance. Many tRNA modifications near the anti-codon have important roles in decoding mRNA (Agris et al. 2007), as evidenced by lethality or slow growth phenotypes (Phizicky and Hopper 2010). For example, loss of 2'-O-methylation at positions 32 and 34 or loss of either m¹G or t⁶A at position 37 results in slow growth (Bjork et al. 2001; Pintard et al. 2002; El Yacoubi et al. 2009; Guy et al. 2012). In addition, a number of modifications in the tRNA body have important roles in stabilizing tRNA

(Kadaba et al. 2004; Alexandrov et al. 2006; Kadaba et al. 2006; Chernyakov et al. 2008b; Phizicky and Hopper 2010; Whipple et al. 2011). However, the precise roles of many modifications are not yet known in detail and are still under investigation.

Although it has been generally assumed that modifications made to tRNAs are constitutively added at similar levels, a number of reports have described altered tRNA modification levels under various conditions in different organisms. *Bacillus subtilis* tRNAs were reported to be hypomethylated during log phase growth (Singhal and Vold 1976), and *Bacillus stearothermophilus* tRNAs have higher 2'-O-methylation levels at 70°C than at 50°C (Agris et al. 1973). Similarly, several types of cancer cell lines have reduced yW (wybutosine) modification of tRNA^{Phe} (Grunberger et al. 1975; Mushinski and Marini 1979, 1983; Kuchino et al. 1982; Grunberger et al. 1983), and tRNA^{Phe} species from a hepatoma and a breast carcinoma have an unexpected additional 1-methylguanosine (m¹G) modification, as well as additional dihydrouridine and 5-methylcytidine (m⁵C) (Kuchino and Borek 1978). In addition, human and murine hepatoma cells have reduced levels of queosine in their tRNA^{Asp} (Kuchino et al. 1981; Randerath et al. 1984; Pathak et al. 2005), while in *Drosophila*, queosine levels of several tRNAs increase with

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age and with the percentage of yeast in the diet (Hosbach and Kubli 1979; Owenby et al. 1979).

Emerging data also suggest growth-dependent changes in tRNA modification levels in the yeast *Saccharomyces cerevisiae*. Mitochondrial tRNA^{Lys(UUU)} has reduced 2-thiolation following growth at elevated temperature (Kamenski et al. 2007). In addition, it was recently reported that oxidative stress mediated by hydrogen peroxide results in increased levels of m⁵C, 2'-O-methylcytidine (Cm), and 2,2-dimethylguanosine (m²G) levels and that a number of other modifications are affected by treatment with several chemicals that induce cellular stress (Chan et al. 2010, 2012).

We previously reported that depletion of the essential tRNA^{His} guanylyltransferase Thg1 results in the loss of G₋₁ from tRNA^{His}, with concomitant accumulation of deacylated tRNA^{His} and the subsequent accumulation of additional m⁵C on tRNA^{His} ~8 h after the loss of G₋₁ (Gu et al. 2005). The additional m⁵C occurs at residues C₄₈ and C₅₀ (~0.5 mol/mol tRNA at each position) (Gu et al. 2005), adjacent to the known m⁵C₄₉ modification, and appears to be due to Trm4 methyltransferase, which catalyzes formation of m⁵C at C₃₄, C₄₀, C₄₈, and C₄₉ in all substrate tRNAs in yeast (Motorin and Grosjean 1999). Interestingly, the additional m⁵C that accumulates following Thg1 depletion was not found on tRNA^{Gly(GCC)}, which also has cytidines at these positions, and tRNA^{His} was observed to accumulate in the nucleus during Thg1 depletion (Gu et al. 2005), presumably by retrograde transport of cytoplasmic tRNA to the nucleus (Shaheen and Hopper 2005; Takano et al. 2005). At the time, we speculated that the additional m⁵C found in tRNA^{His} from Thg1-depleted cells might be due to the increased availability of deacylated tRNA^{His} to Trm4, which is localized to the nucleus (Wu et al. 1998), or possibly due to a regulatory response from the uncharged tRNA^{His} as the cells arrested growth due to loss of Thg1 (Gu et al. 2005). Alternatively, the lack of the G₋₁ modification on tRNA^{His} might trigger the accumulation of m⁵C, much as *Thermus thermophilus* cells lacking either m⁷G₄₆ or ψ ₅₅ have altered modifications under certain conditions (Tomikawa et al. 2010; Ishida et al. 2011).

In this work, we explore the conditions in which tRNA^{His} can be modified with additional m⁵C. We show that m⁵C accumulates in tRNA^{His} under a variety of conditions in which growth is arrested and that this accumulation is not associated with cell death. Although several conditions examined involve nutrient deprivation, the *GCN4* pathway is

not responsible for the additional m⁵C. However, the target of rapamycin (TOR) pathway may play a role in the m⁵C response, since rapamycin treatment results in an increase in tRNA^{His} m⁵C levels. Remarkably, we also show that this additional m⁵C is specific to tRNA^{His}, relative to two other tRNAs with cytidine residues at the same positions, which are capable of being overmodified with m⁵C in vivo. We conclude that m⁵C modification of tRNA^{His} is unusually sensitive to yeast growth conditions, although the cellular function of this phenomenon remains unclear.

RESULTS

m⁵C levels are increased in tRNA^{His} from *thg1*^{ts} strains but not from a *trm4*- Δ strain

Our initial goal was to explore the cause of additional m⁵C on tRNA^{His} observed following depletion of Thg1 (Gu et al. 2005), which adds G₋₁ to the 5' end of tRNA^{His} (Gu et al. 2003). The secondary structure and modifications of tRNA^{His} are depicted in Figure 1A. The m⁵C modification (catalyzed by Trm4) is clearly resolved from other nucleosides by reverse-phase HPLC analysis of tRNA^{His} purified from wild-type and *trm4*- Δ strains (Fig. 1B). Values of nucleoside modifications are quantified from HPLC traces and are expressed as moles of modification per mole of tRNA (mol/mol tRNA).

FIGURE 1. Post-transcriptional modifications of mature tRNA^{His}. (A) Secondary structure of mature *S. cerevisiae* tRNA^{His}. Post-transcriptional modifications are indicated. G₋₁ is added to the 5' end of tRNA^{His} by tRNA^{His} guanylyltransferase (Thg1). Under normal growth conditions, Trm4 methylates position 49 to form 5-methylcytidine (m⁵C, circle). When Thg1 is depleted, Trm4 also methylates adjacent cytidines at positions 48 and 50 (squares). (B) Representative HPLC traces of tRNA^{His} nucleosides. Wild-type (BY4741) and *trm4*- Δ cells were grown to log phase in SD complete media, and tRNA^{His} was purified, digested with P1 nuclease, phosphatase-treated, and separated by reverse-phase HPLC. Insets compare the wild type vs. *trm4*- Δ m⁵C peak (left), with the Am peak (right) as a control.

Based on our previously published data, it seemed plausible that increased levels of m^5C upon Thg1 depletion might be due to the loss of the essential Thg1 protein, the consequent loss of the $tRNA^{His}$ G_{-1} residue, and the accumulation of uncharged $tRNA^{His}$ (Gu et al. 2005). To test this, we examined a *thg1*- Δ strain that was viable due to overexpression of $tRNA^{His}$ and the histidyl-tRNA synthetase *HTS1* (Preston and Phizicky 2010). We find that this strain has normal levels of m^5C on $tRNA^{His}$ relative to wild type (Table 1, WT). Since this *thg1*- Δ strain also has approximately 15-fold more $tRNA^{His}$ than a wild-type strain and the $tRNA^{His}$ is mostly deacylated (Preston and Phizicky 2010), the availability of uncharged $tRNA^{His}$ alone is likely not the cause of additional m^5C levels. In support of this conclusion, increasing the amount of available $tRNA^{His}$ by overexpression of $tRNA^{His}$ in a wild-type strain has no effect on $tRNA^{His}$ m^5C levels (Table 1). Therefore, it is possible that the addition of m^5C to $tRNA^{His}$ is, instead, triggered by growth arrest due to loss of Thg1 function.

To explore the connection between Thg1 depletion-mediated growth arrest and elevated $tRNA^{His}$ m^5C levels, we measured m^5C levels in $tRNA^{His}$ purified from three different *thg1* temperature-sensitive mutants (Y146H, G172D/L233S, and Y8C) before and after shift to 37°C (Table 1). Growth arrest was apparent by 3 h for the *thg1*-Y146H strain and by 4–5 h for the *thg1*-G172D/L233S and *thg1*-Y8C strains (data not shown). Whereas $tRNA^{His}$ from the wild-type strain and each of the *thg1* mutants have near normal amounts of m^5C when grown at 24°C (0.92–1.25 mol/mol $tRNA$), $tRNA^{His}$ m^5C levels increase dramatically to 2.36–2.41 mol/mol $tRNA$ when *thg1* mutants are shifted to 37°C for 7 h. $tRNA^{His}$ from the wild-type strain has normal levels of m^5C after the temperature shift, and levels of control modifications (dihydrouridine (D), pseudouridine (ψ), m^1G , and

Am) remain unchanged in $tRNA^{His}$ isolated from each strain (Table 1; data not shown).

$tRNA^{His}$ m^5C levels increase when temperature-sensitive strains are grown at nonpermissive temperature

Based on the data above, the increase in m^5C levels could be correlated with lack of growth or with cell death, associated with loss of Thg1 function. To determine if an increase in $tRNA^{His}$ m^5C levels is specific to loss of Thg1 function and/or subsequent cell death, we grew a set of temperature-sensitive (ts) strains at permissive or nonpermissive temperatures and analyzed cell viability. We then purified $tRNA^{His}$ and measured modification levels by HPLC analysis. We grew BY4741 (WT) and the *thg1*-Y146H^{ts} strain as a control for additional m^5C . We also grew three temperature-sensitive strains unrelated to tRNA processing: the *fcp1*-1^{ts} strain, which is defective for transcription by RNA polymerase II at nonpermissive temperature (Kobor et al. 1999), the *abf1*-102^{ts} strain, which has a mutation in a DNA binding protein involved in transcriptional regulation, DNA replication, and DNA repair (Buchman et al. 1988; Reed et al. 1999; Miyake et al. 2004), and the *cdc48*-9^{ts} strain, which has a mutation in an ATPase involved in protein export from the ER to the cytoplasm (Ye et al. 2001). Growth arrest was apparent by 2–3 h for the *fcp1*-1^{ts} strain, by 3–4 h for the *thg1*-Y146H^{ts} strain, by 6–7 h for the *cdc48*-9^{ts} strain, and by over 7 h for the *abf1*-102^{ts} strain (data not shown).

For two of the temperature-sensitive strains grown at 37°C, we observe an increase in m^5C levels on $tRNA^{His}$ following growth arrest and without any significant loss of cell viability (Fig. 2; Table 2). Thus, at the 7 h time point at 37°C, the *fcp1*-1^{ts} and *cdc48*-9^{ts} strains are nearly completely viable

TABLE 1. $tRNA^{His}$ modification levels in conditions of reduced Thg1 activity

Strain	2 μ plasmid	Temp. (°C)	ψ	m^5C
<i>thg1</i> - Δ [<i>HTS1</i>]	$tRNA^{His}$	30 ^a	3.03 $\pm$ 0.06	0.99 $\pm$ 0.04
BY4741 (WT)	—	30 ^b	2.87 $\pm$ 0.08	0.94 $\pm$ 0.04
BY4741 (WT)	$tRNA^{His}$	30 ^a	3.00 $\pm$ 0.03	1.05 $\pm$ 0.01
	Vector	30 ^b	2.90 $\pm$ 0.09	0.95 $\pm$ 0.08
<i>THG1</i> (WT)	—	24 ^a	2.91 $\pm$ 0.08	0.92 $\pm$ 0.03
	—	37 ^a	2.97 $\pm$ 0.10	1.12 $\pm$ 0.02
<i>thg1</i> -Y146H	—	24 ^c	2.95 $\pm$ 0.11	0.98 $\pm$ 0.06
	—	37 ^c	3.11 $\pm$ 0.04	2.40 $\pm$ 0.12 ^d
<i>thg1</i> -G172D/L233S	—	24	2.94	0.99
	—	37	3.08	2.41
<i>thg1</i> -Y8C	—	24	3.86	1.25
	—	37	3.24	2.36
Expected mol/mol $tRNA$			3	1

^a $n = 4$, mean $\pm$ standard deviation.

^b $n = 5$, mean $\pm$ standard deviation.

^c $n = 3$, mean $\pm$ standard deviation.

^d $tRNA^{His}$ m^5C from *thg1*-Y146H 24°C vs. 37°C: Student's *t*-test, $P = 2.1 \times 10^{-4}$.

FIGURE 2. Temperature-sensitive strains have additional m^5C on $tRNA^{His}$ when grown at non-permissive temperature. (A) Assessment of viable cell titer following growth at $37^\circ C$. (B) m^5C levels in $tRNA^{His}$ and $tRNA^{Val(AAC)}$ during growth at $37^\circ C$. $tRNA^{His}$ and $tRNA^{Val(AAC)}$ were purified from BY4741, *thg1-Y146H*, and *fcp1-1* strains, and modification levels were measured by HPLC. m^5C levels for $tRNA^{His}$ (black) and $tRNA^{Val(AAC)}$ (gray) during temperature-sensitive strain growth at $37^\circ C$ were plotted. m^5C values during growth at $24^\circ C$ were plotted as the $37^\circ C$ 0-h time point.

(Fig. 2A; Table 2; data not shown) and their $tRNA^{His}$ have 1.75 and 1.51 $mol\ m^5C/mol\ tRNA$, respectively, compared to 0.92 and 0.93 $mol/mol\ tRNA$ at $24^\circ C$ (Fig. 2B; Table 2; data not shown). At the same time point after shift to $37^\circ C$, $tRNA^{His}$ m^5C levels increase only slightly in the wild-type strain (from 0.92 to 1.12 $mol/mol\ tRNA$) and in the *abf1-102^{ts}* mutant (from 0.89 to 1.18 $mol/mol\ tRNA$) (data not shown). We ruled out the possibility that the increased $tRNA^{His}$ m^5C levels in the *fcp1-1^{ts}* strain were somehow due to the absence of the G_{-1} residue of $tRNA^{His}$ by direct exam-

ination of the 5' end of $tRNA^{His}$. As expected, there was no change in the G_{-1} status in *fcp1-1^{ts}* and WT strains and a marked reduction in G_{-1} of $tRNA^{His}$ from the *thg1-Y146H^{ts}* grown at $37^\circ C$ (data not shown). Thus, the additional m^5C on $tRNA^{His}$ is unrelated to the G_{-1} addition activity of Thg1, and we conclude that growth arrest, but not cell death, of temperature-sensitive strains results in additional m^5C on $tRNA^{His}$.

We also find evidence that the increased amount of m^5C is specific to $tRNA^{His}$. $tRNA^{Val(AAC)}$, which also normally has unmodified C_{48} and C_{50} residues adjacent to m^5C_{49} , has only marginally increased levels of m^5C 7 h after temperature shift in the *fcp1-1^{ts}* mutant (from 0.81 to 1.03 mol/mol

$tRNA$) and in the *thg1-Y146H^{ts}* mutant (from 0.82 to 1.01 $mol/mol\ tRNA$) (Fig. 2B; Table 2).

$tRNA^{His}$ m^5C levels increase in several conditions in which a wild-type strain arrests growth and is still viable

Since the accumulation of m^5C in $tRNA^{His}$ occurs in temperature-sensitive strains following growth arrest, we reasoned that wild-type strains might also have elevated m^5C levels in conditions where the cells stop growing. We, therefore,

TABLE 2. Temperature-sensitive strains grown at nonpermissive temperature have additional m^5C on $tRNA^{His}$

Strain	Temp. ($^\circ C$)	Time (h)	$tRNA^{His}$				$tRNA^{Val(AAC)}$				Viable cells/mL ^B
			ψ	m^5C	Gm + m^1G	Am	ψ	m^5C	I	m^1G	
BY4741	24	6 ^a	2.91 $\pm$ 0.08	0.92 $\pm$ 0.03	2.04 $\pm$ 0.04	0.93 $\pm$ 0.04	3.85	0.81	1.19	1.12	9.8 $\times$ 10 ⁶
	37	4	2.97	1.06	2.10	0.94	3.81	0.91	1.25	1.10	4.6 $\times$ 10 ⁷
	37	7 ^a	2.97 $\pm$ 0.10	1.12 $\pm$ 0.02	2.06 $\pm$ 0.08	0.93 $\pm$ 0.02	3.83	0.94	1.10	1.12	7.7 $\times$ 10 ⁷
	37	23 ^b	2.97 $\pm$ 0.01	1.22 $\pm$ 0.06	2.13 $\pm$ 0.02	0.95 $\pm$ 0.02	3.86	0.97	1.30	1.15	8.8 $\times$ 10 ⁷
<i>fcp1-1</i>	24	6 ^c	2.87 $\pm$ 0.02	0.92 $\pm$ 0.03	2.07 $\pm$ 0.02	0.93 $\pm$ 0.03	3.79	0.81	1.14	1.14	6.3 $\times$ 10 ⁶
	37	4	3.07	1.55	2.18	0.94	3.79	0.96	1.09	1.11	3.2 $\times$ 10 ⁶
	37	7 ^c	2.97 $\pm$ 0.09	1.75 $\pm$ 0.04 ^d	2.17 $\pm$ 0.04	0.96 $\pm$ 0.03	3.76	1.03	1.10	1.13	9.7 $\times$ 10 ⁶
	37	23 ^b	2.94 $\pm$ 0.02	2.12 $\pm$ 0.02 ^e	2.12 $\pm$ 0.02	0.88 $\pm$ 0.00	3.90	1.24	1.08	1.10	8.4 $\times$ 10 ⁵
<i>thg1-Y146H</i>	24	6 ^c	2.95 $\pm$ 0.11	0.98 $\pm$ 0.06	1.89 $\pm$ 0.02	0.94 $\pm$ 0.04	3.82	0.82	1.18	1.11	1.4 $\times$ 10 ⁷
	37	4	3.03	1.96	1.79	0.97	3.82	0.94	1.17	1.13	1.9 $\times$ 10 ⁷
	37	7 ^c	3.11 $\pm$ 0.04	2.40 $\pm$ 0.12 ^f	1.95 $\pm$ 0.08	1.02 $\pm$ 0.01	3.78	1.01	1.13	1.14	2.3 $\times$ 10 ⁷
	37	23	2.91	2.65	2.13	0.98	3.81	1.20	1.22	1.13	2.6 $\times$ 10 ⁶
Expected $mol/mol\ tRNA$			3	1	2	1	4	1	1	1	

^a $n = 4$, mean $\pm$ standard deviation.

^b $n = 2$, mean $\pm$ standard deviation.

^c $n = 3$, mean $\pm$ standard deviation.

^d $tRNA^{His}$ m^5C , *fcp1-1* $24^\circ C$ vs. $37^\circ C$ 7 h: Student's *t*-test, $P = 5.4 \times 10^{-6}$.

^e $tRNA^{His}$ m^5C , *fcp1-1* $24^\circ C$ vs. $37^\circ C$ 23 h: Student's *t*-test, $P = 7.0 \times 10^{-6}$.

^f $tRNA^{His}$ m^5C , *thg1-Y146H* $24^\circ C$ vs. $37^\circ C$ 7 h: Student's *t*-test, $P = 2.1 \times 10^{-4}$.

^BViable cells per mL from a representative experiment.

examined modification status of $tRNA^{His}$ in the BY4741 strain after growth into stationary phase and after nutrient starvation.

As BY4741 is grown in SD complete medium, we find that $tRNA^{His}$ m^5C levels increase from 0.92 mol/mol $tRNA$ in mid-log phase growth to 1.55 mol/mol $tRNA$ in the first day of stationary phase and then gradually increase to 1.81 mol/mol $tRNA$ over the next 5 d of stationary phase (Fig. 3, black bars). In contrast, during this time course, the control modifications (Am, Gm+ m^1G , D, and ψ) remain virtually unchanged in the $tRNA^{His}$ (Fig. 3, light and dark gray bars; data not shown). The increase in m^5C seems to correlate with the diauxic shift, which transitions energy production from fermentation to respiration when glucose levels are low (Herman 2002). Accumulation of m^5C is not likely due to cell death since cells in stationary phase are known to be viable (Werner-Washburne et al. 1996; Allen et al. 2006).

We also find that levels of $tRNA^{His}$ m^5C increase following several nutrient starvation treatments of the BY4741 strain, in each case with no significant loss of cell viability (Table 3). We find that m^5C levels increase significantly following 6 h of starvation for histidine (from 0.86 to 1.80 mol/mol $tRNA$) and following 24 h of starvation for histidine (to 2.00 mol/mol $tRNA$), for leucine (to 1.66 mol/mol $tRNA$), and for a combination of amino acids and uracil (SD minimal; to 1.68 mol/mol $tRNA$). m^5C levels also significantly increase to 1.53 mol/mol $tRNA$ following 24 h of growth in SD complete medium, because the cells had reached saturation. In contrast, we observe a more modest increase in m^5C levels following 24 h of starvation for glucose (to 1.32 mol/mol $tRNA$), and a minimal increase following 24 h of starvation for uracil (to 1.23 mol/mol $tRNA$). The BY4741 strain viable cell titer remained

constant during all of these treatments (Table 3), although cell growth ceased by ~ 3 h in each case (data not shown).

It is likely that Trm4 is responsible for the additional m^5C modifications observed in $tRNA^{His}$ under these conditions, for two reasons. First, Trm4 is the only known *S. cerevisiae* m^5C methyltransferase and can catalyze m^5C formation on substrate tRNAs at C_{34} , C_{40} , C_{48} , and C_{49} (Motorin and Grosjean 1999). Second, *trm4*- Δ mutants depleted of Thg1 lack all m^5C , including the additional m^5C found in $tRNA^{His}$ at C_{48} and C_{50} (Gu et al. 2005). Consistent with this, we find that deletion of *TRM4* abolishes the accumulation of m^5C in $tRNA^{His}$ that occurs upon histidine starvation of BY4741 (Table 3), suggesting that Trm4 catalyzes formation of the additional m^5C on $tRNA^{His}$ during starvation conditions.

The GCN4 pathway is not responsible for additional m^5C levels on $tRNA^{His}$

Since we observed significant increases in m^5C on $tRNA^{His}$ after starving BY4741 for amino acids and only a marginal increase following starvation for uracil, we reasoned that additional m^5C might result from activation of the general amino acid starvation pathway. Indeed, we had previously shown that depletion of Thg1 leads to activation of the GCN4 pathway, in addition to the accumulation of m^5C on $tRNA^{His}$ (Gu et al. 2005). Thus, as expected, our amino acid starvation conditions lead to increased levels of both *HIS5* and *LYS1* mRNAs (Table 3; data not shown), which are known to be under GCN4 control (Natarajan et al. 2001).

Two lines of evidence suggest that the increased m^5C levels observed in $tRNA^{His}$ during starvation are not due to the GCN4 pathway. First, we find nearly identical levels of elevated m^5C in $tRNA^{His}$ after 5 h of histidine starvation of wild-type (0.86 to 1.71 mol/mol $tRNA$) and *gcn4*- Δ strains (0.83 to 1.65 mol/mol $tRNA$) (Table 4), while control modifications (D, ψ , Gm, m^1G , and Am) are virtually unchanged (Table 4; data not shown). Second, induction of the GCN4 pathway by overexpression of GCN4 from the P_{GAL} promoter, using the yeast movable ORF (MORF) collection (Gelperin et al. 2005), does not affect m^5C levels of $tRNA^{His}$ (Table 5). Accordingly, although overexpression of GCN4-MORF in galactose dramatically increases mRNA levels of the known Gcn4 targets *HIS5* and *LYS1* relative to levels observed in glucose-containing media, there is only a very limited effect on m^5C levels of $tRNA^{His}$ (from 0.89 to 0.98 mol/mol $tRNA$). Similarly, there is no effect on m^5C levels of $tRNA^{His}$ when the vector control strain is grown in galactose (from 0.87 to 0.89 mol/mol $tRNA$) or when a control MORF construct (*ERV25*-MORF) is expressed in galactose (from 0.86 to 0.97 mol/mol $tRNA$). However, we note that our growth conditions do allow for an increase in $tRNA^{His}$ m^5C , since overexpression of *TRM4*-MORF increases $tRNA^{His}$ m^5C levels to 1.59 mol/mol $tRNA$ (Table 5). Thus, we conclude that $tRNA^{His}$ m^5C levels are not the result of GCN4 pathway induction.

FIGURE 3. $tRNA^{His}$ m^5C levels increase when BY4741 is grown to stationary phase. $tRNA^{His}$ was purified from BY4741 grown in SD complete media in mid-log phase and in stationary phase for the indicated number of days. Nucleoside modifications were analyzed by HPLC, and m^5C (black), Am (dark gray), and m^1G + Gm (light gray) values are plotted over time. Other control modifications (dihydrouridine and pseudouridine) were unchanged during the time course (data not shown). The increase in m^5C levels between log phase growth and Day 1 of stationary phase is highly reproducible (Day 0 log phase, $m^5C = 0.86 \pm 0.03$, $n = 6$; Day 1 stationary phase, $m^5C = 1.53 \pm 0.11$, $n = 7$, mean $\pm$ standard deviation) (see Table 3).

TABLE 3. tRNA^{His} m⁵C levels increase following starvation for amino acids or glucose

Media	Time (h)	BY4741						trm4-Δ	W303/ CRY1	TRM4- cMORF
		ψ	m ⁵ C	Gm + m ¹ G	Am	Viable cells/ mL ^P	<i>HIS5</i> / <i>ACT1</i> ^Q	m ⁵ C	m ⁵ C	m ⁵ C
SD complete	0 ^{a,b}	2.82 ± 0.04	0.86 ± 0.03	2.05 ± 0.02	0.87 ± 0.02	1.5 × 10 ⁶	1.0	0.02	0.73	0.96
	6 ^c	2.86 ± 0.06	0.93 ± 0.11	2.08 ± 0.05	0.90 ± 0.04	1.8 × 10 ⁷	0.3	0.02	0.73	0.85
	24 ^d	2.91 ± 0.06	1.53 ± 0.11 ^g	2.15 ± 0.02	0.91 ± 0.04	3.8 × 10 ⁷	ND	0.01	1.47	1.38
SD minimal	0 ^a	2.82 ± 0.04	0.86 ± 0.03	2.05 ± 0.02	0.87 ± 0.02	5.8 × 10 ⁶	ND	ND	ND	ND
	6 ^e	2.92 ± 0.09	1.31 ± 0.12 ^h	2.11 ± 0.09	0.90 ± 0.06	5.5 × 10 ⁶	27.1	ND	ND	ND
	24 ^e	2.95 ± 0.07	1.68 ± 0.05 ⁱ	2.14 ± 0.05	0.92 ± 0.03	4.2 × 10 ⁶	ND	ND	ND	ND
SD – His	0 ^a	2.82 ± 0.04	0.86 ± 0.03	2.05 ± 0.02	0.87 ± 0.02	6.0 × 10 ⁶	ND	0.02	0.73	0.96
	6 ^b	2.97 ± 0.07	1.80 ± 0.08 ^j	2.12 ± 0.01	0.94 ± 0.03	6.6 × 10 ⁶	55.9	0.03	1.83	1.71
	24 ^d	2.96 ± 0.06	2.00 ± 0.13 ^k	2.13 ± 0.04	0.92 ± 0.03	4.9 × 10 ⁶	ND	0.02	1.94	1.94
SD – Leu	0 ^a	2.82 ± 0.04	0.86 ± 0.03	2.05 ± 0.02	0.87 ± 0.02	5.6 × 10 ⁶	ND	ND	ND	0.96
	6 ^e	2.94 ± 0.07	1.33 ± 0.07 ^l	2.16 ± 0.02	0.92 ± 0.03	5.9 × 10 ⁶	22.2	ND	ND	1.30
	24 ^e	2.92 ± 0.10	1.66 ± 0.04 ^m	2.19 ± 0.00	0.91 ± 0.03	4.5 × 10 ⁶	ND	ND	ND	1.57
SD – Ura	0 ^a	2.82 ± 0.04	0.86 ± 0.03	2.05 ± 0.02	0.87 ± 0.02	5.7 × 10 ⁶	ND	ND	ND	ND
	6 ^f	2.91 ± 0.10	1.16 ± 0.09 ⁿ	2.15 ± 0.02	0.92 ± 0.04	4.9 × 10 ⁶	0.9	ND	ND	ND
	24 ^f	2.89 ± 0.08	1.23 ± 0.08 ^o	2.17 ± 0.02	0.92 ± 0.03	5.5 × 10 ⁶	ND	ND	ND	ND
S complete no glucose	0 ^a	2.82 ± 0.04	0.86 ± 0.03	2.05 ± 0.02	0.87 ± 0.02	5.7 × 10 ⁶	ND	ND	ND	ND
	6	2.87	1.16	2.17	0.90	6.1 × 10 ⁶	ND	ND	ND	ND
	24	2.85	1.32	2.14	0.89	5.6 × 10 ⁶	ND	ND	ND	ND
Expected mol/mol tRNA		3	1	2	1			1	1	1

(ND) Not determined.

^aFor each strain, one starting culture was split into the indicated media. Thus, each media condition has the same modification values at 0 h.^b*n* = 6, mean ± standard deviation.^c*n* = 5, mean ± standard deviation.^d*n* = 7, mean ± standard deviation.^e*n* = 4, mean ± standard deviation.^f*n* = 3, mean ± standard deviation.^{g-o}Student's *t*-test *P* values compare tRNA^{His} m⁵C levels between each growth condition and SD complete 0h. SD complete 24 h: ^g*P* = 8.0 × 10⁻⁷; SD minimal 6 h: ^h*P* = 2.3 × 10⁻³; 24 h: ⁱ*P* = 5.0 × 10⁻⁶; SD – His 6 h: ^j*P* = 6.6 × 10⁻⁸; 24 h: ^k*P* = 7.6 × 10⁻⁸; SD – Leu 6 h: ^l*P* = 2.3 × 10⁻⁴; 24 h: ^m*P* = 1.1 × 10⁻⁷; SD – Ura 6 h: ⁿ*P* = 1.4 × 10⁻²; 24 h: ^o*P* = 6.1 × 10⁻³.^PViable cells per mL from a representative experiment.^QNormalized to *HIS5/ACT1* values of SD complete 0 h.

tRNA^{His} m⁵C levels increase during prolonged inhibition of the target of rapamycin pathway

We also tested the role of the TOR pathway in the induction of additional m⁵C. The TOR pathway is another mechanism by which yeast respond to nutrient deprivation. In yeast, Tor1 and Tor2 can form two distinct multiprotein complexes:

TORC1 (containing either Tor1 or Tor2), which is inhibited by rapamycin; and TORC2 (containing Tor2), which is insensitive to rapamycin (Loewith et al. 2002). To inhibit TORC1, we sought to treat cells with rapamycin, but BY4741 is relatively insensitive to rapamycin treatment when *TOR1* is present (data not shown). Therefore, we used the W303/CRY1 strain, after first showing that BY4741 and W303/CRY1 had virtually

TABLE 4. $tRNA^{His}$ m^5C levels during inhibition of starvation pathways

Strain	Media	Time (h)	ψ	m ⁵ C	Gm + m ¹ G	Viable cells/mL ^e
BY4741	SD complete	0 ^{a,b}	2.83 ± 0.05	0.86 ± 0.03	2.04 ± 0.03	2.5 × 10 ⁶
		3	ND	ND	ND	8.8 × 10 ⁶
		5 ^b	2.85 ± 0.02	0.90 ± 0.04	2.04 ± 0.04	2.3 × 10 ⁷
	SD – His	0 ^{a,b}	2.83 ± 0.05	0.86 ± 0.03	2.04 ± 0.03	6.4 × 10 ⁶
		3	ND	ND	ND	8.3 × 10 ⁶
		5 ^b	2.98 ± 0.04	1.71 ± 0.05 ^c	2.05 ± 0.04	1.1 × 10 ⁷
<i>gcn4-Δ</i>	SD complete	0 ^{a,b}	2.84 ± 0.05	0.83 ± 0.05	2.06 ± 0.02	1.9 × 10 ⁶
		3	ND	ND	ND	9.0 × 10 ⁶
		5 ^b	2.85 ± 0.06	0.83 ± 0.02	2.10 ± 0.05	2.2 × 10 ⁷
	SD – His	0 ^{a,b}	2.84 ± 0.05	0.83 ± 0.05	2.06 ± 0.02	5.0 × 10 ⁶
		3	ND	ND	ND	6.8 × 10 ⁶
		5 ^b	2.91 ± 0.04	1.65 ± 0.10 ^d	2.01 ± 0.06	8.7 × 10 ⁷
W303/CRY1	YPD 0 nM Rapamycin	0	2.89	0.84	2.11	2.5 × 10 ⁵
		4.5	3.03	1.02	2.15	3.0 × 10 ⁶
	YPD 100 nM Rapamycin	0	2.89	0.84	2.11	2.0 × 10 ⁶
		4.5	2.91	1.39	2.16	1.9 × 10 ⁶
Expected mol/mol tRNA			3	1	2	

(ND) Not determined.

^aFor each strain, one starting culture was split into the indicated media. Thus, each media condition has the same modification values at 0 h.^b $n = 3$, mean ± standard deviation.^c $tRNA^{His}$ m^5C , BY4741 SD complete 0 h vs. SD – His 5 h: Student's t -test, $P = 2.8 \times 10^{-5}$.^d $tRNA^{His}$ m^5C , *gcn4-Δ* SD complete 0 h vs. SD – His 5 h: Student's t -test, $P = 5.6 \times 10^{-4}$.^eViable cells per mL from a representative experiment.

identical increases in $tRNA^{His}$ m^5C levels after histidine starvation, while all other modifications (D, ψ , Gm, m^1G , and Am) were unaffected by starvation (Table 3; data not shown). Growth arrest triggered by rapamycin treatment (50, 100, and 150 nM) was apparent by 1.5–2 h, and we observed significant decreases in cell viability after treatment with 100 nM and 150 nM rapamycin for longer than 4.5 h (data not shown). However, we did observe increases in m^5C levels following several treatments with rapamycin that did not lead to loss of viability. We detected a modest increase in $tRNA^{His}$ m^5C levels when cells were treated with 150 nM rapamycin for 3

h (from 0.89 ± 0.01 to 1.18 ± 0.03 mol/mol tRNA, mean ± standard deviation; $n = 2$) (data not shown) and greater increases when cells were treated with 100 nM rapamycin for 4.5 h (from 0.84 to 1.39 mol/mol tRNA) (Table 4) or with 50 nM rapamycin for 24 h (from 0.84 to 1.73 mol/mol tRNA) (data not shown), without loss of viability. In each of these cases, we observe no change in all other $tRNA^{His}$ modifications (Table 4; data not shown). These data suggest that inhibition of the TOR pathway or the resulting G1 cell cycle arrest (Kunz et al. 1993; Barbet et al. 1996; Helliwell et al. 1998) can trigger additional m^5C levels on $tRNA^{His}$.

TABLE 5. Quantification of tRNA nucleoside modifications following *GCN4* and *TRM4* overexpression

BY4741 + MORF plasmid	S – Ura Media	<i>HIS5/ACT1</i> ^a	<i>LYS1/ACT1</i> ^b	$tRNA^{His}$				$tRNA^{Val(AAC)}$			$tRNA^{Gly(GCC)}$		
				ψ	m^5C	Gm + m^1G	Am	ψ	m^5C	m^1G	ψ	m^5C	m^1G
<i>P_{GAL} GCN4</i>	Raff Gal	39.2 ^a	51.7 ^a	2.86	0.98	2.17	0.91	ND	ND	ND	ND	ND	ND
	Glucose	1.2 ^b	1.3 ^b	2.83	0.89	2.11	0.90	ND	ND	ND	ND	ND	ND
<i>P_{GAL} TRM4</i>	Raff Gal	ND	ND	2.84	1.59	2.12	0.92	4.13	1.53	1.13	3.88	1.98	1.05
	Glucose	ND	ND	2.85	0.86	2.08	0.88	4.04	0.80	1.00	3.90	1.56	1.04
<i>P_{GAL} ERV25</i>	Raff Gal	ND	ND	2.78	0.97	2.14	0.88	ND	ND	ND	ND	ND	ND
	Glucose	ND	ND	2.87	0.86	2.09	0.89	ND	ND	ND	ND	ND	ND
BG1766 (vector)	Raff Gal	1.0 ^a	1.0 ^a	2.83	0.89	2.12	0.88	3.70	0.87	1.10	3.96	1.69	1.08
	Glucose	1.0 ^b	1.0 ^b	2.80	0.87	2.07	0.87	4.18	0.96	1.08	3.81	1.54	1.02
Expected mol/mol tRNA				3	1	2	1	4	1	1	4	1	1

(ND) Not determined.

^aNormalized to *HIS5/ACT1* or *LYS1/ACT1* values of BG1766 in S – Ura + Raffinose + Galactose media.^bNormalized to *HIS5/ACT1* or *LYS1/ACT1* values of BG1766 in S – Ura + Glucose media.

Trm4 protein levels do not increase during starvation conditions

A simple explanation for the increase in tRNA^{His} m⁵C levels is that Trm4 is more highly expressed during starvation conditions. To monitor endogenous Trm4 levels during starvation, we first inserted a chromosomal affinity tag derived from the MORF library (Gelperin et al. 2005) immediately 5' of the *TRM4* stop codon to encode a Trm4-cMORF fusion. We then assayed tRNA^{His} m⁵C levels before and after amino acid starvation, demonstrating that the tag did not alter levels of m⁵C in tRNA^{His} compared to those in a wild-type strain (Table 3), and using cells from the same growth, we extracted cell lysate and measured Trm4 protein levels by Western blotting for the HA epitope. Control modifications (D, ψ , Gm, m¹G, and Am) remained constant over the time course of growth (data not shown). However, although tRNA^{His} m⁵C levels are elevated at 24 h in both the wild-type and the *TRM4*-cMORF strain (Table 3), we find that Trm4 protein levels appear to decrease at the 24-h time point (Fig. 4), consistent with experiments that demonstrate reductions in *TRM4* mRNA levels (Natarajan et al. 2001) and ribosome occupancy (Ingolia et al. 2009) following starvation.

Aminoacylation status of tRNA^{His} is variably affected by different starvation conditions

Starvation for a particular amino acid in yeast and in *Escherichia coli* has been shown to affect the charging status of the corresponding tRNA isoacceptors, with some effects on other tRNAs (Dittmar et al. 2005; Zaborske et al. 2009). Therefore, tRNA^{His} aminoacylation status may be affected by the starvation conditions that we examined, and changes in tRNA^{His} aminoacylation levels might correlate with the amount of additional m⁵C added to tRNA^{His}. To address this possibility, we starved the BY4741 strain for histidine, leucine, uracil, or glucose, or grew BY4741 to stationary phase and extracted RNA in acidic conditions to preserve tRNA aminoacylation. We performed the experiment with two in-

FIGURE 4. Trm4-cMORF levels do not increase during starvation. BY4741 and Trm4-cMORF strains were grown in SD complete, SD-His, or SD-Leu media and grown for 24 h at 30°C. Crude extracts were prepared at 0, 7, and 24 h, and 30 μ g of protein were loaded in each lane and resolved by SDS-PAGE, followed by Western blotting. Anti-HA antibody was used to detect the cMORF tag on Trm4. BY4741 crude extract was used as a negative control. The same blot was incubated with anti-enolase antibody as a loading control.

FIGURE 5. Analysis of tRNA^{His} aminoacylation under different starvation conditions. RNA from the wild-type (BY4741) strain grown in the indicated media was isolated in acidic conditions to preserve aminoacylation, and 2 μ g of RNA were resolved on an acidic gel, analyzed by Northern blot for tRNA^{His}, tRNA^{Arg(ICG)}, and 5S rRNA, and quantified using ImageQuant software. A control sample was treated with base (+) to detect migration of deacylated tRNA. (Solid arrows) Aminoacyl-tRNA, (dotted arrows) deacyl-tRNA. Each percent aminoacylation value is the mean $\pm$ standard deviation (SD) from two independent cultures. A representative Northern blot from one of the two independent cultures is shown.

dependent cultures which serve as biological replicates. A Northern blot of samples from one of the replicates is shown in Figure 5. Prior to starvation, when the cultures are growing in log phase (SD complete 0 h), tRNA^{His} is 66% aminoacylated. Following 24 h of starvation for histidine, leucine, uracil, or glucose, tRNA^{His} is 44%, 60%, 55%, and 52% aminoacylated, respectively. Furthermore, following 24 h of growth in SD complete medium (stationary phase), tRNA^{His} is 66% aminoacylated. These data suggest that aminoacylation levels do not directly correlate with additional m⁵C levels. Although histidine starvation induces the most additional m⁵C and the tRNA^{His} is the most deacylated, we observe no change in aminoacylation levels of tRNA^{His} following entry into stationary phase (Fig. 5), although tRNA^{His} m⁵C levels increase by 0.67 mol/mol tRNA (Table 3). In addition, tRNA^{His} is more aminoacylated following leucine starvation than following uracil or glucose starvation (Fig. 5), but leucine starvation induces more additional m⁵C than uracil or glucose (Table 3).

Increases in m⁵C levels are unique to tRNA^{His}

Since additional m⁵C on tRNA^{His} occurs at C₄₈ and C₅₀ (adjacent to m⁵C₄₉) in Thg1-depleted cells (Gu et al. 2005), we examined tRNA^{Gly(GCC)} and tRNA^{Val(AAC)}, two of the four additional tRNAs known to have m⁵C₄₉ and unmodified C₄₈ and C₅₀ (Juhling et al. 2009). Consistent with results from Thg1-depleted cells, we find little change in m⁵C levels of these two tRNA species after starvation of wild-type cells (Table 6). Whereas histidine starvation for 24 h results in a large increase in tRNA^{His} m⁵C levels (from 0.86 to 2.00

TABLE 6. $tRNA^{His}$, $tRNA^{Val(AAC)}$, and $tRNA^{Gly(GCC)}$ m^5C levels during starvation

Media	Time (h)	$tRNA^{His}$ ^a	$tRNA^{Val(AAC)}$ ^b				$tRNA^{Gly(GCC)}$ ^b			
		m^5C	ψ	m^5C	I	m^1G	ψ	m^5C	Cm	m^1G
SD complete	0	0.86 ± 0.03	3.76	0.79	0.96	1.10	3.53	1.44	0.75	1.03
	24 ^c	1.53 ± 0.11	3.86	0.96	1.00	1.12	3.77	1.69	0.80	1.07
SD – His	6	1.80 ± 0.08	3.86	0.87	0.98	1.14	3.73	1.73	0.74	1.06
	24	2.00 ± 0.13	3.78	1.03	1.01	1.13	3.61	1.74	0.75	1.08
SD – Leu	24	1.66 ± 0.04	3.87	0.98	0.99	1.14	3.72	1.70	0.74	1.08
SD – Ura	24	1.23 ± 0.08	3.78	0.90	0.96	1.14	3.62	1.70	0.74	1.06
Expected mol/mol tRNA		1	4	1	1	1	4	1	1	1

^aValues from Table 3.^b $tRNA^{His}$, $tRNA^{Val(AAC)}$, and $tRNA^{Gly(GCC)}$ were purified from bulk RNA extracted from the same growth.^cStationary phase.

mol/mol tRNA), $tRNA^{Gly(GCC)}$ m^5C levels increase modestly (from 1.44 to 1.74 mol/mol tRNA), and $tRNA^{Val(AAC)}$ m^5C levels barely increase (from 0.79 to 1.03 mol/mol tRNA) (Fig. 6; Table 6). Similarly, leucine starvation for 24 h results in a substantial increase in $tRNA^{His}$ m^5C levels (from 0.86 to 1.66 mol/mol tRNA) but only modest increases in m^5C levels of $tRNA^{Gly(GCC)}$ (from 1.44 to 1.70 mol/mol tRNA) and $tRNA^{Val(AAC)}$ (from 0.79 to 0.98 mol/mol tRNA). Control modification levels for each tRNA remain normal during these treatments (Table 6; data not shown). Furthermore, m^5C levels only slightly increase in $tRNA^{Val(AAC)}$ when temperature-sensitive strains are shifted to 37°C, relative to the drastic induction of additional m^5C in $tRNA^{His}$ (Fig. 2B; Table 2). Thus, we infer that m^5C accumulation occurs preferentially on $tRNA^{His}$ in conditions where cells stop growing.

In support of this claim, both $tRNA^{Val(AAC)}$ and $tRNA^{Gly(GCC)}$ have the capacity to acquire additional m^5C after overexpression of *TRM4*-MORF (Table 5). Strikingly, *TRM4* overexpression results in a nearly identical increase in m^5C levels of $tRNA^{His}$ (from 0.86 to 1.59 mol/mol tRNA) and $tRNA^{Val(AAC)}$ (from 0.80 to 1.53 mol/mol tRNA) and a smaller but still significant increase in m^5C levels of $tRNA^{Gly(GCC)}$ (from 1.56 to 1.98 mol/mol tRNA), whereas all other control modifications (D, ψ , Gm, m^1G , I, Cm, and Am) are normal (Table 5; data not shown). Since both $tRNA^{Val(AAC)}$ and $tRNA^{Gly(GCC)}$ can be modified with additional m^5C in vivo by Trm4 but are not substrates for additional m^5C modification (presumably by Trm4) during growth arrest conditions, we conclude that the additional m^5C during growth arrest is specific to $tRNA^{His}$.

DISCUSSION

We have demonstrated here that m^5C levels of $tRNA^{His}$ are unusually responsive to a number of different conditions in which yeast cells stop growing. We had previously shown that $tRNA^{His}$ accumulates additional m^5C at C_{48} and C_{50} in the variable loop, adjacent to m^5C_{49} , following depletion of

Thg1 and the subsequent growth arrest (Gu et al. 2005). We have extended this analysis to show that $tRNA^{His}$ has additional m^5C when either *thg1* temperature-sensitive strains or temperature-sensitive strains unrelated to tRNA function are grown at nonpermissive temperature and when wild-type strains such as BY4741 are grown to saturation or are starved for histidine, amino acids, leucine, or (to a lesser extent) for glucose or uracil. In the majority of these cases, the accumulation of additional m^5C on $tRNA^{His}$ is not caused by loss of cell viability. In addition, we have shown that the accumulation of additional m^5C on $tRNA^{His}$ is not due to induction of the *GCN4* pathway and is not due to an increase in Trm4 protein levels during our starvation conditions, although the additional m^5C modification is absent in a *trm4*- Δ mutant. Remarkably, this additional m^5C specifically occurs on $tRNA^{His}$, and not on two other possible substrates, $tRNA^{Val(AAC)}$ or $tRNA^{Gly(GCC)}$, that also have m^5C_{49} flanked by cytidines, although these tRNA species can acquire more m^5C when Trm4 is overproduced.

The molecular signals that trigger Trm4 to modify $tRNA^{His}$ with additional m^5C during growth arrest conditions remain

FIGURE 6. m^5C increases specifically on $tRNA^{His}$ during starvation. $tRNA^{His}$ (black), $tRNA^{Val(AAC)}$ (dark gray), and $tRNA^{Gly(GCC)}$ (light gray) were purified from BY4741 grown in the indicated starvation conditions. Nucleoside modifications were analyzed by HPLC, and m^5C values are plotted over time for each tRNA.

unclear. Although tRNA^{His} localizes to the nucleus following Thg1 depletion (Gu et al. 2005), and Trm4 is a nuclear protein (Wu et al. 1998), our data suggest that modulation of tRNA subcellular dynamics is not the sole contributor to additional m⁵C levels, since glucose starvation results in rapid accumulation of tRNAs in the nucleus (Shaheen and Hopper 2005; Whitney et al. 2007), but we observe only modest accumulation of m⁵C relative to that observed with histidine or leucine starvation. Three lines of evidence suggest that tRNA charging status is also not the sole contributor to additional m⁵C on tRNA^{His}. First, tRNA^{His} from a *thg1*-Δ strain has normal m⁵C levels, despite the fact that the tRNA is mostly deacylated (Preston and Phizicky 2010). Second, tRNA^{His} has an additional 0.80 mol m⁵C/mol tRNA (Table 3) following 24 h of leucine starvation, although tRNA^{His} aminoacylation status is virtually unaffected (Fig. 5). Third, tRNA^{His} m⁵C increases by 0.67 mol/mol tRNA (Table 3) when wild-type cells are grown to stationary phase and tRNA^{His} aminoacylation levels are unaltered (Fig. 5).

However, it remains possible that both deacylation of tRNA^{His} and its presence in the nucleus contribute to the additional m⁵C that we observe. This would be consistent with the observation that more m⁵C accumulates on tRNA^{His} during histidine starvation than during other starvation conditions. Furthermore, increased m⁵C on tRNA^{His} occurs when tRNAs are known to accumulate in the nucleus: during starvation for amino acids, starvation for glucose, and as cells enter late-log and near-stationary phase (Whitney et al. 2007). Indeed, we previously showed that the additional m⁵C that accumulates upon Thg1 depletion occurs at a time when tRNA^{His} is deacylated and is localized to the nucleus (Gu et al. 2005), and it is known that both charged and uncharged tRNA can accumulate in the nucleus during starvation (Whitney et al. 2007). It is not completely clear why different levels of tRNA^{His} m⁵C arise during leucine starvation compared to glucose starvation; however, it is clear that different stress conditions have different effects on the cell since they elicit transcriptionally distinct types of growth arrest (Gasch et al. 2000; Boer et al. 2008; Brauer et al. 2008; Klosinska et al. 2011).

The specificity of additional m⁵C modification for tRNA^{His} is also puzzling. Although overproduction of Trm4 results in similar levels of additional m⁵C on tRNA^{His}, tRNA^{Val(AAC)}, and tRNA^{Gly(GGC)}, the additional m⁵C is much more specific for tRNA^{His} after temperature shift of *fcp1*^{ts} or *thg1*^{ts} strains, after starvation of a wild-type strain for histidine or leucine, and after growth to stationary phase. Therefore, we presume that, under these conditions, tRNA^{His} somehow becomes more accessible to Trm4, or Trm4 activity is altered, but it is not yet clear how this is accomplished. This specificity for tRNA^{His} might possibly be explained by a unique structural property of tRNA^{His} or by a secondary protein that binds tRNA^{His} during growth arrest. One possibility is that specificity is related to the unique sensitivity of histidyl-tRNA^{His} to deacylation in vitro (Chernyakov et al. 2008a; Preston and

Phizicky 2010), and the observation that tRNA^{His} is partially deacylated when isolated (Gu et al. 2005; Preston and Phizicky 2010), which could mean that this tRNA species is a cellular sensor for poor growth conditions.

Although our data demonstrate that the additional m⁵C levels of tRNA^{His} during histidine starvation accumulate independently of the *GCN4* gene and are not provoked by induction of the *GCN4* pathway, it is not clear if the accumulation of m⁵C of tRNA^{His} involves the TOR pathway, which also plays a role in stationary phase and quiescence (Herman 2002). We observe a distinct increase in tRNA^{His} m⁵C levels when cells are treated with rapamycin, but this increase is modest compared to other treatments. However, because rapamycin treatment results in G1 phase cell cycle arrest (Kunz et al. 1993; Barbet et al. 1996; Helliwell et al. 1998), it is difficult to determine whether the increased m⁵C is due to inhibition of the TOR pathway or to the resulting growth arrest. It is intriguing to speculate that the TOR pathway is connected to the accumulation of m⁵C in tRNA^{His}, because high-throughput studies have reported that deletion of *TRM4* results in increased rapamycin sensitivity (Parsons et al. 2004) and that overexpression of *TRM4* confers rapamycin resistance (Butcher et al. 2006), although we have not been able to observe such effects (data not shown).

The function of the additional m⁵C of tRNA^{His} has not yet been elucidated. Since overexpression of *TRM4*-MORF appears to increase m⁵C levels of several tRNA species and causes a slow-growth phenotype (Yoshikawa et al. 2011; data not shown; JM Dewe and EM Phizicky, unpubl.), we infer that excess m⁵C modification has a deleterious effect on function of one or more tRNAs. Conversely, m⁵C modification of tRNAs can play a protective role during stress conditions. Amino acid starvation in *Tetrahymena* and oxidative stress in yeast, humans, and plants results in cleavage of tRNAs in the anti-codon loop (Lee and Collins 2005; Thompson et al. 2008), and the *Drosophila* m⁵C methyltransferase Dnmt2 is important for viability during oxidative stress and heat shock conditions (Schaefer et al. 2010), because its modification of C₃₈ in the anti-codon loop protects substrate tRNAs from cleavage by angiogenin in both *Drosophila* and mice (Goll et al. 2006; Schaefer et al. 2010). Thus, the additional m⁵C of tRNA^{His} may have a protective function, similar to the role of m⁵C₄₉ of tRNA^{Val(AAC)} in reducing the extent of rapid decay of tRNAs lacking m⁷G₄₆ (Alexandrov et al. 2006; Chernyakov et al. 2008b; Dewe et al. 2012). Intriguingly, while preparing this manuscript, it was reported that hydrogen peroxide treatment leads to alteration of m⁵C levels in yeast tRNA^{Leu(CAA)}, with 70% more m⁵C₃₄ and 20% less m⁵C₄₈, and these changes were associated with increased expression of a ribosomal protein enriched with TTG codons (Chan et al. 2012). Further experiments will be required to fully understand the biology of m⁵C, the cause of the additional m⁵C of tRNA^{His}, and the role of the additional m⁵C during growth arrest.

MATERIALS AND METHODS

Strain construction

Strains used in this study are listed in Table 7.

To construct the *gcn4-Δ::ble^R* (MBY934) strain, we amplified the phleomycin resistance cassette from the pUG66 plasmid (Guedener et al. 2002) with GCN4 Up 179 + Phleo Forward (5'-ATCATGTA CCCGTAGAATTTTATTCAAGATGTTTCCGTAACGGCAGCTG AAGCTTCGTAC-3') and GCN4 Down 312 + Phleo Reverse (5'-G CATTAGCTATAACACGTTAATATGGTGGAGTCAGCTGAGAA GGCATAGGCCACTAGTGG-3') to add 43-nt sequence upstream of and downstream from GCN4 to the Phleo cassette 5' and 3' ends, respectively. We extended the GCN4 homologous regions to 87 nt by amplifying the first PCR product with GCN4 Up 223 (87) Forward (5'-ACTGTCAGTTTTTTGAAGAGTTATTTG TTTTGTACCAATTGCTATCATGTACCCGTAGA-3') and GC N4 Down 356 (87) Reverse (5'-CATGAGTACTCCTAAATAGGG CGATATTTTAAAGTTTCATTCCAGCATTAGCTATAACAC-3'). This extended PCR product was transformed into BY4741 as previously described (Sherman 1991) and selected on YPD media containing 8 μg/mL phleomycin (Chernyak et al. 2008b). The *gcn4-Δ::ble^R* strain was verified by PCR.

The TRM4-cMORF strain, MBY1026B, was constructed in a manner similar to the *gcn4-Δ::ble^R* strain. We used the plasmid, AVA0258, which encodes the cMORF tag (consisting of His6, HA epitope, 3C protease site, ZZ protein A), followed by *Kluyveromyces lactis* URA3 as a marker for insertion into the chromosome. We PCR-amplified this construct using TRM4 C-ter + TAP 5' (5'-GAACCTCTA CTGAAGCTCCTAGCGCTGCTAATAACCCAGCTTTCTTGTA CAAAGTGG-3'; contains 43 nt corresponding to the C terminus of Trm4 without the stop codon and a 5' portion of the cMORF tag sequence), and TRM4 Downstream (w/stop) + TAP 3' (5'-CTTT ACAGTGGAGGGGATAAGAAACATGATAACTATCATACGACT CACTATAGGG-3'; inserts the Trm4 stop codon after the cMORF tag and URA3 marker and contains 43 nt of the region downstream from TRM4). We then used this PCR product to extend the regions homologous to TRM4 by PCR amplification with TRM4 C-ter

extended (TAP) 5' (5'-GACTGAATCTCCCGCAGAACTACTA CCGGAACCTCTACTGAAGCTC-3') and TRM4 Downstream extended (TAP) 3' (5'-AGTATTATATTCTTATTTTGCCTTTTAA TAATATACATTTACTTTACAGTGGAGGGGAT-3'). The resulting strain was verified by PCR.

We constructed *thg1* temperature-sensitive (*thg1^{ts}*) strains, MBY294 (*thg1-Y8C*), MBY289 (*thg1-G172D,L233S*), and MBY303 (*thg1-Y146H*) in two steps. First, we randomly mutagenized a single copy *THG1* plasmid, screened for temperature sensitivity in a *thg1-Δ* strain, and sequenced the resulting alleles. We then inserted these *thg1^{ts}* alleles and wild-type *THG1* into the *THG1* locus by transformation of the SC1300-2 strain in which *THG1* was deleted with a *LEU2,CYH2* cassette in a *cyh^r* background, and selected for cycloheximide resistance, accompanied by leucine auxotrophy. Temperature sensitivity was confirmed and complemented by a wild-type copy of *THG1*.

Strain starvation

Strains were grown to log phase at 30°C in SD complete media, then spun down and resuspended in the following media conditions: SD complete, SD minimal (contains only yeast nitrogen base and glucose), SD – His, SD – Leu, SD – Ura, or S complete no glucose. These cultures were grown for 24 h at 30°C, and cells were harvested for analysis of tRNA modifications at indicated times.

Temperature-sensitive strain growth

Strains were grown to log phase in YPD at 24°C, diluted to OD₆₀₀ = 0.5 and grown at either 24°C or 37°C for the time indicated.

Rapamycin treatment of W303/CRY1

W303 was pretreated with DMSO by growing in YPD media containing 1% DMSO overnight until the strain reached early log phase. Cells were harvested prior to drug treatment and after growth in the

TABLE 7. Strains used in this study

Strain	Genotype	Parent	Source
BY4741	<i>MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>		Open Biosystems
W303/CRY1	<i>MAT α ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1</i>		D. Krysan
SC1300-2	<i>MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cyh^r</i> <i>thg1-Δ0::LEU2,CYH2 [CEN URA3 P_{GAL} THG1]</i>	BY4741	This study
MBY289	<i>MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cyh^r thg1-G172D/L233S</i>	SC1300-2	This study
MBY294	<i>MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cyh^r thg1-Y8C</i>	SC1300-2	This study
MBY303	<i>MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cyh^r thg1-Y146H</i>	SC1300-2	This study
MBY308	<i>MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cyh^r THG1</i>	SC1300-2	This study
MBY480	<i>MAT a thg1-Δ0::kanMX</i> <i>[2μ LEU2 tRNA^{His} A₇₃, 2μ HIS3 P_{HTS1}-HTS1]</i>	BY4741	(Preston and Phizicky 2010)
Y08328	<i>MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 abf1-102</i>	BY4741	C. Boone
Y09266	<i>MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 fcp1-1</i> <i>MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cdc48-9(Y-F)</i>	BY4741	C. Boone
MBY934A,B	<i>MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gcn4-Δ::ble^R</i>	BY4741	This study
MBY1026B	<i>MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TRM4-cMORF(His₆-HA epitope-3C site-ZZ protein A)-K. lactis URA3</i>	BY4741	This study
MBY687A	<i>MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 trm4-Δ::kanMX</i>	BY4741	This study

presence of the indicated concentration of rapamycin or 1% DMSO for the indicated amount of time.

Assessment of viable cell titer

For each condition, a given volume of culture was plated onto YPD plates. Cells were grown for 3 d at 30°C or at 24°C for temperature-sensitive strains, and colonies were counted and normalized to the volume plated to calculate viable cells per milliliter of culture.

Bulk RNA isolation and purification of tRNA

Bulk low molecular weight RNA was isolated from 150 to 300 OD of yeast cells that were grown in conditions described above, using a hot phenol extraction method, as described elsewhere (Kotelawala et al. 2008). Total RNA was extracted from stationary phase cells by lysis with glass beads, phenol-chloroform extraction, and ethanol precipitation, as described previously (Letzring et al. 2010). tRNAs were purified using 5' biotinylated DNA oligomers complementary to the following: nt 48–72 for tRNA^{His} (5' Bio tRNA^{His}: 5'-/Biotin/GCCATCTCCTAGAAATCGAACCAGGG-3') (Preston and Phizicky 2010), nt 52–76 for tRNA^{Gly(GCC)} (BioGly: 5'-/Biotin/TGGTGCGCAAGCCCGGAATCGAACC-3') (Gu et al. 2005), and nt 55–76 for tRNA^{Val(AAC)} (Biotin-tRNA^{Val}1: 5'-/Biotin/TGGTGATTTTCGCCAGGATCGA-3'). For each tRNA purified, 22.5 pmol of oligomer were first bound to streptavidin magnetic particles (Roche). Then, bulk RNA (1–1.25 mg) was added to oligomer-bound beads in the presence of 2.4 M tetraethylammonium chloride (TEACl; Sigma), washed, and the tRNA was melted off the oligomer at 60°C to obtain pure tRNA. The resulting tRNA was desalted and concentrated using Amicon Ultra 4 10,000 MWCO columns (Millipore).

HPLC analysis of tRNA nucleosides

tRNAs (1.25 µg) were digested at 37°C for at least 2 h using 0.5 µg P1 nuclease (MP Biomedicals) in a buffer containing 20 mM NaOAc pH 5.2 and 0.2 mM ZnCl₂ and then treated with calf intestinal phosphatase (Roche) for at least 1 h. Nucleosides were resolved by reverse-phase HPLC essentially as described (Gehrke and Kuo 1989), and each nucleoside was identified and quantified as described previously (Jackman et al. 2003; Kotelawala et al. 2008).

Quantitative RT-PCR

RNA was extracted using the hot phenol extraction method (Kotelawala et al. 2008) and treated with RQ1 RNase-free DNase (Promega), followed by a phenol-chloroform extraction, two chloroform extractions, and ethanol precipitation. This RNA was reverse transcribed with Superscript II Reverse Transcriptase (Invitrogen) using Random Primers (Invitrogen). Next, this DNA was PCR-amplified using Fast SYBR Green master mix (Applied Biosystems) and 0.2 µM each of 5' and 3' primers specific to *ACT1* (*ACT1* Set 1 Forward 5'-ACGTTCCAGCCTTCTACGTTTCCA-3' and *ACT1* Set 1 Reverse 5'-ACGTGAGTAACACCATCACCGGAA-3', *HIS5* (*HIS5* Set 1 Forward 5'-AATGCCATGGACCTACTCCAGTT-3' and *HIS5* Set 1 Reverse 5'-ACACCTAGGCACAGATTGTCAGCA-3'), or *LYS1* (*LYS1* Set 1 Forward 5'-AGCAGACACTACCAA CCCTCACAA-3' AND *LYS1* Set 1 Reverse 5'-CTTGGCAGCAAA

GAAGGCAAGTGA-3'), with the following amplification scheme: 95°C for 20 sec and then 40 cycles of 95°C for 3 sec and 60°C for 30 sec.

Analysis of aminoacylated RNA

RNA was isolated from 50 OD of cells in acidic conditions (pH 4.5), and 2 µg of RNA were resolved by PAGE under acidic conditions, as previously described (Chernyakov et al. 2008a), and transferred to Hybond N+ membrane (Amersham Biosciences). The membrane was UV cross-linked, hybridized with 5'-labeled oligomers tRNA^{His} (40–64) (5'-CTAGAATCGAACCAGGGTTTCATC-3'), ArgP1 (5'-TAGCCAGACGCCGTGAC-3'), and 5S RNA (5'-GGT AGATATGGCCGCAACC-3') to detect tRNA^{His}, tRNA^{Arg} (ICG), and 5S rRNA, and visualized with a Typhoon PhosphorImager (GE Healthcare).

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