

# tRNA thiolation links translation to stress responses in *Saccharomyces cerevisiae*

Jadyn R. Damon, David Pincus, and Hidde L. Ploegh

Whitehead Institute for Biomedical Research, Cambridge, MA 02142

**ABSTRACT** Although tRNA modifications have been well catalogued, the precise functions of many modifications and their roles in mediating gene expression are still being elucidated. Whereas tRNA modifications were long assumed to be constitutive, it is now apparent that the modification status of tRNAs changes in response to different environmental conditions. The *URM1* pathway is required for thiolation of the cytoplasmic tRNAs tGlu<sup>UUC</sup>, tGln<sup>UUG</sup>, and tLys<sup>UUU</sup> in *Saccharomyces cerevisiae*. We demonstrate that *URM1* pathway mutants have impaired translation, which results in increased basal activation of the Hsf1-mediated heat shock response; we also find that tRNA thiolation levels in wild-type cells decrease when cells are grown at elevated temperature. We show that defects in tRNA thiolation can be conditionally advantageous, conferring resistance to endoplasmic reticulum stress. *URM1* pathway proteins are unstable and hence are more sensitive to changes in the translational capacity of cells, which is decreased in cells experiencing stresses. We propose a model in which a stress-induced decrease in translation results in decreased levels of *URM1* pathway components, which results in decreased tRNA thiolation levels, which further serves to decrease translation. This mechanism ensures that tRNA thiolation and translation are tightly coupled and coregulated according to need.

## Monitoring Editor

Sandra Wolin  
Yale University

Received: Jun 24, 2014

Revised: Oct 24, 2014

Accepted: Nov 5, 2014

## INTRODUCTION

The posttranscriptional modification of RNA molecules enhances the functionality of tRNA, mRNA, and rRNA species; such modifications are ubiquitous among the major domains of life (<http://mods.rna.albany.edu>). N<sup>6</sup>-methyladenosine (m<sup>6</sup>A) is a widely occurring mRNA modification that has been recently shown to regulate mRNA stability and play a role in yeast developmental/cell fate determination programs (Agarwala *et al.*, 2012; Schwartz *et al.*, 2013; Wang *et al.*, 2013). Defects in rRNA modifications affect tRNA binding and translational fidelity, as well as ribosome maturation (Decatur and Fournier, 2002; Liang *et al.*, 2009; Jack *et al.*, 2011). The

modifications found on tRNAs are numerous and well characterized, with 25 unique modifications found at 36 different positions in the tRNAs of *Saccharomyces cerevisiae*. Although the well-conserved nature of tRNA modifications would suggest critical roles in cellular processes, the majority of modifications are nonessential. The deletion of many genes encoding modification enzymes causes only mild phenotypes (Phizicky and Hopper, 2010). What, then, do tRNA modifications contribute to the process of gene expression?

Lack of modifications in the body of tRNAs can result in defects in aminoacylation and rapid degradation of hypomodified tRNAs (Alexandrov *et al.*, 2006; Chernyakov *et al.*, 2008; Whipple *et al.*, 2011; Tuorto *et al.*, 2012). Modifications at or near the anticodon appear to affect translation directly by mediating codon–anticodon interactions and facilitating accurate and efficient translation of the genetic code (Yarian, 2002; Murphy *et al.*, 2004; Agris *et al.*, 2007). Still, the specific mechanisms by which hypomodified tRNAs produce specific phenotypes and affect cellular processes remain unclear, even though many modification pathways are genetically and/or biochemically well described (Yacoubi *et al.*, 2012).

In *S. cerevisiae*, formation of the modified anticodon wobble nucleoside mcm<sup>5</sup>s<sup>2</sup>U<sub>34</sub> (5-methoxycarbonylmethyl-2-thiouridine) is dependent on the activities of the *ELP* and *URM1* pathways. Components of the *ELP* pathway, elongator complex (Elp1–Elp6), as well as Kti1-3 and Trm9, are responsible for formation of the mcm<sup>5</sup>

This article was published online ahead of print in MBcC in Press (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E14-06-1145>) on November 12, 2014.

Address correspondence to: Hidde L. Ploegh ([ploegh@wi.mit.edu](mailto:ploegh@wi.mit.edu)).

Abbreviations used: APM, N-acryloylamino phenyl mercuric chloride; AZC, azetidine-2-carboxylic acid; ER, endoplasmic reticulum; HSE, heat shock element; HSR, heat shock response; mcm5s2U, 5-methoxycarbonylmethyl-2-thiouridine; mcm5U, 5-methoxycarbonylmethyluridine; s2U, 2-thiouridine; STRE, stress response element; UPR, unfolded protein response; UPR-Cyto, cytoplasmic unfolded protein response; UPRE, unfolded protein response element.

© 2015 Damon *et al.* This article is distributed by The American Society for Cell Biology under license from the author(s). Two months after publication it is available to the public under an Attribution–Noncommercial–Share Alike 3.0 Unported Creative Commons License (<http://creativecommons.org/licenses/by-nc-sa/3.0>).

“ASCB®,” “The American Society for Cell Biology®,” and “Molecular Biology of the Cell®” are registered trademarks of The American Society for Cell Biology.

Supplemental Material can be found at:  
<http://www.molbiolcell.org/content/suppl/2014/11/10/mbc.E14-06-1145.DC1>

moyety found on tRNAs (Kalhor and Clarke, 2003; Lu et al., 2005). Deletion of genes in the *URM1* pathway result in hypomodified cytoplasmic tGlu<sup>UUC</sup> (GAA codon), tGln<sup>UUG</sup> (CAA codon), and tLys<sup>UUU</sup> (AAA codon) tRNAs, a subset of the tRNAs modified in an *ELP*-dependent manner; these tRNAs contain the mcm<sup>5</sup>U<sub>34</sub> but not the s<sup>2</sup>U<sub>34</sub> modification (Huang et al., 2008; Nakai et al., 2008; Schlieker et al., 2008; Leidel et al., 2009). Central to the *URM1* pathway is the conserved eukaryotic ubiquitin-related modifier Urm1, which shares the  $\beta$ -grasp fold that characterizes the ubiquitin superfamily (Singh et al., 2005; Xu et al., 2006) and was originally identified through sequence similarity to the prokaryotic sulfur carriers Moad and ThiS (Furukawa et al., 2000). The functions of Urm1 reflect the evolutionary link between eukaryotic ubiquitin-like proteins and prokaryotic sulfur carriers. The C-terminal diglycine motif of Urm1 is activated in ATP-dependent manner by Uba4 in *S. cerevisiae* to yield a C-terminal thiocarboxylate (Schmitz et al., 2008). Urm1 then functions both as a noncanonical lysine-directed protein modifier under conditions of oxidative stress (Goehring et al., 2003a,b; Van der Veen et al., 2011) and as a sulfur carrier that is used in tRNA thiolation reactions (Huang et al., 2008; Nakai et al., 2008; Schlieker et al., 2008; Leidel et al., 2009). The effects of tRNA thiolation can be distinguished from effects of protein urmylation through comparison of *urm1* $\Delta$  strains with *ncs2* $\Delta$  and *ncs6* $\Delta$  cells (*Ncs2* and *Ncs6* have no reported functions outside of tRNA modification) or with *ELP* pathway mutants. The mcm<sup>5</sup>s<sup>2</sup>U<sub>34</sub> nucleoside is a well-described modification whose synthesis is well studied in *S. cerevisiae* and thus represents a tractable system for studying the effects of tRNA modifications on cellular processes.

*URM1* pathway mutants display a variety of phenotypes, including increased sensitivity to oxidative stressors and defects in nutrient sensing and invasive growth, many of which are linked to defects in tRNA modification (Goehring et al., 2003a,b; Rubio-Teixeira, 2007; Leidel et al., 2009). Along with recent studies demonstrating that the levels of certain tRNA modifications change in response to different growth conditions (Kamenski et al., 2007; Chan et al., 2010; Preston et al., 2012), the phenotypes of mutant cells suggest that the dynamic regulation of tRNA modification pathways plays an underappreciated role in the response of cells to a variety of stresses. Not much is known about the specific conditions that lead to changes in tRNA modification levels, the mechanisms that might regulate tRNA modifications, or the properties of differentially modified tRNAs.

Here we explore the role of Urm1-dependent tRNA modifications by examining translation in wild-type and *urm1* $\Delta$  cells. We find that *urm1* $\Delta$  mutants have defects in translation and that tRNA modification defects result in a slow-growth phenotype as well as an increased activation of the Hsf1-dependent stress response. We also find that *URM1* pathway mutants are more resistant to certain stresses than wild-type cells, suggesting that the consequences of hypomodified tRNAs result in cellular adaptations that allow cells to better withstand certain stresses. Of interest, we find that wild-type cells grown at an elevated temperature for an extended period of time accumulate a population of unthiolated tRNAs, and we suggest that modulation of tRNA modification pathways is an adaptive response to ongoing stress.

## RESULTS

### The *URM1* pathway links tRNA modification to translation

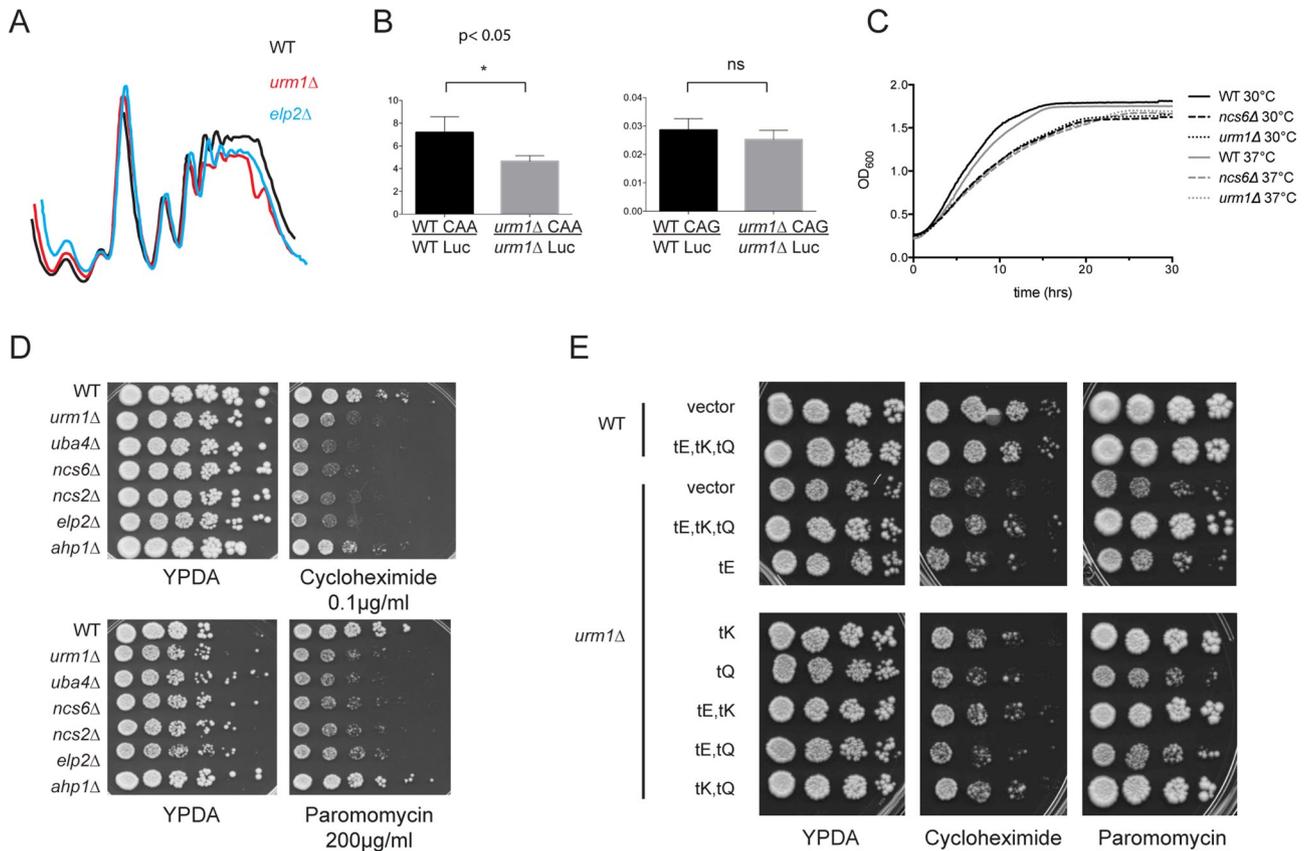
To determine the effect of hypomodified tRNAs on translation, we performed polysome profile analysis as a proxy for bulk translation. We found that *urm1* $\Delta$  cells demonstrated a subtle but significant decrease in the polysome:monosome (P:M) ratio

compared with wild-type cells, indicating a slight global impairment in translation (Figure 1A). As described earlier, disruption of the elongator complex impairs formation of the mcm<sup>5</sup>U modification (Huang et al., 2008). Polysome profiles of elongator-deficient *elp2* $\Delta$  cells were similar to those of *urm1* $\Delta$  cells, consistent with the interpretation that defects in U<sub>34</sub> modifications underlie the differences between wild-type and mutant cells (Figure 1A). To examine directly the effect of hypomodified tRNAs on translation, we measured incorporation of radioactive amino acids ([<sup>35</sup>S] cysteine and [<sup>35</sup>S]methionine). When measured in this manner, wild-type and *urm1* $\Delta$  cells showed no difference in the rate of protein synthesis when grown at 25°C, reinforcing the finding that changes in bulk protein synthesis are minor (Supplemental Figure S1).

Although bulk translation may be largely unaffected in *URM1* pathway mutants, we reasoned that there might be transcript-specific differences in translation in deletion mutants. Specifically, transcripts enriched in AAA, GAA, or CAA codons might be translated at lower levels in cells containing hypomodified tRNAs, since the tRNAs that decode these codons are thiolated. To test this, we examined the translation of mRNAs transcribed in vitro in wild-type or *urm1* $\Delta$  extracts. We monitored the translation of either a leaderless luciferase transcript or of a luciferase transcript engineered to contain a leader sequence of 12x CAA or 12x CAG codons. Although there was no difference between wild-type and *urm1* $\Delta$  extracts with regard to levels of 12x CAG luciferase produced, *urm1* $\Delta$  extracts translated 12x CAA luciferase less efficiently than did wild-type extracts, suggesting that there are codon/tRNA-specific translation defects in *urm1* $\Delta$  cells (Figure 1B). Although defects in translation were subtle, they may significantly affect cellular fitness; mutant cells (*urm1* $\Delta$  and *ncs6* $\Delta$  strains) grew more slowly than wild-type cells at 30 and 37°C (Figure 1C), indicating that there are phenotypic consequences associated with defects in Urm1-dependent tRNA modifications.

We reasoned that compounds known to interfere with protein synthesis might exacerbate the defects in translation of *URM1* pathway mutants. Cycloheximide blocks the elongation step of protein synthesis, whereas aminoglycosides such as paromomycin interfere with translation by binding to the ribosome-decoding site, which increases misreading (Fan-Minogue and Bedwell, 2007; Kramer et al., 2010). Consistent with this notion, *URM1* pathway mutants (*urm1* $\Delta$ , *uba4* $\Delta$ , *ncs2* $\Delta$ , *ncs6* $\Delta$ ) displayed impaired growth in the presence of translation inhibitors. Growth of the *ELP* pathway mutant *elp2* $\Delta$  was also reduced by translation inhibitors, suggesting that the phenotype is likely a function of the tRNA modification function of Urm1 and not attributable to defects in protein urmylation. In agreement with this interpretation, *ahp1* $\Delta$  cells (*Ahp1* is the only known protein to which Urm1 is conjugated in yeast; Goehring et al., 2003a) grow like wild type in the presence of translation inhibitors (Figure 1D).

Overexpression of tRNAs can suppress phenotypes attributable to defects in tRNA modification (Esberg et al., 2006; Bjork et al., 2007). To establish definitively that the observed defects in translation were due to impaired tRNA modification, we overexpressed tGlu<sup>UUC</sup> (tE), tGln<sup>UUG</sup> (tQ), or tLys<sup>UUU</sup> (tK) from a high-copy (2  $\mu$ ) plasmid. We created constructs to express each tRNA alone or in combination with the other tRNAs. Expression of the triplet E/K/Q construct in an *urm1* $\Delta$  background rescued growth in the presence of cycloheximide and paromomycin, as did expression of the double tE/K and tK/Q constructs. Indeed, overexpression of tLys<sup>UUU</sup> (tK) alone was sufficient to rescue growth (Figure 1E).



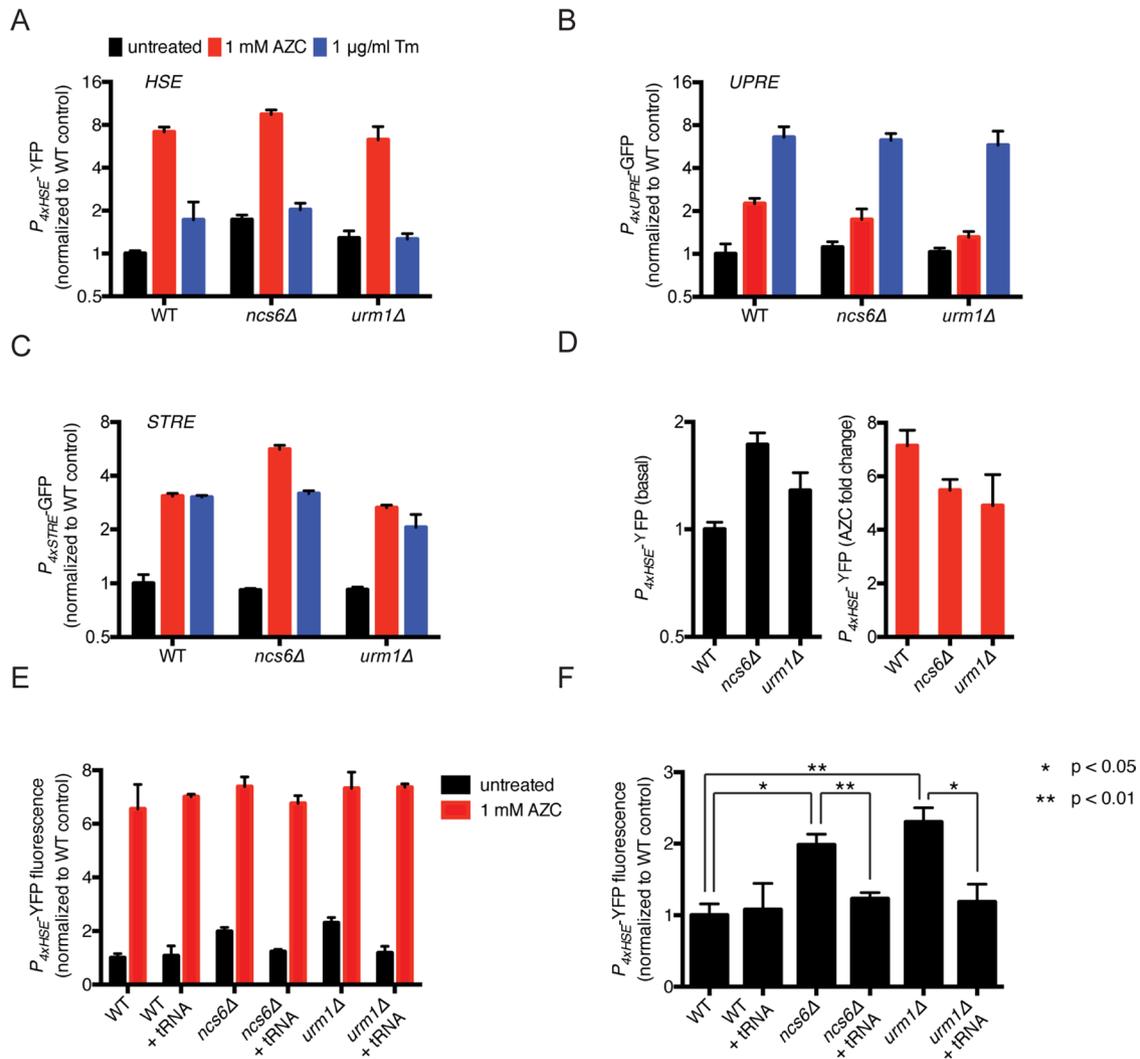
**FIGURE 1:** Translation is mildly affected in *URM1* pathway mutants. (A) Polysome profiles of wild-type, *urm1Δ*, and *elp2Δ* cells. (B) Luciferase mRNA, either leaderless or containing a 12xCAA or 12xCAG leader, was incubated with translation extracts prepared from the indicated strain. Luciferase activity resulting from translation of leader-containing transcript was normalized to that of leaderless transcript. (C) Growth curves of wild-type, *ncs6Δ*, and *urm1Δ* cells grown in yeast extract/peptone/dextrose/adenine (YPDA) at 30 or 37°C. The average of two (for *urm1Δ* 37°C) or three replicates (all other strains/conditions) is shown. (D) Fivefold serial dilutions of wild-type, *urm1Δ*, *uba4Δ*, *ncs6Δ*, *ncs2Δ*, *elp2Δ*, or *ahp1Δ* cells were plated onto YPDA plates containing 0.1 μg/ml cycloheximide or 0.2 mg/ml paromomycin where indicated. (E) Fivefold serial dilutions of wild-type or *urm1Δ* cells harboring empty vector or the plasmid encoding the indicated tRNA(s) were plated onto YPDA plates containing 0.1 μg/ml cycloheximide or 0.2 mg/ml paromomycin.

### The heat shock response is activated in *URM1* pathway mutants

Because strains with deletions in the *URM1* pathway showed increased sensitivity to inhibitors of translation, we asked whether these strains might also display altered cell stress responses. We reasoned that defects in protein synthesis could lead to the production of misfolded or error-containing proteins, which in turn could lead to the activation of pathways that evolved to cope with these stresses. We examined the transcript levels of genes whose transcription is known to be up-regulated in response to a variety of stresses: *UBI4*, the yeast polyubiquitin gene, as well as *SSA4*, and *STI1*, which encode chaperones/cochaperones. We found evidence that *urm1Δ* cells had increased levels of stress-induced transcripts (Supplemental Figure S2A) and that the increased levels of at least a subset of these transcripts were linked to the tRNA modification function of Urm1 (Supplemental Figure S2B). We analyzed the levels of C-terminally triple-hemagglutinin (3xHA)-tagged versions of Ssa4 and Sti1 in wild-type and *urm1Δ* cells by immunoblot analysis and found that differences in protein levels between wild-type and mutant cells were minor and that, when challenged with appropriate stressors, mutant cells were able to up-regulate protein levels (Supplemental Figure S2D). This indicates that tRNA thiolation is unlikely to drastically affect the translation of *SSA4* and *STI1* transcripts and that the stress phenotype

of *URM1* pathway mutants might be mediated primarily through transcriptional changes.

Many stress-responsive genes are transcriptionally regulated by multiple promoter elements that are recognized by distinct transcription factors. The transcription factor Hsf1 recognizes the heat shock element (HSE; Pelham, 1982; Slater and Craig, 1987; Wiederrecht et al., 1988). The unfolded protein response element (UPRE) is recognized by Hac1 in response to endoplasmic reticulum (ER) stress (Mori et al., 1992, 1996, 1998; Kohno et al., 1993; Cox and Walter, 1996; Nikawa et al., 1996), whereas the stress response element (STRE) is recognized by Msn2 and Msn4 (Wieser et al., 1991; Kobayashi and McEntee, 1993; Martínez-Pastor et al., 1996) when cells are exposed to any one of a variety of environmental stresses (Gasch et al., 2000). The promoter of *UBI4*, for example, contains both HSEs and STREs (Simon et al., 1999), and so our Northern blot results did not indicate which pathway(s) were activated in *urm1Δ* cells. Cells in which *TRM9* has been deleted, for example, show signs of activation of both the unfolded protein response (UPR) and the heat shock response (HSR; Patil et al., 2012a). To identify the stress response pathway(s) activated in the *urm1Δ* cells, we used reporter constructs specific for a single response. These reporters consist of a fluorescent protein (yellow fluorescent protein [YFP] or green fluorescent protein [GFP]) whose expression is controlled by a promoter containing four repeats of a motif recognized by a specific transcription factor. For our



**FIGURE 2:** The heat shock response pathway is up-regulated in *URM1* pathway mutants. Indicated strains harboring the (A)  $P_{4xHSE}$ -YFP, (B)  $P_{4xUPRE}$ -GFP, or (C)  $P_{4xSTRE}$ -GFP were left untreated or exposed to 1 mM AZC or 1  $\mu$ g/ml tunicamycin. (D) Data from A replotted. (E) Wild-type, *ncs6Δ*, or *urm1Δ* strains harboring the  $P_{4xHSE}$ -YFP reporter and a 2  $\mu$  plasmid (either empty vector or tE/K/Q plasmid, denoted as +tRNA) were untreated or exposed to 1 mM AZC. (F) Data from untreated samples in E replotted. Fluorescence levels were normalized to wild-type untreated, and values are the averages of three cultures. Error bars denote SDs.

analysis, we used the following constructs, which were integrated as single copies at the *LEU2* locus in wild-type, *urm1Δ*, or *ncs6Δ* yeast strains:  $P_{4xHSE}$ -YFP,  $P_{4xUPRE}$ -GFP, and  $P_{4xSTRE}$ -GFP.

We measured fluorescence levels by flow cytometry in cells that were left untreated or grown in the presence of tunicamycin, which activates of the UPR, or azetidine-2-carboxylic acid (AZC), a proline analogue that, when incorporated into proteins, leads to misfolding and activation of the heat shock response (or UPR-Cyto). We found that in all strain backgrounds, the reporters were responsive to the appropriate stimulus at comparable levels (Figure 2, A–C), indicating that our constructs were functional in all of the strains being used and also that *urm1Δ* and *ncs6Δ* cells were able to respond to specific stressors comparably to wild-type cells. For both the  $P_{4xUPRE}$ -GFP and  $P_{4xSTRE}$ -GFP reporters, we found that basal levels of activation were the same in wild-type, *urm1Δ*, and *ncs6Δ* cells. By contrast, we observed that *urm1Δ* and *ncs6Δ* cells harboring the  $P_{4xHSE}$ -YFP reporter had higher levels of fluorescent protein than wild type when cells were untreated, indicating that the Hsf1-mediated transcriptional response is basally up-regulated in *URM1* pathway mutants (Figure 2, A and D).

Because both *urm1Δ* and *ncs6Δ* strains showed an increase in  $P_{4xHSE}$ -YFP reporter levels, the phenotype is likely linked to the tRNA modification function of Urm1. To test this directly, we expressed either empty vector or the high-copy plasmid expressing tE/K/Q in wild-type, *urm1Δ*, or *ncs6Δ* strains harboring the  $P_{4xHSE}$ -YFP construct. All strains were responsive to AZC (Figure 2E). Whereas overexpressing tRNAs had no effect on the wild-type basal level of Hsf1 activity, in the *urm1Δ* and *ncs6Δ* cells, overexpression of tRNAs was able to restore  $P_{4xHSE}$ -YFP expression to wild-type levels in untreated cells (Figure 2F). Thus the processes that are dysregulated in *urm1Δ* cells lead specifically to activation of the Hsf1-mediated heat shock response and are tRNA dependent, consistent with the defects in translation that *urm1Δ* cells exhibit.

### tGlu<sup>UUC</sup>, tGln<sup>UUG</sup>, and tLys<sup>UUU</sup> become hypomodified at elevated temperature

A recent study of tRNA modifications in *S. cerevisiae* demonstrated that the modification status of many nucleosides changes in response to various chemical agents. Levels of mcm<sup>5</sup>s<sup>2</sup>U were found to be condition specific; treatment with methyl methanesulfonate,

hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and sodium arsenite decreased levels of mcm<sup>5</sup>s<sup>2</sup>U detected by mass spectrometry (Chan *et al.*, 2010). However, this study did not examine the modification status of specific tRNAs.

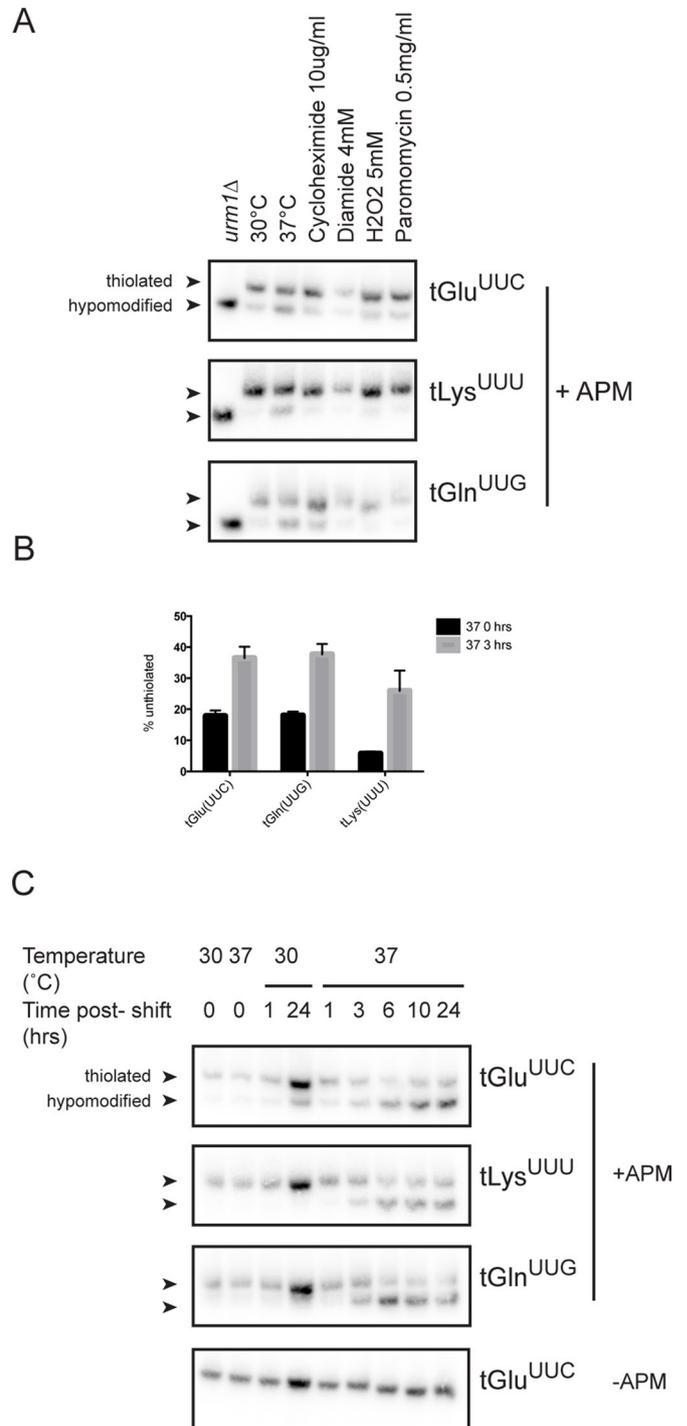
To further define the functions of the mcm<sup>5</sup>s<sup>2</sup>U<sub>34</sub> modification, we examined different conditions for evidence of alterations in the Urm1-dependent thiolation status of tGlu<sup>UUC</sup>, tGln<sup>UUG</sup>, and tLys<sup>UUU</sup> in wild-type cells. To assess the thiolation status of tRNAs, we performed Northern blot analysis on total RNA separated on urea-polyacrylamide gels supplemented with *N*-acryloylamino phenyl mercuric chloride (APM); the sulfur–mercury interaction results in a readily apparent retardation in electrophoretic mobility of thiolated tRNAs (Igloi, 1988). We examined the thiolation status of tRNA isolated from cultures grown under standard conditions (30°C) or at an elevated temperature (37°C) and from cultures exposed to compounds that either inhibit translation (cycloheximide or paromomycin) or result in oxidative stress (diamide or H<sub>2</sub>O<sub>2</sub>). Because oxidative stress causes Urm1 conjugation to proteins (Goehring *et al.*, 2003a; Van der Veen *et al.*, 2011), we were particularly interested to determine whether there was a link between protein urmylation and tRNA thiolation. Consistent with previous studies, under standard growth conditions, the majority of tGlu<sup>UUC</sup>, tGln<sup>UUG</sup>, and tLys<sup>UUU</sup> was thiolated (Nakai *et al.*, 2008; Schlieker *et al.*, 2008; Leidel *et al.*, 2009), and thiolation was dependent on Urm1 (Figure 3A). Neither translation inhibition nor oxidative stress caused a change in the modification status of these tRNAs. By contrast, growth at elevated temperature resulted in accumulation of hypomodified tGlu<sup>UUC</sup>, tGln<sup>UUG</sup>, and tLys<sup>UUU</sup>, all of the tRNAs known to be thiolated by Urm1 (Figure 3, A and B).

To better understand the temporal dynamics of tRNA modification, we performed an extended temperature shift experiment in which cells were grown at 37°C for up to 24 h. An increase in hypomodified tRNA species was evident beginning at 3 h of growth at elevated temperature. At later time points, the proportion of unthiolated tRNA relative to thiolated tRNA continued to increase. By 6 h, the majority of tGlu<sup>UUC</sup>, tGln<sup>UUG</sup>, and tLys<sup>UUU</sup> existed in the hypomodified form (Figure 3C). This suggests that the change in tRNA thiolation is not a transient phenomenon but an ongoing adaptation to growth at high temperatures.

Despite being hypomodified at elevated temperatures, we found that tRNAs were not degraded (Supplemental Figure S3) and continued to associate with translating ribosomes (Figure 4). Thus both fully modified and hypomodified tRNAs can participate in translation *in vivo*. The properties of hypomodified tRNAs are therefore relevant to the process of translation in cells growing at high temperatures.

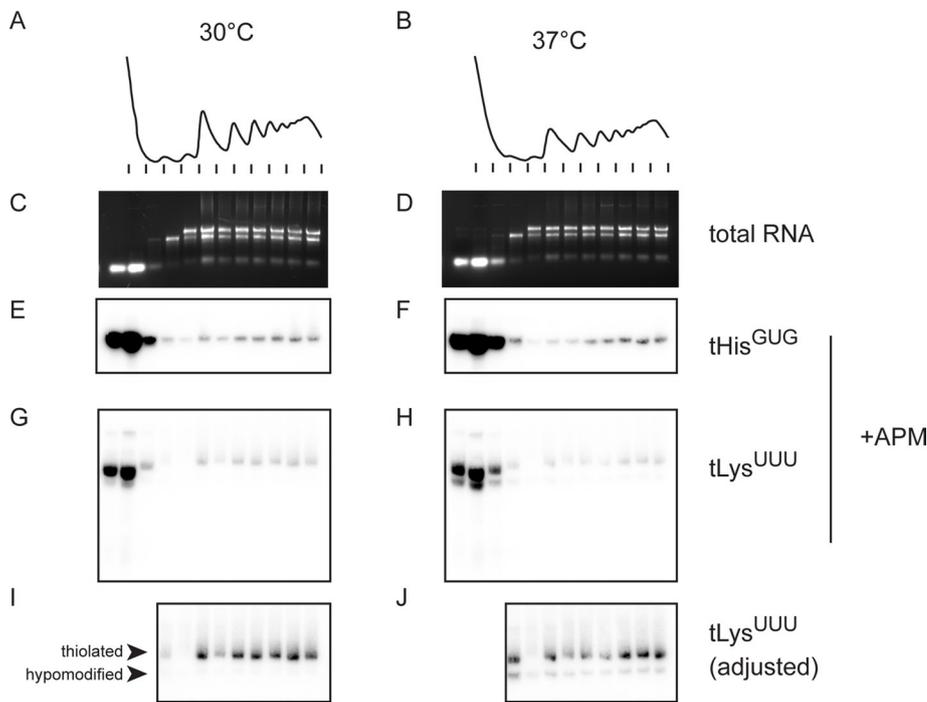
### The appearance of hypomodified tRNA requires the transcription of new tRNA by RNA polymerase III

Temperature-dependent modulation of tRNA modification could in principle result from the removal of the modification from mature tRNAs or the lack of modification of newly synthesized tRNAs. To determine which of these possibilities applies, we examined the role of RNA polymerase III (pol III), which is responsible for tRNA synthesis, in the modification of mcm<sup>5</sup>s<sup>2</sup>U<sub>34</sub>-containing tRNA. To this end, we preincubated wild-type cells with the pol III inhibitor ML-60218 or dimethyl sulfoxide (DMSO) at 30°C for 2 h before shifting cells to 37°C for up to 3 h. APM-Northern blot analysis showed that levels of hypomodified tGlu<sup>UUC</sup>, tGln<sup>UUG</sup>, and tLys<sup>UUU</sup> were decreased in cells when pol III was inhibited (Figure 5A). The relative difference in levels of thiolated and unthiolated tRNA is apparent even though there is less total tRNA in inhibitor-treated cells. We quantified the



**FIGURE 3:** Unthiolated tRNAs accumulate at elevated temperatures. (A) Small RNA APM Northern blot of RNA isolated from *urm1Δ* or wild-type cells grown under indicated conditions for 3 h; membranes were probed for the indicated tRNA. The positions of thiolated and unthiolated tRNA molecules are indicated. (B) Quantification of unthiolated tRNA as a percentage of total tRNA species in wild-type cells grown at 37°C for 0 or 3 h. The average and SD of three replicates are reported. (C) Small RNA (±APM as indicated) Northern blot analysis of RNA extracted from cells grown at 30 or 37°C for the indicated period of time; membranes were probed for the indicated tRNA.

levels of unthiolated tRNAs in each sample and found that incubation with ML-60218 resulted in a decrease of unthiolated tRNAs as a percentage of total tRNA (Figure 5B). We conclude that it is the



**FIGURE 4:** Hypomodified tRNAs associate with actively translating ribosomes. Polysome profiles of wild-type cells grown at (A) 30 or (B) 37°C for 3 h. RNA was extracted from indicated fractions and run on an agarose gel containing ethidium bromide (C, 30°C; D, 37°C) and subsequently analyzed by small RNA APM Northern blot analysis. Membranes were probed with probes specific for tHis<sup>GUG</sup> (E, 30°C; F, 37°C) or tLys<sup>UUU</sup> (G, I, 30°C; H, J, 37°C).

newly synthesized tRNA transcripts that are the substrates for the modification enzymes in the *URM1* pathway mutants.

### The tRNA thiolation components are unstable proteins

The observation that accumulation of hypomodified tRNAs is dependent on the production of new transcripts by pol III suggested that regulation of tRNA modification pathways could be responsible for the observed differences in tRNA thiolation levels. Modulation of the activity and/or abundance of *ELP* (mcm<sup>5</sup>U<sub>34</sub>) or *URM1* (s<sup>2</sup>U<sub>34</sub>) pathway components could explain changes in tRNA thiolation levels.

To examine the abundance of *ELP* and *URM1* pathway components, we generated a collection of strains in which a gene was modified at the endogenous genomic locus in a wild-type background to produce a C-terminally 3xHA tagged protein. We chose *NCS2*, *NCS6*, *TUM1*, and *UBA4* (s<sup>2</sup>U<sub>34</sub>), as well as *ELP2* and *ELP4*, which are required for the formation of the mcm<sup>5</sup>U<sub>34</sub> modification (Huang, 2005). We then examined the levels of tagged proteins in cells growing at 30°C or shifted to 37°C for 1, 3, or 5 h by Western blot. All of the proteins examined decreased in abundance when cells were grown at 37°C, although the magnitude and kinetics of the changes varied (Figure 5C).

Because heat shock is known to decrease translation and ribosome biogenesis (Gasch *et al.*, 2000; Shalgi *et al.*, 2012; Liu *et al.*, 2013), we wondered whether tRNA modification and translation are coordinated through stability of the modification machinery. We performed cycloheximide chase experiments to shut off translation in order to determine the stability of *URM1* pathway proteins. At 30 and 37°C, components of the *URM1* pathway were significantly less stable than Pgk1, which we used as a reference (Figure 5D, S4). Tum1-3HA and Ncs6-3HA in particular were degraded rapidly, although levels of Uba4-3HA and Ncs2-3HA also decreased signifi-

cantly. Thus the components of the *URM1* pathway depend on continuous translation for their stable expression in the cell (Supplemental Figure S4). In this manner, the levels of the thiolation components are matched to the level of translation.

We wanted to determine whether the 3xHA epitope tag affected protein function. We asked whether Ncs2-3HA, Ncs6-3HA, Tum1-3HA, Uba4-3HA, Elp2-3HA, and Elp4-3HA retained functional activity, using tRNA thiolation status as the readout. Impairment of function of any of the proteins would be expected to result in a decrease in the levels of tRNA thiolation. Thiolation in Tum1-3HA- and Elp2-3HA-expressing strains was comparable to tRNA thiolation in a wild-type strain not expressing tagged proteins, indicating that the tagged versions of Tum1 and Elp2 are fully functional. Thiolation in Ncs2-3HA and Elp4-3HA strains was mildly impaired, and tRNA thiolation in the Ncs6-3HA strain was also impaired. tRNA thiolation was absent in the Uba4-3HA strain, suggesting that the C-terminal 3HA tag interferes with the function of Uba4, a phenomenon that has been reported by others (Laxman *et al.*, 2013; Supplemental Figure S4C). Although the C-terminal HA tag in some cases impairs the functionality of proteins,

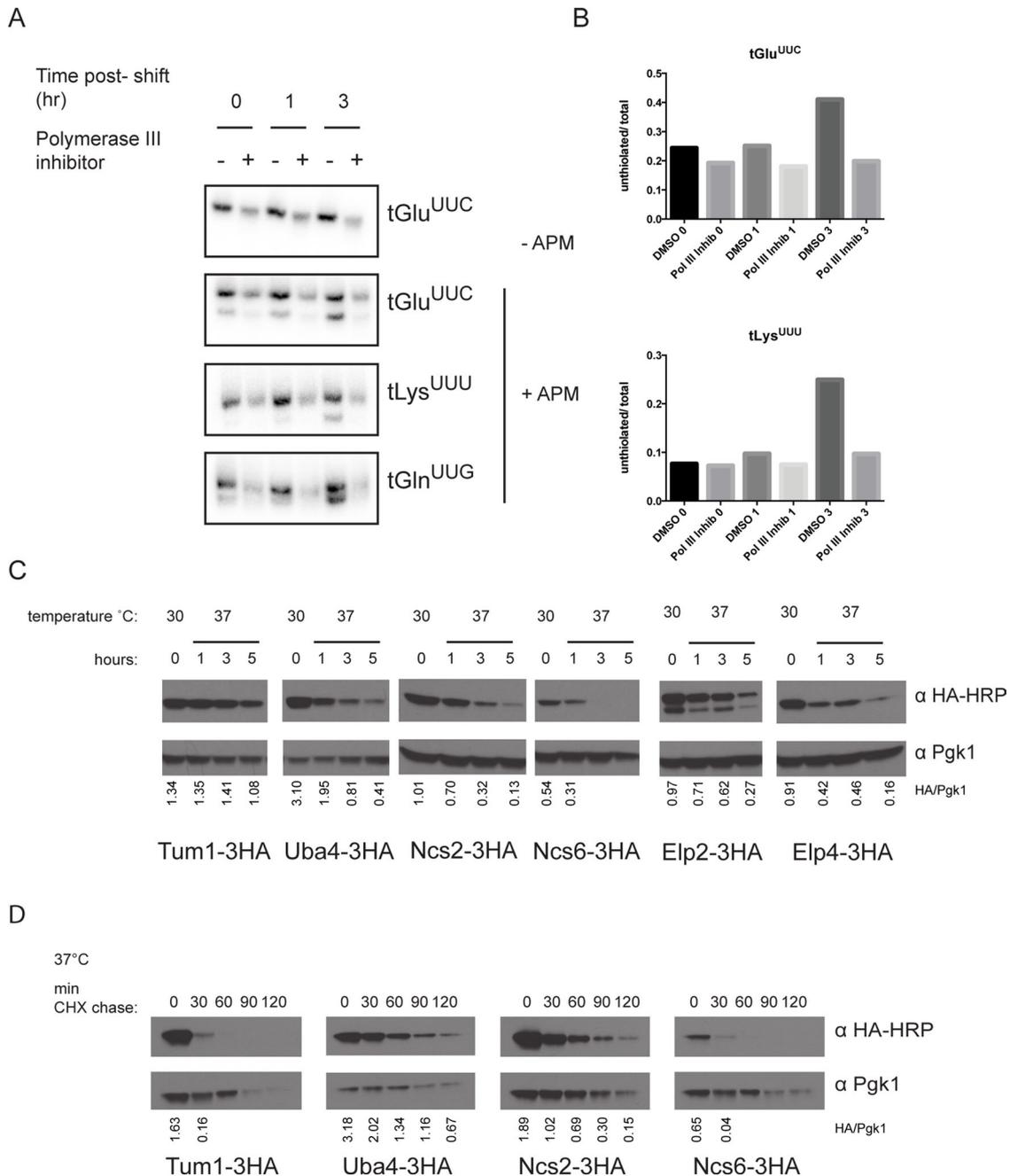
our data, taken as a whole, indicate that components of tRNA modification pathways are most likely coordinately regulated in response to changes in growth conditions.

### *URM1* pathway mutants display a resistance to tunicamycin

What would be the benefit of reducing levels of thiolated tRNAs? Under challenging growth conditions, it may be beneficial for cells to modulate rates of growth and protein synthesis/translation efficiency. While characterizing the stress responses of *URM1* pathway mutants, we exposed cells to tunicamycin. Tunicamycin induces ER stress by inhibition of Alg7, the yeast Dol-PP-GlcNAc transferase, which results in inhibition of N-linked glycosylation and accumulation in the ER of the C<sub>110</sub> isoprenoid dolichol. Tunicamycin induces the UPR through Ire1-mediated splicing of *HAC1* mRNA, an unconventional tRNA ligase-catalyzed reaction required for the production of functional Hac1 transcription factor mRNA. Surprisingly, we found that *urm1Δ* and *nsc6Δ* cells showed an increased resistance to tunicamycin compared with wild-type cells (as did *uba4Δ* and *elp2Δ* strains; unpublished data), both in serial dilution spot assays and in liquid culture (Figure 6, A and B).

To assess whether the tunicamycin-resistant phenotype we observed was a result of the tRNA modification function of Urm1, we expressed tRNA constructs in cells growing in the presence of tunicamycin. Expression of the tK construct in an *urm1Δ* background was sufficient to restore tunicamycin sensitivity; expression of the tE/K and tK/Q construct, as well as the tE/K/Q construct, also restored tunicamycin sensitivity (Figure 6B).

We reasoned that the observed tunicamycin-resistant phenotype of *urm1Δ* cells might be due to aberrant activation of the UPR. We therefore examined *HAC1* splicing by Northern blot analysis. There was no difference between wild-type and mutant cells in kinetics or extent of *HAC1* mRNA splicing in response to tunicamycin

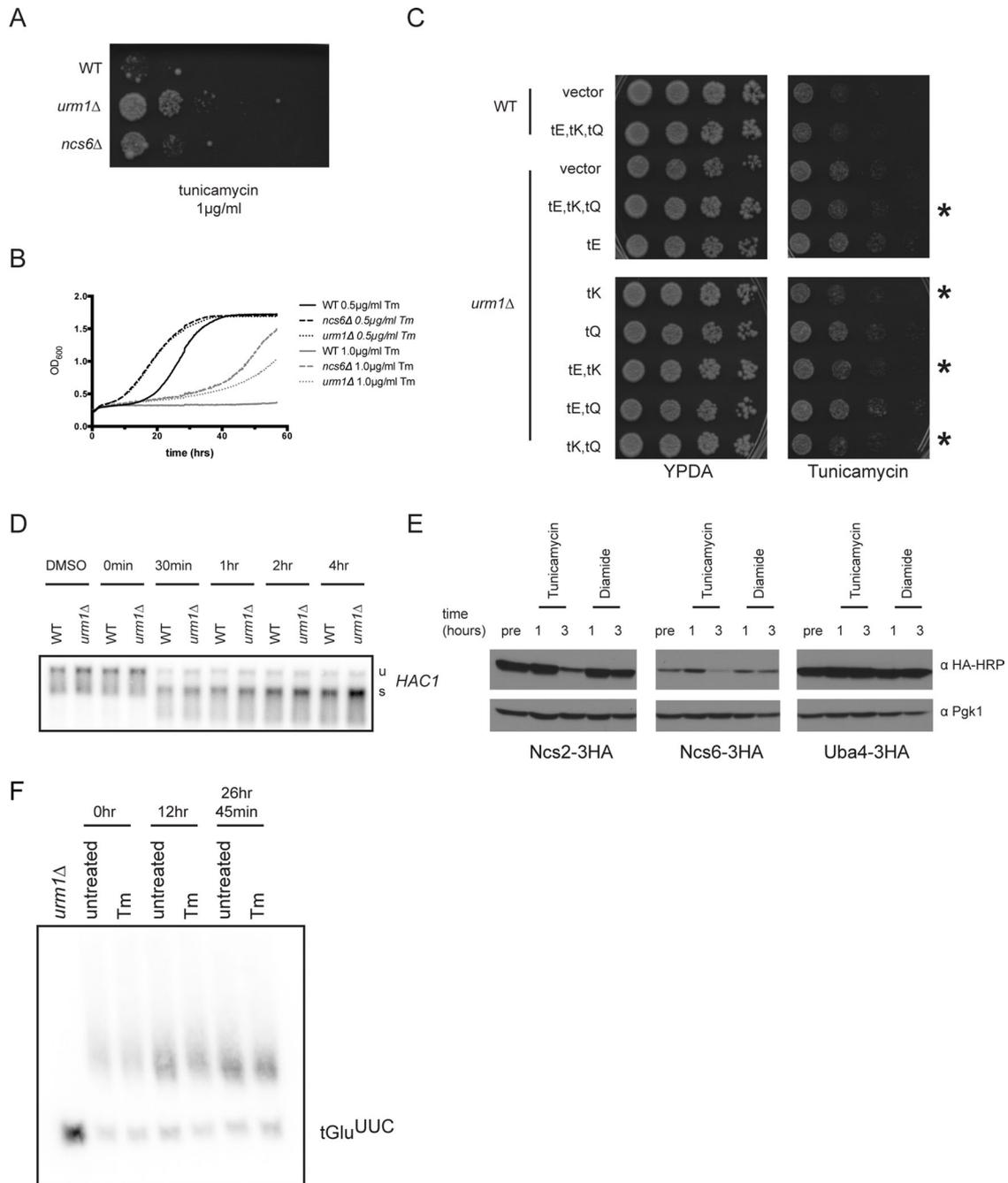


**FIGURE 5:** Accumulation of hypomodified tRNAs requires synthesis of new tRNA transcripts and is dependent on levels of tRNA-modification enzymes. (A) Northern blot analysis of RNA extracted from wild-type cells growing at 37°C for the indicated time points. Cells were pretreated with pol III inhibitor before the temperature shift. (B) Quantification of levels of unthiolated/total tGlu<sup>UUC</sup> or tLys<sup>UUU</sup> from blot in A. (C) Levels of C-terminally 3xHA-tagged Tum1, Uba4, Ncs2, Ncs6, Elp2, or Elp4 protein were determined by Western blot analysis. Indicated strains were grown at 30 or 37°C for the indicated time period. Membranes were probed with antibodies specific for the HA epitope tag or Pgk1 as a loading control. (D) Determination of protein stability using 3HA-tagged strains. Cells growing at 37°C were incubated with 200 µg/ml cycloheximide for indicated periods of time, and protein abundance was determined by Western blot analysis. For C and D, the level of HA-tagged protein relative to the level of Pgk1 at each time point was determined using ImageJ software.

treatment, although mutant cells appeared to have slightly higher levels of *HAC1* mRNA when grown in the presence of tunicamycin for an extended period of time (Figure 6D).

To determine whether there was any sign of differential tRNA modification in wild-type cells growing in the presence of tunicamycin, we used strains expressing epitope-tagged versions of the

*URM1* pathway components Ncs2, Ncs6, and Uba4. Cells treated with 0.5 µg/ml tunicamycin for 3 h had decreased levels of Ncs2-3HA and Ncs6-3HA, although there was no change in Uba4-3HA protein abundance (Figure 6E). As a comparison, we treated cells with diamide, which did not result in modulation of tRNA thiolation levels (Figure 3A); diamide-treated cells did not have altered levels



**FIGURE 6:** *URM1* pathway mutants are resistant to tunicamycin in a tRNA-dependent and *HAC1*-independent manner. (A) Fivefold serial dilutions of wild-type, *urm1* $\Delta$ , or *ncs6* $\Delta$  cells were plated onto YPDA plates containing 1  $\mu$ g/ml tunicamycin. (B) Growth curves of wild-type, *urm1* $\Delta$ , and *ncs6* $\Delta$  cells grown in YPDA containing tunicamycin at 0.5 or 1.0  $\mu$ g/ml final concentration. Average growth of three colonies. (C) Fivefold serial dilutions of wild-type or *urm1* $\Delta$  cells harboring empty vector or a 2  $\mu$  plasmid expressing the tRNA(s) indicated were plated onto YPDA plates containing 0.5  $\mu$ g/ml tunicamycin. Stars indicate tRNA constructs conferring sensitivity to tunicamycin. (D) Northern blot of RNA extracted from wild-type or *urm1* $\Delta$  cells grown in YPDA and treated with DMSO or 1  $\mu$ g/ml tunicamycin for the indicated time period. Membranes were probed for *HAC1*; the locations of unspliced (u) and spliced (s) versions of *HAC1* transcript are indicated. (E) Levels of 3xHA-tagged Ncs2, Ncs6, or Uba4 proteins, determined by Western blot analysis, in cells treated with tunicamycin (0.5  $\mu$ g/ml) or diamide (4 mM) for the indicated time period. (F) Small RNA APM Northern blot analysis of RNA extracted from wild-type cells treated with 0.5  $\mu$ g/ml tunicamycin for the indicated time period; membrane was probed for tGlu<sup>UUC</sup>.

of Ncs2-3HA, Ncs6-3HA, or Uba4-3HA at 1 or 3 h of exposure to diamide compared with the 0-h time point (Figure 6E). Wild-type cells thus down-regulate components of the *URM1* pathway under

challenging growth conditions. We directly examined tRNA thiolation status in cells exposed to tunicamycin to determine whether there was a corresponding decrease in tRNA thiolation levels. We

found, however, that tRNA thiolation did not appear to be decreased when cells were exposed to tunicamycin, even for prolonged time periods (Figure 6F). This indicates that there may be other factors, besides the levels of tRNA modification enzymes, that play a role in determining the final levels of modified tRNAs.

## DISCUSSION

A lack of tRNA modifications is likely to have multiple effects, both direct and indirect. Direct effects would be attributable directly to the altered properties of unmodified tRNAs. Indirect effects would be due to downstream consequences of altered translation and/or compensatory mechanisms associated with defects in tRNA thiolation marshaled by mutant cells.

We demonstrated that bulk translation is subtly diminished by defects in tRNA thiolation under standard growth conditions but that there are codon-specific defects in translation. Our data are consistent with genetic evidence in yeast that indicates that the  $mcm^5s^2U_{34}$  modification enhances the reading of A-ending codons (Johansson *et al.*, 2008; Rezgui *et al.*, 2013; Rodriguez-Hernandez *et al.*, 2013). It seems likely that the primary role of the  $mcm^5s^2U_{34}$  modification is in promoting efficient translation. Recent studies indicate that transcripts enriched in AAA, GAA, and CAA codons (recognized by  $U_{34}$  thiolated tRNAs) correspond to proteins whose levels are down-regulated in *urm1Δ* mutant cells and that these proteins are involved in anabolic/growth-related biological processes such as translation, rRNA processing, and ribosomal subunit biogenesis (Laxman *et al.*, 2013; Rezgui *et al.*, 2013). In fission yeast, specific stress protein transcripts biased toward AAA (vs. AAG) codons were translated less efficiently in cells lacking *URM1*-pathway tRNA-modification enzymes (Fernández-Vázquez *et al.*, 2013). Our results demonstrating that there appear to be codon specific defects in translation are consistent with these observations. Taken together, these results demonstrate that in *URM1* pathway mutants, there are direct effects on the translation of A-ending codons and that these translational defects result in decreased protein production from transcripts that are enriched in these A-ending codons even if bulk protein synthesis is not greatly affected in mutant cells.

Our analysis of *URM1* pathway mutants further revealed that defects in tRNA modification have indirect consequences. Specifically, the Hsf1-dependent HSR is activated in *urm1Δ* cells in a tRNA-dependent manner. The HSR elicits the transcription of chaperones (Gasch *et al.*, 2000), and the HSR overlaps significantly with the cytosolic unfolded protein response (UPR-Cyto) that cells mount when unfolded proteins accumulate in the cytosol (Casagrande *et al.*, 2000; Trotter, 2002; Metzger and Michaelis, 2009; Geiler-Samerotte *et al.*, 2011). How hypomodified tRNAs activate stress pathways remains unclear, but there are several plausible hypotheses. The functional consequences of hypomodified  $U_{34}$  nucleosides may mimic tRNA depletion in cells, causing proteotoxic stress and decreased growth rates (Yona *et al.*, 2013). Hypomodified tRNAs could affect translation elongation and interfere with cotranslational protein folding. *Urm1Δ* cells may experience protein folding-related stress that, while minor, is sufficient to elicit a transcriptional response. Alternatively, differences in gene expression in *urm1Δ* cells (compared with wild-type cells) could trigger activation of the HSR. Further studies are needed to distinguish between these models. There is some evidence that perturbation of tRNA-modification pathways causes indirect effects as well; mutants lacking *TRM9* show signs of stress (Patil *et al.*, 2012a), and a study examining genome-wide mRNA abundance and ribosome profiling data in budding yeast demonstrated that cells defective in formation of the  $mcm^5s^2U_{34}$  modification show a modest translational up-regulation of the nutri-

ent-responsive transcription factor Gcn4 (Zinshteyn and Gilbert, 2013). Defects in translation that result from the loss of this modification are unlikely to affect translation of *GCN4*; its translation is regulated by a series of short upstream open reading frames (uORFs), which are sufficient to recapitulate translational up-regulation in modification-deficient mutants (Zinshteyn and Gilbert, 2013). These uORFs do not contain codons recognized by thiolated tRNAs (Hinnebusch, 1984). Instead, differences in tRNA processing or charging might account for up-regulation of Gcn4 in modification-deficient cells (de Aldana *et al.*, 1994; Qiu *et al.*, 2000; Daugeron *et al.*, 2011). Compensatory changes in gene expression in mutant cells might likewise contribute. Our data are consistent with the notion that defects in tRNA modification have multiple downstream consequences, including activation of multiple stress response pathways.

There is increasing evidence that tRNA modifications are important in the regulation of gene expression at the level of translation (Begley *et al.*, 2007; Bauer *et al.*, 2012; Chan *et al.*, 2012; Patil *et al.*, 2012b). We identified a condition, growth at 37°C, that results in the accumulation of unthiolated tRNAs in wild-type cells. These tRNAs are stable, and thus hypomodified tRNAs constitute a distinct population whose altered physical properties may serve to fine tune gene expression by controlling efficiency of translation. Previous work demonstrated that mitochondrially encoded  $tLys^{UUU}$  was hypomodified at 37°C, although mitochondrial  $tGlu$  and  $tGln$  tRNAs were not differentially modified (Kamenski *et al.*, 2007). This is in contrast to our finding that in the cytoplasm,  $tGlu^{UUC}$ ,  $tGln^{UUG}$ , and  $tLys^{UUU}$  are all differentially modified at high temperatures. This is not surprising, as the modification pathways for mitochondrial and cytoplasmic tRNAs are distinct (Umeda, 2004; Leidel *et al.*, 2009).

The synthesis of tRNA transcripts by pol III is required for the accumulation of hypomodified tRNAs, indicating that differential modification of newly transcribed tRNAs is responsible for the appearance of unthiolated tRNAs. At elevated temperatures, the levels of *URM1* pathway components decrease as levels of unthiolated tRNAs increase; these proteins are unstable and are rapidly degraded. We propose a mechanism for the modulation of tRNA modification levels in which specific stress conditions result in a decrease in translation and a subsequent decrease in levels of tRNA modification enzymes, leading to the accumulation of hypomodified tRNAs, which serves to further modulate translation. These changes in translation affect cellular growth and stress sensitivity; the phenotypes of *urm1Δ* cells indicate that stress sensitivity could be mediated at least in part through tRNA modification-dependent modulation of growth rate and activation of the HSR. In other words, translation and tRNA thiolation are mutually reinforcing processes and are linked to stress sensitivity and resistance phenotypes (Supplemental Figure S5).

Under certain stress conditions, *URM1* mutants have an advantage; *URM1* pathway mutants are more resistant to tunicamycin than wild-type cells in a tRNA-dependent manner. The tunicamycin-resistant phenotype of *URM1* pathway mutants might be attributable to the slow growth that results from hypomodified tRNAs. Resistance to tunicamycin has been observed in ribosomal protein deletion strains; growth rates of deletion strains in the presence of tunicamycin were inversely correlated with growth rates in the absence of tunicamycin (Steffen *et al.*, 2012), supporting a link between decreased translation, decreased growth, and resistance to stress. Although we observed that wild-type cells treated with tunicamycin had decreased levels of Ncs2-3HA and Ncs6-3HA compared to untreated cells, we were unable to detect changes in tRNA thiolation under these same conditions; although this is somewhat puzzling, this could be due to other changes in cellular processes that result

from exposure to tunicamycin and also have an effect on tRNA biology. The link between growth rate and response to stress has been established; the environmental stress response is a generalized transcriptional response mounted by yeast cells in response to a decrease in growth rate that is believed to result from exposure to any of a variety of stresses (Brauer *et al.*, 2008; Lu *et al.*, 2009).

Our observations are consistent with a model in which modulation of tRNA thiolation levels is a long-term adaptation to an ongoing stress. tRNA thiolation levels under a specific condition would be the combined result of a variety of factors, including rate of tRNA synthesis, abundance of *URM1* pathway transcripts, the magnitude and duration of changes in translation, and rate of protein degradation. Different types of stress might thus be expected to have different consequences for tRNA thiolation.

## MATERIALS AND METHODS

### Yeast strains and growth

Yeast strains are derivatives of W303. Strains used are listed in Supplemental Table S1. Yeast were grown according to standard techniques. Strains containing gene deletions of C-terminally 3xHA-tagged versions of protein were generated as described (Longtine *et al.*, 1998) and verified by PCR (and Western blot for tagged strains). When required, strains of the necessary mating type or double mutants were generated using standard yeast genetic techniques.

### Polysome profile analysis

Yeast cultures were grown to mid-log phase; cycloheximide was added to a final concentration of 0.1 mg/ml before collection. Cells were lysed by the addition of polysome lysis buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES]—KOH, pH 7.4, 2 mM MgOAc, and 100 mM KOAc with 0.1 mg/ml cycloheximide, 3 mM dithiothreitol [DTT], and 1% Triton X-10) and acid-washed glass beads, followed by vortexing. Between 5 and 10  $A_{260}$  units of cleared lysate was loaded onto 10–50% sucrose gradients (prepared in polysome lysis buffer containing cycloheximide and DTT) and subjected to ultracentrifugation (35,000 rpm, 3 h, using a SW41-Ti rotor). Gradients were analyzed using a Labconco Auto Densi-Flow Gradient Fractionator connected to an Isco Tris pump with constant monitoring at OD<sub>254</sub>. When required, fractions were collected for RNA preparation.

### Radioactive amino acid incorporation

Cells were grown in minimal media supplemented with 2% glucose and appropriate amino acids. [<sup>35</sup>S]methionine/[<sup>35</sup>S]cysteine (Easy Tag; Perkin Elmer, Waltham, MA) was added to aliquots of actively growing cells, and at indicated time points, 200  $\mu$ l of labeled culture was added to an equal amount of cold 20% trichloroacetic acid (TCA) and incubated on ice. Cells were lysed by boiling for 10 min and then incubated on ice for 10 min. Aliquots of lysate were spotted onto glass microfiber filters (Whatman GF/B; Whatman-GE Healthcare Life Sciences, Little Chalfont, United Kingdom) and allowed to dry completely. Filters were washed twice with 5% TCA, rinsed briefly with acetone, and dried. Scintillation fluid was added to filters and then counts per minute were determined, normalized to OD<sub>600</sub> readings taken using duplicate, nonlabeled cultures.

### Serial dilutions

Overnight cultures of cells were diluted into fresh media and allowed to grow for several hours and then rinsed in phosphate-buffered saline and resuspended to an OD<sub>600</sub> of 0.1. Aliquots of fivefold serial dilutions were plated onto solid media, and plates were scanned after various growth times. For strains containing 2  $\mu$  plasmids

requiring selection/maintenance, yeast were grown until the time of plating in the required selective media and then plated onto the media indicated.

### RNA isolation

Total RNA was prepared by standard acid phenol/chloroform extraction as described (Collart and Oliviero, 2001); after precipitation and rinsing, RNA was resuspended in diethylpyrocarbonate-treated water for storage.

### Northern blots

For small RNA northern blots, RNA samples were denatured at 80°C for 10 min and then run on 8% polyacrylamide–urea gels (Ureagel; National Diagnostics, Atlanta, GA) in 0.5x Tris/borate/EDTA. For gels containing APM, APM was added to a final concentration of 20  $\mu$ M. Gels were transferred to Genescreen Plus membrane (Perkin Elmer) and then ultraviolet (UV) cross-linked. Membranes were probed with end-labeled  $\gamma$ -<sup>32</sup>P (Perkin Elmer) DNA oligonucleotide probes (Integrated DNA Technologies, Coralville, IA) complementary to the tRNA species of interest. Sequences of probes used are listed in Supplemental Table S2. After hybridization and washing steps, membranes were exposed to phosphorimager screens and imaged using BAS/Fuji imager (Fujifilm, Tokyo, Japan). Quantifications were carried out using Multiguage imaging software. For Northern blots, denatured RNA samples were run on formaldehyde-agarose gels. Gels were transferred to Genescreen Plus membrane by overnight capillary transfer in 10x saline-sodium citrate buffer and then UV cross-linked. Probes were prepared using the Amersham Megaprime DNA labeling system (GE Healthcare, Little Chalfont, United Kingdom) and  $\alpha$ -<sup>32</sup>P dTTP (Perkin Elmer); probes were PCR products generated using yeast genomic DNA as a template and using primers specific for the transcript of interest. After hybridization and washing steps, membranes were exposed to phosphorimager screens and imaged using BAS/Fuji imager. Quantifications were carried out using Multiguage imaging software.

### Temperature shift/stress experiments

Yeast cells were grown to mid-log phase at 30°C and resuspended in media prewarmed to 37°C or in 30°C containing the indicated concentration of a given compound. For experiments using polymerase III inhibitor, cells were preincubated with ML-60218 (Wu *et al.*, 2003; EMD Millipore-Merck, Darmstadt, Germany) before temperature shift; alternatively, ML-60218 was added at the time of the temperature shift. For tRNA stability experiments, samples were collected at time 0 immediately after resuspension in 37°C media and after 3 h of growth at high temperatures. For cycloheximide chase experiments, cycloheximide was added to a final concentration of 200  $\mu$ g/ml, and aliquots of cells were harvested at various times.

### Stress response reporter assays

Reporter constructs consisted of GFP (STRE, UPRE constructs) or YFP (HSE constructs) with a series of 4xHSE, 4xSTRE, or 4xUPRE sequences in a crippled *CYC1* promoter in a single integrating vector backbone. Constructs were integrated into wild-type or mutant yeast strain backgrounds at the *leu2* locus. For tRNA expression assays, strains containing the reporter constructs were transformed with the 2  $\mu$  plasmids expressing tEKQ. For assays, cells were grown from single colonies in SD complete medium (or SD –Ura for tRNA strains) overnight, diluted into fresh medium, and then left untreated or treated with 1 mM AZC or 1  $\mu$ g/ml tunicamycin for 4 h at 30°C. Fluorescent reporters were measured by flow cytometry in a BD

LSRFortessa equipped with a high-throughput sampler. Data were analyzed using FlowJo.

### Translation extracts

Translation extracts were prepared from L-A (–) strains of the indicated genotype essentially as described (Iizuka *et al.*, 1994) and treated with micrococcal nuclease (New England Biolabs, Ipswich, MA) immediately before use. m7GpppG (New England Biolabs)-capped transcripts were produced using T7 RNA polymerase (Promega, Madison, WI) using plasmids encoding either a leaderless luciferase or the 12xCAA/12xGAG leader luciferase constructs (which were constructed by ligating annealed oligonucleotides into the luciferase base construct). (The L-A (–) strain and plasmid encoding leaderless luciferase were gifts of the Gilbert Lab, Massachusetts Institute of Technology, Cambridge, MA.) RNA was analyzed by agarose gel electrophoresis before use. Translation reactions (15  $\mu$ l) containing 9.5  $\mu$ l of translation extract and 50 ng of mRNA (final concentrations 22 mM HEPES-KOH, pH 7.4, 120 mM potassium acetate, 1.5 mM magnesium acetate, 0.75 mM ATP, 0.1 mM GTP, 25 mM creatine phosphate, 0.04 mM each amino acid, 1.7 mM DTT, 5  $\mu$ g of creatine kinase, 1  $\mu$ l of 0.6 mM methionine, and 0.5  $\mu$ l of RNasein Plus [Promega]) were incubated at 23°C for 60 min. Reactions were stopped with the addition of GloLysis buffer, and luciferase activities were measured using the Steady-Glo Luciferase Assay System (Promega).

### Western blotting

Protein samples were prepared from equivalent amounts of yeast cultures using an alkaline SDS lysis protocol as described in Haar (2007). Equal volumes of lysates were run on 10% SDS–PAGE gels, and after electrophoresis, proteins were transferred to polyvinylidene difluoride membranes. Anti-HA–Peroxidase, High-Affinity, Rat monoclonal (3F10) was used for detection of 3-HA tagged proteins (12013819001; Roche, Basel, Switzerland), and PGK1 was detected using Phosphoglycerate Kinase Monoclonal Antibody, Mouse (22C5D8; Life Technologies, Carlsbad, CA) and anti-mouse immunoglobulin G–peroxidase (NXA931; GE Healthcare). Quantifications were carried out using ImageJ (Schneider *et al.*, 2012).

### Plasmids

Plasmids for tRNA expression were derivatives of the *URA3* marked 2  $\mu$  pRS426 plasmid (Christianson *et al.*, 1992). For construction of the plasmids, tQ (UUG)B, tE(UUC)B, and tK (UUU)D sequences were PCR amplified from genomic DNA using primers containing *HindIII* and *BamHI* restriction sites and cloned individually into pRS426 (or other vectors in the pRS420 series). Initial constructs were then used for subcloning to create constructs in which combinations of any two or all three tRNAs could be expressed from pRS426. Plasmids included pRS426/tE, pRS426/tK, pRS426/tQ, pRS426/tEK, pRS426/tEQ, pRS426/tKQ, and pRS426/tEKQ. Construction of GFP/YFP reporter plasmids is described in *Stress response reporter assays*.

### ACKNOWLEDGMENTS

We thank C. Schlieker, A. Van der Veen, and K. Stribis for useful discussions and input, N. Yoder for help with the APM synthesis, T. Carlile for useful discussions, technical assistance, and critical reading of the manuscript, and A. Amon for critical reading of the manuscript. We acknowledge the lab of W. Gilbert for strains. D.P. was supported by a National Institutes of Health Early Independence Award (DP5 OD017941-01) and a Cancer Research Fellowship from the Alexander and Margaret Stewart Trust.

### REFERENCES

- Agarwala SD, Blitzblau HG, Hochwagen A, Fink GR (2012). RNA methylation by the MIS complex regulates a cell fate decision in yeast. *PLoS Genet* 8, e1002732.
- Agris PF, Vendeix FAP, Graham WD (2007). tRNAs wobble decoding of the genome: 40 years of modification. *J Mol Biol* 366, 1–13.
- Alexandrov A, Chernyakov I, Gu W, Hiley SL, Hughes TR, Grayhack EJ, Phizicky EM (2006). Rapid tRNA decay can result from lack of nonessential modifications. *Mol Cell* 21, 87–96.
- Bauer F, Matsuyama A, Candiracci J, Dieu M, Scheliga J, Wolf DA, Yoshida M, Hermand D (2012). Translational control of cell division by elongator. *Cell Rep* 1, 424–433.
- Begley U, Dyavaiah M, Patil A, Rooney JP, DiRenzo D, Young CM, Conklin DS, Zitomer RS, Begley TJ (2007). Trm9-catalyzed tRNA modifications link translation to the DNA damage response. *Mol Cell* 28, 860–870.
- Bjork GR, Huang B, Persson OP, Bystrom AS (2007). A conserved modified wobble nucleoside (mcm5s2U) in lysyl-tRNA is required for viability in yeast. *RNA* 13, 1245–1255.
- Brauer MJ, Huttenhower C, Airolidi EM, Rosenstein R, Matese JC, Gresham D, Boer VM, Troyanskaya OG, Botstein D (2008). Coordination of growth rate, cell cycle, stress response, and metabolic activity in yeast. *Mol Biol Cell* 19, 352–367.
- Casagrande R, Stern P, Diehn M, Shamu C, Osario M, Zúñiga M, Brown PO, Ploegh H (2000). Degradation of proteins from the ER of *S. cerevisiae* requires an intact unfolded protein response pathway. *Mol Cell* 5, 729–735.
- Chan CTY, Dyavaiah M, DeMott MS, Taghizadeh K, Dedon PC, Begley TJ (2010). A quantitative systems approach reveals dynamic control of tRNA modifications during cellular stress. *PLoS Genet* 6, e1001247.
- Chan CTY, Pang YLJ, Deng W, Babu IR, Dyavaiah M, Begley TJ, Dedon PC (2012). Reprogramming of tRNA modifications controls the oxidative stress response by codon-biased translation of proteins. *Nat Commun* 3, 937.
- Chernyakov I, Whipple JM, Kotelawala L, Grayhack EJ, Phizicky EM (2008). Degradation of several hypomodified mature tRNA species in *Saccharomyces cerevisiae* is mediated by Met22 and the 5'–3' exonucleases Rat1 and Xrn1. *Genes Dev* 22, 1369–1380.
- Christianson TW, Sikorski RS, Dante M, Shero JH, Hieter P (1992). Multifunctional yeast high-copy-number shuttle vectors. *Gene* 110, 119–122.
- Collart MA, Oliviero S (2001). Preparation of yeast RNA. *Curr Protoc Mol Biol* Chapter 13, Unit 13.12.
- Cox JS, Walter P (1996). A novel mechanism for regulating activity of a transcription factor that controls the unfolded protein response. *Cell* 87, 391–404.
- Daugeron MC, Lenstra TL, Frizzarin M, El Yacoubi B, Liu X, Baudin-Bailieu A, Lijnzaad P, Decourty L, Saveanu C, Jacquier A, *et al.* (2011). Gcn4 misregulation reveals a direct role for the evolutionary conserved EKC/KEOPS in the t6A modification of tRNAs. *Nucleic Acids Res* 39, 6148–6160.
- de Aldana CV, Wek RC, Segundo PS, Truesdell AG, Hinnebusch AG (1994). Multicopy tRNA genes functionally suppress mutations in yeast eIF-2 alpha kinase GCN2: evidence for separate pathways coupling GCN4 expression to unchanged tRNA. *Mol Cell Biol* 14, 7920–7932.
- Decatur WA, Fournier MJ (2002). rRNA modifications and ribosome function. *Trends Biochem Sci* 27, 344–351.
- Esberg A, Huang B, Johansson MJO, Byström AS (2006). Elevated levels of two tRNA species bypass the requirement for elongator complex in transcription and exocytosis. *Mol Cell* 24, 139–148.
- Fan-Minogue H, Bedwell DM (2007). Eukaryotic ribosomal RNA determinants of aminoglycoside resistance and their role in translational fidelity. *RNA* 14, 148–157.
- Fernández-Vázquez J, Vargas-Pérez I, Sansó M, Buhne K, Carmona M, Paulo E, Hermand D, Rodríguez-Gabriel M, Ayté J, Leidel S, *et al.* (2013). Modification of tRNA<sup>LysUUU</sup> by elongator is essential for efficient translation of stress mRNAs. *PLoS Genet* 9, e1003647.
- Furukawa K, Mizushima N, Noda T, Ohsumi Y (2000). A protein conjugation system in yeast with homology to biosynthetic enzyme reaction of prokaryotes. *J Biol Chem* 275, 7462–7465.
- Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D, Brown PO (2000). Genomic expression programs in the response of yeast cells to environmental changes. *Mol Biol Cell* 11, 4241–4257.
- Geiler-Samerotte KA, Dion MF, Budnik BA, Wang SM, Hartl DL, Drummond DA (2011). Misfolded proteins impose a dosage-dependent fitness cost and trigger a cytosolic unfolded protein response in yeast. *Proc Natl Acad Sci USA* 108, 680–685.

- Goehring AS, Rivers DM, Sprague GF (2003a). Attachment of the ubiquitin-related protein Urm1p to the antioxidant protein Ahp1p. *Eukaryot Cell* 2, 930–936.
- Goehring AS, Rivers DM, Sprague GF (2003b). Urymylation: a ubiquitin-like pathway that functions during invasive growth and budding in yeast. *Mol Biol Cell* 14, 4329–4341.
- Haar von der T (2007). Optimized protein extraction for quantitative proteomics of yeasts. *PLoS One* 2, e1078.
- Hinnebusch AG (1984). Evidence for translational regulation of the activator of general amino acid control in yeast. *Proc Natl Acad Sci USA* 81, 6442–6446.
- Huang B, Johansson MJO, Bystrom AS (2005). An early step in wobble uridine tRNA modification requires the Elongator complex. *RNA* 11, 424–436.
- Huang B, Lu J, Bystrom AS (2008). A genome-wide screen identifies genes required for formation of the wobble nucleoside 5-methoxycarbonylmethyl-2-thiouridine in *Saccharomyces cerevisiae*. *RNA* 14, 2183–2194.
- Igloi GLG (1988). Interaction of tRNAs and of phosphorothioate-substituted nucleic acids with an organomercurial. Probing the chemical environment of thiolated residues by affinity electrophoresis. *Biochemistry* 27, 3842–3849.
- Iizuka N, Najita L, Franzusoff A, Sarnow P (1994). Cap-dependent and cap-independent translation by internal initiation of mRNAs in cell extracts prepared from *Saccharomyces cerevisiae*. *Mol Cell Biol* 14, 7322–7330.
- Jack K, Bellodi C, Landry DM, Niederer RO, Meskauskas A, Musalgaonkar S, Kopmar N, Krasnykh O, Dean AM, Thompson SR, et al. (2011). rRNA pseudouridylation defects affect ribosomal ligand binding and translation from yeast to human cells. *Mol Cell* 44, 660–666.
- Johansson MJO, Esberg A, Huang B, Bjork GR, Bystrom AS (2008). Eukaryotic wobble uridine modifications promote a functionally redundant decoding system. *Mol Cell Biol* 28, 3301–3312.
- Kalhor HR, Clarke S (2003). Novel methyltransferase for modified uridine residues at the wobble position of tRNA. *Mol Cell Biol* 23, 9283–9292.
- Kamenski P, Kolesnikova O, Jubenet V, Entelis N, Krashennikov IA, Martin RP, Tarassov I (2007). Evidence for an adaptation mechanism of mitochondrial translation via tRNA import from the cytosol. *Mol Cell* 26, 625–637.
- Kobayashi N, McEntee K (1993). Identification of cis and trans components of a novel heat shock stress regulatory pathway in *Saccharomyces cerevisiae*. *Mol Cell Biol* 13, 248–256.
- Kohno K, Normington K, Sambrook J, Gething MJ, Mori K (1993). The promoter region of the yeast *Kar2* (*Bip*) gene contains a regulatory domain that responds to the presence of unfolded proteins in the endoplasmic reticulum. *Mol Cell Biol* 13, 877–890.
- Kramer EB, Vallabhaneni H, Mayer LM, Farabaugh PJ (2010). A comprehensive analysis of translational missense errors in the yeast *Saccharomyces cerevisiae*. *RNA* 16, 1797–1808.
- Laxman S, Sutter BM, Wu X, Kumar S, Guo X, Trudgian DC, Mirzaei H, Tu BP (2013). Sulfur amino acids regulate translational capacity and metabolic homeostasis through modulation of tRNA thiolation. *Cell* 154, 416–429.
- Leidel S, Pedrioli PGA, Bucher T, Brost R, Costanzo M, Schmidt A, Aebersold R, Boone C, Hofmann K, Peter M (2009). Ubiquitin-related modifier Urm1 acts as a sulphur carrier in thiolation of eukaryotic transfer RNA. *Nature* 457, 228–232.
- Liang XH, Liu Q, Fournier MJ (2009). Loss of rRNA modifications in the decoding center of the ribosome impairs translation and strongly delays pre-rRNA processing. *RNA* 15, 1716–1728.
- Liu B, Han Y, Qian S-B (2013). Cotranslational response to proteotoxic stress by elongation pausing of ribosomes. *Mol Cell* 49, 453–463.
- Longtine MS, McKenzie A, Demarini DJ, Shah NG, Wach A, Brachet A, Philippsen P, Pringle JR (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* 14, 953–961.
- Lu C, Brauer MJ, Botstein D (2009). Slow growth induces heat-shock resistance in normal and respiratory-deficient yeast. *Mol Biol Cell* 20, 891–903.
- Lu J, Huang B, Esberg A, Johansson MJO, Byström AS (2005). The *Kluyveromyces lactis* gamma-toxin targets tRNA anticodons. *RNA* 11, 1648–1654.
- Martínez-Pastor MT, Marchler G, Schüller C, Marchler-Bauer A, Ruis H, Estruch F (1996). The *Saccharomyces cerevisiae* zinc finger proteins *Msn2p* and *Msn4p* are required for transcriptional induction through the stress response element (STRE). *EMBO J* 15, 2227–2235.
- Metzger MB, Michaelis S (2009). Analysis of quality control substrates in distinct cellular compartments reveals a unique role for *Rpn4p* in tolerating misfolded membrane proteins. *Mol Biol Cell* 20, 1006–1019.
- Mori K, Kawahara T, Yoshida H, Yanagi H, Yura T (1996). Signalling from endoplasmic reticulum to nucleus: transcription factor with a basic-leucine zipper motif is required for the unfolded protein-response pathway. *Genes Cells* 1, 803–817.
- Mori K, Ogawa N, Kawahara T, Yanagi H, Yura T (1998). Palindrome with spacer of one nucleotide is characteristic of the cis-acting unfolded protein response element in *Saccharomyces cerevisiae*. *J Biol Chem* 273, 9912–9920.
- Mori K, Sant A, Kohno K, Normington K, Gething MJ, Sambrook JF (1992). A 22 bp cis-acting element is necessary and sufficient for the induction of the yeast *KAR2* (*Bip*) gene by unfolded proteins. *EMBO J* 11, 2583–2593.
- Murphy FV, Ramakrishnan V, Malkiewicz A, Agris PF (2004). The role of modifications in codon discrimination by tRNA<sup>LysUUU</sup>. *Nat Struct Mol Biol* 11, 1186–1191.
- Nakai Y, Nakai M, Hayashi H (2008). Thio-modification of yeast cytosolic tRNA requires a ubiquitin-related system that resembles bacterial sulfur transfer systems. *J Biol Chem* 283, 27469–27476.
- Nikawa J-I, Akiyoshi M, Hirata S, Fukuda T (1996). *Saccharomyces cerevisiae* IRE2/HAC1 is involved in IRE1-mediated *KAR2* expression. *Nucleic Acids Res* 24, 4222–4226.
- Patil A, Chan CTY, Dyavaiah M, Rooney JP, Dedon PC, Begley TJ (2012a). Translational infidelity-induced protein stress results from a deficiency in *Trm9*-catalyzed tRNA modifications. *RNA Biol* 99, 990–1001.
- Patil A, Dyavaiah M, Joseph F, Rooney JP, Chan CTY, Dedon PC, Begley TJ (2012b). Increased tRNA modification and gene-specific codon usage regulate cell cycle progression during the DNA damage response. *Cell Cycle* 11, 3656–3665.
- Pelham H (1982). A regulatory upstream promoter element in the *Drosophila* Hsp 70 heat-shock gene. *Cell* 30, 517–528.
- Phizicky EM, Hopper AK (2010). tRNA biology charges to the front. *Genes Dev* 24, 1832–1860.
- Preston MA, D’Silva S, Kon Y, Phizicky EM (2012). tRNA<sup>His</sup> 5-methylcytidine levels increase in response to several growth arrest conditions in *Saccharomyces cerevisiae*. *RNA* 19, 243–256.
- Qiu H, Hu C, Anderson J, Bjork GR, Sarkar S, Hopper AK, Hinnebusch AG (2000). Defects in tRNA processing and nuclear export induce GCN4 translation independently of phosphorylation of the alpha subunit of eukaryotic translation initiation factor 2. *Mol Cell Biol* 20, 2505–2516.
- Rezgui VAN, Tyagi K, Ranjan N, Konevega AL, Mittelstaet J, Rodnina MV, Peter M, Pedrioli PGA (2013). tRNA<sup>tKUUU</sup>, t<sup>QUUG</sup>, and t<sup>EUUC</sup> wobble position modifications fine-tune protein translation by promoting ribosome A-site binding. *Proc Natl Acad Sci USA* 110, 12289–12294.
- Rodriguez-Hernandez A, Spears JL, Gaston KW, Limbach PA, Gamper H, Hou Y-M, Kaiser R, Agris PF, Perona JJ (2013). Structural and mechanistic basis for enhanced translational efficiency by 2-thiouridine at the tRNA anticodon wobble position. *J Mol Biol* 425, 1–19.
- Rubio-Teixeira M (2007). Urymylation controls *Nil1p* and *Gln3p*-dependent expression of nitrogen-catabolite repressed genes in *Saccharomyces cerevisiae*. *FEBS Lett* 581, 541–550.
- Schlieker CD, Van der Veen AG, Damon JR, Spooner E, Ploegh HL (2008). A functional proteomics approach links the ubiquitin-related modifier Urm1 to a tRNA modification pathway. *Proc Natl Acad Sci USA* 105, 18255–18260.
- Schmitz J, Chowdhury MM, Hänzelmann P, Nitz M, Lee E-Y, Schindelin H, Leimkühler S (2008). The sulfurtransferase activity of *Uba4* presents a link between ubiquitin-like protein conjugation and activation of sulfur carrier proteins. *Biochemistry* 47, 6479–6489.
- Schneider CA, Rasband WS, Eliceiri KW (2012). NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 9, 671–675.
- Schwartz S, Agarwala SD, Mumbach MR, Jovanovic M, Mertins P, Shishkin A, Tabach Y, Mikkelsen TS, Satija R, Ruvkun G, et al. (2013). High-resolution mapping reveals a conserved, widespread, dynamic mRNA methylation program in yeast meiosis. *Cell* 155, 1409–1421.
- Shalgi R, Hurt JA, Krykbaeva I, Taipale M, Lindquist S, Burge CB (2012). Widespread regulation of translation by elongation pausing in heat shock. *Mol Cell* 1–14.
- Simon JR, Treger JM, McEntee K (1999). Multiple independent regulatory pathways control *UBI4* expression after heat shock in *Saccharomyces cerevisiae*. *Mol Microbiol* 31, 823–832.
- Singh S, Tonelli M, Tyler RC, Bahrami A, Lee MS, Markley JL (2005). Three-dimensional structure of the AAH26994.1 protein from *Mus musculus*, a putative eukaryotic Urm1. *Protein Sci* 14, 2095–2102.
- Slater MR, Craig EA (1987). Transcriptional regulation of an hsp70 heat shock gene in the yeast *Saccharomyces cerevisiae*. *Mol Cell Biol* 7, 1906–1916.

- Steffen KK, McCormick MA, Pham KM, MacKay VL, Delaney JR, Murakami CJ, Kaerberlein M, Kennedy BK (2012). Ribosome deficiency protects against ER stress in *Saccharomyces cerevisiae*. *Genetics* 191, 107–118.
- Trotter EW (2002). Misfolded proteins are competent to mediate a subset of the responses to heat shock in *Saccharomyces cerevisiae*. *J Biol Chem* 277, 44817–44825.
- Tuerto F, Liebers R, Musch T, Schaefer M, Hofmann S, Kellner S, Frye M, Helm M, Stoecklin G, Lyko F (2012). RNA cytosine methylation by Dnm2 and NSun2 promotes tRNA stability and protein synthesis. *Nat Struct Mol Biol* 19, 900–905.
- Umeda N (2004). Mitochondria-specific RNA-modifying enzymes responsible for the biosynthesis of the wobble base in mitochondrial tRNAs: implications for the molecular pathogenesis of human mitochondrial diseases. *J Biol Chem* 280, 1613–1624.
- Van der Veen AG, Schorpp K, Schlieker C, Buti L, Damon JR, Spooner E, Ploegh HL, Jentsch S (2011). Role of the ubiquitin-like protein Urm1 as a noncanonical lysine-directed protein modifier. *Proc Natl Acad Sci USA* 108, 1763–1770.
- Wang X, Lu Z, Gomez A, Hon GC, Yue Y, Han D, Fu Y, Parisien M, Dai Q, Jia G, *et al.* (2013). N6-methyladenosine-dependent regulation of messenger RNA stability. *Nature* 505, 1–21.
- Whipple JM, Lane EA, Chernyakov I, D'Silva S, Phizicky EM (2011). The yeast rapid tRNA decay pathway primarily monitors the structural integrity of the acceptor and T-stems of mature tRNA. *Genes Dev* 25, 1173–1184.
- Wiederrecht G, Seto D, Parker CS (1988). Isolation of the gene encoding the *S. cerevisiae* heat shock transcription factor. *Cell* 54, 841–853.
- Wieser RR, Adam GG, Wagner AA, Schüller CC, Marchler GG, Ruis HH, Krawiec ZZ, Bilinski TT (1991). Heat shock factor-independent heat control of transcription of the CTT1 gene encoding the cytosolic catalase T of *Saccharomyces cerevisiae*. *J Biol Chem* 266, 12406–12411.
- Wu L, Pan J, Thoroddsen V, Wysong DR, Blackman RK, Bulawa CE, Gould AE, Ocain TD, Dick LR, Errada P, *et al.* (2003). Novel small-molecule inhibitors of RNA polymerase III. *Eukaryot Cell* 2, 256–264.
- Xu J, Zhang J, Wang L, Zhou J, Huang H, Wu J, Zhong Y, Shi Y (2006). Solution structure of Urm1 and its implications for the origin of protein modifiers. *Proc Natl Acad Sci USA* 103, 11625–11630.
- Yacoubi El B, Bailly M, de Crécy-Lagard V (2012). Biosynthesis and function of posttranscriptional modifications of transfer RNAs. *Annu Rev Genet* 46, 69–95.
- Yarian C (2002). Accurate translation of the genetic code depends on tRNA modified nucleosides. *J Biol Chem* 277, 16391–16395.
- Yona AH, Bloom-Ackermann Z, Frumkin I, Hanson-Smith V, Charpak-Amikam Y, Feng Q, Boeke JD, Dahan O, Pilpel Y (2013). tRNA genes rapidly change in evolution to meet novel translational demands. *Elife* 2, e01339.
- Zinshteyn B, Gilbert WV (2013). Loss of a conserved tRNA anticodon modification perturbs cellular signaling. *PLoS Genet* 9, e1003675.