The Physiological Consequences of Loss of tRNA Thiolation in *Saccharomyces cerevisiae*

by

Jadyn Rose Damon

B. S. Microbial Biology
University of California, Berkeley, 2004

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ABSTRACT

Ubiquitin and ubiquitin-like proteins (UBLs) have a diverse array of functions that serve to regulate many cellular processes in eukaryotic cells. The ubiquitin-related modifier Urm1 is a conserved UBL that, in addition to serving as a protein modifier, functions as a sulfur carrier in tRNA thiolation reactions. Urm1 is required for formation of the \( s^2 \) moiety that is found as part of the more complex \( mc^2 s^2 U_{34} \) modification on the anticodon wobble uridines of \( tGlu^{GUC} \), \( tGln^{UUG} \) and \( tLys^{UUU} \) tRNAs in a variety of organisms.

It has become increasingly clear that tRNA modifications serve to alter the properties of tRNA molecules, and that tRNA modifications can impact the translational regulation of gene expression, but how specific modifications are connected to cellular processes remains largely unknown. This work focuses on Urm1-dependent tRNA modifications in *Saccharomyces cerevisiae*. This thesis describes the phenotypes of *URM1* pathway mutants, and describes the physiological consequences in cells that lack the ability to thiolate tRNAs: slow growth, impaired translation and the increased activation of at least one stress response pathway. This thesis also describes a condition, growth at \( 37^\circ C \), that results in a decrease in tRNA thiolation in wild type cells. This decrease in tRNA thiolation requires the activity of RNA polymerase III and is accompanied by decreased levels of proteins that are involved in the tRNA thiolation pathway. This decrease in tRNA thiolation may be an adaptive strategy used by cells under specific growth conditions, and is an example of the condition specific modulation of tRNA modification levels.

Thesis Supervisor: Hidde Ploegh
Title: Professor of Biology
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Abstract

Introduction

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Introduction
The regulation of gene expression

The regulation of gene expression is complex and involves diverse mechanisms that act at varying stages of gene expression and on varying scales. Genetic information is stored as DNA, which is transcribed to produce messenger RNA (mRNA), an intermediate form of information that is used in the process of translation to produce proteins (the central dogma dictates that the flow of information in cells is from DNA to RNA to protein). The multiple steps involved in producing protein from DNA provide many potential regulatory inputs. The process of transcription is regulated, affecting the levels of mRNA template available, and changes in the processing and stability of mRNA can also affect steady state mRNA levels. The process of translation is also highly regulated via a variety of mechanisms. Once produced, proteins are subject to a number of regulatory processes that affect function and stability.

Ubiquitin and ubiquitin-like modifiers in eukaryotes

Ubiquitin and ubiquitin-like proteins (UBLs) expand the functionality of the eukaryotic proteome by allowing for the post-translational control of protein stability, localization and interaction through the covalent attachment of a UBL to a target protein. Although the functions of ubiquitin and UBLs are diverse, they all share a core β-grasp fold, which consists of a β-sheet with five antiparallel β-strands and one helical segment, and are activated through similar pathways. UBLs are activated in an ATP-dependent fashion by their cognate E1 enzyme, which results in the formation of a C-terminal UBL acyl-adenylate, which is resolved to form a UBL~E1 thioester complex. The UBL is subsequently transferred to an E2 conjugating enzyme, which, with the coordinated actions of an E3 ligase, directs the transfer of the UBL to a target residue, which is most commonly a lysine (Schulman and Harper, 2009). These conjugations are
reversible: ubiquitin can be removed from a target by the enzymatic activities of deubiquitylating enzymes (DUBs) and UBLs can be removed by UBL-specific proteases (ULPs) (Love et al., 2007) (Figure 1A, 1B).
Figure 1: Schematic of the ubiquitin proteasome system. A. Mechanism of ubiquitin activation and conjugation cycle. Ub(A) is ubiquitin that is noncovalently associated with UBA1 at adenylation site, Ub(T) is ubiquitin that is covalently linked to UBA1 through a thioester bond. Steps include (1) adenylate formation, (2) thioester formation, (3) double ubiquitin loading of E1 and (4) ubiquitin transfer to E2. B. The ubiquitin conjugation cascade. Ubiquitin is activated by UBA1 in an ATP dependent fashion, and ubiquitin is subsequently transferred to an E2 ubiquitin-conjugating enzyme. E3 enzymes facilitate the transfer of ubiquitin to specific substrates. Substrates can be mono- or poly- ubiquitylated, and ubiquitylation has a variety of consequences for target proteins depending on factors such as the topology of chain linkage. Lysine- 48 linked chains, for example, target substrate proteins for degradation by the proteasome. Ubiquitin can be removed from substrates through the actions of DUBs. Adapted from (Schulman and Harper, 2009) and (Hochstrasser, 2009).

Ubiquitin and UBLs largely function by mediating protein-protein interactions, serving as binding elements that are recognized by other proteins or complexes, triggering conformational changes that facilitate or inhibit binding to other proteins, allowing for recruitment of specific subsets of proteins or by directly blocking protein-protein interactions (Hochstrasser, 2009; Schulman and Harper, 2009).

Ubiquitin is the most well known of the small protein modifiers, and perhaps most associated with the targeting of polyubiquitylated substrates to the proteasome for degradation. The ubiquitin system is quite versatile: substrates can be mono- or poly-ubiquitylated, and there is variation in ubiquitin linkage (K-48, K-63). These factors dictate the function of ubiquitylation, which plays a role in processes as diverse as signal transduction, membrane-protein trafficking, endocytosis, DNA repair and chromatin-modulated gene transcription (Love et al., 2007; Hochstrasser, 2009).
Since the initial discovery of ubiquitin, many other UBLs have been described. Although the basic mechanisms of activation and conjugation are generally conserved between UBLs, the specific enzymes utilized are pathway/UBL specific, and the functions of UBLs vary greatly, and in some cases are still being elucidated. Some UBLs, like ubiquitin, are conjugated to proteins; this is the case with Rub1 (Nedd8 in vertebrates), which modifies Cullin RING E3 ubiquitin ligases and regulates ubiquitylation. Other UBLs have unconventional targets or functions; Atg8, for example, is a lipid modifier involved in autophagosomal membrane growth (Schulman and Harper, 2009; Van der Veen and Ploegh, 2012). The diverse functions of various UBLs underscore the importance of protein-protein conjugation systems in the regulation of cellular processes (Table 1).
<table>
<thead>
<tr>
<th>UBL</th>
<th>Identity with ubiquitin (%)</th>
<th>E1 (UBL-activating enzyme)</th>
<th>E2 (UBL-conjugating enzyme)</th>
<th>Function / comments on UBL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ubiquitin</td>
<td>100</td>
<td>Uba1 (UBA6)</td>
<td>Many</td>
<td>Many; proteasomal degradation, localization, protein-protein interactions; precursors encoded by multiple genes</td>
</tr>
<tr>
<td>Rub1 (NEDD8)</td>
<td>55</td>
<td>Uba3-Uha1 heterodimer</td>
<td>Ubc12</td>
<td>Modification of Cullin RING E3 ligases, regulation of ubiquitylation</td>
</tr>
<tr>
<td>MNSF-β</td>
<td>38</td>
<td>NI</td>
<td>NI</td>
<td>Unclear function, potential role in immune defense/ antiviral response; derived from ribosomal-protein precursor</td>
</tr>
<tr>
<td>FAT10</td>
<td>32 and 40*</td>
<td>UBA6</td>
<td>NI</td>
<td>Unclear function, potential role in immune defense/ antiviral response; conjugates accumulate upon proteasomal degradation induced by type 1 interferons, host defense against viral infection</td>
</tr>
<tr>
<td>ISG15</td>
<td>32 and 37*</td>
<td>UBE1L</td>
<td>UBC18</td>
<td>Induced by type 1 interferons, host defense against viral infection</td>
</tr>
<tr>
<td>Smt3 (SUMO1, SUMO2, SUMO3)</td>
<td>18</td>
<td>Uha2-Aos1 heterodimer</td>
<td>Ubc9</td>
<td>Many; nuclear transport/ organization, transcription, chromatin remodeling, DNA repair, ribosomal biogenesis; encoded by 3-4 genes in vertebrates</td>
</tr>
<tr>
<td>Atg8</td>
<td>ND</td>
<td>Atg7</td>
<td>Atg3</td>
<td>Lipid modifier, role in autophagosomal membrane growth and expansion; three known isoforms in humans</td>
</tr>
<tr>
<td>Atg12</td>
<td>ND</td>
<td>Atg7</td>
<td>Atg10</td>
<td>Autophagosome formation; ~20% identical to Atg8</td>
</tr>
<tr>
<td>Urm1</td>
<td>ND</td>
<td>Uba4</td>
<td>NI</td>
<td>Protein modification and tRNA thiolation; related to sulfur-carrying proteins MoaD and ThiS</td>
</tr>
<tr>
<td>UFM1</td>
<td>ND</td>
<td>UBA5</td>
<td>UFC1</td>
<td>Unclear function, potential role in differentiation; conserved in metazoan and plants</td>
</tr>
<tr>
<td>Hub1 (UBL5)</td>
<td></td>
<td></td>
<td></td>
<td>No C-terminal glycine (dityrosine motif), nonequivalently involved in splicing</td>
</tr>
</tbody>
</table>

Table 1: Known Ubiquitin-like proteins in eukaryotes. ND, not detectable by standard BLAST searches. NI, not identified. UBLs are listed as the yeast *Saccharomyces cerevisiae* symbol if the UBL is present in yeast, otherwise vertebrate symbols are listed. Known vertebrate orthologues with symbols that differ from yeast proteins are listed in parentheses. For E1s and E2s, yeast symbols are listed if the protein is found in yeast. * The identities listed are for each of
two ubiquitin-related domains. Figure adapted from (Hochstrasser, 2009) with information from (Van der Veen and Ploegh, 2012).

Small protein modifiers in bacteria and archaea

The origins of ubiquitin have been mysterious for some time: while widely conserved in eukaryotes, there is no ubiquitin protein found in members of the other kingdoms of life. Archaea and eubacteria do have small protein modification systems that overlap in function with ubiquitin, but the degree of relatedness of these systems to the ubiquitin system varies. In bacteria (initially observed in *Mycobacterium tuberculosis* and conserved in *Actinobacteria* and *Nitrospirae* (Maupin-Furlow, 2013)), Pup was identified as a small protein modifier that tags proteins for degradation, although the nature of Pup activation and conjugation is biochemically distinct from the mechanisms used in the ubiquitin system (Pearce *et al.*, 2008; Burns *et al.*, 2009; Striebel *et al.*, 2009). Pup is intrinsically disordered and does not share the canonical ubiquitin fold, although Pup does contain a C-terminal GGQ motif that is required for function (Liao *et al.*, 2009; Chen *et al.*, 2009b).

Another protein conjugation system has been described in bacteria that is homologous to the ubiquitin conjugation system. In *Thermus thermophilus*, TtuA, TtuB and TtuC had been identified as being required for tRNA thiolation. Recently, it was discovered that TtuB likely contains a β-grasp fold and possesses some sequence similarity to ubiquitin, suggesting that there might be functional similarities between ubiquitin and TtuB. Indeed, TtuB was observed to form protein conjugates in a TtuC dependent fashion, leading to a model in which TtuB is activated by TtuC and conjugated to target proteins via an activated C-terminal glycine residue (Shigi, 2012).

In archaea (*Haloferax volcanii*), the ubiquitin-like β-grasp proteins SAMP1 and SAMP2 (which also show similarity to sulfur carriers) were shown to form protein conjugates and to
direct at least a subset of target proteins to the proteasome for degradation. SAMPs were found to modify target proteins via an isopeptide linkage, and although activation by an E1 is required for SAMP function, no E2 or E3 homologs have yet been identified (Humbard et al., 2010; Ranjan et al., 2011). Although limited work has been carried out in archaea, a comparison of genome sequences suggests that ubiquitin-fold proteins are widespread in archaea (Maupin-Furlow, 2013).

**Urm1: a “molecular fossil” linking prokaryotic sulfur carriers to eukaryotic UBLs evolutionarily**

The discovery of Urm1 as a putative protein modifier, and the eventual discovery that Urm1 acts as a sulfur carrier, revealed an evolutionary link between prokaryotic sulfur carriers and eukaryotic ubiquitin-like modifiers. Urm1 was initially identified in *Saccharomyces cerevisiae* through sequence similarity to the *Escherichia coli* proteins MoaD and ThiS, sulfur carriers that are involved in molybdopterin and thiamine synthesis, respectively. Urm1 has 23% identity compared to MoaD and 20% identity compared to ThiS (Xu et al., 2006). Though there is very low sequence similarity between Urm1 and ubiquitin, Urm1, along with MoaD and ThiS, contains the C-terminal diglycine motif that is characteristic of ubiquitin-like modifiers and the β-grasp fold (β-GF) that is common to proteins in the ubiquitin superfamily (Furukawa et al., 2000; Rudolph et al., 2001; Wang et al., 2001; Xu et al., 2006). Structurally, Urm1 is most similar to MoaD, and structural and phylogenetic analysis suggests that Urm1, MoaD and ThiS are evolutionarily related to ubiquitin like proteins, with Urm1 and the prokaryotic sulfur carriers having diverged from ubiquitin at an early stage (Xu et al., 2006).
The similarities between prokaryotic sulfur carriers, Urm1 and other eukaryotic UBLs extend from structural characteristics to mode of activation and function. MoaD, ThiS, Urm1 and UBLs require an activating enzyme to render them ready for function. It had been noted that the genes encoding MoeB and Uba1 demonstrated significant sequence similarity to one another, and the diglycine motif of MoaD suggested that it might be activated by MoeB in a process analogous to the activation of ubiquitin by Uba1 (Rajagopalan, 1997). Indeed, a structure of MoaD in complex with MoeB suggested mechanistic similarities to the activation of ubiquitin by Uba1 (Lake et al., 2001). Following activation, thiocarboxylate formation on the C-terminus of MoaD is a requirement for the generation of active molybdopterin synthase, which is a heterotetramer comprised of two heterodimers of MoaD and MoaE; this complex converts a precursor Z into molybdopterin through the addition of dithiolene (Gutzke, 2001; Rudolph et al., 2001). Similarly, in the thiamine biosynthetic pathway, ThiS is activated in an ATP dependent fashion by ThiF, and ThiS-COAMP is subsequently converted to the thiocarboxylate ThiS-COSH by ThiI (Taylor et al., 1998) (Figure 2).

The molybdopterin and thiamine synthesis pathways are widely present in bacteria, suggesting that these sulfur transfer systems might be evolutionary precursors to the ubiquitin conjugation system. Urm1, which has similarities to prokaryotic sulfur carriers and eukaryotic protein modifiers, is viewed as a “molecular fossil” that helps to elucidate the origins of the ubiquitin system and bridges different functions. Indeed, although ubiquitin was the first β-GF protein identified, it is now clear that β-GF proteins are found in prokaryotes and that much functional diversification of β-GF proteins took place in prokaryotes prior to the expansion/diversification of UBLs found in eukaryotes (Burroughs et al., 2012).
Figure 2: **Urm1 is a eukaryotic UBL with similarities to prokaryotic sulfur carriers.** Urm1 has similarities with ubiquitin and MoaD. All are activated in an ATP dependent fashion to form an adenylate. Ubiquitin is subsequently transferred to the ε-amine of a lysine residue of a substrate protein through the activities of E2 and E3 enzymes. Urm1-adenylate and MoaD-adenylate are subsequently thiocarboxylated. MoaD functions in the molybdopterin synthesis pathway, while Urm1 functions as a protein modifier and as a sulfur carrier in tRNA modification reactions. Adapted from (Petroski et al., 2011).
The discovery of Urm1

The expanding number of UBLs in eukaryotes led to the hunt for as-yet unidentified protein conjugation systems. The 99-amino acid ubiquitin-related modifier Urm1 was identified in yeast through sequence similarity to prokaryotic sulfur carrier proteins. A two-hybrid screen using Urm1 as bait identified Uba4 as an interactor with Urm1. Uba4 also had similarities to prokaryotic proteins: while the N-terminal portion of Uba4 showed similarity to Uba1, the E1 for ubiquitin, the protein was also similar to prokaryotic MPT synthase sulfurylases. Uba4, containing a conserved ATP binding motif, was demonstrated to act as the E1 activating enzyme for Urm1 (Furukawa et al., 2000).

The further characterization of Urm1 came from a search for factors involved in cell polarization. The Sprague group conducted a synthetic lethal mutant screen to identify genetic interactors of CLA4. Cla4 is a p21-activated kinase that functions in budding and plays a role in actin cytoskeleton morphogenesis and septin formation. This screen identified UBA4, and both URM1 and UBA4 were shown to be essential in cla4Δ cells. NCS2, NCS6, ELP2 and ELP6 were also identified in the screen and were subsequently linked to URM1 in functional assays (Goehring et al., 2003c; 2003a). uba4Δ, urm1Δ, elp2Δ, elp6Δ, ncs2Δ and ncs6Δ cells had defects in cell elongation and had defects in haploid invasive growth assays. Additionally, uba4Δ/uba4Δ diploid cells had defects in pseudohyphal development in response to nitrogen depletion (Goehring et al., 2003c). uba4Δ, urm1Δ, elp2Δ, elp6Δ, ncs2Δ and ncs6Δ cells were also shown to be sensitive to rapamycin and to interact with the TOR pathway, solidifying a link to nutrient sensing as well as cell morphogenesis (Chan et al., 2000; Goehring et al., 2003c; Rubio-Texeira, 2007).
It was initially assumed that Urm1 would act as a protein modifier analogous to the well-described ubiquitin. Indeed, initial studies identified Urm1-protein conjugates by western blot analysis, and appearance of Urm1-conjugates was dependent upon Uba4 (Furukawa et al., 2000; Goehring et al., 2003b; 2003c). The thiol-specific peroxiredoxin Ahp1 was identified as a target of urmylation, with levels of Ahp1-Urm1 increasing when cells were exposed to the thiol-specific oxidant diamide. Together with the observation that urmlΔ cells were sensitive to oxidative stress, this data suggested that Urm1 was involved in mediating the cellular response to oxidants through a protein modification pathway (Goehring et al., 2003b).

It was surprising, then, when it was discovered that Urm1 also had the capacity to function as a sulfur carrier in tRNA modification reactions. Specifically, Urm1, Uba4, Ncs6 and Ncs2 were found to be required for formation of the s² moiety, which is found in conjunction with the mcm⁵ modification, on wobble uridines of the cytoplasmic tRNAs tGluUUC, tGlnUUG and tLysUUU in S. cerevisiae, C. elegans and mammalian cells (Dewez et al., 2008; Huang et al., 2008; Nakai et al., 2008; Schlieker et al., 2008; Leidel et al., 2009). In S. pombe and C. elegans, Ctu1 (Ncs6) and Ctu2 (Ncs2) were found to be required for thiolation of tRNAs, and a CTU-1 null mutation in C. elegans resulted in delays in germ-line maturation at high temperatures; similarly, deletion of ctu1 or ctu2 in fission yeast resulted in a thermosensitive lethal phenotype as well as morphology defects and ploidy abnormalities (Dewez et al., 2008).

A study from our lab utilized a functional proteomics approach to study the function of Urm1. Use of an HA-tagged Urm1 vinylmethylester (HA-Urm1-VME) suicide inhibitor probe to search for substrates with cognate enzymatic activities led to the identification of ATPBD3 and UPF0432, mammalian homologs of Ncs6 and Ncs2, respectively, and linked Urm1 to tRNA modification pathways. The requirement for Urm1 in tRNA thiolation reactions was
demonstrated in *S. cerevisiae* and mammalian cells, and mass spectrometry was used to demonstrate the presence of an Urm1-thiocarboxylate in mammalian cellular extracts. Additionally, the function of Urm1 in mammalian cells was examined, and treating cells with shRNA constructs to deplete Urm1 levels led to cytokinesis defects, consistent with phenotypes in fission yeast and *C. elegans* (Schlieker *et al.*, 2008).

Multiple approaches led to the in-depth biochemical and genetic description of the role of Urm1 and related proteins in *S. cerevisiae*. A genetic approach utilized the observation that resistance to the toxin zymocin, which is secreted by *Kluveromyces lactis*, was correlated with defects in synthesis of the mcm<sup>5</sup>s<sup>2</sup>- modification. A screen for zymocin sensitivity coupled with HPLC analysis of tRNAs in *S. cerevisiae* identified *URM1, UBA4, NCS2* and *YOR251c* (*TUM1*) as being required specifically for synthesis of the s<sup>2</sup>- modification (Huang *et al.*, 2008). The identification in Ncs6 of motifs known to be present in bacterial tRNA modification enzymes ultimately led to the description of Ncs6, Ncs2, Urm1 and Uba4 as being required for the thiolation of tRNAs (Nakai *et al.*, 2007). Another study employed synthetic genetic array (SGA) analysis using *urmlA* and *uba4A* as query strains revealed interactions with components of the *ELP* pathway/complex, which was already known to be required for tRNA modifications. This analysis led to a description of Urm1 as a sulfur carrier in tRNA modification reactions that also involved Tum1, Ncs2 and Ncs6 (Leidel *et al.*, 2009).

The combination of genetic and biochemical approaches clarified the identities of *URMI* pathway components and elucidated the specific functions of several of the proteins required for Urm1-dependent tRNA thiolation; altogether the *URMI* pathway is comprised of *TUM1, UBA4, URM1, NCS2* and *NCS6* (and *NFS1*).
Genetic and biochemical data also helped to define the ELP (elongator) pathway. Utilizing the finding that tRNAs carrying the full \( \text{mcm}^5\text{s}^2\text{U}_{34} \) modification were efficiently cleaved in the anticodon loop by *Kluveromyces lactis* \( \gamma \)-toxin (Lu et al., 2005), a genetic screen was set up which identified the genes *ELP1-ELP4, ELP6, KTI1-KTI13* as being required for synthesis of the \( \text{mcm}^5 \) modification (Huang, 2005) and *TRM9* was separately identified as being required for \( \text{mcm}^5\text{s}^2 \) formation (Kalhor and Clarke, 2003; Bjork et al., 2007). Additionally, biochemical experiments had identified the components of Elongator, a complex of proteins with histone acetyltransferase activity that was found to associate with RNA polymerase II. Elongator was found to consist of two subcomplexes, one comprised of Elp1, Elp2 and Elp3 and the second comprised of Elp4, Elp5 and Elp6 (Winkler, 2001), and the genes encoding these proteins (and others required for the synthesis of the \( \text{mcm}^5 \) moiety) comprise the *ELP* pathway.

**Sulfur transfer in the *URMI1* pathway**

The sulfur transfer pathway required for thiolation of \( \text{tGlu}^{\text{UUU}}, \text{tGln}^{\text{UUG}} \) and \( \text{tLys}^{\text{UUU}} \) in *S. cerevisiae* has been described in detail, both in genetic and biochemical terms. Generally, the sulfur transfer pathway requires the activity of a desulfurase to mobilize sulfur from a donor molecule, involves the sequential transfer of sulfur to a series of carriers and ultimately requires proteins that facilitate the transfer of sulfur to appropriate substrates. The sulfur transfer pathway in budding yeast is similar to the sulfur relay that has been described for tRNA modification reactions in bacterial cells (Ikeuchi et al., 2006).

*Nfs1* is a mitochondrially located cysteine desulfurase involved in iron-sulfur (Fe/S) cluster biogenesis, and is required for thiolation of both cytoplasmic and mitochondrial tRNA species (Nakai et al., 2004). *Nfs1* dependent Fe/S cluster assembly was also demonstrated to be
required for efficient thiolation of cytosolic tRNAs, as evidenced by the unthiolated tRNAs in cells depleted of Fe/S cluster assembly machine components (Nakai et al., 2007). Elp3, which is involved in formation of the mcm$^5$- group, is an Fe/S cluster containing protein (Paraskevopoulou et al., 2006), and so the requirement for Fe/S clusters in tRNA thiolation may be an indirect consequence of a requirement for mcm$^5$- modification for efficient thiolation. In the cytoplasmic tRNA thiolation pathway, Nfs1 accepts sulfur from a cysteine to form a persulfide group. The sulfur is then transferred as a persulfide to a cysteine residue in the rhodanese-like domain (RLD) of Tum1. Rhodaneses are enzymes that catalyze the transfer of a sulfur atom from thiosulfate to cyanide in vitro, and whose activity is dependent upon a conserved cysteine residue (Bordo and Bork, 2002). In addition to acting as a sulfur carrier, Tum1 appears to enhance the desulfurase activity of Nfs1 (Noma et al., 2008).

Next, the sulfur, still as a persulfide, is transferred to the RLD of Uba4, although it is probable that sulfur can also be transferred directly from Nfs1 to Uba4 (Noma et al., 2008). Following activation of Urm1 by Uba4, Uba4 catalyzes formation of Urm1-thiocarboxylate (Noma et al., 2008; Schlieker et al., 2008; Leidel et al., 2009). Uba4 is notable because of its integrated dual functions. Uba4 contains an N-terminal MoeB/E1 domain and a C-terminal RLD and carries out both the ATP dependent activation of Urm1 and the transfer of sulfur to form Urm1-thiocarboxylate. Specifically, the cysteine at position 397 of the RLD is required for the sulfurtransferase activity of Uba4, while the MoeB/E1 domain of Uba4 is required for activation of Urm1 (Furukawa et al., 2000). As is the case for ubiquitin, the C-terminal diglycine motif of Urm1 is critical for function, as the C-terminal glycine is required for thiocarboxylate formation (Furukawa et al., 2000; Van der Veen et al., 2011).
The Urm1-thiocarboxylate then acts as a sulfur donor, and Ncs6 and Ncs2 facilitate the transfer of sulfur to the U₃₄ of a tRNA substrate. Although the precise mechanism of sulfur transfer at this step is unknown, it is known that Ncs6 and Ncs2 are capable of forming a complex, that both proteins are required for thiolation of tRNAs, and that Ncs6 is capable of binding to tRNAs (Dewez et al., 2008; Leidel et al., 2009). It is probable that sulfur transfer is primarily a function of Ncs6, which contains multiple CXXC motifs as well as a PP-motif, which are motifs that function in ATP binding and adenylation of target nucleotides; indeed, Ncs6 can bind to and adenylate tRNAs (Nakai et al., 2007; Shigi, 2014) (Figure 3).

**Figure 3: Sulfur transfer and the URM1 tRNA modification pathway.** Sulfur is removed from cysteine by the desulfurase Nfs1, and then transferred to Tum1 and then to Uba4, the E1 activating enzyme for Urm1. Uba4 contains an E1 domain, which functions to activate Urm1 in an ATP dependent fashion, as well as a rhodanese-like domain, which functions to form Urm1-thiocarboxylate. Urm1-thiocarboxylate serves as a protein modifier (not shown here) or as a sulfur donor for formation of the mcm⁵⁵²U₃₄ modification found on a subset of tRNAs. The proteins Ncs6 and Ncs2 mediate transfer of sulfur to tRNA molecules. Formation of mcm⁵ modification facilitates tRNA thiolation; this modification requires the activities of Trm9,
Trm112, the Elongator complex as well as other gene products. Adapted from (Noma et al., 2008).

**Urm1 as a protein modifier**

As mentioned previously, Urm1 was initially assumed to be a protein modifier, and there was evidence that Urm1 could form conjugates with at least one protein, Ahp1, in yeast. The discovery that Urm1 functioned in tRNA modification reactions, along with the lack of urmylation substrates aside from Ahp1, led to the question of how widespread a phenomenon protein urmylation was. The exact mechanism of protein urmylation and the chemical nature of the Urm1-protein bond remained undefined, and although Uba4 had been identified as the E1 for Urm1, no proteins with Urm1 specific E2 or E3 functionalities had been identified. When considering the protein modification and tRNA modification functions of Urm1, it is important to note that, at least here, use of the term “URM1 pathway” refers to the components required for tRNA modification, and not all components are required for protein urmylation. Although components of the sulfur transfer pathway that act upstream of Urm1 are required for protein and tRNA modification, Ncs2 and Ncs6 are involved only in tRNA modification reactions.

A study was able to confirm that Urm1 was conjugated to proteins in response to oxidative stress (exposure to diamide or H$_2$O$_2$); Ahp1 was confirmed as an urmylation target in budding yeast, and a number of protein targets were identified in mammalian cells. Targets included known components of the Urm1 pathway as well as deubiquitylating proteins, proteins involved in RNA processing and components of the nuclear import/export pathway (Van der Veen et al., 2011) (Table 2).
Table 2: Mammalian protein targets of urmylation. Urmylated proteins identified by LC-MS/MS analysis of immunoprecipitates from cells expressing HA-Urm1 that were left untreated or exposed to diamide or H₂O₂. For components of tRNA modification pathways, yeast homolog is listed. Adapted from (Van der Veen et al., 2011).

This same study also elucidated the biochemical requirements for, and details of, protein urmylation. It is now known that protein urmylation requires Urm1-thiocarboxylate formation, which itself requires functional Uba4/ MOCS3 (in mammalian cells) and the C-terminal glycine of Urm1, involves a thioester intermediate and results in the covalent modification of proteins at specific lysine residues (Figure 4). Only monourmylated substrates have been detected. Experiments utilizing in vitro generated thiocarboxylate proteins revealed that while HA-Urm1-COSH was used in protein conjugation, HA-EGFP-COSH could not be conjugated to proteins, indicating that proteins were specifically modified by Urm1 and not more generally by
thiocarboxylated proteins. This observation, along with the mapping of target lysines, demonstrated that there is specificity to urmylation (Van der Veen et al., 2011).

![Diagram of protein urmylation]

**Figure 4: Schematic of protein urmylation.** Protein urmylation requires activation and thiocarboxylation of Urm1, both of which are accomplished by the enzymatic activities of Uba4. Urm1 is subsequently conjugated to lysine residues on target proteins. It is unknown whether E2- and/or E3- like enzymes function in the protein urmylation pathway. It is also unknown if deurmylases, capable of removing Urm1 from substrates, exist.

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**Eukaryotic translation**

Translation is the process by which cells use the information contained in messenger RNA (mRNA) molecules to produce proteins, which are by and large the effector molecules in cells. This process is carried out by the ribosome, a molecular machine that is composed of protein and RNA; the ribosome is the environment in which the triplet codons of mRNAs are recognized by corresponding aminoacylated tRNAs, and it also catalyzes the peptidyl transferase reaction that joins amino acids together to form a growing polypeptide chain. Translation is a complex and highly regulated process, as fidelity is important to ensuring the integrity of the cellular proteome. Translation is divided into several steps: initiation, elongation, termination and sometimes ribosome recycling. Each step is regulated, requiring various protein factors.
(many of which are GTPases); this allows the ribosome to maintain accuracy and also allows for the condition specific control of gene expression.

Translation initiation, the first step in protein synthesis, is highly controlled, and for most mRNAs is the regulated, and rate limiting step in translation. The cap-dependent scanning model describes initiation for the majority of cellular messages. In this model, the preinitiation complex (PIC) is recruited to the capped 5' end of an mRNA. The PIC is comprised of the 40S subunit of the ribosome in complex with the initiation factors eIF 1, 1A, 3, and 5, along with the initiator tRNA Met-tRNAi•eIF2•GTP ternary complex (TC). The 5' cap is bound by eIF4E, eIF4G and eIF4A (the eIF4F complex); eIF4G is itself bound to poly(A) binding protein PABP, which recognizes the poly(A) tail of the mRNA and is thought to result in circularization of the mRNA (although circularization is not universally accepted). The eIF4F complex facilitates binding of the PIC to the 5’ end of the mRNA; after binding, the PIC scans the mRNA until an AUG enters the P-site. When the anticodon of Met-tRNAi encounters a complementary codon, GTP is hydrolyzed and eIF2-GDP, along with other eIFs, are released from the complex. The 60S ribosomal subunit then joins with the 40S subunit on the mRNA to form the 80S initiation complex (Sonenberg and Hinnebusch, 2009). There are many variations on the theme of initiation: initiation may begin at a non-AUG codon, for example, and some mRNAs (cellular and viral) bypass cap-dependent initiation by utilizing highly structured internal ribosome entry sites (IRESs) to recruit translation machinery. Initiation of other transcripts is regulated by the presence of short upstream ORFs (uORFs) (Hinnebusch, 1984).

The process of elongation consists of decoding, peptidyl transfer and translocation steps. Once initiation occurs, the ribosome is bound to both mRNA and Met-tRNAi, which occupies the ribosomal P site (peptidyl site). The A site (aminoacyl site) and the E site (exit site) are
unoccupied. Elongation commences when the ternary complex (TC), comprised of aminoacylated tRNA (aa-tRNA) in complex with GTP-bound eEF1A, arrives at the A site of the ribosome. TCs rapidly associate with and dissociate from the A site in a process termed tRNA sampling. When a tRNA anticodon binds its cognate codon, the geometry of the codon-anticodon interaction results in conformational changes in the rRNA such that specific rRNA residues form interactions with the codon-anticodon duplex. The codon-anticodon “fit” is primarily determined by interactions between the first two bases of the codon and the corresponding anticodon bases. Codon recognition triggers conformational changes in the ribosome, the tRNA, and in eEF1A; these changes activate the GTPase activity of eEF1A. When GTP is hydrolyzed to GDP, eEF1A dissociates from the ribosome, and the aa-tRNA is held on the ribosome mainly through the codon-anticodon interaction; at this point the aa-tRNA can either dissociate or be accommodated into the A site. The rate of accommodation is greater for cognate compared to non-cognate interactions, and so this step further contributes to maintenance of translational fidelity.

Next, the rRNA in the large subunit of the ribosome catalyzes the peptidyl-transfer reaction in which the peptidyl group from the tRNA in the P site is transferred onto the A site aa-tRNA; the P site tRNA is deacylated and the growing peptide, one amino acid longer, is attached to the A site tRNA. Following peptide bond formation, translocation, the coordinated movement of tRNAs and mRNA with respect to the ribosome, takes place. Translocation ensures that the ribosome is ready for a subsequent round of elongation and that the proper reading frame of the mRNA is maintained (Figure 5A). Translocation proceeds through the so-called hybrid state, in which ratcheting of the ribosomal subunits with respect to each other results in the 3’ ends of the A and P site tRNAs positioned in the P and E sites of the large subunit (tRNAs in the hybrid A/P state).
and P/E states, respectively). Subsequently, tRNAs and the mRNA move with respect to the small subunit in a step that is catalyzed by the GTPase eEF2. The ribosome is “reset” with tRNAs fully in the P and E sites (the E site tRNA will dissociate), an empty A site, and the mRNA presenting the next triplet for decoding (Figure 5B). In fungi, but not in prokaryotes or higher eukaryotes, the translation factor eEF3 is required during elongation, apparently for aa-tRNA•eEF1A•GTP binding to the ribosome and for release of tRNA from the E site (Noble and Song, 2008; Voorhees and Ramakrishnan, 2013).

Elongation continues until a stop codon (UAA, UAG or UGA) enters the A site. The stop codon is recognized by the class I release factor eRF1, which resembles a tRNA molecule in size and shape. eRF1 recognizes the stop codon via a peptide-codon interaction and induces hydrolysis of the ester bond that links the peptide chain to the P site tRNA. The GTPase eRF3 is also required for termination; although its role is less clear, it may have a role in ensuring the accuracy of eRF1. Ribosome recycling occurs after termination (Noble and Song, 2008).
A

1. Codon recognition

2. Peptidyl transfer

3. Translocation

B

hybrid state
Figure 5: Eukaryotic translation elongation. A. The eukaryotic translation elongation cycle consists of codon recognition, peptidyl transfer and translocation steps, and requires the hydrolysis of GTP at several steps. The ternary complex (TC) is comprised of an aa-tRNA in complex with GTP bound eEF1A. Codon recognition results in conformational changes that result in GTP hydrolysis and eEF1A dissociation from the ribosome. Next, the peptidyl group from the P site tRNA is transferred onto the A site aa-tRNA, extending a polypeptide chain. Following peptidyl transfer, the translocation step ensures that the ribosome is conformationally ready for subsequent rounds of elongation. B. Translocation proceeds through the so-called hybrid state. The 3' ends of the A and P site tRNAs are repositioned into the P and E sites of the large ribosomal subunit (and are in the A/P and P/E states) as a consequence of the movement of ribosomal subunits with respect to one another. eEF2 catalyzes the movement of tRNAs and mRNA with respect to the small subunit in a GTP-dependent process. Adapted from (Schneider-Poetsch et al., 2010) and (Voorhees and Ramakrishnan, 2013).

The role of tRNAs in translation

Cells have multiple mechanisms for ensuring translational accuracy. In yeast, mistranslation rates vary by codon, producing errors at rates of $4 \times 10^{-5}$ to $6.9 \times 10^{-4}$ (Salas-Marco and Bedwell, 2005; Kramer et al., 2010). There are multiple factors that have an impact on translational fidelity, and the properties of tRNAs are critical for the faithful decoding of information contained in mRNA.

tRNA aminoacylation is an important step in ensuring translational fidelity, as a given tRNA/anticodon must result in delivery of one specific amino acid to the ribosome for incorporation into the growing polypeptide chain. Aminoacyl-tRNA synthetases (aaRSs) discriminate at the levels of amino acid recognition and tRNA recognition during aminoacyl-tRNA synthesis. tRNAs contain a number of elements known as determinants and antideterminants; these can be single nucleotides, base pairs, modified nucleotides or structural motifs. These elements ensure that a tRNA is aminoacylated by a specific aaRS and at the same
time prevent mischarging by other aaRSs (Giege, 2008). Editing by aaRSs further ensures accuracy; pre-transfer editing results in the hydrolysis of mischarged aminoacyl adenylates by the aaRS, post-transfer editing results in the hydrolysis of mischarged aminoacyl adenylates in the editing site of aaRSs, and trans editing occurs after an incorrectly charged aa-tRNA dissociates from the aaRS (Reynolds et al., 2010).

As mentioned previously (and as will be discussed later in further detail), tRNA identity is important for proper codon-anticodon interactions at the ribosome both for initial tRNA selection as well as for accommodation in the ribosomal A site. tRNAs even play a role in quality control after peptide bond formation, at least in bacterial cells; if an improper tRNA is used in translation, a mismatched codon-anticodon in the ribosomal P site can result in loss of A site specificity, which in turn results in further misincorporation events and the premature termination of elongation (Zaher and Green, 2008; Reynolds et al., 2010).

Although fidelity and speed have generally been considered to be of paramount importance in maintaining a functional proteome, there are instances in which modulation of these parameters may actively occur to the benefit of cells. Misacylation of nonmethionyl tRNAs with methionine has been described in mammalian, yeast and bacterial cells (Netzer et al., 2009; Jones et al., 2011; Wiltrout et al., 2012). In mammalian cells, misacylation of tRNAs with methionine was up-regulated in response to the presence of reactive oxygen species (ROS), and it has been hypothesized that Met-misacylation is a mechanism that results in increased incorporation of methionine into proteins and results in increased protection against ROS-induced damage (Netzer et al., 2009). There is growing evidence that the misincorporation of amino acids into a protein may be a mechanism that cells use to their advantage to cope with stress (termed “adaptive translation”) (Pan, 2013). Additionally, mistranslated proteins or
proteins that, due to defects in translation are not folded correctly, have the potential to trigger cellular stress response pathways (Ruan et al., 2008; Silva et al., 2009; Paredes et al., 2012; Patil et al., 2012a; de Pouplana et al., 2014).

The abundance of tRNAs, which is correlated with tRNA gene copy number (Percudani et al., 1997), coupled with codon usage in genes, also influences the expression of genes. In *E. coli*, the speed at which certain codon sets are translated serves to separate the translation of segments of proteins, which results in the coordination of protein folding in a process termed cotranslational protein folding. Changing the speed at which portions of a protein are translated, either by altering tRNA concentration or by making synonymous substitutions to codons (which results in the use of a different isoacceptor tRNA that may be present at a different abundance), results in the reduced folding efficiency of proteins (Zhang et al., 2009).

Recent studies have highlighted the importance, as well as the complexities, of the tRNA pool in *S. cerevisiae*, which must be able to accommodate the transcriptome and coordinate translation and protein folding. A deletion library consisting of strains with deletions in 204 of the 275 nuclear-encoded tRNA genes revealed that individual tRNA genes, even different copies of tRNA genes in the same family, contributed differentially to fitness levels under various growth conditions. Altering the tRNA pool, either by deleting or overexpressing rare tRNAs, affected growth. When low-copy tRNAs were overexpressed, cells experienced proteotoxic stress and demonstrated decreased growth rates; when low-copy tRNAs were deleted, cells experienced proteotoxic stress that appeared offer cross-protection when cells were challenged with proteotoxic agents (Yona et al., 2013; Bloom-Ackermann et al., 2014).
tRNA biogenesis

The eukaryotic RNA polymerases (pols) transcribe distinct sets of genes; pol I synthesizes large ribosomal RNAs, pol II synthesizes mRNAs and pol III synthesizes a variety of small noncoding RNAs, including 5S rRNA, U6 snRNAs and tRNAs. The transcription of tRNAs is controlled by type II promoters consisting of the conserved intragenic sequence elements known as A blocks and B blocks. Although spatially separate, the A and B blocks are bound simultaneously by the multi-subunit transcription factor TFIIIC, which in turn recruits TFIIIB. TFIIIB recruits pol III, which initiates transcription at an optimal initiating sequence within the area to which it has been recruited. Transcription continues until pol III encounters a termination site comprised of a series of four or more T residues. After recruitment and the initial round of tRNA transcription, pol III remains associated with the tRNA gene, allowing for multiple rounds of transcription (Paule and White, 2000). Although the ~274 tRNA genes are distributed across the yeast genome, tRNA genes appear to be spatially localized to the nucleolus during transcription, where ribosomal genes are also transcribed (Thompson, 2003).

After transcription, tRNAs undergo a series of processing steps, the first of which involves a series of end-processing steps. Pre-tRNA transcripts contain 5’ leader sequences and 3’ trailing sequences. Cleavage of the 5’ leader occurs in the nucleolus and is catalyzed by the ribonucleoprotein complex RNase P (Frank and Pace, 1998; Walker and Engelke, 2006; Hopper et al., 2010). Cleavage of the 3’ trailing sequence is accomplished through the actions of the 3’ to 5’ exonuclease Rex1 and the endonuclease RNase Z; tRNA binding by the La protein (Lhp1) is thought to determine which nuclease will be involved in the processing of a given tRNA (Hopper et al., 2010; Hopper, 2013). The 3’ terminal CCA sequence present in all tRNAs is then added by the nucleotidyl transferase activity of Cca1 (Aebi et al., 1990)
Following 5’ and 3’ end cleavages, tRNAs undergo further processing events including modifications and, for some tRNAs, splicing. tRNAs are highly modified, with different modification enzymes acting at distinct subcellular locations and on distinct substrates (pre-tRNAs, intron-containing tRNAs or spliced tRNAs) (Jiang et al., 1997). tRNA modifications will be discussed in depth later. In yeast, 10 tRNA families (59 of 274 individual tRNA genes) contain introns of 14 to 60 nucleotides in length. Introns are located 1 nucleotide 3’ to the anticodon and must be spliced out in order to produce functional tRNAs (Hopper et al., 2010; Hopper, 2013). tRNA splicing is a three step process in which (1) the tRNA splicing endonuclease removes the intron to generate tRNA half molecules, which are then (2) joined in a phosphodiester bond by the tRNA splicing ligase; finally (3) an extra 2’ phosphate is removed from the splice junction by tRNA 2’ phosphotransferase (Abelson et al., 1998; Hopper et al., 2010). While tRNA splicing was initially thought to be a nuclear event, it has since been shown that tRNA splicing occurs in the cytoplasm; specifically, components of the splicing endonuclease complex are localized to the cytoplasmic surface of the mitochondria (Yoshihisa et al., 2003).

The subcellular trafficking of tRNA molecules is complicated. As mentioned previously, pre-tRNAs containing introns must be transported to the cytoplasm for splicing, and all tRNAs must ultimately end up in the cytoplasm to be used in protein synthesis; additionally some nuclear encoded tRNAs function in mitochondrial protein synthesis and so must be imported into this organelle. This is far from the end of the story, however, as tRNA movement is neither unidirectional nor permanent. tRNAs can be transported in a retrograde fashion from the cytoplasm to the nucleus, and can subsequently be re-exported to the cytosol (Hopper et al., 2010; Hopper, 2013).
The Ran pathway governs tRNA nuclear export. tRNAs bind to an importin-β family member (an exportin) in a Ran-GTP dependent fashion in the nucleus, and the exportin, which also binds nuclear pore components, mediates the transfer of cargo to the cytoplasm, where Ran-GTP is hydrolyzed to GDP and the tRNA is released. The directionality of transport is dictated by the gradient of Ran-GTP, which is high in the nucleus and low in the cytoplasm (Hopper et al., 2010; Hopper, 2013). The exportins Los1 (Hellmuth et al., 1998; Sarkar and Hopper, 1998) and Msn5 (Takano, 2005; Murthi et al., 2010) are involved in tRNA nuclear export and re-export, although based on genetic and biochemical data, there are as-yet unidentified pathways/proteins that also function in tRNA nuclear-cytoplasmic transport. Mtr10 is one of the factors involved in tRNA retrograde nuclear import (Shaheen and Hopper, 2005), which appears to be a constitutive process (Murthi et al., 2010). The regulation of nuclear export/re-export and retrograde import of tRNAs determines the distribution of tRNAs in a cell, and these processes are governed by the nutrient status of cells (Whitney et al., 2007). The retrograde import of tRNAs is thought to have multiple functions, including regulation of tRNA modification, regulation of protein synthesis and tRNA quality control (Hopper, 2013) (Figure 6).
Figure 6: tRNA biogenesis and trafficking.

A schematic of tRNA biogenesis and trafficking in *S. cerevisiae*. (1) tRNA genes are transcribed by pol III in the nucleolus; transcription is regulated by intragenic promoter sequences. Note that not all tRNA genes contain introns. (2) 5′ end-processing occurs in the nucleolus and is catalyzed by RNaseP. (3) 3′ end-processing is catalyzed by Rex1 and RNaseZ. (4) Following end-processing, the 3′ terminal CCA sequence is added by Cca1. (5) and (6) Some modifications are carried out in the nucleus prior to export into the cytoplasm. (7) tRNA export into the cytoplasm is controlled by the exportin Los1. (8) Intron-containing tRNA transcripts are spliced; components of the tRNA splicing endonuclease are localized to the cytoplasmic surface of the mitochondria, suggesting that the splicing reaction occurs here. (9) tRNAs are further modified by cytoplasmically localized modification enzymes. (10) tRNAs are charged by cognate aminoacyl-tRNA synthetases and are functional for use in translation. (11) and (12) Under certain conditions, tRNAs can traffic into the nucleus (retrograde import), a process that is controlled by Mtr10, and be re-exported into the cytoplasm, a process that is dependent on Los1 and Msn5. Adapted from (Phizicky and Hopper, 2010).
tRNA molecules are highly stable, but there are multiple pathways known to be involved in tRNA turnover. Some hypomodified tRNAs can be degraded by the 3' to 5' exonucleolytic activity of the nuclear exosome following association with and polyadenylation by the TRAMP (Trf4/Air2/Mtr4) complex (Kadaba, 2004; LaCava et al., 2005; Kadaba, 2006). Some tRNA molecules can be degraded by the rapid tRNA decay (RTD) pathway, which functions to monitor correct structure of the acceptor and T-stems of mature tRNAs (Whipple et al., 2011). tRNAs lacking stabilizing modifications are rapidly degraded by the 5' to 3' exonucleases Rat1 (nuclear) and Xrn1 (cytoplasmic) (Alexandrov et al., 2006; Chernyakov et al., 2008). In addition to these tRNA quality control pathways, during oxidative and other stresses, tRNAs are cleaved into 5' and 3' half molecules by the endonuclease Rny1, which is normally vacuolar (Thompson et al., 2008; Thompson and Parker, 2009). Interestingly, the phenomenon of stress-induced cleavage is conserved in higher eukaryotes, with the stress-activated endoribonuclease angiogenin responsible for the cleavage of tRNAs; in this system, tRNA fragments appear to inhibit protein synthesis (Fu et al., 2009; Yamasaki et al., 2009; Ivanov et al., 2011).

The posttranscriptional modification of tRNA

The posttranscriptional modification of RNAs is a widespread phenomenon that occurs in all kingdoms of life. Chemical modifications of the four RNA bases-adenine, cytosine, guanine and uracil- serve to alter, expand or enhance the various functionalities of tRNAs, snoRNAs, rRNAs and mRNAs. Modifications can change the geometries of bases and/or alter the capacity of a base to interact with other bases. Here we will focus specifically on the diverse modifications found in tRNAs. It has been estimated that between 1% and 10% of an organism’s
genome encodes tRNA modification enzymes, and this, along with the evolutionary conservation of many modifications, underscores the critical functions of tRNA modifications.

While modifications on tRNAs have long been catalogued, it has taken more time for the majority of the tRNA modification genes in model organisms such as *S. cerevisiae* to be identified and validated. Many modifications are complex and require the sequential activities of multiple enzymes and/or pathways to produce the final modification. Modifications are found at many different positions in tRNAs, and various modifications have different functions (Table 3).

<table>
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<tr>
<th>Yeast gene</th>
<th>Modification</th>
<th>Null mutant phenotype</th>
</tr>
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<tbody>
<tr>
<td>DUS1</td>
<td>D16, D25</td>
<td>Not essential</td>
</tr>
<tr>
<td>DUS2</td>
<td>D28</td>
<td>Not essential</td>
</tr>
<tr>
<td>DUS3</td>
<td>D47</td>
<td>Not essential</td>
</tr>
<tr>
<td>DUS4</td>
<td>D202, D206</td>
<td>Not essential</td>
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<td>mcm1'U34, mcm's'U34, mcm2'U34, mcm2'Um34</td>
<td>Many phenotypes</td>
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<td>MOD5</td>
<td>f'A</td>
<td>Loss of suppression</td>
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<tr>
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</tr>
<tr>
<td>TYY1, TYY2, TYY3, TYY4</td>
<td>yW57</td>
<td>Not essential, reading frame maintenance</td>
</tr>
</tbody>
</table>

Table 3: Genes from *Saccharomyces cerevisiae* whose products catalyze steps in tRNA modifications. Adapted from (Phizicky and Hopper, 2010).
Many modifications enhance the stability of tRNA molecules, which must maintain a specific conformation to ensure proper function. Lack of these modifications leads to recognition and degradation of hypomodified tRNAs by quality surveillance pathways, including the RTD pathway and the TRAMP complex. Modifications also serve as recognition elements: some modifications serve as identity determinants for tRNA recognition by aaRSs, and a modification has been identified which affects aa-tRNA recognition by EF-Tu. Some complex tRNA modifications require the sequential actions of enzymes, and some modifications ensure that steps occur in the proper order by affecting the recognition of tRNA molecules by proteins. Modifications can also play a role in tRNA trafficking (Hopper, 2003; Motorin and Helm, 2010; Phizicky and Hopper, 2010; Phizicky et al., 2010; Yacoubi et al., 2012).

Modifications that have the most direct impact on protein synthesis are found on nucleosides in the anticodon stem loop (ASL) positions, namely positions 34 and 37. These modifications directly impact decoding by affecting how the tRNA recognizes/interacts with the mRNA codon (Phizicky and Hopper, 2010; Yacoubi et al., 2012), and so can impact aminoacylation, reading frame maintenance, speed of translation and codon recognition. Modifications at position 34 allow for “wobble,” the non-canonical base pairing between the first base in the anticodon loop and the third base in an mRNA codon. Francis Crick’s Wobble Hypothesis proposed that tRNA anticodon positions 36 and 35 would pair with the first two bases of a codon according to standard pyrimidine- purine (Watson-Crick) pairing rules, but that the remaining base pair could be a canonical or a non-canonical pair, which could be facilitated by modified nucleosides (Crick, 1966). This would allow the limited number of tRNAs (40) that cells have evolved to accurately decode the greater number of codons (61) that encode the 20
amino acids plus the three stop codons used by cells (Agris et al., 2007). It has been determined that modifications on nucleoside 34 can either expand or restrict wobble (the “modified-wobble hypothesis” (Agris, 1991)); in some instances a tRNA must be able to decode multiple codons that encode a given amino acid that may share the same first two bases/positions but differ at the third position. In other instances, codons for different amino acids differ only at the third base position and it is important that a given tRNA recognizes one codon but not the other. It is also the case that for some amino acids with multiple codons, each codon has its own isoacceptor tRNA, and modifications on isoacceptor tRNAs can serve to either expand or restrict recognition of other codons that encode the same amino acid.

The \( mcm^5s^2U_{34} \) modification

Lysine, glutamine and glutamic acid are each encoded by two different codons, and each codon is predominantly recognized by its own isoacceptor tRNA. Lysine codons are AAA (tLys\(^{UUU}\)) and AAG (tLys\(^{CUU}\)), glutamine codons are CAA (tGln\(^{UUG}\)) and CAG (tGln\(^{CUG}\)), and glutamic acid codons are GAA (tGlu\(^{UUC}\)) and GAG (tGlu\(^{CUC}\)). tRNAs recognizing lysine, glutamine and glutamic acid decode codons in split codon boxes (a set of codons that have the same first two bases and differ at the third position), which code for more than one amino acid (Gln/His, Lys/Asn and Glu/Asp). The tRNAs reading the A ending codons of these amino acids, tLys\(^{UUU}\), tGln\(^{UUG}\) and tGlu\(^{UUC}\), all contain modified U\(_{34}\) nucleosides. Specifically, these tRNAs contain the 5-methoxycarbonylmethyl-2-thiouridine (\( mcm^5s^2U_{34} \)) modified nucleoside (Figure 7).
Figure 7: The mcm\textsuperscript{5}s\textsuperscript{2}U\textsubscript{34} modification. A. A schematic of the tRNA anticodon loop illustrating the location of the wobble position, where the mcm\textsuperscript{5}s\textsuperscript{2}U\textsubscript{34} modification is found. B. The sequence of the tLys\textsuperscript{UUU} tRNA, one of the tRNAs that are thiolated in an Urm1-dependent fashion, with the location of the mcm\textsuperscript{5}s\textsuperscript{2}U\textsubscript{34} indicated. Image courtesy of T. Carlile. C. Structures of the mcm\textsuperscript{5} U\textsubscript{34} nucleoside and the mcm\textsuperscript{5}s\textsuperscript{2}U\textsubscript{34} nucleoside. Formation of the mcm\textsuperscript{5}-moiety is dependent on the \textit{ELP} pathway and formation of the s\textsuperscript{2}-moiety is dependent on the \textit{URMI} pathway.

In bacterial and eukaryotic systems, modifications of U\textsubscript{34} provide structure to the ASL and promote ribosome binding in both the A and P sites. Modifications allow at least some
degree of binding of the modified U to A and G, but not to C and U, and result in stabilization of
the codon-anticodon interaction (Ashraf et al., 1999; Sundaram et al., 2000; Yarian et al., 2000;
Yarian, 2002; Murphy et al., 2004; Durant et al., 2005; Vendeix et al., 2012). The mcm5
modification appears to facilitate recognition of G residues, while the s2 modification appears to
promote recognition of A and G (although primarily A) ending codons (Murphy et al., 2004;
Vendeix et al., 2012).

In bacterial cells, the s2 modification has multiple functions. Thiolation increases the rate
of aminoacylation of tRNAs, but slows release of the aa-tRNA from the aaRS; although these
parameters vary, there is no overall difference in steady state aminoacylation levels between
fully modified and hypomodified tRNAs lacking the s2 modification. The s2 modification also
facilitates binding to cognate A-ending codons (but also G ending codons to a lesser extent) in
the ribosomal A site and increases the rate of peptide bond formation as measured by GTP
hydrolysis (Rodriguez-Hernandez et al., 2013). Modifications have been shown to affect the
translation rate of reporters containing glutamate codons; the s2 modification increases the
affinity of tRNAs for GAA codons with little effect on GAG recognition, while the mnm5 (5-
methylaminomethylene, analogous to the eukaryotic mcm5) modification increases the affinity
of tRNAs for GAG codons. Additionally, changes in translation rate correspond to changes in the
growth rates of mutant cells (Kruger, 1998). There may also be a role for tRNA thiolation in
controlling frameshifting in bacterial cells (Maynard et al., 2012).

In eukaryotic cells, both the mcm5 and s2 modifications are important for tRNA binding to
the A site of the ribosome; tRNAs lacking these modifications have lower $k_{on}$ rates and higher $k_{off}$
rates compared to fully modified tRNAs, and hypomodified tRNAs lead to decreased rates of
ribosome catalyzed peptide bond formation. In yeast cells, these defects in protein synthesis were
linked to decreased levels of proteins whose transcripts were enriched in AAA, CAA, GAA and, to some extent, AAG, codons (Rezgui et al., 2013). Genetic data suggests that the primary role of modifications in yeast cells is to increase the efficiency of recognition of cognate codons by tRNAs. Mutants lacking both the mcm⁵ modification and the s² modifications (elp2Δ/ ncs6Δ double mutants) are not viable, but can be rescued by overexpression of tLys¹₈⁶. As with the bacterial system, the mcm⁵ modification enhances recognition and decoding of G ending codons and the s² modification enhances recognition of A, and to a lesser extent G, ending codons. Although mcm⁵s² modified tRNAs can under some conditions recognize G ending codons in vivo, this decoding is not efficient. The primary effect of the mcm⁵s² modification is in codon recognition, as in yeast cells there are no differences between hypomodified and modified tRNAs with regard to steady state aminoacylation or abundance/stability (Bjork et al., 2007; Johansson et al., 2008).

It is worth noting that while much data from prokaryotic systems seems to apply to eukaryotic systems, there are chemical differences between the modifications, specifically the mnm⁵ and the mcm⁵ modifications, and this may lead to differences between the properties of modified prokaryotic and eukaryotic tRNAs. There are certainly differences with regard to the functions of modifications, as prokaryotic cells and eukaryotic cells have evolved different requirements with regard to speed and fidelity in translation. Nonetheless, there are conclusions that can be drawn from the large body of research on prokaryotic and eukaryotic U₃₄ modifications found on tLys¹₈⁶, tGln¹₈⁶ and tGlu¹₈⁶ tRNAs. Modifications are essential for the efficient recognition of codons in the context of the ribosome, and the mcm⁵/mnm⁵ modification enhances recognition of G and A ending codons (primarily G ending), while the s² modification
enhances recognition of A ending codons, especially in eukaryotic systems. The specific consequences of hypomodified tRNAs on gene expression will be discussed later.

**tRNA thiolation systems exist in all kingdoms of life**

Thiolated nucleosides are found in tRNA molecules in eukaryotes, prokaryotes and archaea. The thiolated nucleosides 4-thiouridine (s^4U), 2-thiocytidine (s^2C), 5-methylaminomethyl-2-thiouridine (mmn^5s^2U), 5-carboxymethylaminomethyl-2-thiouridine (cmnm^5s^2U), and 2-methylthio-N^6-isopentenyladenosine (ms^2i^6A) are found in *E. coli* tRNAs. This is in contrast to *S. cerevisiae*, for which there are two known thionucleosides: 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U_{34}) in cytosolic tRNAs and 5-carboxymethylaminomethyl-2-thiouridine (cmnm^5s^2U_{34}) in mitochondrial tRNAs. In addition, although not well characterized, thiolated tRNAs have been detected in archaea (Miranda *et al.*, 2011; Chavarria *et al.*, 2014; Shigi, 2014).

**The dynamics of tRNA modifications**

Much effort has been expended in cataloguing tRNA modifications and in identifying the genetic pathways and biochemical activities required for formation of modifications, but little attention has been paid to determining whether tRNA modifications are constitutive or dynamic. While it is clear that certain modifications critically impact tRNA function or stability and hence are likely to be required under all growth conditions, other modifications may alter tRNA properties in ways that are advantageous under certain conditions.

There is some evidence from different organisms that tRNA modifications fluctuate. Initially, differentially modified tRNA species were detected by chromatography (either liquid or
thin layer chromatography); subsequently, comparison of tRNA sequences established that nucleotide differences were the result of posttranscriptional modifications and not of different genetic sequences. tRNA methylation levels in *Bacillus subtilis* were found to be lower in exponentially growing cells, as compared to the levels in stationary phase cells or in spores (Singhal and Vold, 1976). In *Bacillus stearothermophilus*, increased levels of tRNA methylation (2'-O-methylribose moieties) were detected when cells were grown at high temperatures (Agris et al., 1973). In *Drosophila melanogaster*, the levels of queuosine in tyrosine, histidine, aspartic acid and asparagine tRNAs were found to vary with age and diet (Owenby et al., 1979).

In mammalian cells, several studies indicated that levels of tRNA modifications in tumor cells differed from modification levels in normal tissues. Phenylalanine tRNAs were found to be differentially modified in tumor cells: tumor derived tRNAs lacked the Y modification (wybutosine), were found to contain altered levels of 1-methylguanine modification, and had elevated levels of the 5-methylcytidine and dihydrouridine modifications (Grunberger et al., 1975; Kuchino and Borek, 1978; Kuchino et al., 1982). Additionally, both cytosolic and mitochondrial aspartate tRNAs derived from tumor tissues were found to lack queuosine modifications (Kuchino et al., 1981; Randerath and Agrawal, 1984).

These studies established that tRNAs were differentially modified in a condition/tissue specific fashion, but for a long time, the functional characterization of tRNA modification dynamics remained a relatively unexplored area. More recently, there have been advances in understanding how tRNA modifications contribute to gene expression. Using a liquid chromatography-coupled mass spectrometry (LC-MS/MS) method, Chan and colleagues were able to detect and quantify levels of 23 tRNA modifications in *S. cerevisiae* cells treated with various toxicants (MMS, hydrogen peroxide, sodium arsenite and sodium hypochlorite)
compared to untreated controls (Chan et al., 2010). Levels of various tRNA modifications were found to increase or decrease in response to treatment with various toxicants in both a dose and toxicant specific manner, with some modifications demonstrating similar patterns in response to specific toxicants. These results demonstrate the potential for a role of tRNA modifications in dynamic, regulatory processes.

**Gene specific translation mediated by tRNA modifications**

The dynamic modification of tRNAs appears to be yet another mechanism that cells use to modulate gene expression. There are several studies, conducted using *S. cerevisiae* unless otherwise noted, indicating that for specific modifications, changes in modification levels are linked to the condition specific expression of proteins.

Trm9 is a methyltransferase required for the last step in the synthesis of the mcmm\(^5\) modification, which is found on tArg\(^{UCU}\), tLys\(^{UUU}\), tGln\(^{UUG}\) and tGlu\(^{UUC}\) (Kalhor and Clarke, 2003; Huang, 2005; Bjork et al., 2007). trm9Δ cells showed an increase in sensitivity to MMS and so the role of Trm9 in the DNA damage response was investigated. It was found that trm9Δ cells had defects in translating codons recognized by modified tRNAs (specifically tArg\(^{UCU}\) and tGlu\(^{UUC}\) tRNAs). An analysis of gene specific codon usage revealed that transcripts enriched in codons recognized by Trm9-modified tRNAs were translated less efficiently in trm9Δ cells. Interestingly, transcripts enriched in these codons were overrepresented in functional categories including protein synthesis, energy and metabolism and stress and damage responses. The DNA damage phenotype was specifically linked to the translation of *RNR1* and *RNR3* transcripts, which were enriched in AGA and GAA codons (Begley et al., 2007; Patil et
Trm9 deficiency was also been linked to translational infidelity-misreading and frameshifting- and to increased levels of protein stress (Patil et al., 2012a).

Analysis of another methyltransferase, Trm4, also revealed a relationship between the condition-specific modulation of tRNA modifications and the condition-specific translation of specific transcripts. trm4Δ mutants are defective in synthesis of the 5-methylcytidine (m⁵C) modification found on tLeuCAA tRNAs. A study found that treatment with H₂O₂ led to an increase in m⁵C modification levels of tLeuCAA and that certain genes showed a biased use of TTG codons. Among these genes was the ribosomal paralog RPL22A, the expression of which is induced by exposure to H₂O₂ and is also impaired in trm4Δ cells. An analysis of protein levels demonstrated that the treatment of wild type cells with H₂O₂ resulted in increased levels of proteins whose transcripts were enriched for TTG codons (Chan et al., 2012).

A study examining the m⁵C modification found on tHisGUG found that levels of the modification, found on positions C₄₈ and C₅₀, increased under specific growth conditions. Cells that were grown to stationary phase, starved for amino acids, treated with rapamycin, or, to a lesser extent, starved of glucose or uracil, accumulated additional m⁵C modifications on tRNAs. This modification was also dependent on the activity of Trm4 (Preston et al., 2012).

In S. pombe, Elp3/Sin3 (a subunit of Elongator complex, equivalent to S. cerevisiae Elp3) is required for formation of the mcm⁵s²U₃₄ modification, and cells in which sin3/elp3 was deleted were sensitive to oxidative stress. Core environmental stress response (CESR) genes, including those whose expression was induced by H₂O₂, were found to have codon usage biased to AAA (vs. AAG), and were less efficiently translated in mutant cells (Fernández-Vázquez et al., 2013).
A series of studies has revealed that tRNA thiolation is also linked to the condition specific control of gene expression. Levels of thiolated mitochondrial tLys\textsuperscript{UUU} were found to decrease when cells were grown at an elevated temperature, leading to defects in mitochondrial translation. No changes in the thiolation status of mitochondrially encoded tGlu or tGln were detected (Kamenski \textit{et al.}, 2007), and it is important to note that the thiolation systems for cytoplasmic and mitochondrial tRNAs are separate and comprised of different components (Umeda, 2004; Leidel \textit{et al.}, 2009). A recent study using a SILAC based proteomics approach found that in \textit{urml}Δ and \textit{elp3}Δ cells, proteins encoded by mRNAs rich in AAA, CAA, AAG and GAA codons were down-regulated at the level of translation. In mutant cells, proteins that were down-regulated were enriched for anabolic processes (including translation initiation and elongation) while up-regulated proteins were enriched in catabolic processes (such as proteasomal degradation) (Rezgui \textit{et al.}, 2013).

A separate study focusing on sulfur metabolism and cellular homeostasis found that intracellular levels of methionine and cysteine controlled tRNA thiolation levels, and that \textit{URMI} pathway mutants, as a function of defects in tRNA thiolation, had defects in metabolic cycling and in sensing of intracellular amino acid levels. Interestingly, mutants incapable of synthesizing thiolated tRNAs showed an increase in chronological lifespan, suggesting that hypomodified tRNAs, under certain conditions, might be advantageous (Laxman \textit{et al.}, 2013). In agreement with the study described previously (Rezgui \textit{et al.}, 2013), Laxman et al. found that \textit{uba4}Δ mutants had decreased levels of proteins enriched in lysine, glutamine and glutamic acid, and that these codons were overrepresented in genes coding for proteins involved in processes such as rRNA processing, ribosomal subunit biogenesis, translation and growth specific biological processes. A proteomic analysis revealed that \textit{uba4}Δ and \textit{ncs2}Δ mutants had down-
regulated levels of proteins involved in sugar and carbohydrate metabolism and up-regulated levels of proteins involved in cellular amino acid biosynthesis, small molecule metabolism and sulfur compound metabolism (Laxman et al., 2013).

**The role of tRNA modifications in health and human disease**

A variety of tRNA modifications have been linked to neurological, cardiac, respiratory and metabolic disorders, as well as various types of cancers (Towns and Begley, 2012; Torres et al., 2014), and several studies have implicated the mcm$^5$s$^2$U$_{34}$ modification in disease. Because tRNA modifications are highly conserved, the use of model organisms to study the biogenesis and functions of tRNA modifications offers the chance to gain insight into diseases.

Genome wide linkage analysis linked SNPs in *ELP4* to Rolandic epilepsy (Strug et al., 2009), and a study using both genetic association studies in amyotrophic lateral sclerosis patients and a genetic screen in *D. melanogaster* suggested a role for *ELP3* in axonal biology and neuronal degeneration (Simpson et al., 2008). SNP variants of the *IKBKAP* gene (which encodes IKAP, the human homolog of *ELP1*) have been linked to bronchial asthma (Takeoka et al., 2001) and *IKBKAP* has also been linked to the neurological condition familial dysautonomia (FD). Specifically, mutations resulting in splicing or phosphorylation defects were found in *ELP1* mRNA isolated from patient samples (Anderson et al., 2001; Slaugenhaupt et al., 2001).

On a mechanistic level, depletion of IKAP/ELP1 was found to result in a decrease in histone H3 acetylation as well as a decrease in the translation of a subset of genes involved in cell motility, suggesting a mechanistic basis for neuropathology in FD (Close et al., 2006).

It was initially assumed that the IKAP/ELP1 phenotype was due to defects in transcription and histone acetylation, as the elongator complex was initially described as an RNA
polymerase II associated transcription elongation factor (Otero et al., 1999; Wittschieben et al., 1999). Recent studies, however, suggest that defects in tRNA modification and the resulting translational changes could be the underlying cause of pathology. Defects in tRNA modification were shown to be responsible for multiple phenotypes associated with Elongator-deficient cells (Esberg et al., 2006). A study in *C. elegans* verified that Elp1 and Elp3 were required for formation of the mcm$^5$ moiety and that Tuc1 (Ncs6) was required for tRNA thiolation; further, Elp1 and Elp3 were expressed in subsets of neurons, and deletion mutants had neuronal defects that resulted in impaired chemotaxis (Chen et al., 2009a). This suggested a mechanism whereby tRNA modifications could lead to FD symptoms.

**Conclusions**

The discovery that Urml serves as a sulfur carrier in tRNA modification reactions, in addition to serving as a protein modifier, has led to many questions regarding the role of Urml-dependent tRNA modifications in the regulation of translation and other cellular processes. This thesis presents work that describes the functions of Urml-dependent tRNA modifications and characterizes the phenotypes of cells lacking components of the *URM1* pathway. This thesis also describes how modulation of tRNA thiolation levels may be part of a strategy that cells use to adapt to growth under stressful conditions.
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Chapter 2:

tRNA Thiolation Links Translation to Stress Responses in *Saccharomyces cerevisiae*

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

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The experiments in Figure 4 were performed in collaboration with David Pincus.
ABSTRACT

Although tRNA modifications have been well catalogued, the precise functions of many modifications and the role they play in mediating gene expression are still being elucidated. While 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 \textit{URM1} pathway is required for thiolation of the cytoplasmic tRNAs $\text{tGlu}^{\text{UUC}}$, $\text{tGln}^{\text{UUG}}$ and $\text{tLys}^{\text{UUU}}$ in \textit{Saccharomyces cerevisiae}. We demonstrate that \textit{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. \textit{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 \textit{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 co-regulated according to need.
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\(^6\)-methyladenosine (m\(^6\)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. While the well-conserved nature of tRNA modifications would suggest critical roles in cellular processes, the majority of modifications are nonessential and the deletion of many genes encoding modification enzymes results in 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 impact 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 impact 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$^5$s$^2$U$_{34}$ (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$^5$ moiety found on tRNAs (Kalhor and Clarke, 2003; Lu *et al.*, 2005). Deletion of genes in the *URM1* pathway result in hypomodified cytoplasmic tGlu$^{UUC}$ (GAA codon), tGln$^{UG}$ (CAA codon) and tLys$^{UUG}$ (AAA codon) tRNAs, a subset of the tRNAs modified in an *ELP*-dependent fashion; these tRNAs contain the mcm$^5$-, but not the s$^2$-, U$_{34}$ 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 β-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 an ATP-dependent fashion by Uba4 in *Saccharomyces 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; 2003b; Van der Veen *et al.*, 2011) and as a sulfur carrier that is utilized 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 differentiated from potential effects of protein urmylation through comparison of *urml*Δ strains with *ncs2*Δ and *ncs6*Δ cells (Ncs2 and Ncs6 have no reported functions outside of tRNA modification), or with *ELP* pathway mutants. The mcm$^5$s$^2$U$_{34}$ 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.

*URMI* pathway mutants display a variety of phenotypes, including increased sensitivity to oxidative stressors as well as defects in nutrient sensing and invasive growth, many of which are linked to defects in tRNA modification (Goehring *et al.*, 2003a; 2003b; Rubio-Texeira, 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 unappreciated 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 establish the role of Urm1-dependent tRNA modifications by examining translation in wild type and *urmlΔ* cells. We find that *urmlΔ* 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 *URMI* pathway mutants are more resistant to certain stresses compared to wild type cells, suggesting that the consequences of hypomodified tRNAs result in cellular adaptations that allow cells to better withstand certain stresses. Interestingly, 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 *URMI* pathway links tRNA modification to translation

In order to determine the impact of hypomodified tRNAs on translation, we performed polysome profile analysis as a proxy for bulk translation. We found that *urm1Δ* cells demonstrated a subtle but significant decrease in the polysome:monosome (P:M) ratio compared to wild type cells, indicating a slight global impairment in translation (Figure 1A). As described above, disruption of the elongator complex impairs formation of the mcm^4^U modification (Huang *et al.*, 2008). Polysome profiles of elongator-deficient *elp2Δ* cells were similar to those of *urm1Δ* cells, consistent with the interpretation that defects in U^34^ modifications underlie the differences between wild type and mutant cells (Figure 1A). In order to directly examine the impact of hypomodified tRNAs on translation, we measured incorporation of radioactive amino acids (^35^S-cysteine and ^35^S-methionine) in different strains. Wild type and *urm1Δ* cells showed no difference in the rate of protein synthesis measured in this manner when grown at 25°C, reinforcing the finding that changes in bulk protein synthesis are minor (Figure 2).

We reasoned that while bulk translation may be largely unaffected in *URMI* pathway mutants, 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 *urm1Δ* 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. While there was no difference between wild type and *urm1Δ* extracts with regard to levels of 12x CAG luciferase produced, *urm1Δ* extracts translated 12x CAA luciferase less efficiently.
compared to wild type extracts, suggesting that there are codon/tRNA specific translation defects in urm1Δ cells (Figure 1B). While defects in translation were subtle, they may significantly impact cellular fitness; mutant cells (urm1Δ and ncs6Δ strains) grew more slowly than wild type cells at 30°C 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 URMI 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, URMI pathway mutants (urm1Δ, uba4Δ, ncs2Δ, ncs6Δ) displayed impaired growth in the presence of translation inhibitors. Growth of the ELP pathway mutant elp2Δ 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, ahplΔ cells – Ahpl is the only known protein to which Urmi 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). In order to establish that the observed defects in translation were due to impaired tRNA modification, we overexpressed tGluUUC (tE), tGlnUUG (tQ) or tLysUUU (tK) from a high copy (2 micron) plasmid. We created constructs to express each tRNA alone or in combination with the other tRNAs. Expression of the triple tE/K/Q construct in an urm1Δ 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 tLysUUU (tK) alone was sufficient to rescue growth (Figure 1E).

**Figure 1:** Translation is mildly affected in **URMI** 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 YPDA at 30°C or 37°C. The average of 2 (for **urm1Δ 37°C**) or 3 replicates (all other strains/conditions) is shown. D. Five-fold 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. Five-fold serial dilutions of wild type or **urm1Δ**
cells harboring empty vector the plasmid encoding the indicated tRNA(s) were plated onto YPDA plates containing 0.1 µg/ml cycloheximide or 0.2 mg/ml paromomycin.

**Figure 2: Bulk protein synthesis is not impaired in urm1Δ cells.** A. Wild type or urm1Δ cells were incubated with $^{35}$S cysteine and methionine for the indicated times, and radioactive amino acid incorporation into proteins was determined following TCA precipitation.

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**The heat shock response is activated in URMI pathway mutants**

Since strains with deletions in the URMI 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, *SSA4* and *STI1*, which encode chaperones/cochaperones. We found evidence that urm1Δ cells had increased levels of stress-induced transcripts (Figure 3A), and that the
increased levels of at least a subset of these transcripts was linked to the tRNA modification function of Urm1 (Figure 3B, 3C).

Figure 3: Levels of stress induced mRNA transcripts are up-regulated in urm1Δ cells. A. Northern blot of RNA extracted from wild type or urm1Δ cells grown at 30°C, shifted to 37°C or treated with the following compounds (final concentration) for 1 hour: paromomycin (0.2 mg/ml), tunicamycin (1 μg/ml), cycloheximide (10 μg/ml) or diamide (2 mM). Membranes were probed for SSA4, STI1, UBI4 or ACT1 transcripts as indicated (note that the UBI4 specific probe cross reacts with UBI1, UBI2, and UBI3 transcripts). B. Northern blot of RNA extracted from wild type cells or urm1Δ cells harboring empty vector or a 2 micron plasmid expressing tE, tK
and tQ as indicated. Cells were grown at 30°C, shifted to 37°C or treated with 0.2 mg/ml of paromomycin for 1 hour. Membranes were probed for UBI4 or ACT1. C. Quantification of UBI4 mRNA levels, normalized to ACT1 levels. The average and SD of 2 independent experiments is shown.

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 ER stress (Mori et al., 1992; Kohno et al., 1993; Cox and Walter, 1996; Mori et al., 1996; Nikawa et al., 1996; Mori et al., 1998), while 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 urmlΔ 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 urmlΔ cells, we utilized reporter constructs specific for a single response. These reporters consist of a fluorescent protein (YFP or GFP) whose expression is controlled by a promoter containing four repeats of a motif recognized by a specific transcription factor. For our analysis, we used the following constructs, which were integrated as a single copy at the LEU2 locus in wild type, urmlΔ or ncs6Δ yeast strains: P4xHSE-YFP, P4xUPRE-GFP and P4xSTRE-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 analog 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 4A-C), indicating that our constructs were functional in all of the strains being used, and also that urmlΔ and ncs6Δ cells were able to respond to specific stressors comparably to wild type cells. For both the P4xUPRE-GFP and P4xSTRE-GFP reporters, we found that basal levels of activation were the same in wild type, urmlΔ and ncs6Δ cells. By contrast, we observed that urmlΔ and ncs6Δ cells harboring the P4xHSE-YFP reporter had higher levels of fluorescent protein compared to wild type when cells were untreated, indicating that the Hsfl mediated transcriptional response is basally up-regulated in URMI pathway mutants (Figure 4A, 4D).

Since both urmlΔ and ncs6Δ strains showed an increase in P4xHSE-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, urmlΔ or ncs6Δ strains harboring the P4xHSE-YFP construct. All strains were responsive to AZC (Figure 4E). While overexpressing tRNAs had no effect in the wild type basal level of Hsfl activity, in the urmlΔ and ncs6Δ cells, overexpression of tRNAs was able to restore P4xHSE-YFP expression to wild type levels in untreated cells (Figure 4F). Thus the processes that are dysregulated in urmlΔ cells lead specifically to activation of the Hsfl-mediated heat shock response and are tRNA-dependent, consistent with the defects in translation that urmlΔ cells exhibit.
Figure 4: The heat shock response pathway is up-regulated in \textit{URM1} pathway mutants.

Indicated strains harboring the \textbf{A}. \( P_{4xHSE}\)-\textit{YFP}, \textbf{B}. \( P_{4xUPRE}\)-\textit{GFP} or \textbf{C}. \( P_{4xSTRE}\)-\textit{GFP} were left untreated, exposed to 1 mM AZC or 1 \( \mu \text{g/ml} \) tunicamycin. \textbf{D}. Data from (A) replotted. \textbf{E}. Wild type, \textit{ncs6}\Delta or \textit{urm1}\Delta strains harboring the \( P_{4xHSE}\)-\textit{YFP} reporter and a 2 micron plasmid (either empty vector or \texttt{tE/K/Q} plasmid, denoted as +\text{tRNA}) were untreated or exposed to 1 mM AZC. \textbf{F}. Data from untreated samples in (E) replotted. Fluorescence levels were normalized to WT untreated, and values are the averages of three cultures. Error bars denote standard deviations.
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 *Saccharomyces 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 (MMS), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and sodium arsenite (NaAsO<sub>2</sub>) 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 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>). As oxidative stress causes Urm1 conjugation to proteins (Goehring et al., 2003a; Van der Veen et al., 2011), we were particularly interested to determine if 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 5A). Neither translation inhibition nor oxidative stress caused any change in the modification status of these tRNAs. By contrast, growth at elevated temperature resulted in an accumulation of
hypomodified tGlu\textsuperscript{UUC}, tGln\textsuperscript{UUG} and tLys\textsuperscript{UUU}, all of the tRNAs known to be thiolated by Urm1 (Figure 5A, 5B).

To better understand the temporal dynamics of tRNA modification, we next performed an extended temperature shift experiment in which cells were grown at 37°C for up to 24 hours. An increase in hypomodified tRNA species was evident beginning at 3 hours of growth at elevated temperature. At later time points, the proportion of unthiolated tRNA relative to thiolated tRNA continued to increase. By 6 hours, the majority of tGlu\textsuperscript{UUC}, tGln\textsuperscript{UUG} and tLys\textsuperscript{UUU} existed in the hypomodified form (Figure 5C). This suggests that the change in tRNA thiolation is not a transient phenomenon but an ongoing adaptation to growth at high temperatures.
Figure 5: 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 hours, membranes were probed for the indicated tRNA. The positions of thiolated and
unthiolated tRNA molecules are indicated. B. Quantification of unthiolated tRNA as a percent of total tRNA species in wild type cells grown at 37°C for 0 or 3 hours. The average and standard deviation of three replicates are reported. C. Small RNA (+/- APM as indicated) Northern blot analysis of RNA extracted from cells grown at 30°C or at 37°C for the indicated period of time, membranes were probed for the indicated tRNA.

Despite being hypomodified at elevated temperatures, we found that tRNAs were not degraded by the rapid tRNA decay pathway (Figure 6) and continued to associate with translating ribosomes (Figure 7). Thus, both fully modified and hypomodified tRNAs can participate in translation in vivo, and that the properties of hypomodified tRNAs are relevant to the process of translation in cells growing at high temperatures.

Figure 6: Stability of hypomodified tRNA species. A. Quantification of steady-state levels of tRNA species in wild type or urm1Δ cells. RNA was extracted from cells grown at 37°C for 0 or 3 hours, analyzed by small RNA Northern blot analysis and levels were quantified. tRNA levels
were normalized to levels of 5S rRNA. The average and standard deviation of three replicates is reported. Levels of B. tGluUUC (tE), C. tLysUUU (tK) or D. tGlnUUG (tQ) were determined in wild type, urm1Δ or elp2Δ cells by Northern blot analysis, cells were treated with pol III inhibitor prior to incubation at the indicated temperature. Levels normalized to wild type 0 hr level.

![Figure 7: Hypomodified tRNAs associate with actively translating ribosomes.](image)

Polysome profiles of wild type cells grown at A. 30°C or B. 37°C for 3 hours. 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^{GUG} E. 30°C, F. 37°C or tLys^{UUU} G. and I. 30°C, H. and J. 37°C.
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 process is occurring, we examined the role of RNA polymerase III (pol III), which is responsible for tRNA synthesis, in the modification of $\text{mcm}^5\text{s}^2\text{U}_{34}$-containing tRNA. To this end, we pre-incubated wild type cells with the pol III inhibitor ML-60218 or DMSO at 30$^\circ$C for 2 hours prior to shifting cells to 37$^\circ$C for up to 3 hours. APM-Northern blot analysis showed that levels of hypomodified tGlu$^{\text{UUC}}$, tGln$^{\text{UUG}}$ and tLys$^{\text{UUU}}$ were decreased in cells when the activity of pol III was inhibited (Figure 8A). 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 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 8B). We conclude that it is the newly synthesized tRNA transcripts that are the substrates for the modification enzymes in the $URMI$ 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 $ELP$ ($\text{mcm}^5\text{U}_{34}$) or $URMI$ ($\text{s}^2\text{U}_{34}$) 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, TUMI and UBA4 (s2-U34) as well as ELP2 and ELP4, which are required for the formation of the mcm5-U34 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 hours 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 8C).

Since heat shock is known to decrease translation and ribosome biogenesis (Gasch et al., 2000; Shalgi et al., 2012; Liu et al., 2013), we wondered if tRNA modification and translation are coordinated through stability of the modification machinery. To test this notion, we performed cycloheximide chase experiments to shut off translation in order to determine the stability of URM1 pathway proteins. We found that at 30°C and 37°C, components of the URM1 pathway were significantly less stable than Pgk1, which we used as a reference (Figure 8D, Figure 9). Tum1-3HA and Ncs6-3HA in particular were degraded rapidly, although levels of Uba4-3HA and Ncs2-3HA also decreased significantly. Thus the components of the URM1 pathway depend on constant translation for their stable expression in the cell (Figure 9). In this manner, the levels of the thiolation components are matched to the level of translation.
Figure 8: Accumulation of hypomodified tRNAs requires synthesis of new tRNA transcripts and is dependent on levels of tRNA modification enzymes. A. Small RNA (+/- APM as indicated) Northern blot analysis of RNA extracted from wild type cells growing at 37°C for the indicated timepoints. Cells were pre-treated with pol III inhibitor prior to the temperature shift. B. Quantification of levels of unthiolated/total tGlu\textsuperscript{UUC} or tLys\textsuperscript{UUU} from blot in (A). C. Levels of C-terminally 3xHA tagged Tum1, Uba4, Ncs2, Ncs6, Elp2 or Elp4 proteins were determined by Western blot analysis. Indicated strains were grown at 30°C 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 200μg/ml of cycloheximide for indicated periods of time, and protein abundance was determined by Western blot analysis.
A

30°C

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- **Ncs2-3HA**
  - α HA
  - α PGK

- **Ncs6-3HA**
  - α HA
  - α PGK

- **Tum1-3HA**
  - α HA
  - α PGK

- **Uba4-3HA**
  - α HA
  - α PGK

B

**Graph**

- Ncs2x3HA
- Ncs6x3HA
- Tum1x3HA
- Uba4x3HA
- Pgk1

**Axes**

- Y-axis: ln (intensity)
- X-axis: Time (min)
Figure 9: Stability of 3HA tagged proteins at 30°C. A. Cells expressing the indicated tagged protein were grown at 30°C, and cycloheximide was added to a final concentration of 200 μg/ml. Protein abundance was determined by Western blot analysis using antibodies detecting the 3HA tag or Pgkl. B. Quantification of protein levels from (A), values were determined using ImageJ software.

**URMI 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 URMI 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_{110} 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 ncs6Δ cells showed an increased resistance to tunicamycin compared to wild type cells (as did uba4Δ and elp2Δ strains, data not shown), both in serial dilution spot assays and in liquid culture (Figure 10A, 10B).

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 10B).

We reasoned that the observed tunicamycin resistant phenotype of urm1Δ cells might be due to aberrant activation of the UPR. To explore this possibility, we next 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 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 10D).

To determine if there was any sign of differential tRNA modification in wild type cells growing in the presence of tunicamycin, we made use of the strains expressing epitope tagged versions of the URM1 pathway components Ncs2, Ncs6 and Uba4. Cells treated with 0.5ug/ml tunicamycin for 3 hours had decreased levels of Ncs2-3HA and Ncs6-3HA, although there was no change in Uba4-3HA protein abundance (Figure 10E). As a comparison, we treated cells with diamide, which did not result in modulation of tRNA thiolation levels (Figure 5A); diamide treated cells did not have altered levels of Ncs2-3HA, Ncs6-3HA or Uba4-3HA at 1 or 3 hours of exposure to diamide compared to the 0 hour time point (Figure 10E). These results indicate that wild type cells down-regulate components of the URM1 pathway under challenging growth circumstances in which hypomodified tRNAs confer a growth advantage.
**Figure 10:** *URMI* pathway mutants are resistant to tunicamycin in a tRNA dependent and *HAC1* independent manner.  

A. Five-fold serial dilutions of wild type, *urmiΔ* or *ncs6Δ* cells were plated onto YPDA plates containing 1 μg/ml tunicamycin. B. Growth curves of wild type, *urmiΔ* and *ncs6Δ* cells grown in YPDA containing tunicamycin at 0.5 or 1.0 μg/ml final concentration. Average growth of three colonies is shown. C. Five-fold serial dilutions of wild type or *urmiΔ* cells harboring empty vector or a 2 micron plasmid expressing the tRNA(s) indicated were plated onto YPDA plates containing 0.5 μg/ml tunicamycin. Stars indicate tRNA constructs conferring sensitivity to tunicamycin. D. Northern blot of RNA extracted from wild type or *urmiΔ* cells grown in YPDA and treated with DMSO or 1 μg/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 μg/ml) or diamide (4 mM) for the indicated time period.
DISCUSSION

Here we have demonstrated that bulk translation is subtly diminished by defects in tRNA thiolation under standard growth conditions, but we have also shown that there are codon specific defects in translation. Our data are consistent with genetic evidence in yeast that indicates that the mcm\(^5\)s\(^2\)U\(_{34}\) 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\(^5\)s\(^2\)U\(_{34}\) modification is not in maintaining translational fidelity but 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 urmlA 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). Our results are consistent with these observations.

Our analysis of URMI pathway mutants further revealed that defects in tRNA modification lead to the activation of the Hsf1-dependent heat shock response (HSR). The HSR specifically 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 (Trotter, 2002; Metzger and Michaelis, 2009; Geiler-Samerotte et al., 2011). Although the exact mechanism whereby hypomodified tRNAs lead to the activation of stress pathways remains unclear, there are several plausible hypotheses. It is possible that the functional consequences of hypomodified U\(_{34}\) nucleosides mimic tRNA depletion in cells, which can result in proteotoxic stress and decreased growth rates (Yona et al., 2013). Hypomodified tRNAs could affect translation elongation and interfere with
cotranslational protein folding. It is possible that urmlΔ cells experience protein-folding related stress that, while minor, is sufficient to elicit a transcriptional response. Alternatively, changes in gene expression could trigger activation of the HSR. Further studies are needed to differentiate between these models.

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 have identified a condition, growth at 37°C, which 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. Interestingly, studies to identify quantitative trait loci associated with thermotolerance in yeast identified NCS2 as a causative gene (Sinha et al., 2008; Yang et al., 2013); specifically, an allele variant consisting of the nonsynonomous A212T SNP was identified as the underlying cause of NCS2 related thermotolerance. It is possible that altered Ncs2 protein function results in variations in tRNA thiolation levels that contribute to thermotolerance. 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).

We have demonstrated that the synthesis of tRNA transcripts by pol III is required for the accumulation of hypomodified tRNAs, indicating that the differential modification of newly transcribed tRNAs is responsible for the appearance of unthiolated tRNAs. We find that at
elevated temperatures, the levels of URMI pathway components decrease as levels of unthiolated tRNAs increase. We have demonstrated that components of the URMI pathway are unstable and rapidly degraded, particularly at 37°C. 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, resulting in 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 (Figure 11).

Figure 11. A model for the link between tRNA thiolation, translation, growth and stress sensitivity. A proposed model for the connection between tRNA thiolation, translation, growth and stress sensitivity in wild type and urm1Δ cells.
Our observations are consistent with a model in which modulation of tRNA thiolation levels constitutes a long-term adaptation to an ongoing stress, helping to perpetuate a slow down of protein synthesis under less than optimal conditions. tRNA thiolation levels under a specific condition would reflect a variety of factors, including rate of tRNA synthesis, abundance of \textit{URMI} pathway transcripts, the magnitude and duration of changes in translation, and rate of protein degradation. Under this model, different stresses might be expected to have different consequences for tRNA thiolation.

We have demonstrated that cells with tRNA thiolation defects phenocopy cells that have been exposed to stress. \textit{URMI} pathway mutants have defects in translation and grow more slowly than wild type cells. Additionally, the Hsfl-dependent heat shock response is activated in \textit{URMI} pathway mutants. Under certain stress conditions, \textit{URMI} mutants have an advantage; \textit{URMI} pathway mutants are more resistant to tunicamycin compared to wild type cells in a tRNA dependent fashion. The tunicamycin-resistant phenotype of \textit{URMI} pathway mutants might be attributable to the slow growth that results from hypomodified tRNAs. There is precedence for this, as resistance to tunicamycin has been observed in ribosomal protein deletion strains; growth rates of deletion strains in the presence of tunicamycin were generally inversely correlated with growth rates in the absence of tunicamycin (Steffen \textit{et al.}, 2012), supporting a link between decreased translation, decreased growth and resistance to stress. We also observed that wild type cells treated with tunicamycin had decreased levels of Ncs2-3HA and Ncs6-3HA compared with untreated cells, suggesting that treatment with tunicamycin might lead to decreased levels of tRNA thiolation, perhaps through decreased translation as part of an adaptive strategy. The link between growth rate and response to stress has been established; the environmental stress response (ESR) is a generalized transcriptional response that yeast cells mount in response to a
decrease in growth rate that is thought to result from exposure to any of a wide variety of stresses (Brauer et al., 2008; Lu et al., 2009).

As mentioned previously, Urm1 also acts as a protein modifier in yeast and mammalian cells. Although we did not find evidence of a direct link between the dual functions of Urm1, the coordination of tRNA thiolation and protein urmylation remains a possibility. The components of the URM1 pathway could be regulated differentially or coordinately. We observed, in the case of wild type cells treated with tunicamycin, that levels of Ncs2 and Ncs6 decreased over time, while levels of Uba4 remained unaffected. The existence of deurmylases and/or other proteins required for protein urmylation would add further complexity to the regulatory landscape of Urm1-dependent functions, as would additional proteins involved in tRNA modification or demodification.

We have characterized the effects of the mcm$^{5s}$U$_{34}$ modification on cellular physiology. While there is still much to be discovered, we have demonstrated that tRNA modifications are dynamic and modifications play a role in maintaining homeostasis in cells.
# MATERIALS AND METHODS

Yeast Strains used in this study (derivatives of W303)

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Yeast Strains and Growth

Yeast strains are derivatives of W303. Strains used are listed in Table S1. Yeast were grown according to standard techniques. Strains containing gene deletions or C-terminally 3HA tagged versions of protein were generated as described in (Longtine et al., 1998) and verified by...
PCR (and Western blot for presence of HA tag). 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 prior to collection. Cells were lysed by the addition of polysome lysis buffer (20 mM Hepes-KOH pH 7.4, 2 mM MgOAc, 100 mM KOAc, with 0.1 mg/ml cycloheximide, 3 mM DTT, 1% Triton-X 10) and acid-washed glass beads followed by vortexing. 5-10 A_{260} units of cleared lysate were loaded onto 10-50% sucrose gradients (prepared in polysome lysis buffer containing cycloheximide and DTT) and subjected to ultracentrifugation (35,000 rpm, 3 hr using 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 254 nm. When required, fractions were collected for RNA preparation.

**Radioactive amino acid incorporation**

Cells were grown in minimal media supplemented with 2% glucose appropriate amino acids. $^{35}$S Met/$^{35}$Cys (Perkin Elmer EasyTag) was added to aliquots of actively growing cells, and at indicated timepoints, 200ul of labeled culture was added to an equal amount of cold 20% TCA and incubated on ice. Cells were lysed by boiling for 10min and then incubated on ice for 10min. Aliquots of lysate were spotted onto glass microfiber filters (Whatman GF/B) 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 CPM (counts per minute) were
determined. CPM were normalized to OD600 readings taken using duplicate non-labeled cultures.

**Serial Dilutions**

Overnight cultures of cells were diluted into fresh media and allowed to grow for several hours, then rinsed in PBS and resuspended to an OD$_{600}$ of 0.1. Aliquots of fivefold serial dilutions were plated onto solid media, plates were scanned after various growth times. For strains containing 2 micron 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); following precipitation and rinsing, RNA was resuspended in DEPC 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 system, National Diagnostics) in 0.5x TBE. For gels containing N-acryloylamino phenyl mercuric chloride, APM was added to gel mixture. Gels were transferred to Genescreen Plus membrane and then UV-crosslinked. Membranes were probed with end-labeled with $\gamma^{32}$[P] (Perkin Elmer) DNA oligonucleotide probes (IDT) complementary to the tRNA species of interest. Sequences of probes used are listed in Table S2. Following hybridization and washing steps, membranes were exposed to phosphorimager
screens and imaged using BAS/Fuji imager. 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 SSC buffer and then UV crosslinked. Probes were prepared using the Amersham Megaprime DNA labeling system (GE Healthcare) and $\alpha^{32}$P dITTP (Perkin Elmer); probes were PCR products generated using yeast genomic DNA as a template and using primers specific for the transcript of interest. Following hybridization and washing steps, membranes were exposed to phosphorimager screens and imaged using BAS/Fuji imager. Quantifications were carried out using Multiguage imaging software.

**Small RNA northern blot probe sequences used in this study**

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<thead>
<tr>
<th>Probe</th>
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<td>tHis-cyto</td>
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<td>tGln-probe</td>
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<td>tGlu-probe</td>
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<td>tQ(UUG)</td>
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<td>B-probe</td>
<td>CACTATAGGACC</td>
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<td>tGlu-probe</td>
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**Temperature shift/stress experiments**

Yeast cells were grown to mid-log phase at 30°C and resuspended in media pre-warmed to 37°C or in 30°C containing the indicated concentration of a given compound. For experiments utilizing polymerase III inhibitor, cells were pre-incubated with ML-60218 (Wu et al., 2003) prior to temperature shift; alternatively, ML-60218 was added at the time of the temperature shift.
shift. For tRNA stability experiments, samples were collected at time= 0 immediately after resuspension in 37°C media and after 3hrs of growth at high temperatures. For cycloheximide chase experiments, cycloheximide was added to a final concentration of 200 μg/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 micron plasmids expressing tEKQ. For assays, cells were grown from single colonies in SD complete media (or SD –Ura for tRNA strains) overnight, diluted into fresh media and then left untreated or treated with 1 mM L-azetidine-2-carboxylic acid or 1μg/ml tunicamycin for 4 hours 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 (lizuka et al., 1994) and treated with micrococcal nuclease immediately prior to use. m7GpppG capped transcripts were produced using T7 RNA polymerase using plasmids encoding either a leaderless luciferase or the 12xCAA/12xCAG leader luciferase constructs (which were constructed by ligating annealed oligonucleotides into the luciferase base
construct). RNA was analyzed by agarose gel electrophoresis prior to use. Translation reactions (15 μl) containing 9.5 μl of translation extract and 50 ng 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 μg creatine kinase, 1 μl 0.6 mM methionine and 0.5 μl RNasein Plus (Promega)) were incubated at 23°C for 60 minutes. 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 following electrophoresis proteins were transferred to PVDF membranes. Anti-HA-Peroxidase, High Affinity Rat monoclonal (3F10) was used for detection of 3-HA tagged proteins (Roche 12013819001) and PGK1 was detected using Phosphoglycerate Kinase Monoclonal Antibody, Mouse (Life Technologies 22C5D8) and anti-mouse IgG-Peroxidase (GE NXA931). Any quantifications were carried out using ImageJ (Schneider et al., 2012).

Plasmids:

Plasmids for tRNA expression were derivatives of the URA3 marked 2-micron 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 elsewhere in this section.

ACKNOWLEDGEMENTS

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Preston, M. A., D'Silva, S., Kon, Y., and Phizicky, E. M. (2012). tRNAHis 5-methylcytidine levels increase in response to several growth arrest conditions in Saccharomyces cerevisiae. RNA 19, 243–256.

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Chapter 3:

Discussion and Future Directions
Key findings

tRNA modifications are found in all kingdoms of life, and while the genetic and biochemical pathways required for the synthesis of modified nucleosides are increasingly well-described, the exact functions that these modifications serve in cells are still being elucidated. The work in this thesis has focused on defining the functions of the Urm1-dependent thiomodification found on a subset of tRNAs containing the mcm*5's2U34 nucleoside in the model organism *Saccharomyces cerevisiae*.

We were able to demonstrate that defects in tRNA thiolation have physiological consequences in cells. We were able to link loss of the *URMI* pathway to defects in translation; although these defects were mild under standard growth conditions, they may be magnified under different growth or stress conditions. Additionally, we were able to demonstrate that cells exhibit a transcriptional adaptation to defects in tRNA thiolation; we observed mutant cells to have an increased basal activation of the Hsf1-dependent heat shock response pathway and to have increased levels of stress induced transcripts.

Analysis of mutant phenotypes allowed us to gain insight into the functions of tRNA modifications in wild type cells and also led to the identification of conditions in which modulation of tRNA modification levels might be utilized by cells to adapt to different growth conditions. We have described a condition, growth at 37°C, under which wild type cells down-regulate tRNA thiolation, and we have described a potential mechanism that cells use to bring about changes in levels of modified tRNAs. The work in this thesis has added to the body of knowledge regarding the role of tRNA modifications in cellular processes, and our results lead to further questions and areas of inquiry that could help to elucidate the functions of the *URMI* pathway in even more detail.
Examining tRNA modifications at elevated temperatures

It is now clear that tRNA modifications play a role in mediating gene expression at the level of translation, and that the genetic pathways controlling tRNA modification status are influenced by the environment and growth conditions. Our APM-Northern blot assays allowed us to specifically examine the thiolation status of tGlu\(^{\text{UUC}}\), tGln\(^{\text{UG}}\) and tLys\(^{\text{UUU}}\) tRNAs. We found that growth of cells at 37°C results in a decrease in the levels of thiolation of these tRNAs. We were able to demonstrate that these hypomodified tRNAs associate with polysomes, suggesting that they can be used in translation. Additionally, we observed that accumulation of hypomodified tRNAs depends upon pol III transcription, suggesting a decrease in the activity of the URM1 pathway under these conditions. Consistent with this hypothesis, we observed a decrease in the protein levels of both URM1 and ELP pathway components upon a shift to elevated temperatures. The decrease in URM1 pathway components provides a possible explanation for the observed decrease in thiolation, however the decrease in protein levels of ELP pathway components suggests that in addition to changes in tRNA thiolation, tGlu\(^{\text{UUC}}\), tGln\(^{\text{UG}}\) and tLys\(^{\text{UUU}}\) tRNAs may also have decreased levels of the mcm\(^{5}\) modification when cells are grown at elevated temperatures.

Examining levels of mcm\(^{5}\)U\(_{34}\) modifications of tRNAs would provide additional insight into how tRNA modification status changes during growth at elevated temperatures. Although the mcm\(^{5}\) and s\(^{2}\) modifications occur on the same nucleoside, and both modifications are important for overall tRNA function, the functions of the modifications do not overlap completely, and so it is unclear whether the modifications might be regulated independently or coordinately. Additionally, while the s\(^{2}\) modification occurs only on tGlu\(^{\text{UUC}}\), tGln\(^{\text{UG}}\) and tLys\(^{\text{UUU}}\) tRNAs, the mcm\(^{5}\) modification is found on these tRNAs and on the U\(_{34}\) nucleosides of

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other tRNAs as well (Kalhor and Clarke, 2003; Huang, 2005; Bjork et al., 2007). Thus, examining the modification status of all tRNAs containing the mcmt\(^5\) modification would give an expanded view of tRNA modifications at high temperatures and would provide additional insights into the regulation of gene expression under these conditions.

A more comprehensive analysis of the tRNA modification landscape in cells grown at elevated temperatures would be even more informative in determining how widespread changes in tRNA modification are under specific growth conditions. Analyzing a subset of tRNA modifications is useful, but it is possible that phenotypes arising in cells grown under various conditions are a consequence of the cumulative effects of changes in the modification status of a number of different tRNAs/nucleosides. These changes might occur on nucleosides at different tRNA positions and could affect various aspects of tRNA biology, from decoding properties to conformation and stability; these changes would be expected to impact the translational capacity of cells. It could be the case that multiple tRNA modifications are coordinately regulated under specific conditions in order to optimize gene expression.

Mass spectrometry has been used to monitor changes in tRNA modification status at the global level and at the level of individual tRNA species (Chan et al., 2010; 2012), and this approach could be used to examine how a multitude of tRNA modifications fluctuate when cells are grown at an elevated temperature (or any other condition). Thin layer chromatography and HPLC analysis of nucleosides are two other methods that have been widely used to analyze tRNA modifications, and these methods could also be appropriate methods for examining specific modifications.

The global analysis of tRNA modifications could be complimented by using a genetic approach to study the functions of specific modifications by utilizing mutants that have
deficiencies in modification pathways. The analysis of mutants can provide information regarding the effects of specific modifications on tRNA structure and function; studies have demonstrated that the lack of specific modifications results in tRNAs that are rapidly degraded (Alexandrov et al., 2006), and tRNAs produced in mutant cells have altered decoding properties as assessed by in vivo and in vitro assays (Phizicky and Hopper, 2010; Yacoubi et al., 2012).

Effects of loss of mcm$^5$S$^2$U$_{34}$ modification on global gene expression under different growth conditions

Our results indicate that changes in translation result in cells lacking an intact URMI pathway. Bulk translation is only mildly affected, as assessed by polysome profiles and radioactive amino acid incorporation, but experiments utilizing luciferase reporter constructs demonstrate that urm1Δ cells translate mRNAs containing codons that are normally recognized by thiolated tRNAs less efficiently. Our data also demonstrate that URMI pathway mutants are likely to have altered patterns of transcription due to the effects of hypomodified tRNAs on cellular processes; we observed an increase in the basal expression of an Hsf1 transcription factor-reporter in urm1Δ and ncs6Δ cells as well as altered levels of stress induced transcripts.

In addition to the specific examples of altered translation in URMI pathway mutants that we describe, recent studies have demonstrated, using ribosome profiling (Zinshteyn and Gilbert, 2013) and proteomic approaches (Laxman et al., 2013; Rezgui et al., 2013), that there are wide-scale changes in gene expression in cells lacking the ability to thiolate tRNAs. These studies were conducted using wild type and URMI pathway mutants grown under standard growth conditions. Our data demonstrates that tRNA thiolation levels are decreased when cells are grown at 37°C, and many studies indicate that URMI pathway mutants have multiple chemical
sensitivities that are related to tRNA modifications. This indicates that it would be relevant to examine gene expression patterns of wild type and mutant cells grown under different conditions.

Mass spectrometry in combination with analysis of RNA levels could be used to analyze the proteomes of mutant cells and to determine how specific tRNA modifications contribute to gene expression at the level of translation. Ribosome profiling is another technique that can provide high-resolution data on ribosome location on mRNA transcripts as well as information regarding the translation efficiency of specific transcripts in wild type and mutant cells (Ingolia et al., 2009). By combining the analysis of tRNA properties in mutant cells with global profiling of tRNA modifications, it should be possible to gain a more detailed understanding of how tRNA modifications coordinate gene expression when cells are subjected to specific stressors. Additionally, total levels of tRNA species in cells, as well as levels of aminoacylated tRNAs, could be determined (Zaborske et al., 2009), adding to the information on the regulation of various aspects of tRNA biology in cells.

The biological significance of changes in tRNA modification status

Urm1-dependent tRNA modifications decrease in abundance when cells are grown at elevated temperatures, but the full significance of the modulation of tRNA modification levels remains unknown. We speculate that a decrease in tRNA thiolation is linked to decreased translation, which is an adaptive response to challenging conditions. In addition to our observations, there are other indications that tRNA thiolation plays a role in the ability of cells to grow under challenging conditions.
Quantitative trait loci mapping studies designed and carried out to find genetic factors contributing to high temperature growth (Htg) in clinical isolates of *S. cerevisiae* suggest that modulation of the *URM1* pathway may be adaptive for high temperature growth. This method identified an allele of *NCS2* underlying the Htg phenotype, growth at 41°C in the case of this study. The allele variant associated with Htg, *NCS2-421*, had an A212T substitution compared to the sequence of the S288c lab strain, resulting in a leucine to histidine substitution at position 71 (H71L) of the Ncs2 protein. The 212T allele was common, found in 19 out of 32 sequenced *S. cerevisiae* strains, but not in the commonly used lab strains S288c and W303 (Sinha *et al.*, 2008; Yang *et al.*, 2013).

These data points to a role for tRNA thiolation in mediating thermotolerance in yeast cells, although the initial genetic studies did not examine the mechanism behind *NCS2* related thermotolerance. It is possible that the 212T allele results in an Ncs2 protein that has altered activity, at least under specific conditions, compared to the 212A allele, resulting in differential levels of tRNA thiolation in strains harboring the different alleles, which could lead to differences in thermotolerance. To examine the role of *NCS2* variants in thermotolerance, the 212T allele could be introduced into the W303 lab strain used in our experiments, and then both the thermotolerance phenotype and tRNA thiolation levels at normal and at elevated growth temperatures could be examined. Additionally, tRNA thiolation levels in different yeast strains could also be determined. Alternatively, the different variants of Ncs2 could also be expressed in a bacterial system, purified, and used to carry out in vitro tRNA thiolation reactions to determine if the variants have different enzymatic activities (Noma *et al.*, 2008). Decreased thiolation in the 212T mutant, in yeast strains containing the 212T allele or using the 212T mutant in in vitro assays would suggest that a decrease in thiolation is adaptive. Such studies would help to
demonstrate that modulation of tRNA thiolation is a strategy cells use to control growth and gene expression under specific conditions.

The regulation of tRNA modification pathways

Since many of the genetic pathways required for specific tRNA modifications have been described (Phizicky and Hopper, 2010; Yacoubi et al., 2012), examining how these pathways are regulated would also be valuable in determining how a cell integrates input from the environment to modulate tRNA modification levels. We have demonstrated that proteins required for Urm1-dependent tRNA thiolation are down-regulated when cells are grown at elevated temperatures, and that this decrease in protein abundance is correlated with the appearance of unthiolated tRNA species. We postulate that this difference is due to changes in the translational capacity of cells coupled with the inherent instability of these proteins, which makes them responsive to changes in translation that occur when cells are exposed to stresses.

While we focused on regulation of the tRNA thiolation, there are multiple mechanisms that could regulate various modification pathways, and it is possible that not all of the mechanisms controlling tRNA thiolation have been identified. The activity of tRNA modification enzymes might be affected by multiple factors. The levels of tRNA modification enzymes might be regulated at the level of synthesis, either at the transcriptional or translational level, or at the level of protein stability or degradation. In addition, the enzymatic activities of tRNA modification enzymes might also be affected by posttranslational modifications such as phosphorylation or potentially urmylation (Van der Veen et al., 2011).

There are other aspects of tRNA biology that could also contribute to overall changes in tRNA modification, including thiolation, levels. Changes in tRNA synthesis could affect levels
of tRNA modifications, either by increasing or decreasing the levels of tRNA substrates relative to modification enzymes. The degradation of tRNAs could also affect overall levels of modifications. Although tRNAs are generally very stable, lack of certain modifications leads to rapid tRNA degradation, and growth conditions that result in decreases in these modifications would be expected to result in decreased levels of some tRNAs. The subcellular localization of both tRNAs and enzymes could affect tRNA modification levels. It has been demonstrated, for example, that tRNAs accumulate in the nucleus in response to nutrient starvation (Shaheen and Hopper, 2005). Additionally, although presently none have been identified, the potential existence of demodification enzymes provides yet another mechanism that could control tRNA modification levels in cells. Thus, the regulation of tRNA modification levels, as well as the timescale on which modification levels change, is likely to reflect changes in a number of different pathways in a cell.

**Further characterization of the URMI tRNA modification pathway**

While many components of the *URMI* pathway are by this point well described, there are still mechanistic details of the tRNA thiolation pathway that must be addressed. The precise biochemical functions of Ncs2 and Ncs6 are unknown, for example. While Ncs6 has been shown to bind directly to tRNAs (Leidel *et al.*, 2009), whether Ncs2 also binds directly to tRNAs has not been determined. Both Ncs6 and Ncs2 contain PP-loop ATPase domains, which play a role in the activation of uracil through the formation of an acyl-adenylate intermediate prior to formation of the modified nucleoside (Numata *et al.*, 2006; Nakai *et al.*, 2008). Either or both proteins might be required for this process, and it also remains to be seen if sulfur is transferred directly from Urm1 to tRNA, or if it is transferred to Ncs6 or Ncs2 prior to transfer to tRNA.
Additionally, since Ncs6 (and potentially Ncs2) binds directly to tRNA, it is likely that these proteins are responsible for the specificity of the URM1 pathway in thiolating tGlu<sup>UUC</sup>, tGln<sup>UUG</sup> and tLys<sup>UUU</sup> tRNAs. It is unclear how these proteins specifically recognize and direct modification of a subset of tRNAs. While it is possible that the mcm<sup>5</sup> modification, which is added to tRNAs prior to thiolation, could serve as an identity determinant, this is unlikely to be the sole mechanism directing specificity, as this modification is also found on the wobble uridines of tRNAs that are not subsequently thiolated. It is possible that specific sequences or structural elements play a role in determining specificity of Ncs2/Ncs6 binding; examining tRNA sequences for conserved primary sequences could reveal motifs that are recognized by modification enzymes, which could then be verified experimentally by mutational analysis.

**Exploring potential links between tRNA thiolation and protein urmylation**

In mammalian cells, the URM1 pathway components MOCS3 (Uba4), ATPBD3 (Ncs6), CTU2 (Ncs2) were determined to be urmylated, as was Elongator complex protein 1 (Elp1). Although no functional consequences of urmylation were described for these targets, the observation that they were urmylated raised the intriguing possibility that the dual functions of Urm1 as a protein modifier and as a tRNA modification enzyme might be coordinated in some fashion. Links between the oxidative stress response and both protein urmylation and tRNA modification are also suggestive of some level of coordination. Ahp1 is involved in the oxidative stress response and is urmylated, and urm1<sup>Δ</sup> cells are sensitive to a variety of oxidative stress in a tRNA dependent fashion (Leidel et al., 2009) (and our observations). Although there is currently no functional data to support such a model, it is possible that the urmylation of tRNA modification proteins affects their enzymatic activity, and so conditions that result in an increase
in protein urmylation might ultimately result in altered tRNA modification levels. This in turn could lead to altered translation either of all mRNAs in a cell or of a subset of transcripts; these changes in translation would be expected to be codon specific effects.

Our observations also suggest mechanisms that might coordinate protein urmylation and tRNA thiolation. When cells were grown at 37°C, levels of the URM1 pathway proteins Tum1, Uba4, Ncs2 and Ncs6 decreased, and this corresponded to decreases in tRNA thiolation that we observed in cells grown at elevated temperatures. This suggests that protein urmylation might also be down-regulated in cells grown at 37°C. In contrast, cells exposed to other stressors show different patterns of protein expression. When we treated cells with tunicamycin, we observed a decrease in Ncs2 and Ncs6 protein levels, while Uba4 levels were unchanged. This suggests that the differential regulation of protein levels could lead to the differential control of urmylation and tRNA thiolation pathways. In tunicamycin treated cells, for example, protein urmylation might be unaffected, while tRNA thiolation would be decreased as a result of altered Ncs2 and Ncs6 protein levels.

Exploring the mechanism and functions of protein urmylation

Urm1 has been shown to modify proteins in yeast and mammalian cells upon exposure to oxidative stress (Goehring et al., 2003a; 2003b; Van der Veen et al., 2011), but how widespread this phenomenon is remains unclear. The consequences of urmylation remain unknown, but the existing data suggest multiple ways in which the posttranslational modification of proteins with Urm1 might function. In yeast, the only known target of protein urmylation is Ahp1, a peroxiredoxin that plays a role in the oxidative stress response (Lee et al., 1999), and structural studies suggest that urmylation might serve to decrease or abolish the peroxidase activity of
Ahpl (Lian et al., 2012). In mammalian cells, one of the proteins identified as a target of urmylation was cellular apoptosis susceptibility protein, or CAS, which is involved in the shuttling of importin α between the nucleus and cytosol. Urmylated CAS was stable and appeared exclusively in the cytoplasmic fraction of cells, suggesting that urmylation of CAS might disrupt nuclear trafficking.

It is possible that protein urmylation has multiple consequences, as is the case for protein ubiquitylation, and further studies are required to define the functions of urmylation. If mammalian cells serve as an indicator, it is unlikely that all of the urmylation targets in yeast have been identified. A more complete inventory of urmylation substrates would prove useful; it is possible that conditions not yet examined might yield more urmylation targets. Conditions that induce urmylation should also be examined to determine if these conditions affect tRNA thiolation, and this type of analysis would help to determine the relationship between protein and tRNA modification. The identification of specific urmylation sites, which are expected to be lysine residues (Van der Veen et al., 2011), coupled with mutational analysis, could help to elucidate how urmylation affects the function of specific substrates. In mammalian cells, a list of potential urmylation targets exists, and at least for a subset of these targets, assays based on the known functions of proteins, such as those used for analysis of CAS, could be used to determine the function of urmylation.

A more complete understanding of protein urmylation would be furthered by determining whether there are as-yet-unknown components that are required for the conjugation of Urm1 to target proteins. Although the E1 activating enzyme for Urm1 has been identified, it is unclear if there are E2 conjugating enzymes and/ or E3 ligases whose activities are required for urmylation. It is unclear how transfer of Urm1 to the target lysine residues is accomplished and the factors, if
any, that dictate substrate specificity are unknown. It is also unknown whether urmylation is reversible, and if so what enzymes are responsible for de-urmylation and how they might function.

Activity-based protein profiling is a strategy that utilizes active-site directed chemical probes to identify proteins with specific enzymatic activities. The use of ubiquitin-based probes incorporating various electrophiles as suicide inhibitors has allowed for the function-dependent identification and description of proteins spanning a range of functions in the ubiquitin-proteasome system, including E3 ligases and deubiquitylating enzymes (Borodovsky et al., 2002; Love et al., 2009). Ubiquitin-based probes have been used very successfully in viral (Kattenhorn et al., 2005), bacterial (Misaghi et al., 2006; Catic et al., 2007) and apicomplexan (Artavanis-Tsakonas et al., 2006; Frickel et al., 2007) systems to retrieve proteins with ubiquitin-directed activities from complex cellular mixtures.

Using an Urm1-based probe, this method was successfully used to identify ATPBD3 (Ncs6), an enzyme with Urm1-specific activity, in mammalian cells (Schlieker et al., 2008). This type of approach could be utilized with an expanded set of labeling conditions and/or using an expanded set of probes that incorporate different electrophiles to identify proteins that might have Urm1-specific activities. Urm1-based probes were used in a mammalian system, but have not yet been utilized in a yeast system, and examining the reactivity of Urm1-based probes in yeast lysates could also prove to be informative. This type of function-based approach would be especially useful in the study of the Urm1 system, components of which might have little, if any, homology to known classes of enzymes. Ubiquitin-based probes were used, for example, to identify a deubiquitylase (M48^USP) encoded in the N-terminal portion of the large tegument protein of murine cytomegalovirus that demonstrated no homology to host deubiquitylases.
(Schlieker et al., 2007). While relatively little is known about protein urmylation, a combination of approaches, both targeted and broad, should be useful in elucidating the extent and mechanisms of protein urmylation.

Investigating the mechanism of Hsf1 activation in *URM1* pathway mutants

The work in this thesis described, in part, a cellular response to the effects of hypomodified tRNAs. *URM1* pathway mutants had increased levels of stress-associated transcripts, and under standard growth conditions had an increased basal activation of the Hsf1-dependent heat shock response (HSR) compared to wild type cells. How is the HSR activated in *URM1* pathway mutants? What are the effects of HSR activation in mutant cells? These are open questions that would be interesting to explore further.

There are many stress response pathways in cells that affect changes in gene expression in response to environmental stimuli in order to optimize growth under new conditions and to maintain cellular homeostasis. These stress responses are coordinated via the actions of specific transcription factors. In budding yeast, the HSR is mediated via the transcription factor (TF) Hsf1, the environmental stress response (ESR) is mediated via the TFs Msn2 and Msn4, and the endoplasmic reticulum unfolded protein response (UPR) is mediated via TF Hac1. Of note is the fact that some stress responses, such as the HSR and ESR, are not completely separate; many genes are targets of multiple transcription factors, which means that stress responses can be overlapping. Interestingly, although these responses are generally mounted in response to a stress, they result in changes to cells that confer resistance, which can be cross-protective, to future stresses (Berry and Gasch, 2008; Liu and Chang, 2008; Verghese et al., 2012).
The HSR generally involves physiological and metabolic adaptations such as a reduction in protein biosynthetic capacity and the up-regulation of chaperones, which are thought to prevent the aggregation of proteins, refold damaged proteins or target damaged proteins for degradation (Verghese et al., 2012). Although a change in temperature is the best-known trigger of the HSR, defects in protein synthesis can also result in at least a partial activation of the HSR. Treatment of yeast cells with the proline analog azetidine-2-carboxylic acid (AZC) or with ethanol produce misfolded or denatured proteins and results in the repression of ribosomal protein genes and the activation of heat shock factor (Hsf1) targets (Trotter, 2002). Treatment of cells with the aminoglycoside antibiotic paromomycin, which causes translational misreading, also results in the activation of the HSR (Grant et al., 1989). Expression of misfolded variants of cytosolic proteins in cells results in the activation of a cytosolic unfolded protein response that overlaps significantly with the HSR (Metzger and Michaelis, 2009; Geiler-Samerotte et al., 2011).

The mechanism whereby the HSR is activated in URMI pathway mutants is unclear, although the observation that overexpression of tRNAs can reduce expression of the Hsf1-induced reporter indicates that HSR activation is linked to the tRNA modification function of Urm1. It is possible that defects in tRNA modification result in the aberrant expression of a subset of genes, which results in the activation of the HSR. It is also possible that defects in translation elongation in URMI pathway mutants result in cotranslational folding defects that result in a pool of misfolded proteins that must be dealt with. It is also possible that defects in tRNA thiolation result in misreading, which produces error-containing proteins that misfold. Although misreading is a possibility, especially given the sensitivity of URMI pathway mutants to paromomycin, preliminary experiments with misreading reporters have not indicated that there
are any differences in misreading in wild type and mutant cells (our observations/ experiments). Examining modification deficient cells for signs of protein aggregation would provide information regarding the potential mechanism of HSR activation. The Hsp104 chaperone colocalizes with protein aggregates in cells, and using microscopy to examine cells for Hsp104-eGFP foci could help to determine if mutant cells experience increased levels of protein stress (Lum et al., 2004). Alternatively, one could look for the presence of protein aggregates using semidenaturing detergent-agarose gel electrophoresis (SDD-AGE) to detect protein aggregates (Kryndushkin et al., 2003; Halfmann and Lindquist, 2008).

Another remaining question is whether the increased basal activation of the HSR in URM1 pathway mutants is of benefit to cells, and if it is linked in any way to the specific stress-resistant phenotypes of mutant cells. Given that exposure to stresses can provide cross-protection against future challenges, it is possible that the increased basal activation of the HSR observed in URM1 pathway mutants is linked to the resistance of mutant cells to tunicamycin, and perhaps other stresses.

Another, related, question is whether/how slow growth is related to the observed phenotypes of URM1 pathway mutants. Slow growth has been reported to result in a relative increase in resistance to certain stresses such as tunicamycin (Steffen et al., 2012), and since URM1 pathway mutants grow slower than wild type cells, it is possible that growth rate and tunicamycin resistance are linked. It has been reported that the growth rate of cells is linked to the expression of stress response genes (Brauer et al., 2008; Lu et al., 2009), and so it is possible that the activation of the HSR in mutant cells is linked to the slow growth that results from defects in tRNA modification.
Conclusions

Since the initial discovery of ubiquitin, it has become clear that ubiquitin and UBLs play a wide variety of functions in a wide variety of cellular processes. The description of some of these UBLs has provided unexpected results, such as the discovery that Urm1 functions in tRNA modification reactions as well as serving as a protein-modifier. This discovery provided insight into the evolutionary relationship between eukaryotic ubiquitin/UBLs and prokaryotic sulfur carriers, and has added to the list of roles that UBLs play in cells. The work in this thesis has focused on the tRNA modification function of Urm1, placing tRNA thiolation in a physiological context and describing conditions under which cells modulate tRNA thiolation levels. The work in this thesis has added to the growing body of evidence describing the functions and importance of tRNA modifications in cells.
REFERENCES


Sinha, H. et al. (2008). Sequential elimination of major-effect contributors identifies additional


Appendix A:

Urm1-dependent tRNA modification and cell cycle progression in *Saccharomyces cerevisiae*
ABSTRACT

Urm1 is a eukaryotic ubiquitin-like protein with similarities to prokaryotic sulfur carriers. Urm1 can function as a lysine directed protein modifier as well as a sulfur carrier in tRNA modification reactions. URM1 pathway mutants display a variety of phenotypes, including slow growth and multiple stress sensitivities. We observe that URM1 pathway mutants have cell cycle progression defects. Specifically, synchronized cultures of URM1 pathway mutant cells demonstrate a delay in budding, which is associated with G1/S progression defects. We also observe that URM1 pathway mutants have an increased cell volume compared to wild type cells. These phenotypes can be rescued by the overexpression of tRNAs that are normally modified in an Urm1-dependent fashion. Since cell growth and cell cycle progression are coordinated processes in Saccharomyces cerevisiae, we hypothesize that the phenotypes we observe are linked, and that both are a consequence of defects in tRNA thiolation.
INTRODUCTION

In *Saccharomyces cerevisiae*, cell growth, the increase in cellular biomass, and the cell cycle are separate but coordinated processes (Jorgensen and Tyers, 2004). Cells must accumulate sufficient biomass and cellular machinery (such as ribosomes) and grow to a critical cell size prior to initiation of the cell division process; in budding yeast commitment to cell division occurs in G1 with initiation of the process known as START. As cells enter S phase, DNA replication is accompanied by bud formation, and buds continue to grow as cells progress through the G2 and M phases of the cell cycle. In *S. cerevisiae*, cell division is regulated in response to growth, which is itself governed by nutrient availability; cells must integrate extracellular cues with data about intracellular events and status in order to divide under appropriate conditions.

Protein synthesis is intricately linked to cell growth and cell cycle progression; at a basic level protein synthesis must occur for cells to produce the biological macromolecules needed for growth. Additionally, specific proteins that carry out specialized functions in the cell cycle, such as cyclins, must be synthesized (and degraded) with appropriate kinetics and timing for cells to progress through the cell cycle. In budding yeast, it has been proposed that the rate of protein synthesis determines progression through the cell cycle (Turner et al., 2012).

*URM1* pathway mutants have defects in tRNA modifications; specifically, *URM1* pathway mutants lack the $\text{s}^2$thio-modification found on the U$_{34}$ nucleosides of tGlu$^{\text{UUC}}$, tGln$^{\text{UG}}$ and tLys$^{\text{UUU}}$ tRNAs in budding yeast(Huang et al., 2008; Nakai et al., 2008; Schlieker et al., 2008; Leidel et al., 2009). The *ELP* pathway is required for synthesis of the mcM$_5$-modification found on the U$_{34}$ nucleosides of tGlu$^{\text{UUC}}$, tGln$^{\text{UG}}$ and tLys$^{\text{UUU}}$ and tArg$^{\text{UCU}}$ tRNAs (Kalhor and Clarke, 2003; Huang, 2005; Bjork et al., 2007). Many mutants with defects in tRNA
modification pathways do not have obvious growth defects under standard growth conditions, but do have more pronounced phenotypes under specific growth or stress conditions. Because of the potential for tRNA modifications, or lack thereof, to impact protein synthesis, and because protein synthesis and cell cycle progression are linked, we examined the role of Urm1-dependent tRNA modifications in cell cycle progression.

Experiments using shRNA constructs to reduce Urm1 levels in HeLa cells demonstrated that cells depleted of Urm1 had increased DNA content, suggesting that Urm1 might play a role in cell cycle progression, specifically cytokinesis, in mammalian cells (Schlieker et al., 2008). In addition it was shown that C. elegans in which ctu1 or ctu2, homologs of the URM1 pathway components NCS6 and NCS2 in S. cerevisiae respectively, had been deleted demonstrated temperature-dependent ploidy abnormalities. We therefore wondered whether Urm1-dependent tRNA modifications were linked to cell cycle progression in S. cerevisiae. We find that in budding yeast, URM1 pathway mutants display a budding defect consistent with a G1/S progression defect and are larger than wild type cells. These phenotypes appear to be linked to the tRNA modification function of Urm1, suggesting that tRNA thiolation plays a role in ensuring that cell cycle progression proceeds appropriately.
RESULTS

_URMI pathway mutants have defects in cell cycle progression_

We sought to determine if defects in tRNA modification pathways affected cell cycle progression. To examine cell cycle progression in synchronized cultures of cells, we synchronized cells in G1 by adding the mating pheromone α-factor to cultures; when the arrest was complete, we released cells into pheromone free medium and followed cell cycle progression by examining several markers of cell cycle stage. We examined budding, which is indicative of the G1 to S transition, by examining cell morphology microscopically. We also, in some cases, assessed progression through the cell cycle by examining spindle morphology using immunofluorescence against tubulin to determine if cells were in metaphase or anaphase. We found that urm1Δ, ncs6Δ and uba4Δ cells showed an approximately 15-20 minute delay in progression from G1 to S, as assessed by degree of budding in synchronous cultures (Figure 1A, 1B). As Uba4 is the E1 activation enzyme for Urml and is required for all the described functions of Urml, uba4Δ mutant cells could have phenotypes attributable to either the tRNA modification or the protein urmylation function of Urml. In contrast, the only known function of Ncs6 is in tRNA modification, and thus a shared phenotype of urm1Δ and ncs6Δ cells is likely to be attributable to the tRNA modification functions of Urml.

![Graph A](image1.png)

![Graph B](image2.png)
Figure 1: *URMJ* pathway mutants exhibit a delay in budding indicative of G1/S progression defects. Wild type, *urm1Δ*, *ncs6Δ* or *ncs2Δ* cell cultures were synchronized using the alpha-factor block release method. Samples were taken at indicated time points and the extent of budding was assessed by examining the morphology of fixed cells. A. Comparison of wild type, *urm1Δ* and *ncs6Δ* cells. B. Comparison of wild type, *urm1Δ* and *ubq4Δ* cells.

Although budding was delayed in *urm1Δ* cells, final percentages of cells characterized as having budded were ultimately equivalent (Figure 1A, 1B, 2A). Additionally, assessment of spindle morphology indicated that *urm1Δ* cells proceeded through metaphase (Figure 2B) and anaphase (Figure 2C), with delays that matched the timescale of the initial budding delays observed in mutant cells, roughly 15 minutes. Additionally, FACS analysis of DNA content revealed that *urm1Δ* cells were able to replicate DNA prior to cell division and were also ultimately able to undergo cytokinesis (as judged by changes in DNA content from 1N $\rightarrow$ 2N $\rightarrow$ 1N) (Figure 2D). Analysis of asynchronously growing wild type and *urm1Δ* cells growing at 30°C or 37°C did not indicate significant differences in ploidy between strains (Figure 2E). Our data indicate that while *URMJ* pathway mutant cells demonstrate a G1/S transition defect, they are unimpaired in other aspects of cell cycle progression.
Figure 2: Cell cycle progression in urm1Δ cells. urm1Δ cells exhibit a budding delay but are not impaired in other aspects of cell cycle progression. Cells of the indicated genotype were synchronized using the alpha-factor block release method. Samples were taken at indicated time points and assessed for progression through the cell cycle: A. the extent of budding was assessed by examining morphology, and B. anaphase and C. metaphase cells were identified by examining spindle morphology. D. A separate synchronization experiment in which FACS analysis was used to determine DNA content in wild type or urm1Δ cells at various times post alpha-factor release. E. FACS analysis of unsynchronized wild type or urm1Δ cells at 30°C or 37°C.
The G1/S delay in urm1Δ cells can be rescued by overexpression of tRNAs

It has been demonstrated that multiple phenotypes associated with defects in tRNA modification pathways can be rescued by overexpression of the tRNAs that are normally modified in wild type cells (Esberg et al., 2006; Bjork et al., 2007; Dewez et al., 2008; Leidel et al., 2009). Because of our observation that urm1Δ, uba4Δ and ncs6Δ cells all demonstrated the same cell cycle progression defect, we wondered if overexpression of tRNAs normally modified in an Urm1-dependent fashion, tGlu\textsuperscript{UUC} (tE), tLys\textsuperscript{UUU} (tK) and tGln\textsuperscript{UUG} (tQ), would be able to rescue the observed G1/S delay.

We transformed urm1Δ cells with either empty vector or with a high copy plasmid encoding a single tRNA (tE, tK or tQ) (Figure 3A), a combination of two tRNAs (tEK, tEQ, or tKQ) (Figure 3B) or all three tRNAs (tEKQ) (Figure 3C). We wanted to determine if a single tRNA or a combination of tRNAs was sufficient to overcome the G1/S delay in urm1Δ cells. Cells were synchronized in G1 using the previously described α-factor block-release protocol. In our experiment, no single tRNA species was capable of fully rescuing the urm1Δ G1/S delay (Figure 3A). Similarly, no combination of any two tRNAs was able to fully rescue the G1/S delay of mutant cells, although the tKQ construct seemed to partially rescue the cell cycle progression defect, as cells expressing this construct appeared to bud faster than other urm1Δ tRNA overexpression strains but not as quickly as wild type cells harboring empty vector (Figure 3B). Expressing all three tRNAs (tEKQ) rescued the cell cycle delay in urm1Δ cells (Figure 3C). These results indicate that the G1/S delay observed in URM1 pathway mutants is most likely a consequence of hypomodified tRNAs in these strains.
Figure 3: Budding delay in urm1Δ cells can be rescued by overexpressing tRNAs. Wild type or urm1Δ cells harboring either empty vector or a construct for expression of the indicated tRNA(s) were synchronized using the alpha-factor block release method. Samples were taken at ...
indicated time points and the extent of budding was assessed by examining morphology. A. Budding in *urm1Δ* cells expressing single tRNAs tE, tK or tQ. B. Budding in *urm1Δ* cells expressing two tRNAs: tEK, tEQ or tKQ. C. Budding in *urm1Δ* cells expressing all three tRNAs modified in an Urm1-dependent fashion, tEKQ.

**_urm1Δ* cells have an increased cell volume that can be rescued by overexpression of tRNAs**

Since cell growth and division are connected processes in budding yeast, we wondered whether, in addition to the observed defects in cell cycle progression, there were any defects in cell growth in *URMI* pathway mutants. We measured cell volumes of actively growing asynchronous cells and observed *urm1Δ* cells to be larger in size compared to wild type cells (Figure 4A). We also examined *uba4Δ*, *ncs6Δ* and *ncs2Δ* cells, and these *URMI* pathway components were also larger than wild type cells (Figure 4A).

We examined the cell sizes of other mutants to determine if the differences in cell size of *URMI* pathway mutants is linked to the tRNA modification function of Urm1 or to the protein modification function of Urm1. We examined the sizes of an *elp2Δ* strain, which has defects in mcm<sup>5</sup>-U<sub>34</sub> modification (Huang, 2005), and an *ahplΔ* strain, which lacks Ahp1, the only verified protein urmylation target in budding yeast (Goehring *et al.*, 2003a; Van der Veen *et al.*, 2011). *ELP2*, and the mcm<sup>5</sup>-modification, is required for tRNA thiolation, while *AHPL1* is not required for tRNA thiolation (Figure 4D). *elp2Δ* cells showed an x fL increase in cell size compared to wild type cells, an increase comparable to *urm1Δ* cells, while *ahplΔ* cells were not significantly larger than wild type cells (Figure 4B). This further demonstrates that the cell size phenotype of *URMI* pathway mutants is likely attributable to defects in tRNA modification. Deletion of *DUS2*, which is required for dihydrouridine synthesis on the U<sub>20</sub> position of cytoplasmic tRNAs
(Phizicky and Hopper, 2010), did not have an effect on cell volume, indicating that the cell size phenotype is specific to a subset of tRNA modifications (Figure 4C).

![Graph A](image)

**Figure 4**: The cell volume of *URMI* pathway mutants is increased compared to wild type cells. Unsynchronized cells of the indicated genotype were sized using a Coulter counter. **A.** A comparison of cell volumes of wild type, *urm1Δ, uba4Δ, ncs6Δ* and *ncs2Δ* cells. **B.** A comparison of cell volumes of wild type, *urm1Δ, elp2Δ* and *ahp1Δ* cells. **C.** A comparison of cell volumes of wild type, *urm1Δ, uba4Δ, ncs6Δ, ncs2Δ, elp2Δ* and *dus2Δ* cells. **D.** RNA isolated from wild type, *urm1Δ, uba4Δ, ncs6Δ, ncs2Δ, elp2Δ* or *ahp1Δ* cells was analyzed by APM-Northern blot analysis, membranes were hybridized by a probe specific for the tLys^{UUU} tRNA.
We next wanted to know if overexpression of tRNAs in mutant strains could restore cell size to wild type volume. We examined wild type cells harboring empty vector, urm1Δ cells harboring empty vector or urm1Δ cells harboring the indicated tRNA construct and examined cell volume in asynchronous, actively growing cultures. No single tRNA was able to rescue the cell size phenotype of urm1Δ deletion cells (Figure 5B). Expression of tE in combination with tK or tK in combination with tQ (but not tE with tQ) was sufficient to restore wild type cell size to urm1Δ cells (Figure 5C). Overexpression of all three tRNAs (tEKQ) also restored urm1Δ cells to wild type cell size (Figure 5D). This result supports the conclusion that the increased cell size we observe is due to the effects of hypomodified tRNAs.
Figure 5: The increased cell volume phenotype of URMI pathway mutants can be suppressed by expression of tRNAs. Unsynchronized wild type or urm1Δ strains harboring either empty vector or a construct for expression of the indicated tRNA(s) were sized using a Coulter counter. A. A comparison of cell volumes of wild type and URMI pathway mutants. B. A comparison of cell volumes of wild type and urm1Δ cells expressing single tRNAs tE, tK or tQ. C. A comparison of cell volumes of wild type and urm1Δ cells expressing two: tRNAs tEK, tEQ or tKQ. D. A comparison of cell volumes of wild type and urm1Δ cells expressing all three tRNAs: tEKQ.

**tRNA thiolation does not vary during the cell cycle**

We wondered whether tRNA thiolation levels might fluctuate during different stages of the cell cycle in wild type cells. This might reflect a differential requirement for modified tRNAs at different stages of the cell cycle which, when disrupted (as in urm1Δ cells incapable of thiolating tRNAs), might lead to the G1/S progression defect that we observed in URMI pathway mutants. We synchronized wild type cells using the α-factor pheromone block release method, and monitored cell cycle progression by examining budding and metaphase/anaphase spindle morphology from samples taken at regular intervals (Figure 6A, 6B). We assessed the levels of thiolated tLys^{UUU}, tGln^{UUG} and tGlu^{UUC} across the cell cycle using APM-Northern blot analysis, and did not observe changes in tRNA thiolation throughout the course of cell cycle progression (Figure 6C, 6D, 6E). At all time points, the majority of tRNAs were thiolated, as we have observed is the case for asynchronously growing wild type cells. This result indicates that there are no significant changes in tRNA thiolation during different stages of the cell cycle, and that differential tRNA thiolation likely does not play a critical role in cell cycle progression.
A

![Graph A: % budded cells vs. time (min)]

B

![Graph B: Percent cells vs. time (min)]

C

![Image C: tE + APM (IQ blot re-probed)]

D

![Image D: tK + APM](Image)

E

![Image E: tQ + APM](Image)
Figure 6: tRNA thiolation levels are steady throughout the cell cycle. Wild type cells were synchronized using the alpha-factor block release method and assessed at indicated timepoints for A. degree of budding, B. percentage of cells in metaphase and C. percentage of cells in anaphase. D. Samples were taken at each timepoint for total RNA isolation; RNA was subsequently analyzed by APM-Northern blotting to determine thiolation levels, membranes were hybridized for probes specific to the tRNA indicated.
DISCUSSION

We have characterized the cell cycle progression and cell size phenotypes of cells lacking components of the \textit{URM1} pathway. Both of these phenotypes appear to be linked to the tRNA modification function of Urm1, as both the G1/S delay and the increased cell size phenotypes could be rescued by overexpression of tRNAs in an \textit{urm1A} genetic background. We, and others (Laxman \textit{et al.}, 2013; Rezgui \textit{et al.}, 2013), have demonstrated that the \textit{URM1} pathway and Urm1-dependent tRNA modifications are linked to translation in \textit{S. cerevisiae}. This link, our results demonstrating that \textit{ncs2A}, \textit{ncs6A} and \textit{elp2A} cells phenocopy \textit{urm1A} cells, and the observation that \textit{ahplA} cells do not share at least a subset of \textit{urm1A} phenotypes, in this case cell size, suggest that defects in translation may underlie the phenotypes that we have described.

Importantly, while we suggest that there is a link between the cell size and cell cycle progression defects in \textit{urm1A} cells, this link is not yet definitively established. Our cell cycle experiments were carried out using a-factor to arrest cells in G1 prior to release into pheromone free medium. In these conditions arrested cells continue to grow, decoupling the G1/S transition from cell growth (Manukyan \textit{et al.}, 2011). This means that we cannot state that the cell cycle entry delay observed in \textit{urm1A} cells is caused by defects in cell growth, or vice versa. The use of centrifugal elutriation to separate cells based on size would allow for the enrichment of G1 cells from an asynchronous population; this would allow us to follow cell cycle progression and cell growth in an un-perturbed cell cycle, and to determine if there is a link between the cell growth and cell cycle progression defects in \textit{URM1} pathway mutants. Specifically, determining the critical cell size of \textit{URM1} pathway mutants would be informative, as this would provide information about cell cycle progression and growth/volume accumulation in mutant cells.
Although the specific mechanism by which cell growth and cell cycle progression are coupled is unknown, it is thought the biosynthetic status of a cell acts as a sizing mechanism. In this model, the overall translation rate of a cell is reflected by the synthesis of “translational sizers” that are critical for cell cycle progression, such as the G1 cyclin Cln3 (Jorgensen, 2004; Turner et al., 2012); cell division and growth are thought to be coupled at least in part through the translational control of CLN3 (Polymenis and Schmidt, 1997). It is possible that the cell cycle progression defect and the cell size defect in URM1 pathway mutants are linked through translation. Indeed, URM1 pathway mutants grow more slowly compared to wild type cells and have translational defects (Laxman et al., 2013; Rezgui et al., 2013) and chapter 2 of this thesis.

In S. cerevisiae, aneuploid strains exhibit slow cell volume accumulation, an increase in critical cell size and stay in G1 for an extended period of time compared to wild type cells (Thorburn et al., 2013). Examining the timing of expression, at the RNA and protein levels, of the G1 cyclins CLN1, CLN2 and CLN3 in synchronized cells would also help to better characterize URM1 pathway mutants. This would allow us to determine if the synthesis of specific factors required for cell cycle progression is affected in URM1 pathway mutants.

Interestingly, URM1 pathway mutants display genetic interactions with the TOR pathway, and the URM1 pathway has been implicated in nutrient sensing (Goehring et al., 2003a; Rubio-Texeira, 2007). As nutrient availability/sensing plays a role in determining cell growth and cell cycle progression, and is mediated partly through the TOR pathway (Jorgensen and Tyers, 2004; Turner et al., 2012), it is possible that the defects we observed in URM1 pathway mutants is due at least in part to defects in nutrient sensing. It is unclear what the nature of the interaction is between the two pathways, but a role for nutrient sensing would be consistent with the observed phenotypes.
A study in fission yeast found that cells defective in synthesis of the mcm5s2U34 modification, specifically cells lacking components of the Elongator complex, had phenotypes consistent with defects in cell cycle progression. These included an elongated cell phenotype (larger cells), thermosensitivity, and misplaced septa. Mutant cells demonstrated codon-specific defects in protein synthesis. Translation of Cdr2, a kinase involved in the G2/M transition and cytokinesis, was found to be subject to translational control by Elongator (Bauer et al., 2012). These results are consistent with the cell cycle defects that we have described in *S. cerevisiae*, although there are differences, which could be attributable to differences between the two organisms. The *S. pombe* study indicated that defects in the synthesis of specific proteins was the underlying cause of cell cycle defects, and it is possible that in *S. cerevisiae* there are specific proteins, such as Cln3, that are responsible for the cell cycle and cell size phenotypes of URM1 pathway mutants.

Another possibility is that Urm1, either directly or indirectly, plays a role in cell morphogenesis and/or cytoskeletal rearrangements. URM1 pathway components were described in a screen for genetic interactors of *CLA4*; deletion of *URM1* pathway components was synthetically lethal with a *cla4Δ* mutant. Cla4 is a kinase that functions in budding, and URM1 pathway mutants were also described as having defects in cell elongation and in invasive/pseudohyphal growth (Goehring et al., 2003b), indicating potential links to cell polarization. We noticed that when cells were arrested, *urm1Δ* had a shmoo morphology that differed from that of wild type cells (shmoos were not as well defined in *urm1Δ* cells), and when cells were released from α-factor arrest, *urm1Δ* cells generally seemed to have smaller buds, even taking into account the delays between wild type and mutant cells) (data not shown, observations). It is possible that the G1/S delay is at least partially attributable to defects in cell
morphogenesis. In order to evaluate this possibility, a first step would be to examine actin localization in cells to determine if there are differences between wild type and $urml\Delta$ cells with regard to cytoskeletal morphology. Different actin structures are linked to specific cellular processes; actin patches function in endocytosis and exocytosis, actin cables are involved with polarized cell growth, and actomyosin rings function in cell division (Moseley and Goode, 2006). Any differences could be indicative of the functional consequences of defects in the $URM1$ pathway, and specific processes or phenotypes could be studied in relation to the tRNA modification functions of Urm1.

In summary, we have described a cell cycle progression phenotype and a cell size defect in $URM1$ pathway mutant cells. Although we have been able to link these phenotypes to the tRNA modification functions of Urm1, the exact relationship between tRNA modification, cell size and cell cycle progression remain to be elucidated.
MATERIALS AND METHODS

Yeast Strains used in this study (derivatives of W303)

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Yeast Growth and Generation of Gene Deletions

Yeast were grown according to standard techniques. Unless specified, cells were grown in medium containing 1% yeast extract, 2% peptone, and 2% glucose supplemented with adenine. Deletion mutants were generated by homologous recombination using PCR generated fragments according to the method described in (Longtine et al., 1998). Gene deletions were verified using PCR to verify insertion of the selection cassette at the desired genomic locus.
When required, strains of the necessary mating type or double mutants were generated using standard yeast genetic techniques.

**Plasmids:**

Plasmids for tRNA expression are described in Chapter 2 of this thesis and include pRS426/tE, pRS426/tK, pRS426/tQ, pRS426/tEK, pRS426/tEQ, pRS426/tKQ and pRS426/tEKQ.

**Cell Cycle Synchronizations (α-factor release)**

Overnight cultures of cells were grown to an OD\textsubscript{600} between 0.3-2.0 at room temperature (if cells were over this density, they were diluted back and allowed to double at least once before proceeding). For the arrest, cells were diluted to OD\textsubscript{600} = 0.19 and α-factor (in DMSO) was added to a final concentration of 5ug/ml and cultures were shifted to 30°C for the remainder of the experiment. After 1.5 hours, α-factor was re-added to the culture (one half of the original volume added) and degree of arrest was determined microscopically. When >95% of cells were non-budded and shmooed, cultures were released from arrest by filtering in Konte filtration apparatus and washing with 10x volumes of pre-warmed media. Filters containing cells were then resuspended in fresh pre-warmed media free of α-factor. Aliquots of cultures were taken at 15min intervals. Degree of synchronicity and progression through the cell cycle was determined by counting buds (assessed by morphology of cells) and by counting metaphase and anaphase spindles (as assessed by spindle morphology after immunofluorescence staining for tubulin). For wild type and deletion strains, YPDA media was used throughout experiment. For cells harboring 2micron plasmids, the appropriate selective media was used throughout. For budding
and immunofluorescence, 1ml culture was added to tubes containing 37% formaldehyde (and further processed as necessary). When samples were collected for RNA preparations, samples were spun down, rinsed once with DEPC treated water and then flash-frozen in liquid N$_2$.

**Cell Sizing**

Aliquots of actively growing cells were sonicated and added to isotonic buffer. Between 30,000- 50,000 cells were analyzed using a Coulter Counter to determine cell size. Cells were either grown in YPDA or in the appropriate selective media for cells harboring plasmids.

**Small RNA Northern Blots**

RNA was isolated using the hot acid-phenol chloroform method and samples were denatured by heating at 80°C for 10 min and then loaded onto a pre-run 8% polyacrylamide-urea gel (Ureagel system, National Diagnostics) containing N-acryloylamino phenyl mercuric chloride and run in 0.5x TBE. Gels were transferred to Genescreen Plus membrane (semi-dry transfer in 0.5x TBE at 25V for 1hr) and then crosslinked. Membranes were prehybridized in 5x SSC, 20mM Na-phosphate pH 7.2, 7% SDS, 2x Denhardt’s solution, 1mg single stranded salmon sperm DNA at 50°C for a least 2hrs. For hybridization, DNA oligonucleotide probes complementary to the sequence of interest were end-labeled with $\gamma^{32}$[P] (Perkin Elmer) using T4-polynucleotide kinase (NEB) and then added to the prehybridization buffer. Hybridization was carried out for at least 8hrs at 50°C and then blots were washed first with non-stringent wash buffer (3x SSC, 25mM Na-phosphate pH 7.5, 5% SDS) and then with stringent wash buffer (1x SSC, 1% SDS). Membranes were exposed to phosphorimager screens and imaged using BAS/Fuji imager.
Small RNA northern blot probe sequences used in this study

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ACKNOWLEDGEMENTS

We wish to thank the Amon lab for assistance with cell cycle arrest experiments, particularly Christian Gonzalez, Jill Falk and Jeremy Rock.
REFERENCES


Appendix B
A functional proteomics approach links the ubiquitin-related modifier Urm1 to a tRNA modification pathway

Christian D. Schlieker¹, Annemarie G. Van der Veen, Jadyn R. Damon, Eric Spooner, and Hidde L. Ploegh¹

Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, MA 02142

Edited by Alexander Rich, Massachusetts Institute of Technology, Cambridge, MA, and approved October 6, 2008 (received for review September 4, 2008)

Urm1 is a highly conserved ubiquitin-related modifier of unknown function. A reduction of cellular Urm1 levels causes severe cytokinesis defects in HeLa cells, resulting in the accumulation of enlarged multinucleated cells. To understand the underlying mechanism, we applied a functional proteomics approach and discovered an enzymatic activity that links Urm1 to a tRNA modification pathway. Unlike ubiquitin (Ub) and many Ub-like modifiers, which are commonly conjugated to proteinaceous targets, Urm1 is activated by an unusual mechanism to yield a thiocarboxylate intermediate that serves as sulfur donor in tRNA thiolation reactions. This mechanism is reminiscent of that used by prokaryotic sulfur carriers and thus defines the evolutionary link between ancient Ub progenitors and the eukaryotic Ub/Ub-like modification systems.

Keywords: cytokinesis | thiolation | urmylation | wobble nucleoside | MOC53

The covalent attachment of ubiquitin (Ub) and ubiquitin-like modifiers (Ubls) to target molecules serves many different functions: Ub and Ubls have been implicated in processes as diverse as protein degradation, modulation of enzymatic activity, control of subcellular localization, and mobilization of defined subunits from complex assemblies (1). Although the functional outcomes are quite diverse, most if not all Ubls employ a similar activation mechanism before conjugation to their targets. Accordingly, Ub/Ubls exhibit remarkably similar structural properties, a feature that also applies to the enzymes that act on them.

The Ub-like β-grasp fold is the structural hallmark common to all representatives of the Ub family (supporting information SI Fig. S1). In the first of several steps that constitute an enzymatic cascade, Ub/Ubl is activated by an activating enzyme (E1) to form an acyl-adenylate at the C terminus. This is followed by thioester formation with the active-site cysteine of the E1 enzyme, and, eventually, further transesterification events to downstream activities (E2s and E3s). The latter confer substrate specificity to the process by conjugating the activated Ub/Ubl to its selected target (2, 3). Ub, NEDDS, SUMO-1, and other Ubls are ultimately transferred to the ε-amino group of a lysine residue in a proteinaceous target to yield an isopeptide-linked Ub/Ubl conjugate. Atg8 and LC3 are attached to phospholipids, and other classes of molecular targets might exist (for a current review, see ref. 1).

Ub, Ubls, and the corresponding enzymatic cascades appear to be restricted to eukaryotes, although a number of bacterial proteins adopt a Ub-like β-grasp fold (4). ThiS and MoeA are the best-characterized bacterial representatives of this type. Both molecules show little if any sequence identity to Ubis but are structurally similar to Ub (Fig. S1). ThiS and MoeA are activated by adenylation reactions, and the corresponding enzymatic activities, ThiF and MoeB, respectively, are structurally similar to eukaryotic E1 enzymes (5–7). ThiS and MoeB can thus be considered as molecular ancestors of the Ub system (4). Unlike eukaryotic Ubls, these proteins serve as sulfur carriers in thiamine and molybdopterin synthesis pathways, respectively. Consequently, their mode of action is distinct from that of the canonical E2–E3 machinery. Instead, both ThiS and MoeA acquire an activated sulfur atom from ThiF/MoeB to yield a thiocarboxylate at their C terminus, and downstream enzymatic activities are responsible for incorporation of the sulfur into the precursors of the respective enzymatic cofactors (4 and references therein).

Furukawa et al. (8) explored the functional similarities between the prokaryotic and eukaryotic systems by using the Escherichia coli ThiS and MoeA as query sequences to search for homologous proteins in Saccharomyces cerevisiae. An uncharacterized ORF of 99 aa was identified and designated URM1 (Ub-related modifier 1). URM1 is conjugated to yield high-molecular weight, supposedly proteinaceous Urm1 adducts, a process that was termed urmylation. Urmylation depends on Uba4, the putative E1 enzyme required for Urm1 activation. To our knowledge, other Urm1-directed enzymatic activities have not been reported.

What is the function of this enigmatic modifier? Urm1 deficiency causes pleiotropic phenotypes in S. cerevisiae: defects in invasive growth and pseudohyphal development (9), rapamycin sensitivity (10), and derepression of nitrogen catabolite-repressed genes (11) are all suggestive of a promiscuous role in nutrient sensing. In addition, urm1Δ strains are temperature-sensitive and hypersensitive toward oxidative stress (8, 10), suggesting a broader role in stress tolerance. The antioxidant protein Ahp1p is the only urmylation target that has been identified to date, and urm1Δ strains are hypersensitive toward the oxidant diamide (10). The high degree of sequence conservation suggests an important role for Urm1 in higher eukaryotes as well, but no function has been assigned to it in mammalian cells.

In this work, we investigate the function of Urm1 in human cells. We used a small hairpin RNA (shRNA)-mediated approach to reduce cellular Urm1 levels and observed a strong cytokinesis defect as one of the phenotypic consequences. Furthermore, the application of an Urm1-based suicide inhibitor allowed us to identify ATPBD3, a yet uncharacterized Urm1-dependent enzyme and a constituent of a multiprotein complex. By detecting a cellular Urm1 thiocarboxylate as catalytic intermediate, we show that the underlying mechanism relies on an unexpected sulfur carrier function for Urm1. The activated sulfur is required for the ATPBD3-catalyzed thiolation of certain wobble nucleosides, as evidenced by the hypomodification of tRNAs in absence of Urm1 or any of the catalytic activities that act on it. We show that this mode of action is operative in S. cerevisiae and in human cells, suggesting its strict conservation throughout all eukaryotes.

Author contributions: C.D.S. designed research; C.D.S., A.G.V.D.V., J.R.D., and E.S. performed research; C.D.S., A.G.V.D.V., J.R.D., and E.S. contributed new reagents/analytic tools; C.D.S., A.G.V.D.V., J.R.D., E.S., and H.L.P. analyzed data; and C.D.S., A.G.V.D.V., J.R.D., and H.L.P. wrote the paper.

The authors declare no conflict of interest.

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1To whom correspondence may be addressed. E-mail: schlieker@wi.mit.edu or ploegh@wi.mit.edu.

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Reduced Urm1 Levels Cause Cytokinesis Defects in Human Cells. To investigate the effects of Urm1 deficiency in mammalian cells, we targeted the Urm1-coding sequencing in HeLa cells with a shRNA by means of lentiviral transduction. We obtained a significant (~95%) and highly reproducible reduction of Urm1 protein levels 4 days after infection (Fig. 1A). The unrelated control shRNA (shLuciferase or shGFP) did not affect Urm1 protein levels (Fig. 1A and data not shown). ShUrm1 cells were viable but showed a reduced growth rate compared with shLuciferase cells. After ~3 days of drug selection, shUrm1 cells exhibited remarkable morphology anomalies: we observed an increase in cell size and the presence of multiple nuclei within single cells (Fig. 1B). In contrast, shLuciferase or mock-infected cells were phenotypically normal (Fig. 1B and data not shown).

To measure the DNA content of these cells, asynchronously growing cells transduced with shUrm1 or shGFP were stained with propidium iodide and analyzed by flow cytometry. Consistent with the multinucleated phenotype, shUrm1 cells showed a significant increase in the 4n population, and in addition a population containing ~4n was present. The DNA content of shGFP cells was essentially unchanged compared with wild-type HeLa cells. The effects of Urm1 deficiency on DNA content were even more pronounced after cell cycle synchronization by imposition of a double-thymidine block (Fig. 1C). Twelve hours after release from this block, 53.3% of shUrm1 cells contained ~4n, compared with 31.0% and 31.2% for shGFP and mock-infected cells, respectively. Similar results were observed in asynchronously growing U2OS cells in which Urm1 protein levels were successfully down-regulated (data not shown).

Taken together, our results suggest that Urm1 is required for cytokinesis and thus for orderly cell cycle progression.

A Functional Proteomics Approach Links Urm1 to tRNA Modification. To gain insights into the molecular mechanism(s) that underlie this remarkable phenotype, we used a functional proteomics approach. We synthesized an electrophilic Urm1-based suicide inhibitor, HA-tagged Urm1 vinyl methylester (HA-Urm1-VME). The analogous Ub/Ubl-based electrophilic probes form a stable thioether linkage to the active site cysteine of Ub/Ubl-directed enzymes (12, 13) and can thus be used to selectively retrieve cognate enzymatic activities from crude cell extracts to allow their identification (14).

HA-Urm1-VME was added to HEK293T cell lysates and incubated at 37 °C for 30 min. Covalent HA-Urm1-VME adducts were retrieved by immunoprecipitation and resolved by SDS/PAGE. After silver staining, a prominent band migrating at an apparent molecular mass of ~47 kDa appeared as unique to the HA-Urm1-VME-derived sample (Fig. S2). This material was excised and processed for liquid chromatography–tandem mass spectrometry (LC-MS/MS). A database search revealed ATPBD3 (ATP-binding domain 3, gi 21687159, theoretical molecular mass 36.4 kDa) as the major HA-Urm1-VME-reactive species (33% sequence coverage, Fig. S2). As expected, Urm1 was also identified in this sample (Fig. S2). The function of ATPBD3 is unknown, but it is annotated as a cancer-associated gene and appears to be differentially expressed in breast and prostate cancer (15).

To confirm this interaction, we constructed a FLAG-tagged version of the ATPBD3 cDNA via RT-PCR and expressed the 35S-labeled protein by a coupled in vitro transcription–translation system. The translation product was incubated in presence or absence of N-ethylmaleimide (NEM), and then exposed to HA-Urm1-VME. A band of the expected ATPBD3 molecular mass was readily detectable, and a shift in electrophoretic mobility indicative of covalent modification was observed upon addition of HA-Urm1-VME (Fig. 2A). This modification was sensitive to NEM, suggesting that a cysteine residue in ATPBD3 is the site of modification. We confirmed these findings by transiently transfecting FLAG-ATPBD3 into HEK293T cells followed by detergent lysis and anti-HA immunoprecipitation in the absence or presence of HA-Urm1-VME. Although FLAG-ATPBD3 was barely detectable in the control lane, a prominent FLAG-reactive band was retrieved in the presence of HA-Urm1-VME (Fig. 2B). In addition, a higher-molecular mass doublet is seen in presence of HA-Urm1-VME, which we interpret as the covalent adduct formed between FLAG-ATPBD3 and the suicide inhibitor.

Finally, we used an HA-tagged variant of ATPBD3 to retrieve and identify additional interaction partners. To this end, HEK293T cells were transiently transfected either with a control vector or with ATPBD3-HA. Cell extracts were prepared 48 h after transfection, and ATPBD3-associated proteins were retrieved by immunoprecipitation, separated by SDS/PAGE, and subjected to silver staining. A total of 4 bands appeared to be unique species compared with the negative control (Fig. 2C). These were excised and processed for identification by mass spectrometry (MS). Notably, Urm1 was readily detectable as interaction partner in this experiment (Fig. 2C, sequence coverage 48.5%). As expected, ATPBD3 accounted for the major band, migrating at the expected apparent molecular mass of ~38 kDa. The 2 major species recovered from the other bands correspond to UPP0432 protein/C16or184, a yet uncharacterized protein (gi 121941955, molecular mass 56 kDa, 36% sequence coverage), and Hsp70 (gi 167466173, molecular mass 70 kDa, 66% sequence coverage), a common contaminant that is encountered in many MS-based approaches.

Because no function was assigned to either human ATPBD3 or UPP0432, we used a structure prediction-based algorithm (16) to search for structural homologues. Mesl [Protein Data Bank (PDB) ID code 1n1s], an ATP α-hydrolase of unknown function, and TiIS (PDB ID code 1wy5), a tRNA-modifying enzyme and likewise an ATP α-hydrolase, were predicted as close structural neighbors for both ATPBD3 and UPP0432 (data not shown). A Pfam search (17) consistently assigned both proteins to the PP-loop ATP hydrolase superfamily.
Urm1 and MoaD, we speculate that Urm1 could serve as sulfur carrier in the context of tRNA thiolation in mammalian cells. To test this possibility, HEK293T cells were cotransfected with MOCS3-HA (the human Uba4 homolog) and HA-Urm1. Soluble cell extracts were prepared 48 h after transfection, HA-Urm1 was retrieved by immunoprecipitation, further purified by SDS/PAGE (Fig. 3A), and digested with the endoprotease Asp-N to yield peptides of a size suitable for analysis by LC-MS/MS. Database searches of the resulting MS data revealed 2 overlapping peptides from the C terminus, indicating that either the Asp-88 or Asp-90 cleavage sites were used (Fig. 3B). In addition, database searching also identified 2 peptides with a mass 16 Da greater than these, with the additional mass localized at the C terminus (Fig. 3B), as shown by the masses of the y ion series (Fig. 3C). This shift in mass is consistent with a C-terminal modification by sulfur in the form of a thioether adduct. We conclude that Urm1 can serve as sulfur carrier, akin to the prokaryotic sulfur donors ThiS and MoaD.

**Cytosolic tRNAs Are Hypomodified in the Absence of Urm1.** In *S. cerevisiae*, 3 cytosolic tRNAs, tRNA^Gln(UUG), tRNA^Glu(UUC), and tRNA^Leu(UUR) contain a 5-methylcarboxamidomethyl-2-thiouridine (mcm^5^S^2^U) at the wobble position (19) (Fig. 4A). Several enzymatic activities act on the 2- and 5-positions of the uridine ring in yeast: Elp3 has been implicated in the derivatization of the 5-position (20), and Ctul/NCS6 is required for the 2-thiolation (19). However, the source of the sulfur atom that is used by Ctul/NCS6 remains enigmatic. With the exception of *Mycoplasma*, 2-thiolated uridines have been identified in all kingdoms of life. Notably, the simultaneous loss of Elp3 and Ctul/NCS6 lethality, indicating a crucial role for these modifications (19).

Can Urm1 serve as sulfur donor for the Ctul/NCS6-catalyzed thiolation reaction? Because the enzymatic pathway responsible for uridine modification is well characterized in yeast, we chose to address this question by introducing chromosomal deletions in *S. cerevisiae*. Four deletion strains were constructed: urm1Δ, uba4Δ, ncs6Δ, and cla4Δ. To assess the extent of tRNA^Gln(UUG), tRNA^Glu(UUC), and tRNA^Leu(UUR) thiolation, RNA was isolated from all deletion strains; a wild-type (WT) strain served as control (Fig. 4B). The RNA was separated on a denaturing polyacrylamide gel supplemented with N-acryloylamino phenyl mercuric (APM) chloride and transferred to a nylon membrane. Small radiolabeled DNA oligonucleotides complementary to the 3 tRNAs in question were used as probes; a probe recognizing the nonthiolated tRNA^His^ served as negative control. The thiolation of RNA molecules is readily detectable in APM gels by transient mercury–sulfur interactions, which cause a considerable shift in electrophoretic mobility (21). In agreement with the established role of Nes2/Ctul in 2-thiolation (19), the deletion of NCS6 caused an increase in tRNA^Gln(UUG)^ mobility, indicative of quantitative hypomodification. RNA isolates from cla4Δ behaved like the WT control, suggesting that Cla4 is not directly involved in this process (Fig. 4B).

**Urm1 Can Serve as Sulfur Carrier by Virtue of a C-Terminal Thiocarboxylate.** What could be the function of Urm1 in the context of tRNA modifications? Given the involvement of Nes6 (Ctul) in tRNA thiolation and based on the structural similarity between phophatase family, which comprises a plethora of tRNA-modifying enzymes. From a structural perspective, these findings collectively suggest a link between Urm1 and tRNA modifications. Genetic evidence likewise suggests that the budding yeast homologs of URM1 and ATPBD3 were designated Ctu2 and Ctul (cytosolic thiouridine). We conclude that Urm1 is required for the thiolation of 3 cytosolic tRNA species, namely tRNA^Gln(UUG), tRNA^Glu(UUC), and tRNA^Leu(UUR).

To establish that tRNA thiolation is Urm1-dependent in higher Schleier et al.
A related modifier. Urmi-deficient yeast strains show a pleiotropic phenotype, attributed to a lack of Urmi conjugation to target proteins, a process that was termed urmylation (8–11). Apart from the identity of Uba4, the putative E1 enzyme responsible for Urmi activation (8), next to nothing is known about the molecular mechanism and the enzymatic machinery that constitute the Urmi pathway.

By using an Urmi-based suicide inhibitor, we discovered ATPBD3 as an Urmi-directed enzymatic activity (Fig. S2 and Fig. 2). Urmi is part of a protein complex that contains ATPBD3 and UPF0432 (Fig. 2C and data not shown). However, neither of these proteins has been assigned a function. A bioinformatics approach revealed similarities of both ATPBD3 and UPF0432 to tRNA-modifying enzymes, which commonly adenylate specific tRNA nucleosides to facilitate their subsequent derivatization. What could be the function of Urmi in the context of tRNA modifications?

The S. cerevisiae homolog of ATPBD3, Ncs6, is required for the thiolation of uracil at the wobble position of U-rich anticondons of tRNAs, exemplified by the human tRNA thiolation in S. cerevisiae. RNA was isolated from the indicated S. cerevisiae strains, resolved by denaturing PAGE, and subjected to Northern blotting. Polyacrylamide gels supplemented with APM served as control.

### Discussion

Urmi is a highly conserved, yet poorly understood, ubiquitin-related modifier. Urmi-deficient yeast strains show a pleiotropic evolutionarily conserved, yet poorly understood, ubiquitin-related modifier.
of uridine modifications is a prerequisite for the killer toxin's nuclease activity. The Kluyveromyces lactis derivatization of the 5-position of the uridine, confer resistance to Urmi, Ncs6 in budding yeast), or those that are required for the Urmi in the tRNA thiolation pathway in S. cerevisiae cultures, thus excluding dynamic fluctuations in this modification as key regulatory mechanism (data not shown). One interpretation would be that the cytokinesis defect is a mere consequence of cellular senescence, which is in turn promoted by translational defects. Alternatively, some of the transcripts that encode key regulators of the cell cycle may be particularly enriched in codons that are read by 2-thiolated tRNAs. Interestingly, skewed codon usage patterns occur in functionally related genes, or groups, and have been linked to tRNA modifications (36 and references cited therein).

The sulfur donor function identified here is not necessarily restricted to tRNAs. The involvement of Urmi in protein conjugation and a role in tRNA thiolation are not mutually exclusive, and further experiments are required to identify or to rule out additional Urmi functions.

**Experimental Procedures**

**Cell Lines, Antibodies, Constructs, and Lentiviral Transduction.** HeLa cells were cultured in DMEM containing 10% fetal calf serum and penicillin/streptomycin at 37 °C, 5% CO₂.

**Antibodies.** Human Urmi 1 was expressed as an N-terminal His-tagged fusion protein in E. coli. BL21 (DE3) Rosetta cells, purified, and sent to Covance Research Products to generate rabbit polyclonal antibodies. The antisera was affinity-purified as described in ref. 37. Anti-p97 was purchased from Fitzgerald Industries International. Anti-FLAG and anti-HA (3F10) were purchased from Sigma and Roche, respectively. Phalloidin-647 and Hoechst 33342 were from Molecular Probes.

** Constructs.** Human CDNA was prepared from HeLa cells with the SuperScript first-strand synthesis system (Invitrogen) and used as template for PCRs to amplify the human Urmi, MOCS3, and ATPBD3 cDNAs. All constructs were cloned into pcDNA3.1+ (Invitrogen) according to standard procedures.

** Lentivirus.** Human Urmi, ATPBD3 and control (GFP and luciferase) shRNA constructs were obtained from the TRC Consortium at the Broad Institute of MIT and Harvard (Boston, MA). Lentivirus production was achieved following the procedure provided by the Broad Institute (www.broad.mit.edu-genome-bio/trc/publicProtocols.html). HeLa cells were infected in a 6-well plate with 100–500 μl of viral supernatant supplemented with 4 μg/mL Polybrene (Sigma), spun at 1,020 × g for 90 min at room temperature and placed for 5 h in the incubator before medium replacement. Antibiotic selection (1 μg/mL puromycin) was started 24 h after infection.

** Transfections.** All transfections were carried out by using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions.

**Confocal Microscopy.** Cells were grown on coverslips, fixed in 4% paraformaldehyde, quenched with 20 mM glycine, 50 mM NH₄Cl, and permeabilized in 0.1% Triton X-100. Fixed and permeabilized cells were blocked in 4% BSA and stained with phallolidin-647 and Hoechst 33342 for 30 min. Images were acquired by using a spinning disk confocal microscope as described in ref. 38 by using a Nikon 60× magnification, 1.4 numerical aperture oil lens.

**Flow Cytometry.** Cell synchronization by double-thymidine block was performed as described in ref. 34. Cells were harvested by trypsinization at 12 h after release from cell cycle block, fixed in ice-cold 70% ethanol, and stained with propidium iodide (50 μg/ml in the presence of 0.1 mg/ml RNase A and 0.05% Triton X-100 for 40 min at 37 °C. Cells were analyzed using a Becton Dickinson FACSSCAlibur, and cell cycle profiles were generated by using FlowJo 8.5.3 software.
Anti-IA Affinity Purification and MS/MS Analysis. HA-Urm1-VME was produced as described for Ub-VME (39). HEK293T cells (107) were harvested by scraping in ice-cold PBS and centrifugation. The pellet was resuspended in 10 mL of lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM MgCl2, 0.5% Nonidet P-40, 0.5 mM DTT, pH 7.5) and incubated on ice for 10 min. The lysate was clarified by centrifugation (14,000 × g for 15 min at 4 °C). The supernatant was applied to anti-HA-agarose (80 μL of staphylococcal affinity beads; Roche), and agitated for 2 h at 4 °C. The beads were washed 4 times in wash buffer (50 mM Tris, 150 mM NaCl, 5 mM MgCl2, 0.1% Nonidet P-40), eluted by boiling in 60 μL of SDS-sample buffer, and applied to a SDS/PAGE and silver staining. For protein identification, bands were excised, processed, and analyzed by LC-MS/MS as described in ref. 37. For thio-O-arboxylate detection, samples were digested with endoproteinase Asp-N and analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS).

**In Vitro Transcription/Translation (IVT).** IVT reactions were performed using the TNT system (Promega), according to the manufacturer's instructions. Five micrograms of HA-Urm1-VME were added to 1 mL of clarified lysate for small-scale reactions and reacted for 30 min at 37 °C before immunoprecipitation.

**Yeast Techniques and RNA Analysis.** Yeast strains. All strains are W303 derivatives and are described in Table 1. Gene deletions were constructed by using the method described (40) to replace the desired ORF with either the H3S36X or the KAM6X cassette. All deletions were confirmed by PCR. cba4Δ was a kind gift from the laboratory of Angelika Amon (Massachusetts Institute of Technology, Cambridge, MA).

**Yeast RNase A and enzymatic hydrolysis of yeast tRNA.** Yeast cultures were grown at 30 °C and harvested in mid-log phase. RNA was prepared using the RNeasy kit (Qiagen) and processed for Northern blotting as described (41, 42).

**Northern Blot Analysis.** RNA for analysis by Northern blotting was prepared by using Trizol reagent (Invitrogen) to isolate total RNA according to the manufacturer's instructions. Ten micrograms of RNA (isolated from yeast or HeLa cells) were run on either 8% PAGE gels (Sequalab; National Diagnostics) or on APM gels. Gels were transferred to GeneScreen Plus membrane (PerkinElmer), UV-cross-linked, and processed according to standard procedures. DNA probes complementary to the RNA sequences (either mammalian or yeast) of Lys-K(UUJ), Glu-([UUJ] or Gin-[UUJ]) were end-labeled with γ-[32P]ATP (PerkinElmer) by T4 polynucleotide kinase (NEB).

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**REFERENCES**


Table 1. Yeast strains and genotypes used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
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<tbody>
<tr>
<td>8015 (wild-type) (from A. Amon)</td>
<td>MATa</td>
</tr>
<tr>
<td>urmtΔ</td>
<td>MATa urmtΔ::KAM6X</td>
</tr>
<tr>
<td>nc6Δ</td>
<td>MATa nc6Δ::HIS3M3X</td>
</tr>
<tr>
<td>uba4Δ</td>
<td>MATa uba4Δ::HIS3M3X</td>
</tr>
<tr>
<td>cba4Δ</td>
<td>MATa cba4Δ::HIS3M3X</td>
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Fig. S1. Urm1 is related to prokaryotic sulfur donors. Shown is a structural catalog of ubiquitin-related proteins and their prokaryotic ancestors, depicted as cartoon representations by using Pymol. A phylogram representing the sequence relationships between these molecules is shown in the center. The amino acid sequences of the depicted molecules were applied to ClustaW to create a phylogram in Unrooted.
Identification of ATPBD3, a Urm1-VME-reactive protein, by mass spectrometry (MS). (Left) Cell lysates were incubated in absence and presence of HA-Urm1-VME, immunoprecipitated with anti-HA antibodies, and subjected to SDS/PAGE and silver staining. MS analysis of the indicated band revealed ATPBD3 as Urm1-VME-reactive species. Peptides that were identified in conjunction with database searches are highlighted in red.
Fig. S3. The thiolation of cytosolic tRNAs is Urm1-dependent in Saccharomyces cerevisiae. RNA was isolated from the indicated S. cerevisiae strains, resolved by denaturing PAGE, and subjected to Northern blotting. Polyacrylamide gels supplemented with acrylamido phenyl mercuric chloride (APM) were used to discriminate between thiolated and hypomodified tRNAs (A) and (B); a gel devoid of APM served as control (C). Radioactive oligonucleotides with the indicated specificities served as probes.
Fig. 54. HPLC-MS analysis of wild-type (WT) and urmΔ RNA hydrolysates. (A) Ion chromatograms of BH2+ and MH+ ions. RNA was isolated from WT (Left) and urmΔ strains (Right), hydrolyzed, and subjected to HPLC-MS. Axes are time (minutes) (abscissa) vs. relative intensity (ordinate). (B) Average spectra across peaks. (C) Structure and masses of mcm5U and mcm5S2U as intact ion, and the corresponding BH2+ fragmentation product.