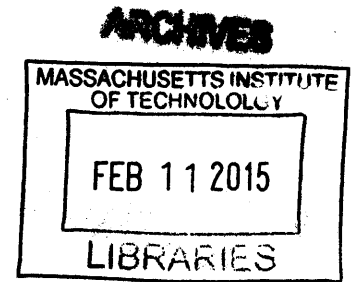# The Physiological Consequences of Loss of tRNA Thiolation in *Saccharomyces cerevisiae*

by

Jadyn Rose Damon

B. S. Microbial Biology
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SUBMITTED TO THE DEPARTMENT OF BIOLOGY IN PARTIAL
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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 $mcm^5s^2U_{34}$ modification on the anticodon wobble uridines of $tGlu^{UUC}$, $tGln^{UUG}$ 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°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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Chapter 1:

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).

A

$$E1_{SH} + ATP + Ub \rightleftharpoons E1_{SH}^{-AMP\sim Ub(A)} + PPi$$

$$E1_{SH}^{-AMP\sim Ub(A)} \rightleftharpoons E1_{S\sim Ub(T)} + AMP$$

$$E1_{S\sim Ub(T)} + ATP + Ub \rightleftharpoons E1_{S\sim Ub(T)}^{-AMP\sim Ub(A)} + PPi$$

$$E1_{S\sim Ub(T)}^{-AMP\sim Ub(A)} + E2_{SH} \rightleftharpoons E1_{SH}^{-AMP\sim Ub(A)} + E2_{S\sim Ub}$$
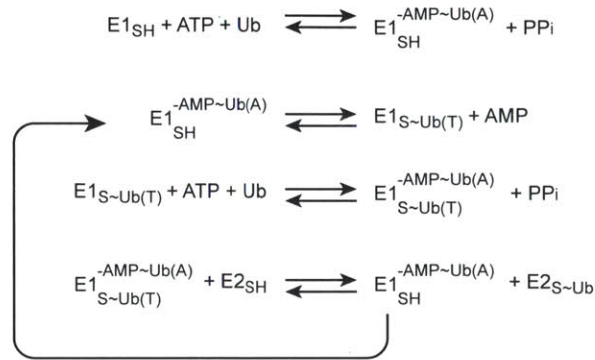
B
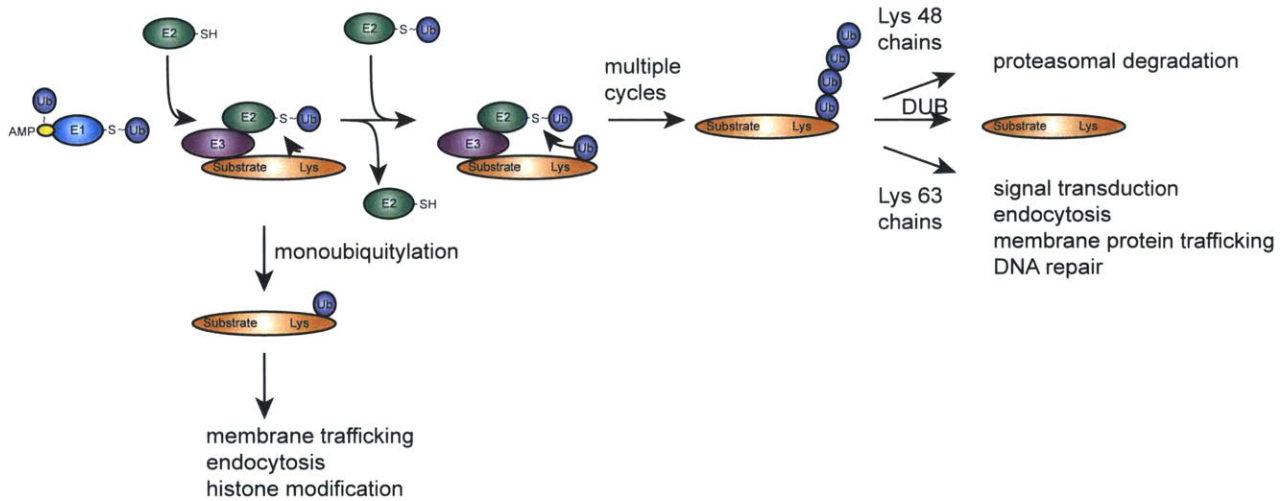
Figure 1: Schematic of the ubiquitin proteasome system. A. Mechanism of ubiquitin activation and conjugation cycle. Ub(A) is ubiquitin that is noncovalently associated with UBA1 at adenylation site, Ub(T) is ubiquitin that is covalently linked to UBA1 through a thioester bond. Steps include (1) adenylate 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).

UBL	Identity with ubiquitin (%)	E1 (UBL-activating enzyme)	E2 (UBL-conjugating enzyme)	Function/ comments on UBL
Ubiquitin	100	Uba1 (UBA6)	Many	Many: proteasomal degradation, localization, protein-protein interactions; precursors encoded by multiple genes
Rub1 (NEDD8)	55	Uba3-Ula1 heterodimer	Ubc12	Modification of Cullin RING E3 ligases, regulation of ubiquitylation
MNSF-β	38	NI	NI	Unclear function, potential role in immune defense/ antiviral response; derived from ribosomal-protein precursor
FAT10	32 and 40*	UBA6	NI	Unclear function, potential role in immune defense/ antiviral response; conjugates accumulate upon proteasomal degradation
ISG15	32 and 37*	UBE1L	UBCH8	Induced by type I interferons, host defense against viral infection
Smt3 (SUMO1, SUMO2, SUMO3)	18	Uba2-Aos1 heterodimer	Ubc9	Many: nuclear transport/ organization, transcription, chromatin remodeling, DNA repair, ribosomal biogenesis; encoded by 3-4 genes in vertebrates
Atg8	ND	Atg7	Atg3	Lipid modifier, role in autophagosomal membrane growth and expansion; three known isoforms in humans
Atg12	ND	Atg7	Atg10	Autophagosome formation; ~20% identical to Atg8
Urm1	ND	Uba4	NI	Protein modification and tRNA thiolation; related to sulfur-carrying proteins MoaD and ThiS
UFM1	ND	UBA5	UFC1	Unclear function, potential role in differentiation; conserved in metazoan and plants
Hub1 (UBL5)				No C-terminal glycine (dityrosine motif), noncovalently involved in splicing

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).
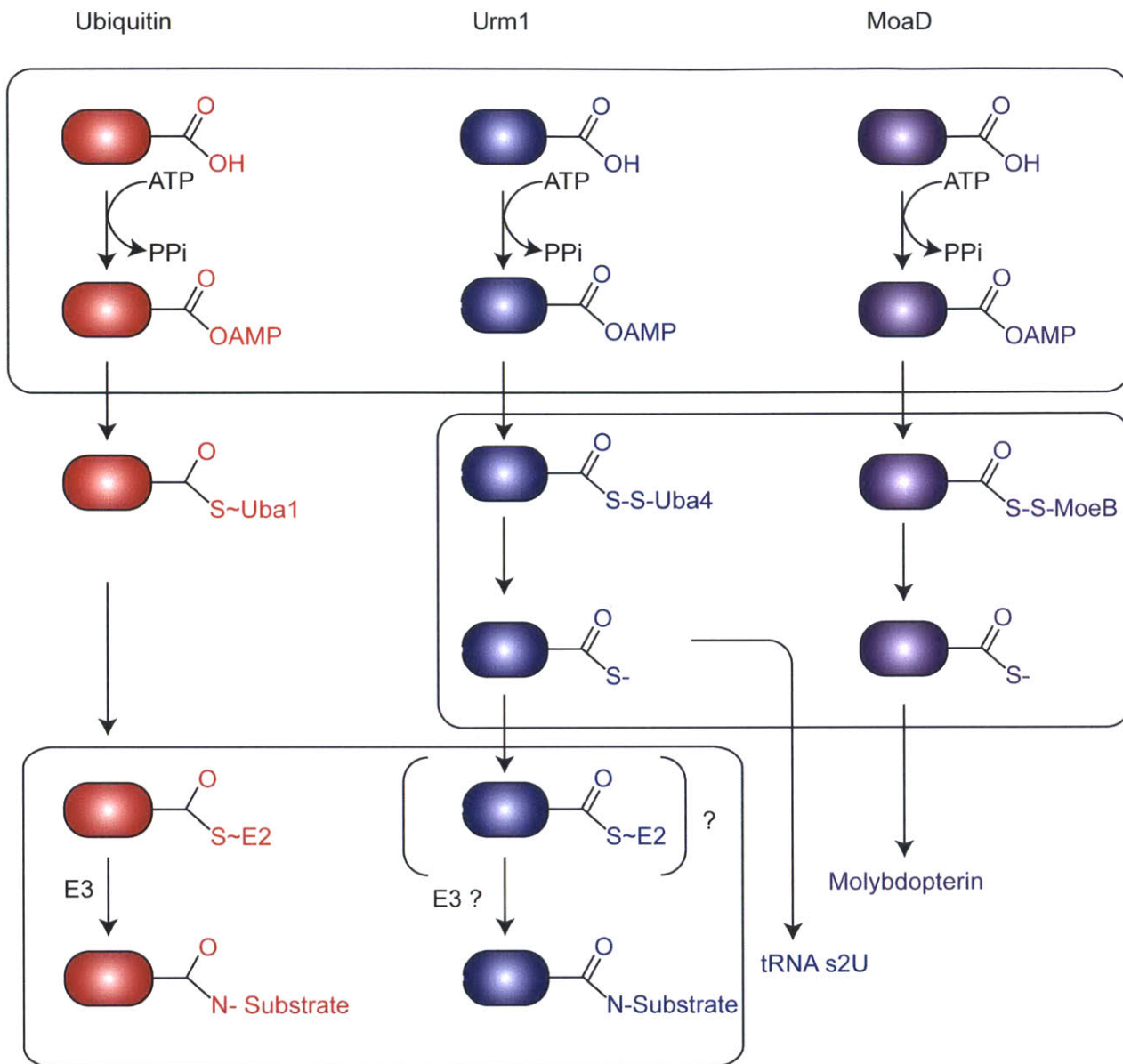
Figure 2: Urm1 is a eukaryotic UBL with similarities to prokaryotic sulfur carriers. Urm1 has similarities with ubiquitin and MoaD. All are activated in an ATP dependent fashion to form an adenylate. Ubiquitin is subsequently transferred to the ε-amine of a lysine residue of a substrate protein through 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 *urm1Δ* 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 s^2 moiety, which is found in conjunction with the mcm^5 modification, on wobble uridines of the cytoplasmic tRNAs $tGlu^{UUC}$, $tGln^{UUG}$ and $tLys^{UUU}$ 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^5s^2- 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^2- 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 *urm1Δ* and *uba4Δ* 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 *URM1* pathway components and elucidated the specific functions of several of the proteins required for Urm1-dependent tRNA thiolation; altogether the *URM1* 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 $mcm^5s^2U_{34}$ modification were efficiently cleaved in the anticodon loop by *Kluveromyces lactis* γ-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 mcm^5 modification (Huang, 2005) and *TRM9* was separately identified as being required for mcm^5s^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 mcm^5 moiety) comprise the *ELP* pathway.

Sulfur transfer in the *URM1* pathway

The sulfur transfer pathway required for thiolation of $tGlu^{UUC}$, $tGln^{UUG}$ and $tLys^{UUU}$ 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 U_{34} 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).
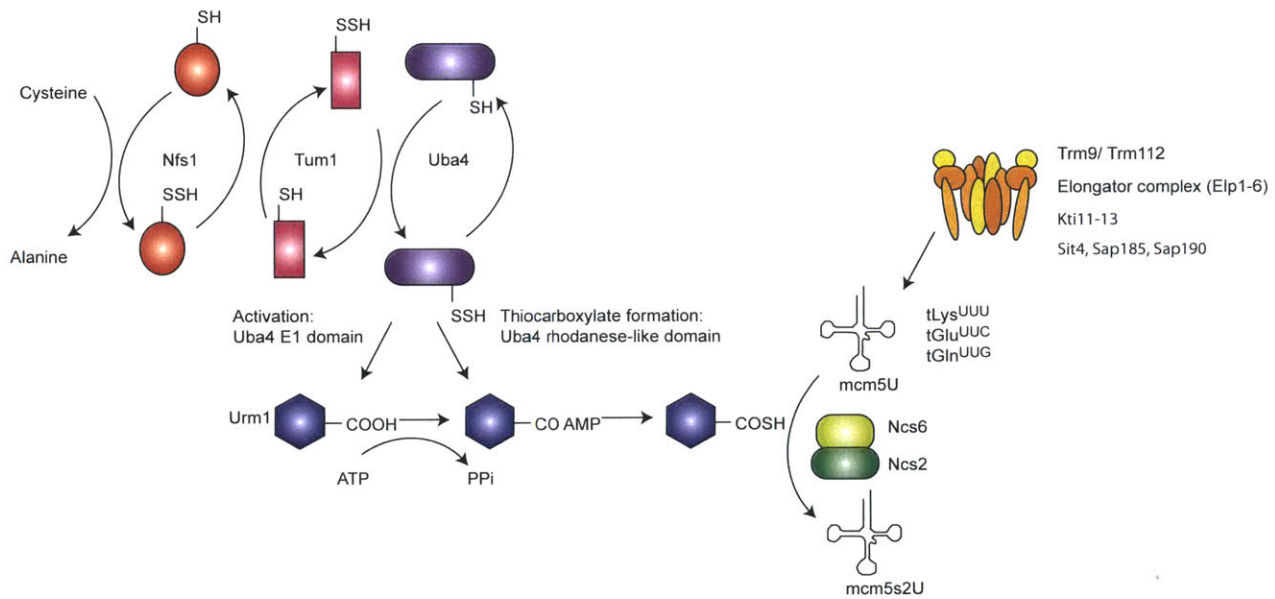
Figure 3: Sulfur transfer and the *URM1* tRNA modification pathway. Sulfur is removed from cysteine by the desulfurase Nfs1, and then transferred to Tum1 and then to Uba4, the E1 activating enzyme for Urm1. Uba4 contains an E1 domain, which functions to activate Urm1 in an ATP dependent fashion, as well 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 $mcm^5s^2U_{34}$ modification found on a subset of tRNAs. The proteins Ncs6 and Ncs2 mediate transfer of sulfur to tRNA molecules. Formation of mcm^5 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_2O_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).

Protein		Diamide	H$_2$O$_2$
Urm1/Ub pathway			
ATP-binding domain 3	Ncs6	+	+
Molybdenum cofactor synthesis 3	Uba4	+	+
Ub-specific peptidase 15		-	+
Ub-specific protease 47		+	+
E3 Ub-protein ligase BRE1A		-	+
Nuclear Transport			
Chromosome segregation 1-like		-	+
Ran GTPase-activating protein 1		-	+
Nuclear RNA export factor isoform 1/2		-	+
Nucleoporin 160kDa		-	+
tRNA modification			
Nol1/Nop2/Sun2 domain family member 2		+	+
Elongator complex protein 1	Elp1	-	+
RNA binding, processing, and transport			
Serpine 1 mRNA-binding protein 1		-	+
ELAV-like protein 1		+	-
Regulator of nonsense transcripts 1		+	-
U5-snRNP-specific protein		+	-
Probable ATP-dependent RNA helicase DDX6		-	+
Oxidative stress			
Hemopexin precursor		+	-
Carbonyl reductase isoform 1/3		-	+
Miscellaneous			
O-linked GlcNac transferase isoform 2		+	-
Transferrin precursor		+	-
Lipocalin 2 precursor		+	-

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 H$_2$O$_2$. 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).
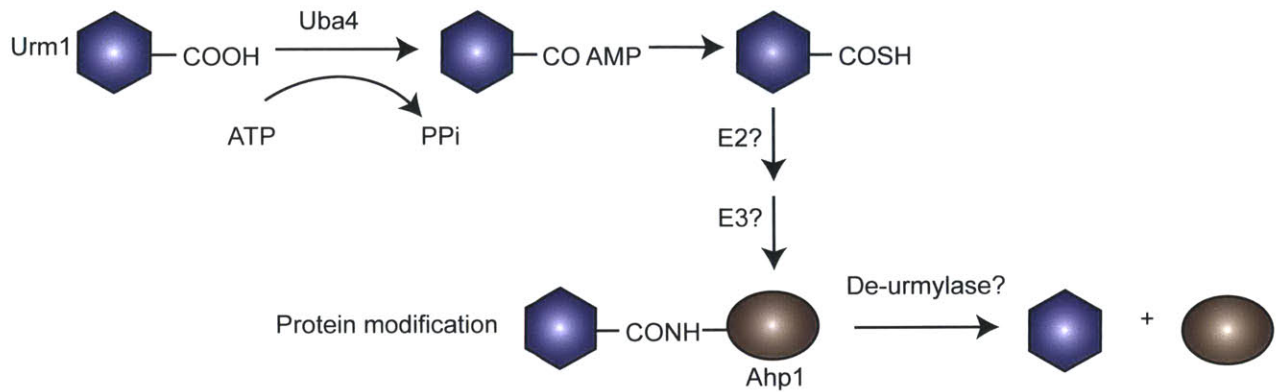
Figure 4: Schematic of protein urmylation. Protein urmylation requires activation and thiocarboxylation of Urm1, both of which are accomplished by the enzymatic activities of Uba4. Urm1 is subsequently conjugated to lysine residues on target proteins. It is unknown whether E2- and/ or E3- like enzymes function in the protein urmylation pathway. It is also unknown if deurmylases, capable of removing Urm1 from substrates, exist.

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

29

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

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).
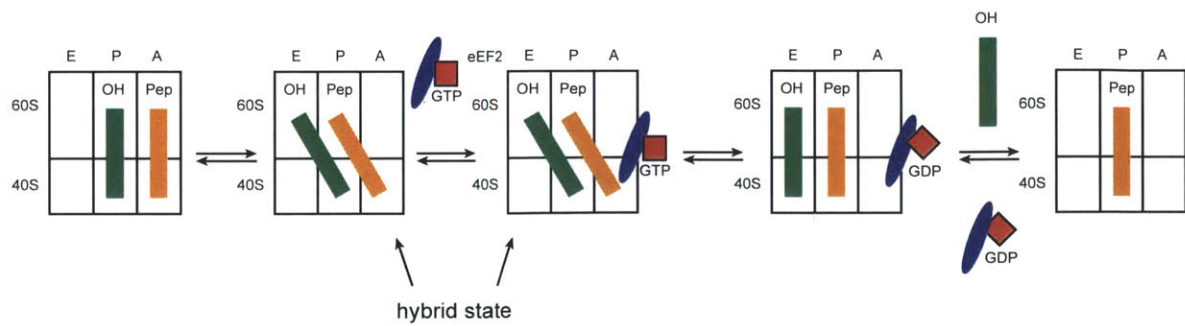
A

1. Codon recognition

2. Peptidyl transfer

3. Translocation

B

hybrid state
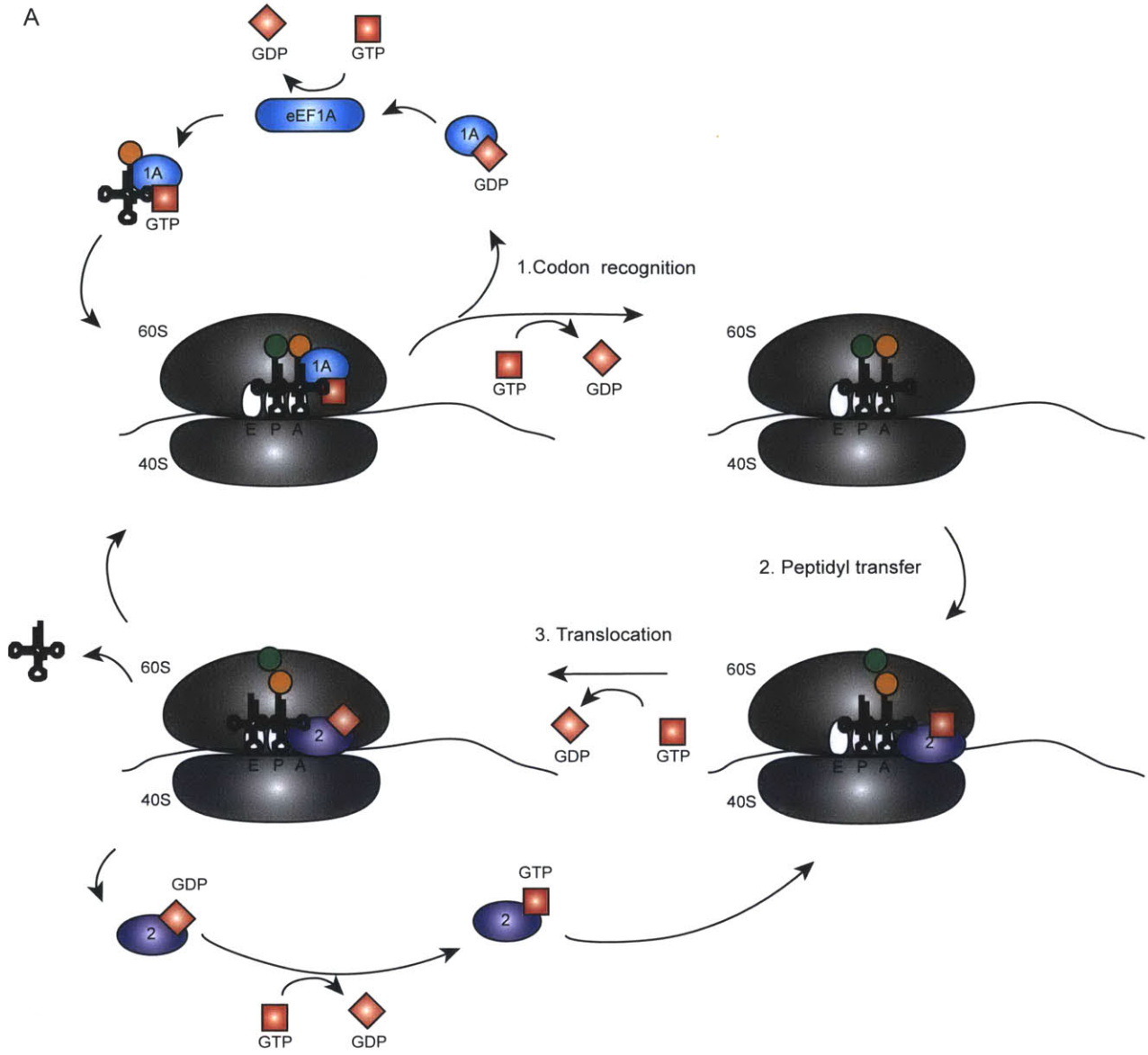
Figure 5: Eukaryotic translation elongation. A. The eukaryotic translation elongation cycle consists of codon recognition, peptidyl transfer and translocation steps, and requires the hydrolysis of GTP at several steps. The ternary complex (TC) is comprised of an aa-tRNA in complex with GTP bound eEF1A. 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 4×10^{-5} to 6.9×10^{-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 (Giegé, 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).
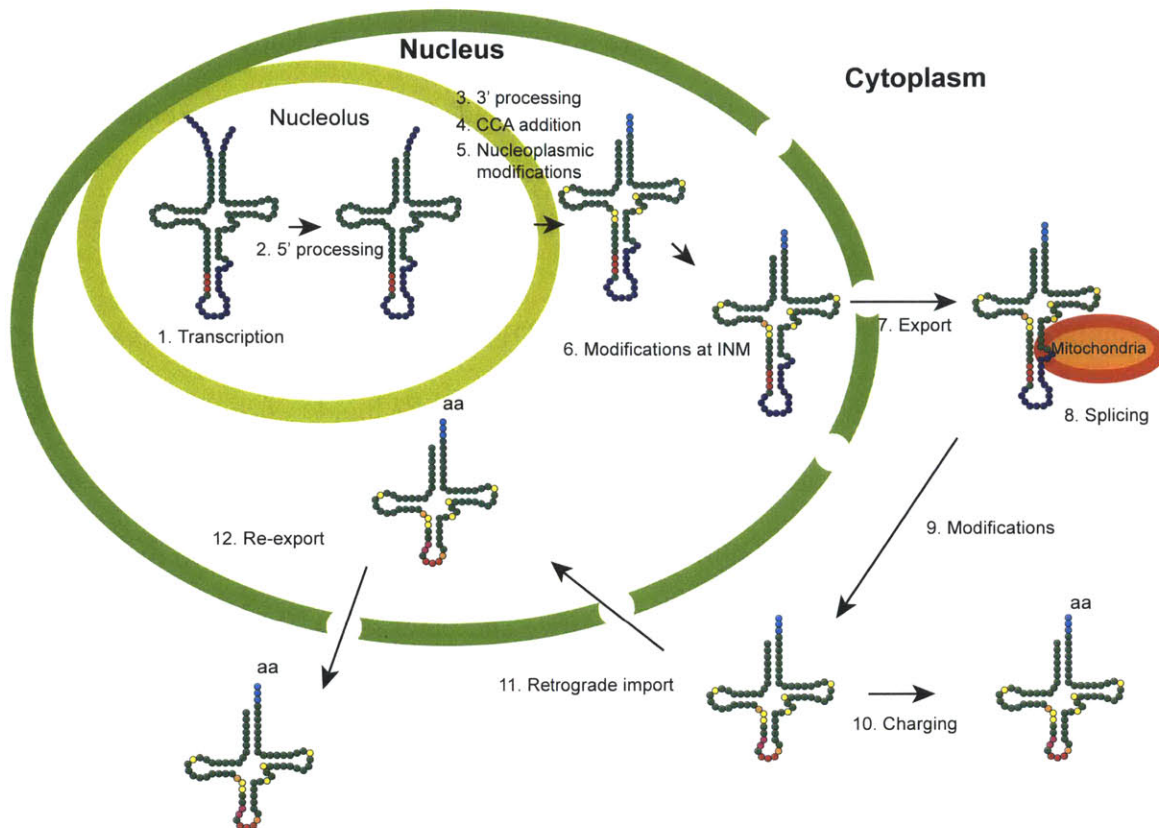
Figure 6: tRNA biogenesis and trafficking.

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).

Yeast gene	Modification	Null mutant phenotype
DUS1	D_{16}, D_{17}	Not essential
DUS2	D_{20}	Not essential
DUS3	D_{47}	Not essential
DUS4	D_{20a}, D_{20b}	Not essential
ELP1 (IKI3), ELP2, ELP3, ELP4, ELP5 (IKI1), ELP6, KTI11 (DPH3), KTI12, KTI13, KTI14, SIT4, SAP185, SAP190	mcm^5U_{34}, $mcm^5s^2U_{34}$, ncm^5U_{34}, ncm^5Um_{34}	Many phenotypes
MOD5	i^6A_{37}	Loss of suppression
NFS1, ISU1, ISU2, CFD1, NBP35, CIA1, URM1, UBA4, NCS2, NCS6, TUM1	$mcm^5s^2U_{34}$	Not essential
PUS1	Ψ_{26}, Ψ_{27}, Ψ_{28}, Ψ_{34}, $\Psi_{(35)}$, Ψ_{36}, Ψ_{65}, Ψ_{67}	Not essential
PUS3	Ψ_{38}, Ψ_{39}	Slow growth
PUS4	Ψ_{55}	Not essential
PUS6	Ψ_{31}	Not essential
PUS7	Ψ_{13}, Ψ_{35}	Not essential
PUS8	Ψ_{32}	Essential
RIT1	$Ar(p)_{64}$, $tRNA_i^{Met}$	Not essential
SUA5	t^6A_{37}	Very sick
TAD1	I_{37}	Not essential
TAD2, TAD3	I_{34}	Essential
TAN1	ac^4C_{12}	Temperature-sensitive
THG1	G_{-1}	Essential
TRM1	$m^{2,2}G_{26}$	Not essential
TRM2	m^5U_{54}	Not essential
TRM3	Gm_{18}	Not essential
TRM4	m^5C_{34}, m^5C_{40}, m^5C_{48}, m^5C_{49}	Not essential
TRM5	m^1G_{37}, m^1I_{37}, yW_{37}	Very sick
TRM6, TRM61	m^1A_{58}	Essential
TRM7	Cm_{32}, Cm_{34}, Gm_{34}, ncm^5Um_{34}	Slow growth, paromomycin-sensitive
TRM8, TRM82	m^7G_{46}	Not essential
TRM9, TRM112	mcm^5U_{34}, $mcm^5s^2U_{34}$	Not essential, paromomycin-sensitive
TRM10	m^1G_9	Not essential
TRM11, TRM112	m^2G_{10}	Not essential
TRM13	Am_4, Gm_4, Cm_4	Not essential
TRM44	Um_{44}	Not essential
TYW1, TYW2, TYW3, TYW4	yW_{37}	Not essential, reading frame maintenance

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, $tLys^{\underline{UUU}}$, $tGln^{\underline{UUG}}$ and $tGlu^{\underline{UUC}}$, all contain modified U_{34} nucleosides. Specifically, these tRNAs contain the 5-methoxycarbonylmethyl-2-thiouridine ($mcm^5s^2U_{34}$) modified nucleoside (Figure 7).
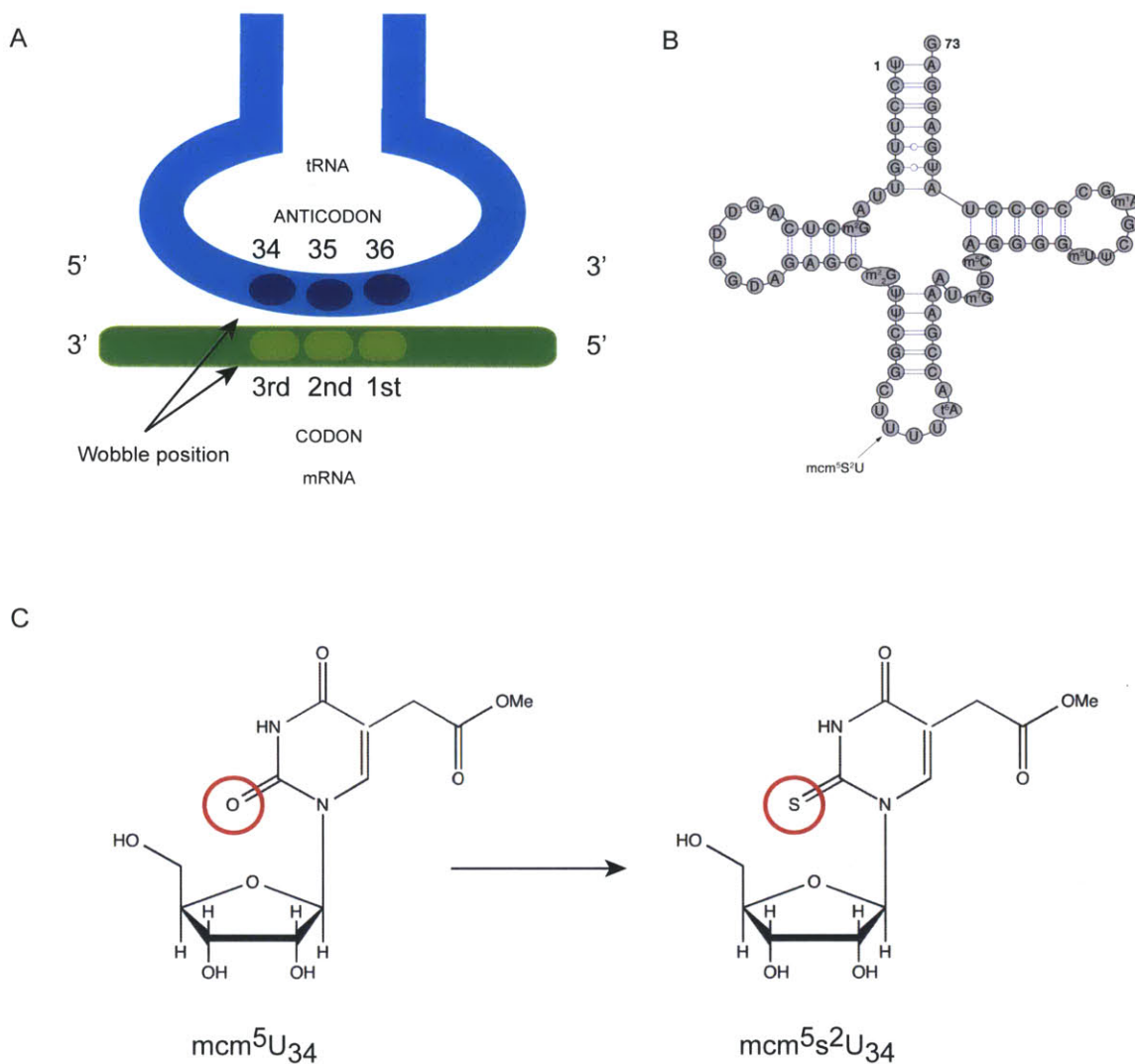
Figure 7: The $mcm^5s^2U_{34}$ modification. A. A schematic of the tRNA anticodon loop illustrating the location of the wobble position, where the $mcm^5s^2U_{34}$ modification is found. **B.** The sequence of the $tLys^{UUU}$ tRNA, one of the tRNAs that are thiolated in an Urm1-dependent fashion, with the location of the $mcm^5s^2U_{34}$ indicated. Image courtesy of T. Carlile. **C.** Structures of the mcm^5 U_{34} nucleoside and the $mcm^5s^2U_{34}$ nucleoside. Formation of the mcm^5-moiety is dependent on the *ELP* pathway and formation of the s^2- moiety is dependent on the *URM1* pathway.

In bacterial and eukaryotic systems, modifications of U_{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 mcm^5 modification appears to facilitate recognition of G residues, while the s^2 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 s^2 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 s^2 modification. The s^2 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 s^2 modification increases the affinity of tRNAs for GAA codons with little effect on GAG recognition, while the mnm^5 (5-methylaminomethylene, analogous to the eukaryotic mcm^5) 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 mcm^5 and s^2 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 mcm^5 modification and the s^2 modifications (*elp2Δ/ ncs6Δ* double mutants) are not viable, but can be rescued by overexpression of $tLys^{UUU}$. As with the bacterial system, the mcm^5 modification enhances recognition and decoding of G ending codons and the s^2 modification enhances recognition of A, and to a lesser extent G, ending codons. Although mcm^5s^2 modified tRNAs can under some conditions recognize G ending codons *in vivo*, this decoding is not efficient. The primary effect of the mcm^5s^2 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 mnm^5 and the mcm^5 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 U_{34} modifications found on $tLys^{UUU}$, $tGln^{UUG}$ and $tGlu^{UUC}$ tRNAs. Modifications are essential for the efficient recognition of codons in the context of the ribosome, and the mcm^5/mnm^5 modification enhances recognition of G and A ending codons (primarily G ending), while the s^2 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 (mnm^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 mcm^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*

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al., 2012b). 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 (m^5C) modification found on tLeu^{CAA} tRNAs. A study found that treatment with H_2O_2 led to an increase in m^5C modification levels of tLeu^{CAA} 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 H_2O_2 and is also impaired in *trm4Δ* cells. An analysis of protein levels demonstrated that the treatment of wild type cells with H_2O_2 resulted in increased levels of proteins whose transcripts were enriched for TTG codons (Chan *et al.*, 2012).

A study examining the m^5C modification found on tHis^{GUG} found that levels of the modification, found on positions C_{48} and C_{50}, 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 m^5C 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 $mcm^5s^2U_{34}$ 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 H_2O_2, 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^{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 *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 *et al.*, 2009). A recent study using a SILAC based proteomics approach found that in *urm1Δ* and *elp3Δ* 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 *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 *URM1* 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 *et al.*, 2013). In agreement with the study described previously (Rezgui *et al.*, 2013), Laxman et al. found that *uba4Δ* 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 *uba4Δ* and *ncs2Δ* 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 $mcm^5s^2U_{34}$ 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 Urm1 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 Urm1-dependent tRNA modifications in the regulation of translation and other cellular processes. This thesis presents work that describes the functions of Urm1-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*

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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 $URM1$ pathway is required for thiolation of the cytoplasmic tRNAs $tGlu^{UUC}$, $tGln^{UUG}$ and $tLys^{UUU}$ in $Saccharomyces$ $cerevisiae$. We demonstrate that $URM1$ pathway mutants have impaired translation, which results in increased basal activation of the Hsf1-mediated heat shock response; we also find that tRNA thiolation levels in wild type cells decrease when cells are grown at elevated temperature. We show that defects in tRNA thiolation can be conditionally advantageous, conferring resistance to endoplasmic reticulum stress. $URM1$ pathway proteins are unstable, and hence are more sensitive to changes in the translational capacity of cells, which is decreased in cells experiencing stresses. We propose a model in which a stress-induced decrease in translation results in decreased levels of $URM1$ pathway components, which results in decreased tRNA thiolation levels, which further serves to decrease translation. This mechanism ensures that tRNA thiolation and translation are tightly coupled and 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^6A) 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 $mcm^5s^2U_{34}$ (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), $tGln^{UUG}$ (CAA codon) and $tLys^{UUU}$ (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 noncannonical 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 *urm1*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 $mcm^5s^2U_{34}$ 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.

URM1 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 *urm1*Δ cells. We find that *urm1*Δ have defects in translation, and that tRNA modification defects result in a slow growth phenotype as well as an increased activation of the Hsf1-dependent stress response. We also find that *URM1* pathway mutants are more resistant to certain stresses 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 *URM1* 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 *urm1*Δ 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^5U modification (Huang *et al.*, 2008). Polysome profiles of elongator-deficient *elp2*Δ cells were similar to those of *urm1*Δ 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 *urm1*Δ 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 *URM1* 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 *urm1*Δ 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 *urm1*Δ extracts with regard to levels of 12x CAG luciferase produced, *urm1*Δ extracts translated 12x CAA luciferase less efficiently

compared to wild type extracts, suggesting that there are codon/tRNA specific translation defects in *urm1*Δ cells (Figure 1B). While defects in translation were subtle, they may significantly impact cellular fitness; mutant cells (*urm1*Δ and *ncs6*Δ 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 *URM1* pathway mutants. Cycloheximide blocks the elongation step of protein synthesis whereas aminoglycosides such as paromomycin interfere with translation by binding to the ribosome decoding site, which increases misreading (Fan-Minogue and Bedwell, 2007; Kramer *et al.*, 2010). Consistent with this notion, *URM1* pathway mutants (*urm1*Δ, *uba4*Δ, *ncs2*Δ, *ncs6*Δ) displayed impaired growth in the presence of translation inhibitors. Growth of the *ELP* pathway mutant *elp2*Δ was also reduced by translation inhibitors, suggesting that the phenotype is likely a function of the tRNA modification function of Urm1 and not attributable to defects in protein urmylation. In agreement with this interpretation, *ahp1*Δ cells – Ahp1 is the only known protein to which Urm1 is conjugated in yeast (Goehring *et al.*, 2003a) – grow like wild type in the presence of translation inhibitors (Figure 1D).

Overexpression of tRNAs can suppress phenotypes attributable to defects in tRNA modification (Esberg *et al.*, 2006; Bjork *et al.*, 2007). In order to establish that the observed defects in translation were due to impaired tRNA modification, we overexpressed tGlu^{UUC} (tE), tGln^{UUG} (tQ) or tLys^{UUU} (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 *urm1*Δ background rescued growth in the presence of cycloheximide and

paromomycin, as did expression of the double tE/K and tK/Q constructs. Indeed, overexpression of tLys^{UUU} (tK) alone was sufficient to rescue growth (Figure 1E).
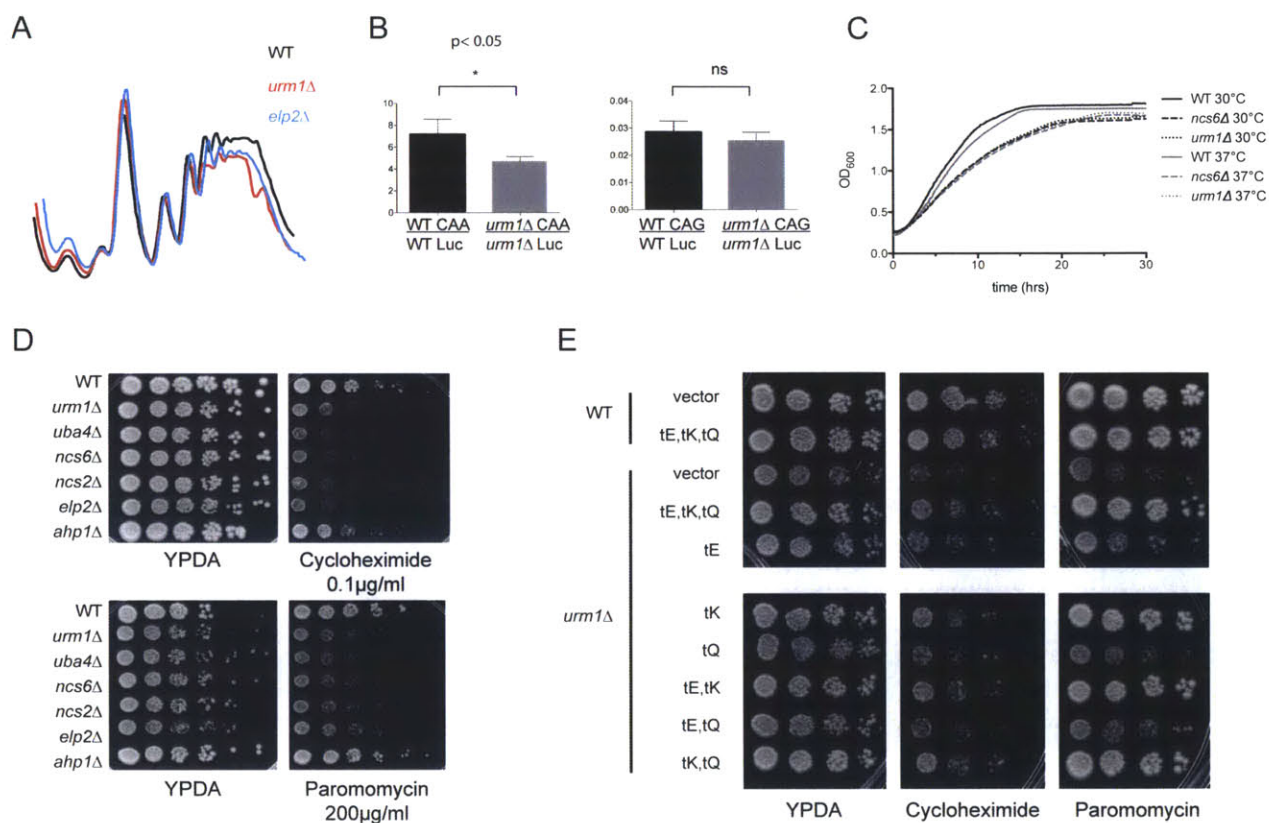

Figure 1: Translation is mildly affected in *URM1* pathway mutants. A. Polysome profiles of wild type, *urm1Δ* and *elp2Δ* cells. **B.** Luciferase mRNA, either leaderless or containing a 12xCAA or 12xCAG leader, was incubated with translation extracts prepared from the indicated strain. Luciferase activity resulting from translation of leader containing transcript was normalized to that of leaderless transcript. **C.** Growth curves of wild type, *ncs6Δ* and *urm1Δ* cells grown in 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.

A

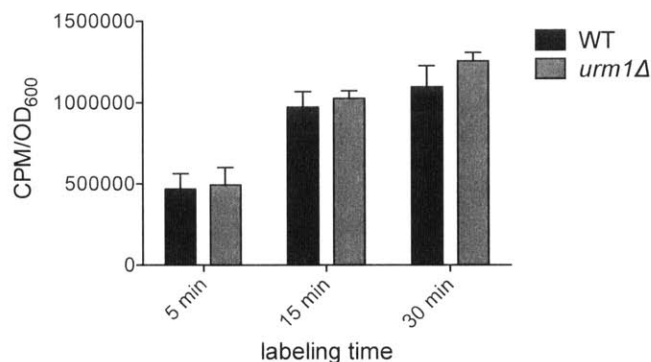**Figure 2: Bulk protein synthesis is not impaired in *urm1Δ* cells. A.** Wild type or *urm1Δ* cells were incubated with ^{35}[S] cysteine and methionine for the indicated times, and radioactive amino acid incorporation into proteins was determined following TCA precipitation.

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

Since strains with deletions in the *URM1* pathway showed increased sensitivity to inhibitors of translation, we asked whether these strains might also display altered cell stress responses. We reasoned that defects in protein synthesis could lead to the production of misfolded or error-containing proteins, which in turn could lead to the activation of pathways that evolved to cope with these stresses. We examined the transcript levels of genes whose transcription is known to be up-regulated in response to a variety of stresses: *UBI4*, the yeast polyubiquitin gene, *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).
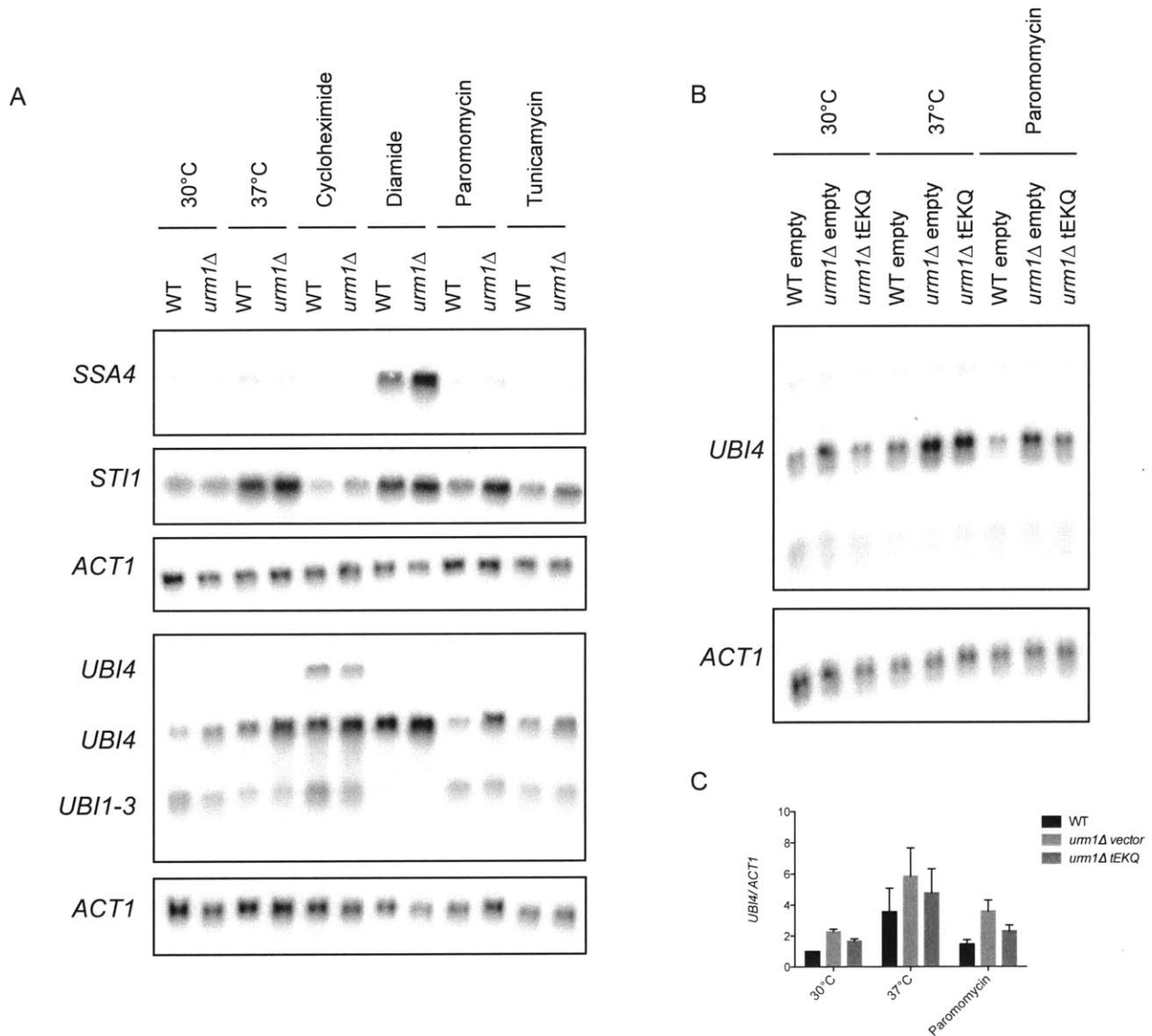

Figure 3: Levels of stress induced mRNA transcripts are up-regulated in *urm1Δ* cells. A. Northern blot of RNA extracted from wild type or *urm1Δ* cells grown at 30°C, shifted to 37°C or treated with the following compounds (final concentration) for 1 hour: paromomycin (0.2 mg/ml), tunicamycin (1 µg/ml), 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 *urm1*Δ 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 *urm1*Δ 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, *urm1*Δ or *ncs6*Δ yeast strains: P_{4xHSE}-YFP, P_{4xUPRE}-GFP and P_{4xSTRE}-GFP.

We measured fluorescence levels by flow cytometry in cells that were left untreated or grown in the presence of tunicamycin, which activates of the UPR, or azetidine-2-carboxylic acid (AZC), a proline 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 $urm1\Delta$ and $ncs6\Delta$ cells were able to respond to specific stressors comparably to wild type cells. For both the P_{4xUPRE}-GFP and P_{4xSTRE}-GFP reporters, we found that basal levels of activation were the same in wild type, $urm1\Delta$ and $ncs6\Delta$ cells. By contrast, we observed that $urm1\Delta$ and $ncs6\Delta$ cells harboring the P_{4xHSE}-YFP reporter had higher levels of fluorescent protein compared to wild type when cells were untreated, indicating that the Hsf1 mediated transcriptional response is basally up-regulated in $URM1$ pathway mutants (Figure 4A, 4D).

Since both $urm1\Delta$ and $ncs6\Delta$ strains showed an increase in P_{4xHSE}-YFP reporter levels, the phenotype is likely linked to the tRNA modification function of Urm1. To test this directly, we expressed either empty vector or the high copy plasmid expressing tE/K/Q in wild type, $urm1\Delta$ or $ncs6\Delta$ strains harboring the P_{4xHSE}-YFP construct. All strains were responsive to AZC (Figure 4E). While overexpressing tRNAs had no effect in the wild type basal level of Hsf1 activity, in the $urm1\Delta$ and $ncs6\Delta$ cells, overexpression of tRNAs was able to restore P_{4xHSE}-YFP expression to wild type levels in untreated cells (Figure 4F). Thus the processes that are dysregulated in $urm1\Delta$ cells lead specifically to activation of the Hsf1-mediated heat shock response and are tRNA-dependent, consistent with the defects in translation that $urm1\Delta$ cells exhibit.
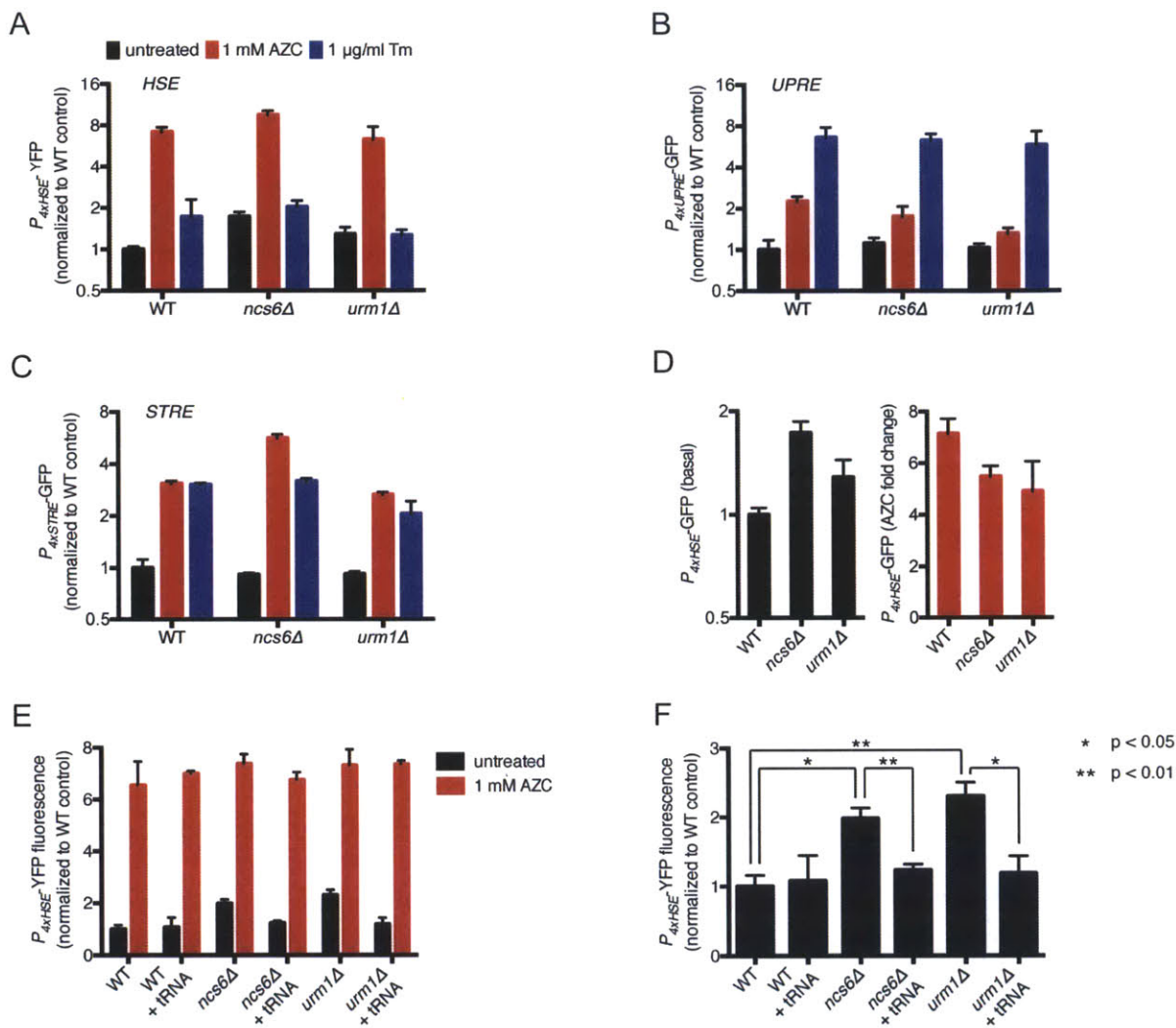
Figure 4: The heat shock response pathway is up-regulated in *URM1* pathway mutants.
Indicated strains harboring the **A.** P_{4xHSE}-YFP, **B.** P_{4xUPRE}-GFP or **C.** P_{4xSTRE}-GFP were left
untreated, exposed to 1 mM AZC or 1 µg/ml tunicamycin. **D.** Data from (A) replotted. **E.** Wild
type, *ncs6Δ* or *urm1Δ* strains harboring the P_{4xHSE}-YFP reporter and a 2 micron plasmid (either
empty vector or tE/K/Q plasmid, denoted as +tRNA) were untreated or exposed to 1 mM AZC.
F. Data from untreated samples in (E) replotted. Fluorescence levels were normalized to WT
untreated, and values are the averages of three cultures. Error bars denote standard deviations.

tGlu^{UUC}, tGln^{UUG} and tLys^{UUU} 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^{5}s^{2}U were found to be condition-specific; treatment with methyl methanesulfonate (MMS), hydrogen peroxide (H$_2$O$_2$) and sodium arsenite (NaAsO$_2$) decreased levels of mcm^{5}s^{2}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^{5}s^{2}U$_{34}$ modification, we examined different conditions for evidence of alterations in the Urm1-dependent thiolation status of tGlu^{UUC}, tGln^{UUG} and tLys^{UUU} 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$_2$O$_2$). 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^{UUC}, tGln^{UUG} and tLys^{UUU} 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^{UUC}, tGln^{UUG} and tLys^{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^{UUC}, tGln^{UUG} and tLys^{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.
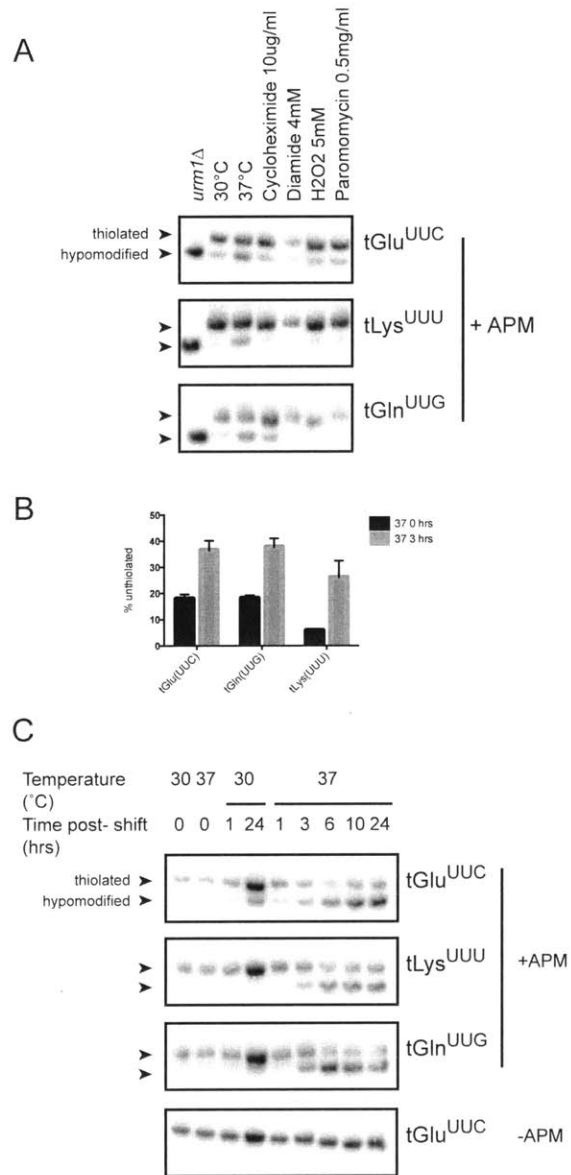
Figure 5: Unthiolated tRNAs accumulate at elevated temperatures. A. Small RNA APM Northern blot of RNA isolated from *urm1Δ* or wild type cells grown under indicated conditions for 3 hours, membranes were probed for the indicated tRNA. The positions of thiolated and

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.
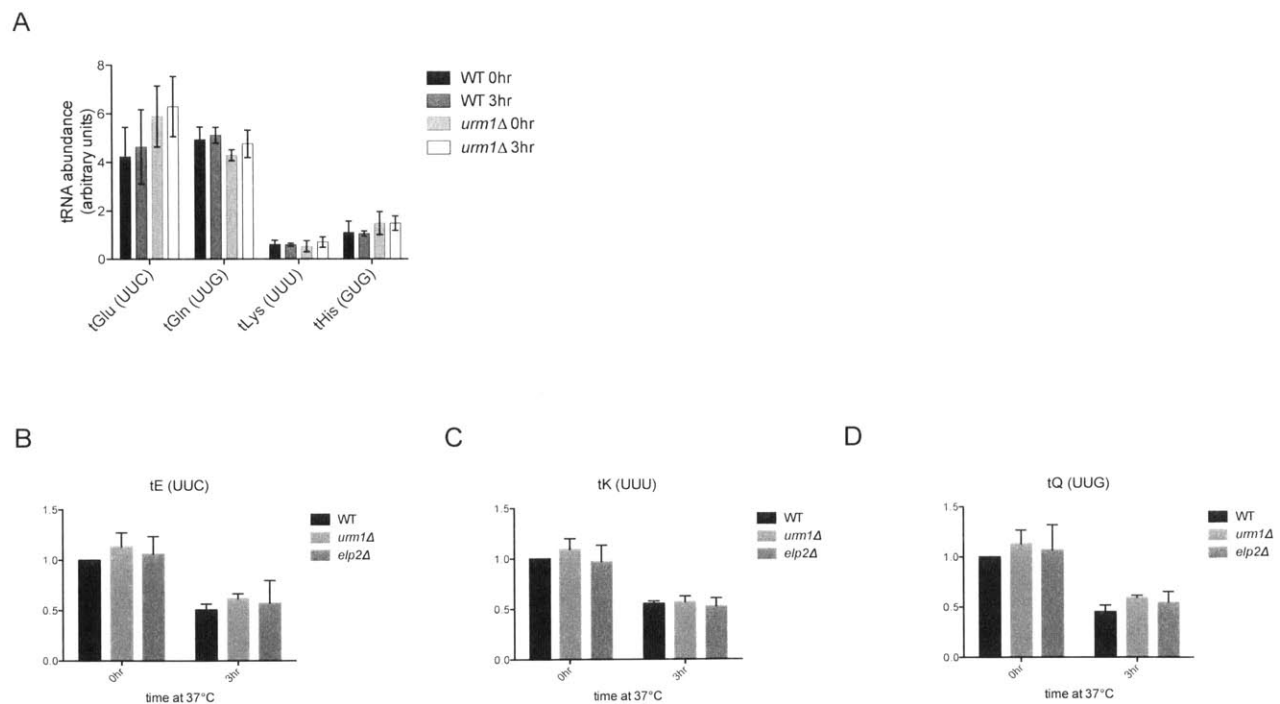
Figure 6: Stability of hypomodified tRNA species. A. Quantification of steady-state levels of tRNA species in wild type or *urm1Δ* cells. RNA was extracted from cells grown at 37°C for 0 or 3 hours, analyzed by small RNA Northern blot analysis and levels were quantified. tRNA levels

were normalized to levels of 5S rRNA. The average and standard deviation of three replicates is reported. Levels of **B.** tGlu^{UUC} (tE), **C.** tLys^{UUU} (tK) or **D.** tGln^{UUG} (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.
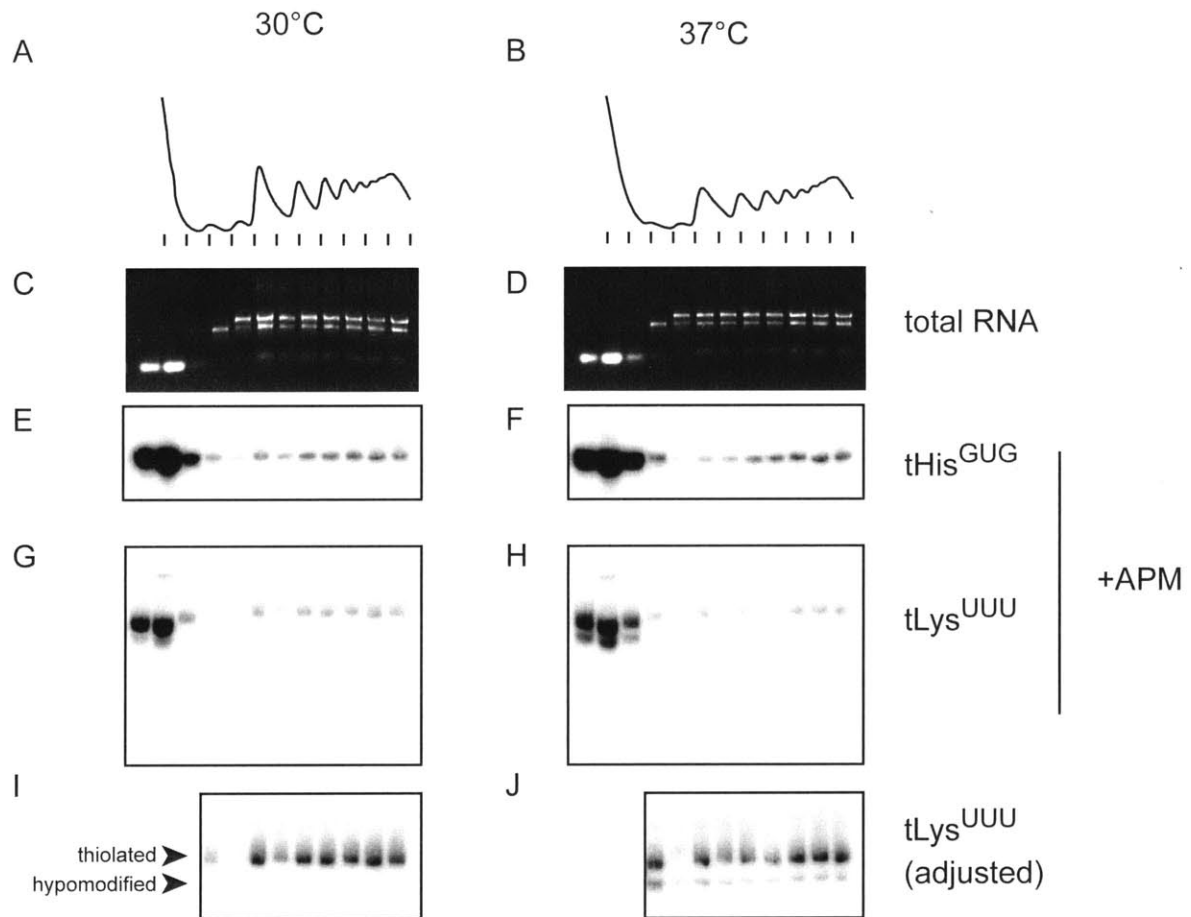

Figure 7: Hypomodified tRNAs associate with actively translating ribosomes. Polysome profiles of wild type cells grown at **A.** 30°C or **B.** 37°C for 3 hours. RNA was extracted from indicated fractions and run on an agarose gel containing ethidium bromide **C.** 30°C, **D.** 37°C, and subsequently 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 $mcm^5s^2U_{34}$ -containing tRNA. To this end, we pre-incubated wild type cells with the pol III inhibitor ML-60218 or DMSO at 30°C for 2 hours prior to shifting cells to 37°C for up to 3 hours. APM-Northern blot analysis showed that levels of hypomodified $tGlu^{UUC}$, $tGln^{UUG}$ and $tLys^{UUU}$ 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 *URM1* pathway mutants.

The tRNA thiolation components are unstable proteins

The observation that accumulation of hypomodified tRNAs is dependent on the production of new transcripts by pol III suggested that regulation of tRNA modification pathways could be responsible for the observed differences in tRNA thiolation levels. Modulation of the activity and/or abundance of *ELP* (mcm^5-U_{34}) or *URM1* (s^2-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*, *TUM1* and *UBA4* (s^2-U$_{34}$) as well as *ELP2* and *ELP4*, which are required for the formation of the mcm^5-U$_{34}$ 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.
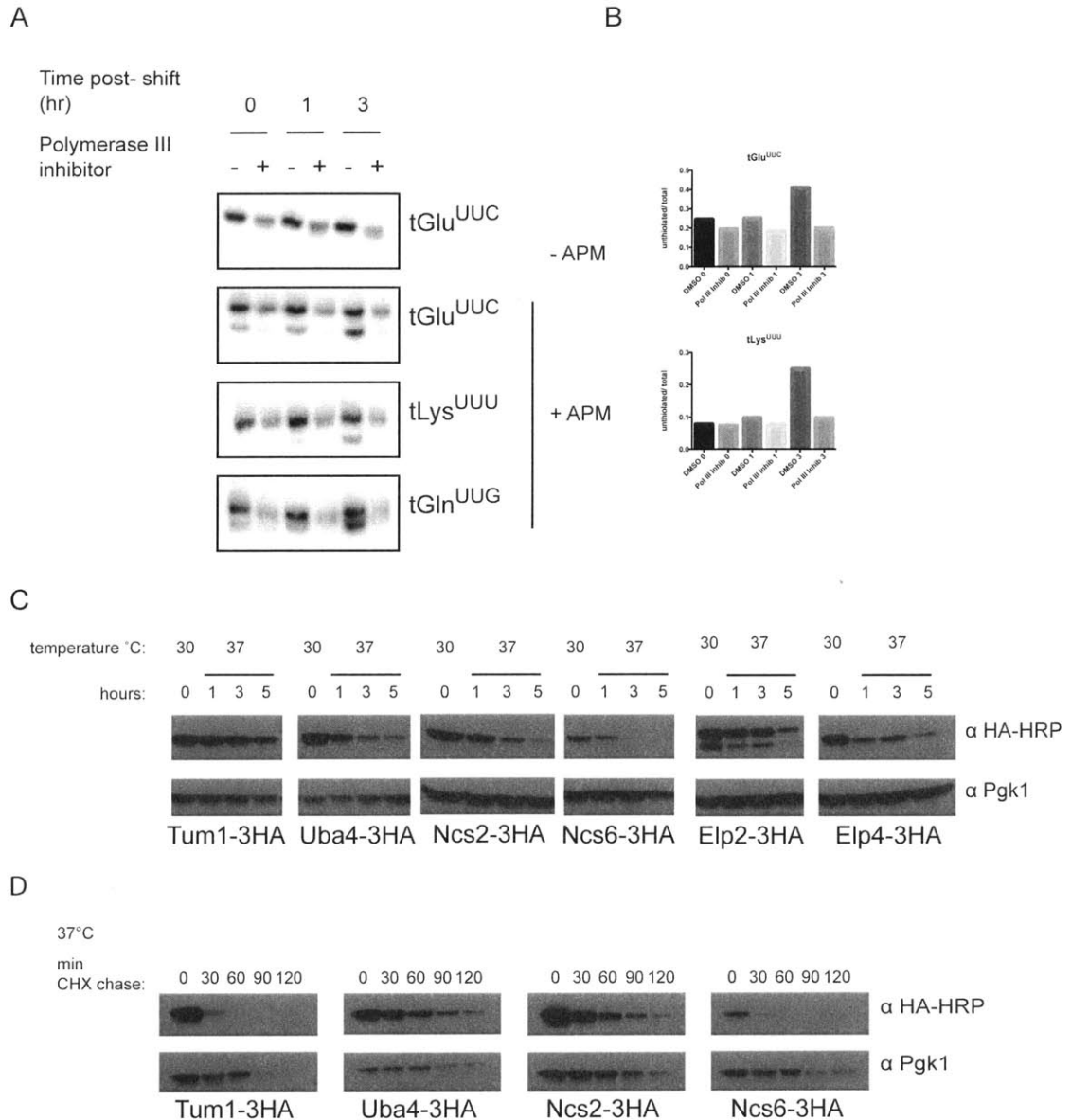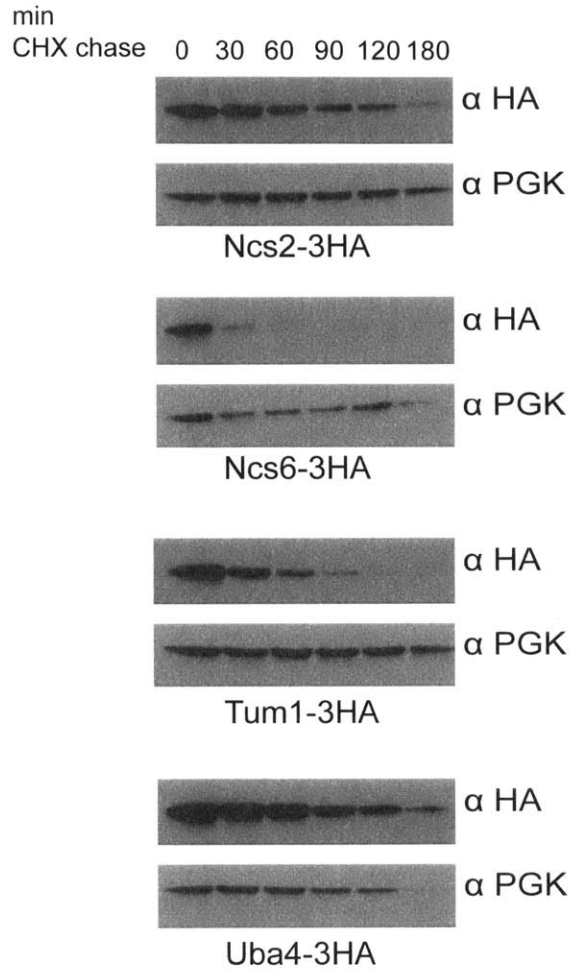
Figure 8: Accumulation of hypomodified tRNAs requires synthesis of new tRNA transcripts and is dependent on levels of tRNA modification enzymes. A. Small RNA (+/- APM as indicated) Northern blot analysis of RNA extracted from wild type cells growing at 37°C for the indicated timepoints. Cells were pre-treated with pol III inhibitor prior to the temperature shift. **B.** Quantification of levels of unthiolated/total tGlu^{UUC} or tLys^{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 200ug/ml of cycloheximide for indicated periods of time, and protein abundance was determined by Western blot analysis.
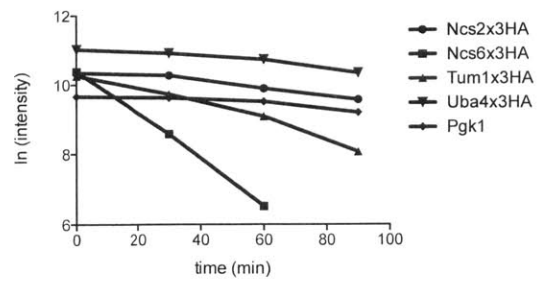
A

30°C

min
CHX chase   0   30   60   90 120 180

α HA

α PGK

Ncs2-3HA

α HA

α PGK

Ncs6-3HA

α HA

α PGK

Tum1-3HA

α HA

α PGK

Uba4-3HA

B

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 Pgk1. **B.** Quantification of protein levels from (A), values were determined using ImageJ software.

URM1 pathway mutants display a resistance to tunicamycin

What would be the benefit of reducing levels of thiolated tRNAs? Under challenging growth conditions, it may be beneficial for cells to modulate rates of growth and protein synthesis/translation efficiency. While characterizing the stress responses of *URM1* pathway mutants, we exposed cells to tunicamycin. Tunicamycin induces ER stress by inhibition of Alg7, the yeast Dol-PP-GlcNAc transferase, which results in inhibition of N-linked glycosylation and accumulation in the ER of the C_{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.
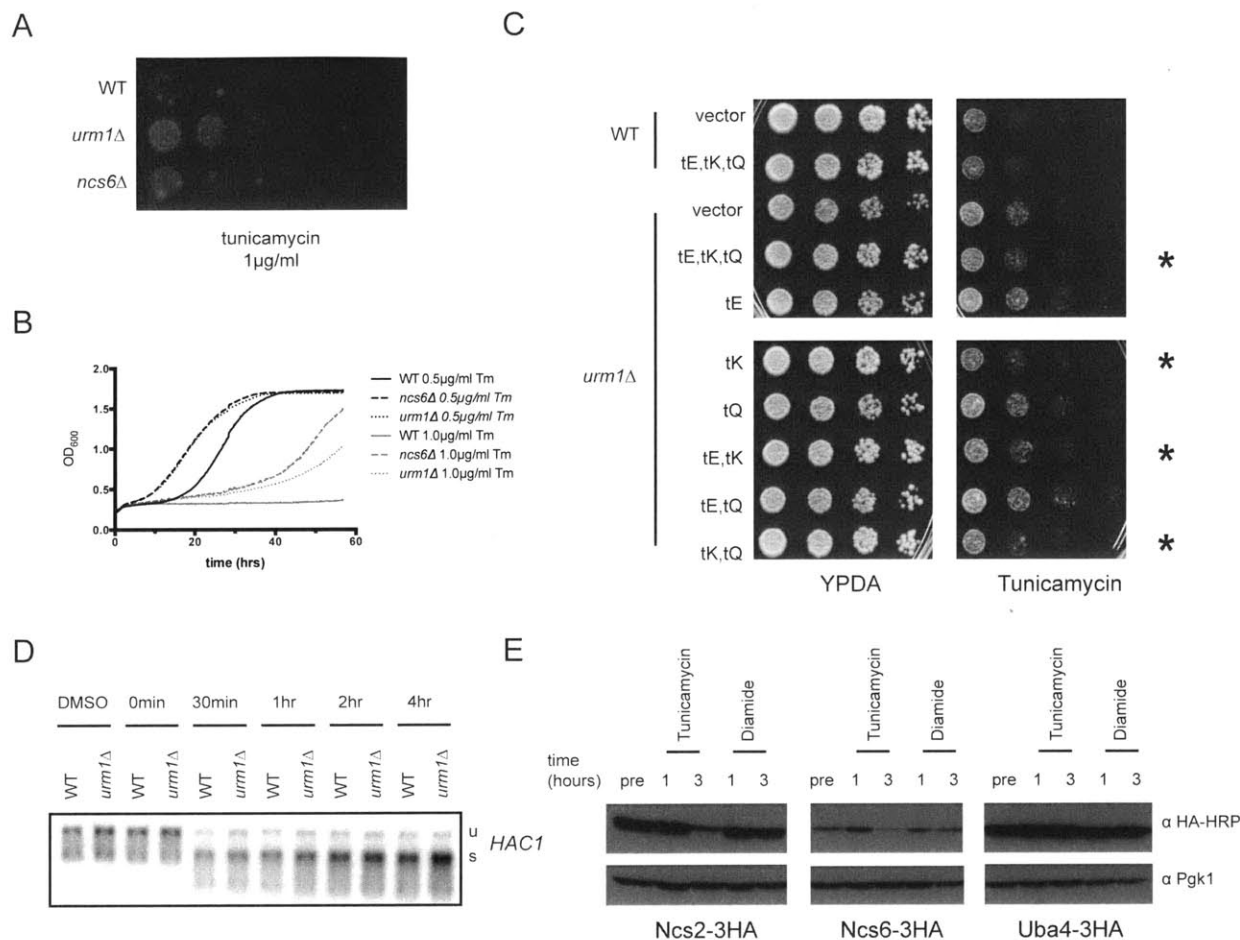
Figure 10: *URM1* **pathway mutants are resistant to tunicamycin in a tRNA dependent and** *HAC1* **independent manner. A.** Five-fold serial dilutions of wild type, *urm1Δ* or *ncs6Δ* cells were plated onto YPDA plates containing 1ug/ml tunicamycin. **B.** Growth curves of wild type, *urm1Δ* and *ncs6Δ* 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 *urm1Δ* 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 *urm1Δ* 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 $mcm^5s^2U_{34}$ modification enhances the reading of A-ending codons (Johansson *et al.*, 2008; Rezgui *et al.*, 2013; Rodriguez-Hernandez *et al.*, 2013). It seems likely that the primary role of the $mcm^5s^2U_{34}$ modification is 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 *urm1*Δ 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 *URM1* 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 *urm1Δ* 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 *URM1* pathway components decrease as levels of unthiolated

tRNAs increase. We have demonstrated that components of the *URM1* 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
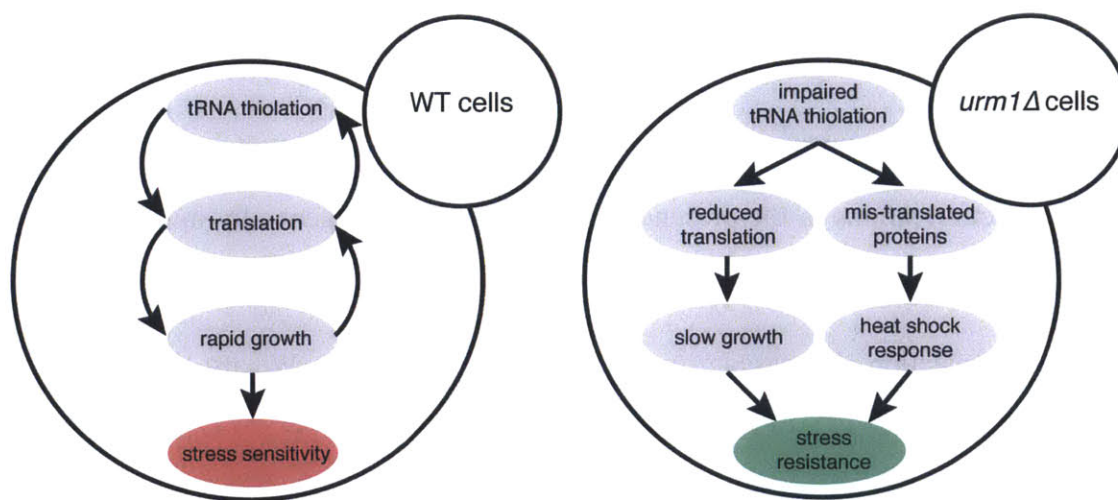
(Figure 11).

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.

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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 *URM1* 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. *URM1* pathway mutants have defects in translation and grow more slowly than wild type cells. Additionally, the Hsf1-dependent heat shock response is activated in *URM1* pathway mutants. Under certain stress conditions, *URM1* mutants have an advantage; *URM1* pathway mutants are more resistant to tunicamycin compared to wild type cells in a tRNA dependent fashion. The tunicamycin-resistant phenotype of *URM1* 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 *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 $mcm^5s^2U_{34}$ 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)

Strain	Genotype
2587	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+]
2588	MATalpha, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+]
yJD180	MATalpha, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], urm1::His3MX6
yJD181	MATalpha, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], uba4::His3MX6
yJD182	MATalpha, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], ncs6Δ::His3MX6
yJD187	MATalpha, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], ncs2::His3MX6
yJD188	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], elp2::KanMX6
yJD189	MATalpha, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], elp2::KanMX6
yJD226	MATalpha, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], ahp1::His3MX6
yJD234	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], ncs6::His3MX6
yJD240	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], urm1::His3MX6
yJD284	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], pRS426
yJD285	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], pRS426/tEKQ
yJD290	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], urm1Δ::His3MX6, pRS426
yJD291	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], urm1Δ::His3MX6, pRS426/tEKQ
yJD305	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], urm1Δ::His3MX6, pRS426/tE
yJD306	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], urm1Δ::His3MX6, pRS426/tK
yJD307	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], urm1Δ::His3MX6, pRS426/tQ
yJD308	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], urm1Δ::His3MX6, pRS426/tEK
yJD309	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], urm1Δ::His3MX6, pRS426/tEQ
yJD310	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], urm1Δ::His3MX6, pRS426/tKQ
yJD330	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], NCS2-3HA::kanMX6
yJD331	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], NCS6-3HA::kanMX6
yJD332	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], TUM1-3HA::kanMX6
yJD334	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-

	100, GAL, [psi+], UBA4-3HA::kanMX6
yJD337	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], ELP2-3HA::kanMX6*
yJD338	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], ELP4-3HA::kanMX6*
yJD340	*MATa, ade2-1, leu2-3::P$_{4xHSE}$-YFP::LEU2, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+]*
yJD341	*MATa, ade2-1, leu2-3::P$_{4xSTRE}$-GFP::LEU2, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+]*
yJD342	*MATa, ade2-1, leu2-3::P$_{4xUPRE}$-GFP::LEU2, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+]*
yJD343	*MATa, ade2-1, leu2-3::P$_{4xHSE}$-YFP::LEU2, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], ncs6::His3MX6*
yJD344	*MATa, ade2-1, leu2-3::P$_{4xSTRE}$-GFP::LEU2, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], ncs6::His3MX6*
yJD345	*MATa, ade2-1, leu2-3::P$_{4xUPRE}$-GFP::LEU2, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], ncs6::His3MX6*
yJD346	*MATa, ade2-1, leu2-3::P$_{4xHSE}$-YFP::LEU2, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], urm1::His3MX6*
yJD347	*MATa, ade2-1, leu2-3::P$_{4xSTRE}$-GFP::LEU2, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], urm1::His3MX6*
yJD348	*MATa, ade2-1, leu2-3::P$_{4xUPRE}$-GFP::LEU2, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], urm1::His3MX6*
yJD370	*MATa, ade2-1, leu2-3::P$_{4xHSE}$-YFP::LEU2, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], pRS426*
yJD371	*MATa, ade2-1, leu2-3::P$_{4xHSE}$-YFP::LEU2, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], pRS426/tEKQ*
yJD372	*MATa, ade2-1, leu2-3::P$_{4xHSE}$-YFP::LEU2, ura3, trp1-1, his3-11,15, can1-100,GAL,[psi+], ncs6::His3MX6, pRS426*
yJD373	*MATa, ade2-1, leu2-3::P$_{4xHSE}$-YFP::LEU2, ura3, trp1-1, his3-11,15, can1-100,GAL,[psi+], ncs6::His3MX6, pRS426/tEKQ*
yJD374	*MATa, ade2-1, leu2-3::P$_{4xHSE}$-YFP::LEU2, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], urm1::His3MX6, pRS426*
yJD375	*MATa, ade2-1, leu2-3::P$_{4xHSE}$-YFP::LEU2, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], urm1::His3MX6, pRS426/tEKQ*
yWG3	*MATa, his3, trp1, ura3, L-A-0, M-0*
yJD183	*MATa, his3, trp1, ura3, L-A-0, M-0, urm1::KanMX6*

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 resuspened 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 γ-^{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 α-^{32}P dTTP (Perkin Elmer); probes were PCR products generated using yeast genomic DNA as a template and using primers specific for the transcript of interest. 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

Probe	Sequence (5' to 3')
tHis-cyto	TGCCATCTCCTAGAATCGAACCAGGGTTTCATCGGCCACAACGATGTGTACTAACCACTATACTAAGATGGC
tH(GUG) E1	CATCGGCCACAACGATGTGTACTAACCACTATACT
tLys-probe	CTCCTCATAGGGGGCTCGAACCCCTGACATTTCGGTTAAAAGCCGAACGCTCTACCAACTGAGCTAACAAGGA
tGln-probe	AGGTCCTACCCGGATTCGAACCGGGGTTGTCCGGATCAAAACCGAAAGTGATAACCACTACACTATAGGACC
tQ(UUG) B-probe	AGGTCTTACCCGGATTCGAACCGGGGTTGTCCGGATCAAAACCGAAAGTGATAACCACTACACTATAAAACC
tGlu-probe	CTCCGATACGGGGAGTCGAACCCCGGTCTCCACGGTGAAAGCGTGATGTGATAGCCGTTACACTATATCGGA

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. 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 (Iizuka *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.

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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 $mcm^5s^2U_{34}$ 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 *URM1* 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 *URM1* 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 $tGlu^{UUC}$, $tGln^{UUG}$ and $tLys^{UUU}$ 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, $tGlu^{UUC}$, $tGln^{UUG}$ and $tLys^{UUU}$ tRNAs may also have decreased levels of the mcm^5 modification when cells are grown at elevated temperatures.

Examining levels of mcm^5U_{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 $tGlu^{UUC}$, $tGln^{UUG}$ and $tLys^{UUU}$ tRNAs, the mcm^5 modification is found on these tRNAs and on the U_{34} nucleosides of

other tRNAs as well (Kalhor and Clarke, 2003; Huang, 2005; Bjork *et al.*, 2007). Thus, examining the modification status of all tRNAs containing the mcm^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 *URM1* 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 *urm1*Δ cells translate mRNAs containing codons that are normally recognized by thiolated tRNAs less efficiently. Our data also demonstrate that *URM1* 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 *urm1*Δ and *ncs6*Δ cells as well as altered levels of stress induced transcripts.

In addition to the specific examples of altered translation in *URM1* 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 *URM1* 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 *URM1* 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 *URM1* tRNA modification pathway

While many components of the *URM1* 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 $tGlu^{UUC}$, $tGln^{UUG}$ and $tLys^{UUU}$ tRNAs. It is unclear how these proteins specifically recognize and direct modification of a subset of tRNAs. While it is possible that the mcm^5 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 *urm1Δ* 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

Ahp1 (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 has 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 *URM1* 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 *URM1* pathway mutants results 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 *URM1* 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.

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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 s^2 thio-modification found on the U_{34} nucleosides of tGlu^{UUC}, tGln^{UUG} and tLys^{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^{UUC}, tGln^{UUG} and tLys^{UUU} and tArg^{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

URM1 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 Urm1 and is required for all the described functions of Urm1, *uba4Δ* mutant cells could have phenotypes attributable to either the tRNA modification or the protein urmylation function of Urm1. 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 Urm1.

A

B
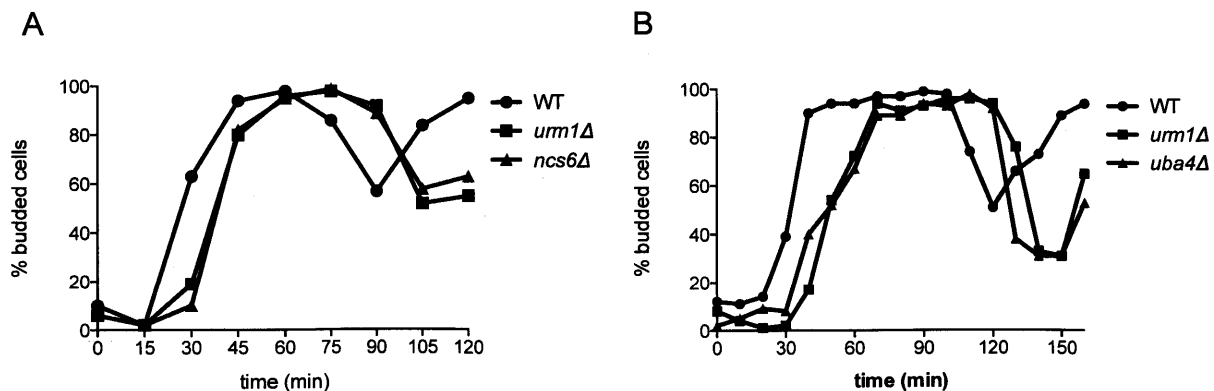
Figure 1: *URM1* **pathway mutants exhibit a delay in budding indicative of G1/S progression defects.** Wild type, *urm1Δ*, *ncs6Δ* or *ncs2Δ* cell cultures were synchronized using the alpha-factor block release method. Samples were taken at indicated time points and the extent of budding was assessed by examining the morphology of fixed cells. **A.** Comparison of wild type, *urm1Δ* and *ncs6Δ* cells. **B.** Comparison of wild type, *urm1Δ* and *uba4Δ* 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 → 2N → 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 *URM1* pathway mutant cells demonstrate a G1/S transition defect, they are unimpaired in other aspects of cell cycle progression.
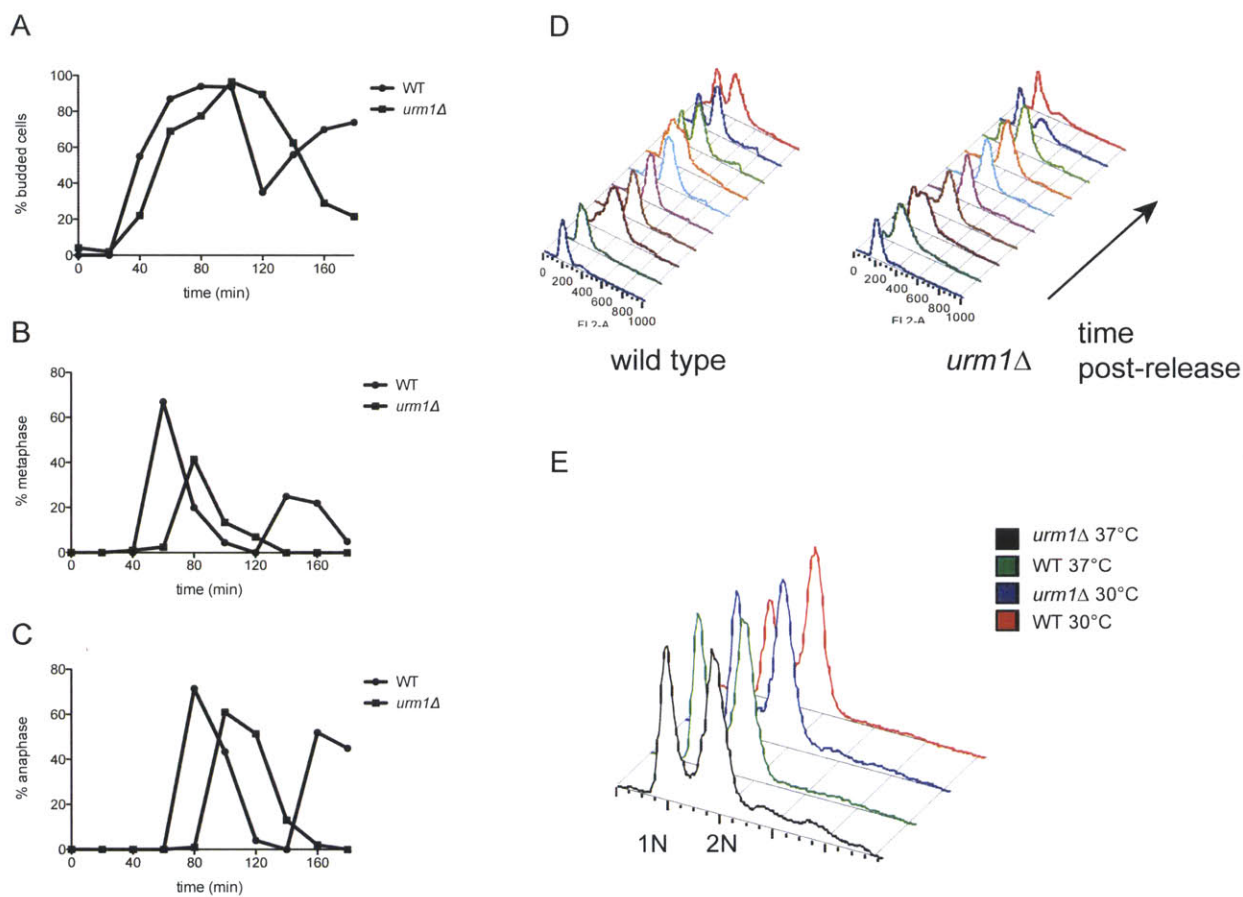
Figure 2: Cell cycle progression in *urm1Δ* cells. *urm1Δ* cells exhibit a budding delay but are not impaired in other aspects of cell cycle progression. Cells of the indicated genotype were synchronized using the alpha-factor block release method. Samples were taken at indicated time points and assessed 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 *urm1*Δ 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 *urm1*Δ, *uba4*Δ and *ncs6*Δ cells all demonstrated the same cell cycle progression defect, we wondered if overexpression of tRNAs normally modified in an Urm1-dependent fashion, tGlu^{UUC} (tE), tLys^{UUU} (tK) and tGln^{UUG} (tQ), would be able to rescue the observed G1/S delay.

We transformed *urm1*Δ 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 *urm1*Δ 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 *urm1*Δ 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 *urm1*Δ 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 *urm1*Δ 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 in these strains.
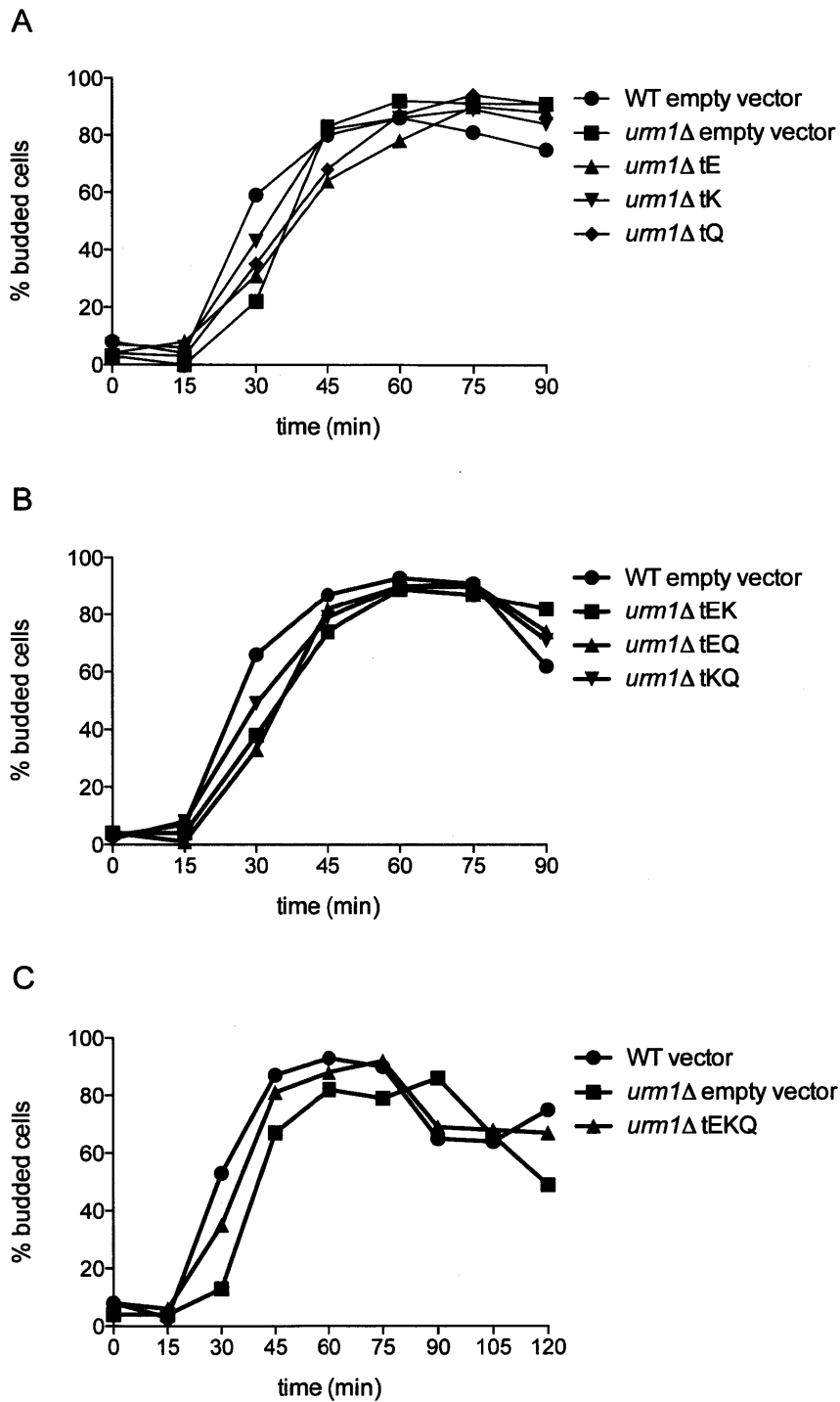
Figure 3: Budding delay in *urm1*Δ cells can be rescued by overexpressing tRNAs. Wild type or *urm1*Δ cells harboring either empty vector or a construct for expression of the indicated tRNA(s) were synchronized using the alpha-factor block release method. Samples were taken at

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 *URM1* 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 *URM1* 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 *URM1* 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^5-U_{34} modification (Huang, 2005), and an *ahp1Δ* 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^5-modification, is required for tRNA thiolation, while *AHP1* 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 *ahp1Δ* cells were not significantly larger than wild type cells (Figure 4B). This further demonstrates that the cell size phenotype of *URM1* pathway mutants is likely attributable to defects in tRNA modification. Deletion of *DUS2*, which is required for dihydrouridine synthesis on the U_{20} position of cytoplasmic tRNAs

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(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).
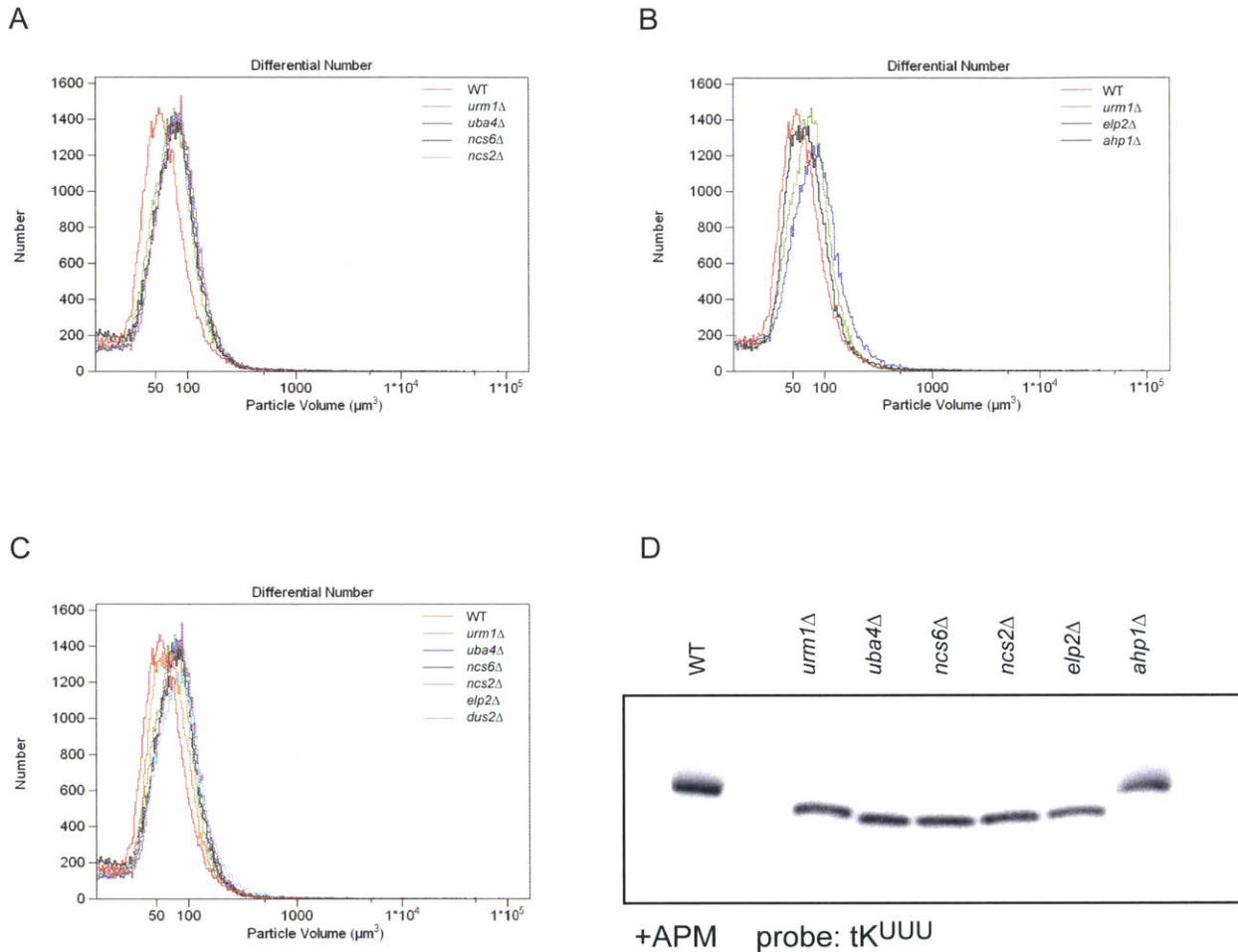
Figure 4: The cell volume of *URM1* pathway mutants is increased compared to wild type cells. Unsynchronized cells of the indicated genotype were sized using a Coulter counter. **A.** A comparison of cell volumes of wild type, *urm1*Δ, *uba4*Δ, *ncs6*Δ and *ncs2*Δ cells. **B.** A comparison of cell volumes of wild type, *urm1*Δ, *elp2*Δ and *ahp1*Δ cells. **C.** A comparison of cell volumes of wild type, *urm1*Δ, *uba4*Δ, *ncs6*Δ, *ncs2*Δ, *elp2*Δ and *dus2*Δ cells. **D.** RNA isolated from wild type, *urm1*Δ, *uba4*Δ, *ncs6*Δ, *ncs2*Δ, *elp2*Δ or *ahp1*Δ 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.

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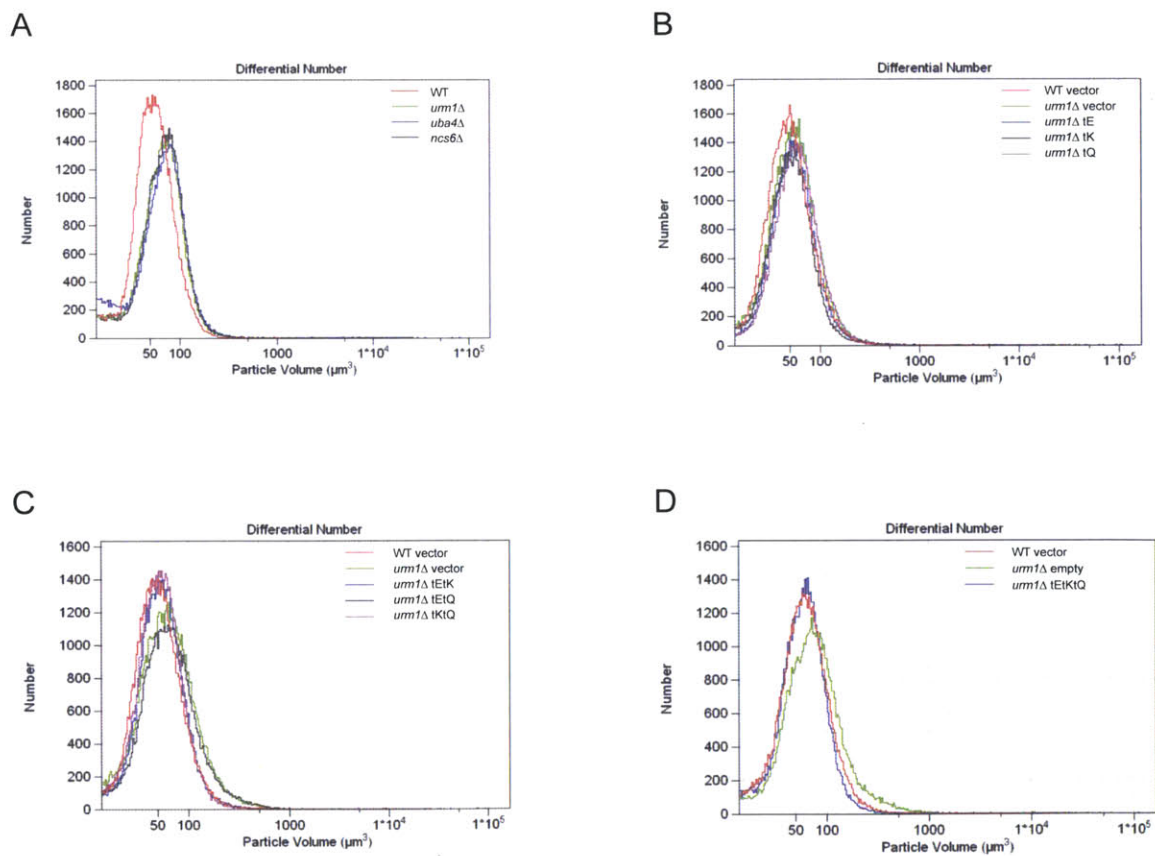
Figure 5: The increased cell volume phenotype of *URM1* pathway mutants can be suppressed by expression of tRNAs. Unsynchronized wild type or *urm1*Δ strains harboring either empty vector or a construct for expression of the indicated tRNA(s) were sized using a Coulter counter. **A.** A comparison of cell volumes of wild type and *URM1* pathway mutants. **B.** A comparison of cell volumes of wild type and *urm1*Δ cells expressing single tRNAs tE, tK or tQ. **C.** A comparison of cell volumes of wild type and *urm1*Δ cells expressing two: tRNAs tEK, tEQ or tKQ. **D.** A comparison of cell volumes of wild type and *urm1*Δ 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 *urm1*Δ cells incapable of thiolating tRNAs), might lead to the G1/S progression defect that we observed in *URM1* 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

B

metaphase cells
anaphase cells
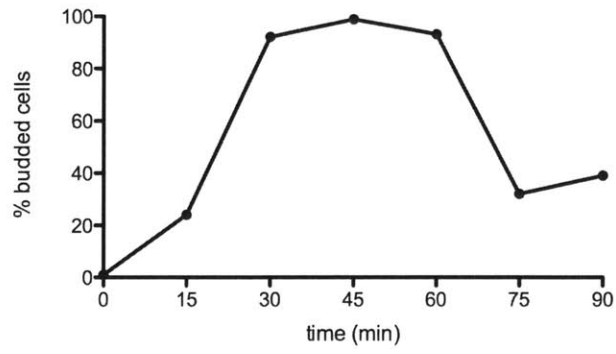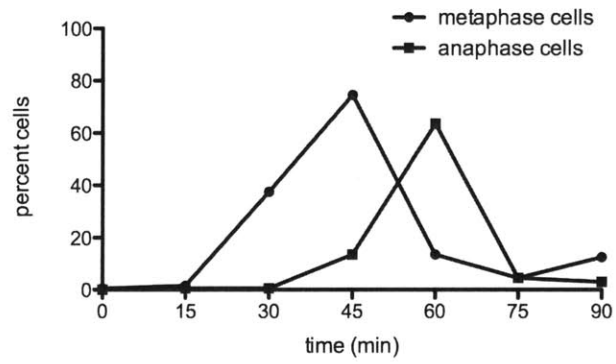
C
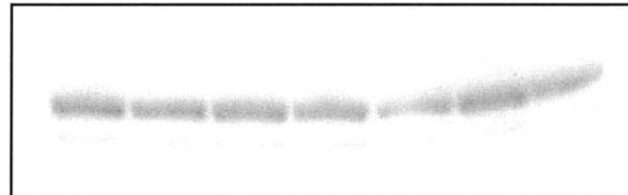
tE +APM
(tQ blot re-probed)

D
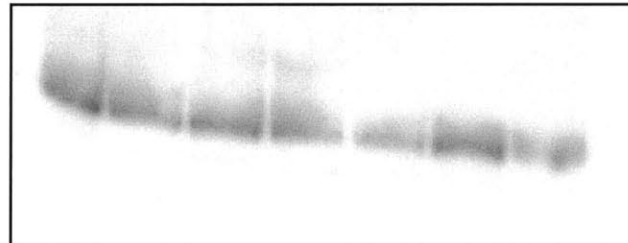
tK +APM

E
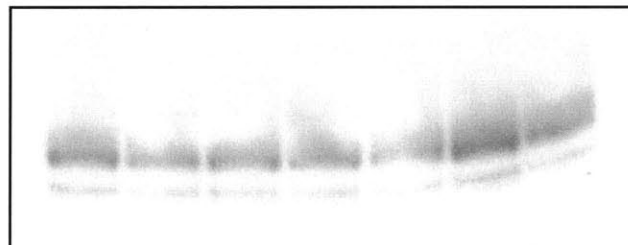
tQ +APM

Time (min)   0   15   30   45   60   75   90

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 *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 *urm1Δ* genetic background. We, and others (Laxman *et al.*, 2013; Rezgui *et al.*, 2013), have demonstrated that the *URM1* pathway and Urm1-dependent tRNA modifications are linked to translation in *S. cerevisiae*. This link, our results demonstrating that *ncs2Δ*, *ncs6Δ* and *elp2Δ* cells phenocopy *urm1Δ* cells, and the observation that *ahp1Δ* cells do not share at least a subset of *urm1Δ* 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 *urm1Δ* cells, this link is not yet definitively established. Our cell cycle experiments were carried out using α-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 *et al.*, 2011). This means that we cannot state that the cell cycle entry delay observed in *urm1Δ* 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 *URM1* pathway mutants. Specifically, determining the critical cell size of *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 $mcm^5s^2U_{34}$ 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 *urm1Δ* 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)

Strain	Genotype
8015	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, CAN1, LYS2, GAL, [psi+]
yJD72	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, CAN1, LYS2, GAL, [psi+], urm1::KanMX6
yJD80	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, CAN1, LYS2, GAL, [psi+], uba4::His3MX6
2587	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+]
yJD234	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], ncs6::His3MX6
yJD240	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], urm1::His3MX6
yJD284	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], pRS426
yJD290	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], urm1Δ::His3MX6, pRS426
yJD291	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], urm1Δ::His3MX6, pRS426/tEKQ
yJD305	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], urm1Δ::His3MX6, pRS426/tE
yJD306	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], urm1Δ::His3MX6, pRS426/tK
yJD307	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], urm1Δ::His3MX6, pRS426/tQ
yJD308	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], urm1Δ::His3MX6, pRS426/tEK
yJD309	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], urm1Δ::His3MX6, pRS426/tEQ
yJD310	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, [psi+], urm1Δ::His3MX6, pRS426/tKQ

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_{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_{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 γ-^{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

Probe	Sequence (5' to 3')
tLys-probe	CTCCTCATAGGGGGCTCGAACCCCTGACATTTCGGTTAAAAGCCGAACGCTCTACCAA CTGAGCTAACAAGGA
tGln-probe	AGGTCCTACCCGGATTCGAACCGGGGTTGTCCGGATCAAAACCGAAAGTGATAACCAC TACACTATAGGACC
tGlu-probe	CTCCGATACGGGGAGTCGAACCCCGGTCTCCACGGTGAAAGCGTGATGTGATAGCCGT TACACTATATCGGA

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Appendix B

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

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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.

cytokinesis | thiolation | urmylation | wobble nucleoside | MOCS3

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 ($E2$s and $E3$s). The latter confer substrate specificity to the process by conjugating the activated Ub/Ubl to its selected target (2, 3). Ub, NEDD8, 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 MoaD are the best-characterized bacterial representatives of this type. Both molecules show little if any sequence identity to Ubls but are structurally similar to Ub (Fig. S1). ThiS and MoaD 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 MoaD 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 MoaD 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) exploited the functional similarities between the prokaryotic and eukaryotic systems by using the Escherichia coli ThiS and MoaD 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, $urm1\Delta$ 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 $urm1\Delta$ 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 anticodon 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.
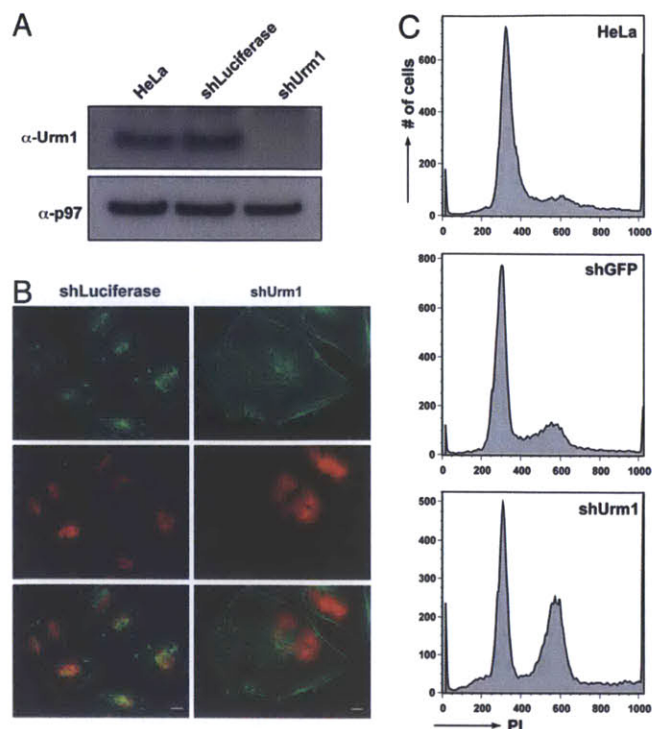
BIOCHEMISTRY

Fig. 1. Depletion of Urm1 results in a cytokinesis defect. (*A*) Efficiency of Urm1 knockdown in HeLa cells 4 days after lentiviral transduction of luciferase (control) or Urm1 shRNA constructs. (*B*) Confocal microscopy of Urm1-depleted HeLa cells. Red, Hoechst; green, actin. (Scale bars, 10 μm.) (*C*) Progress through cell cycle was assessed by quantification of DNA content in wild-type, shGFP, and shUrm1 HeLa cells. The DNA content of cells was quantified by flow cytometry 12 h after release from a double-thymidine block and plotted in a histogram.

Results

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 ($\approx 95\%$) and highly reproducible reduction of Urm1 protein levels 4 days after infection (Fig. 1*A*). The unrelated control shRNA (shLuciferase or shGFP) did not affect Urm1 protein levels (Fig. 1*A* 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 morphological anomalies: we observed an increase in cell size and the presence of multiple nuclei within single cells (Fig. 1*B*). In contrast, shLuciferase or mock-infected cells were phenotypically normal (Fig. 1*B* 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. 1*C*). Twelve hours after release from this block, 53.3% of shUrm1 cells contained $\geq 4n$, 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 ^{35}S-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. 2*A*). 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. 2*B*). 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. 2*C*). These were excised and processed for identification by mass spectrometry (MS). Notably, Urm1 was readily detectable as interaction partner in this experiment (Fig. 2*C*, 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 UPF0432 protein/C16orf84, 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 UPF0432, we used a structure prediction-based algorithm (16) to search for structural homologues. MesJ [Protein Data Bank (PDB) ID code 1ni5], an ATP α-hydrolase of unknown function, and TilS (PDB ID code 1wy5), a tRNA-modifying enzyme and likewise an ATP α-hydrolase, were predicted as closest structural neighbors for both ATPBD3 and UPF0432 (data not shown). A Pfam search (17) consistently assigned both proteins to the PP-loop ATP pyro-
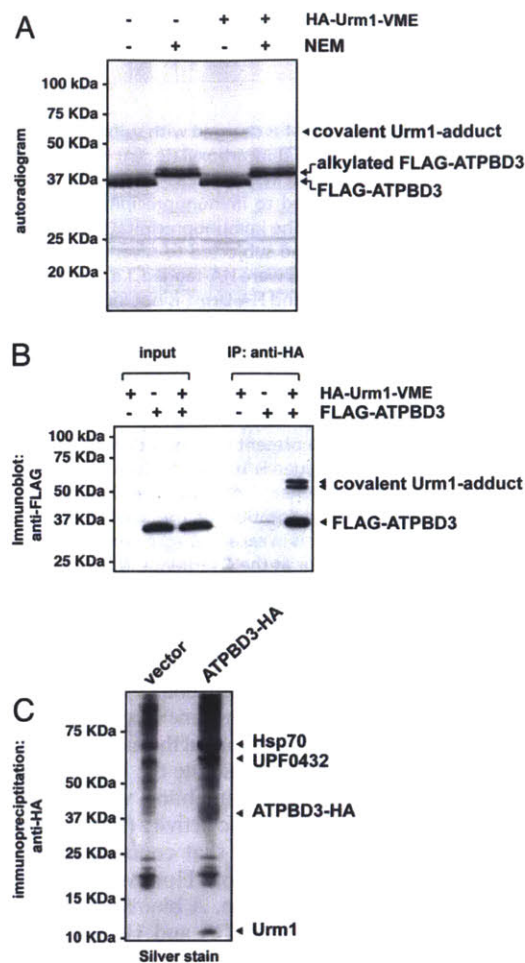
Fig. 2. A Urm1-based functional probe reacts with ATPBD3, a Urm1-directed enzymatic activity. (*A*) HA-Urm1-VME reacts with ATPBD3 to yield a covalent adduct. Radiolabeled ATPBD3 was synthesized by in vitro translation, reacted with HA-Urm1-VME in the absence or presence of NEM, and resolved by SDS/PAGE. (*B*) HA-Urm1-VME retrieves ATPBD3 from cell lysates. HEK293T cells were transfected with FLAG-ATPBD3 or control vector (−), lysed, exposed to HA-Urm-VME, and immunoprecipitated (IP) with anti-HA antibodies. Anti-FLAG antibodies were used for detection. (*C*) Overexpressed ATPBD3 associates with endogenous UPF0432 and Urm1 in vivo. HEK293T cells were transfected with ATPBD3-HA, lysed, and immunoprecipitated with anti-HA antibodies. After SDS/PAGE and silver staining, the indicated ATPBD3-associated proteins were identified by MS.

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 UPF0432 and ATPBD3, *NCS2* and *NCS6*, respectively, are linked to the Urm1 pathway: *NCS2* (needs Cla4 to survive) and *NCS6* deletions are both synthetic lethal in the absence of *Cla4*, a protein kinase involved in septin phosphorylation. This phenotype is mirrored by *Uba4* and *Urm1* deletions, which are essential when *Cla4* is absent (9). More recently, the fission yeast homologs of UPF0432 and ATPBD3 were designated Ctu2 and Ctu1 (cytosolic thiouridylase), respectively. They were shown to form a heterodimeric complex, required for the thiolation of the uridine at the first anticodon position of cytosolic tRNAs (18, 19).

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 Ncs6 (Ctu1) in tRNA thiolation and based on the structural similarity between

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. 3*A*), 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. 3*B*). 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. 3*B*), as shown by the masses of the y ion series (Fig. 3*C*). This shift in mass is consistent with a C-terminal modification by sulfur in the form of a thiocarboxylate. 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$^{\text{Gln(UUG)}}$, tRNA$^{\text{Glu(UUC)}}$, and tRNA$^{\text{Lys(UUU)}}$ contain a 5-methylcarboxymethyl-2-thiouridine (mcm^{5}S^{2}U) at the wobble position (19) (Fig. 4*A*). 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 Ctu1/NCS6 is required for the 2-thiolation (19). However, the source of the sulfur atom that is used by Ctu1/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 Ctu1is lethal, indicating a crucial role for these modifications (19).

Can Urm1 serve as sulfur donor for the Ctu1/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$^{\text{Gln(UUG)}}$, tRNA$^{\text{Glu(UUC)}}$, and tRNA$^{\text{Lys(UUU)}}$ thiolation, RNA was isolated from all deletion strains; a wild-type (WT) strain served as control (Fig. 4*B*). 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$^{\text{His}}$ 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 Ncs2/Ctu1 in 2-thiolation (19), the deletion of *NCS6* caused an increase in tRNA$^{\text{Lys(UUU)}}$ 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. 4*B*). Remarkably, the *Urm1* deletion mirrored the effect seen in a *ncs6Δ* background, indicative of a quantitative loss of thiolation (Fig. 4*B*). Virtually identical results were obtained for tRNA$^{\text{Gln(UUG)}}$ and tRNA$^{\text{Glu(UUC)}}$ (Fig. S3). As an additional control, the RNA samples were applied to denaturing PAGE devoid of APM: all samples showed an identical electrophoretic behavior (Fig. S3), thus excluding the formal possibility that a difference in length is responsible for the differential migration behavior as seen in Fig. 4*B*.

To substantiate our findings, we hydrolyzed RNA isolated from WT and *urm1Δ* strains for analysis by LC-MS. For the WT control, we observed masses corresponding to 5-methylcarboxymethyluridine (mcm^{5}U) and mcm^{5}S^{2}U. We did not detect mcm^{5}S^{2}U in *urm1Δ*-derived hydrolysates, whereas mcm^5 was clearly detectable (Fig. S4). We conclude that Urm1 is required for the thiolation of 3 cytosolic tRNA species, namely tRNA$^{\text{Gln(UUG)}}$, tRNA$^{\text{Glu(UUC)}}$, and tRNA$^{\text{Lys(UUU)}}$.

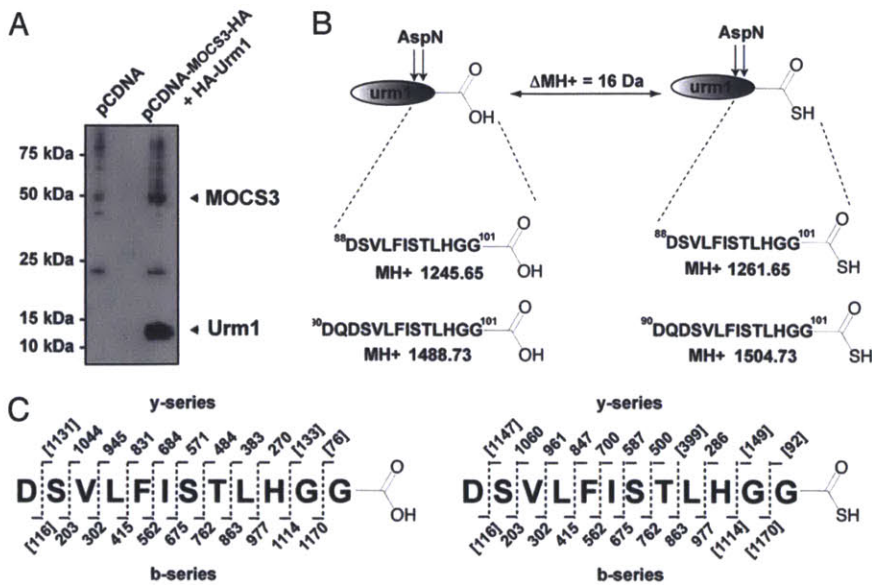To establish that tRNA thiolation is Urm1-dependent in higher

Fig. 3. Urm1 is charged with sulfur in vivo to form of a C-terminal thiocarboxylate. (*A*) HEK293T cells were cotransfected with MOCS3-HA and HA-Urm1, lysed, and subjected to immunoprecipitation with anti-HA antibodies. The immunoprecipitates were resolved by SDS/PAGE and subjected to silver staining. Note that both proteins were HA-tagged, i.e., the appearance of MOCS3-HA and HA-Urm1 is not indicative of a mutual interaction. (*B*) The band marked in *A* was excised, digested with Asp-N, and analyzed by LC-MS/MS. The observed parent ion masses are consistent with Asp-N-released peptides from the C terminus of Urm1 and suggest that both unmodified and thiocarboxylated Urm1 were present (the mass difference between sulfur and oxygen is 16 Da). (*C*) Note that the y-ion series (i.e., the series containing the C terminus), derived from fragmentation of the smaller parent ions depicted in *B*, is in excellent agreement with the location of the sulfur at the C terminus. Masses in brackets are theoretical and were not observed in this experiment.

eukaryotes, we reduced the levels of Urm1 and ATPBD3 via lentivirus-tranduced shRNA in HeLa cells. Total RNA was extracted from the resulting HeLa cell lines propagated under selective pressure for 3 days. Because appropriate antisera were not available for ATPBD3, we cannot assess the knockdown efficacy achieved by the respective shRNA. However, an accumulation of hypomodified cytosolic tRNA$^{Lys(UUU)}$ was seen in all cell lines relative to the shGFP control, as judged by APM-PAGE/Northern blot analysis (Fig. 4 *C* and *D*). Taken together, the levels of thiolated tRNAs are correlated to cellular Urm1-levels in 2 distinct organisms, indicating that the role for Urm1 in these pathways is evolutionarily conserved.

Discussion

Urm1 is a highly conserved, yet poorly understood, ubiquitin-related modifier. Urm1-deficient yeast strains show a pleiotropic phenotype, attributed to a lack of Urm1 conjugation to target proteins, a process that was termed urmylation (8–11). Apart from the identity of Uba4, the putative E1 enzyme responsible for Urm1 activation (8), next to nothing is known about the molecular mechanism and the enzymatic machinery that constitute the Urm1 pathway.

By using an Urm1-based suicide inhibitor, we discovered AT-PBD3 as an Urm1-directed enzymatic activity (Fig. S2 and Fig. 2). Urm1 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 Urm1 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 anticodons of
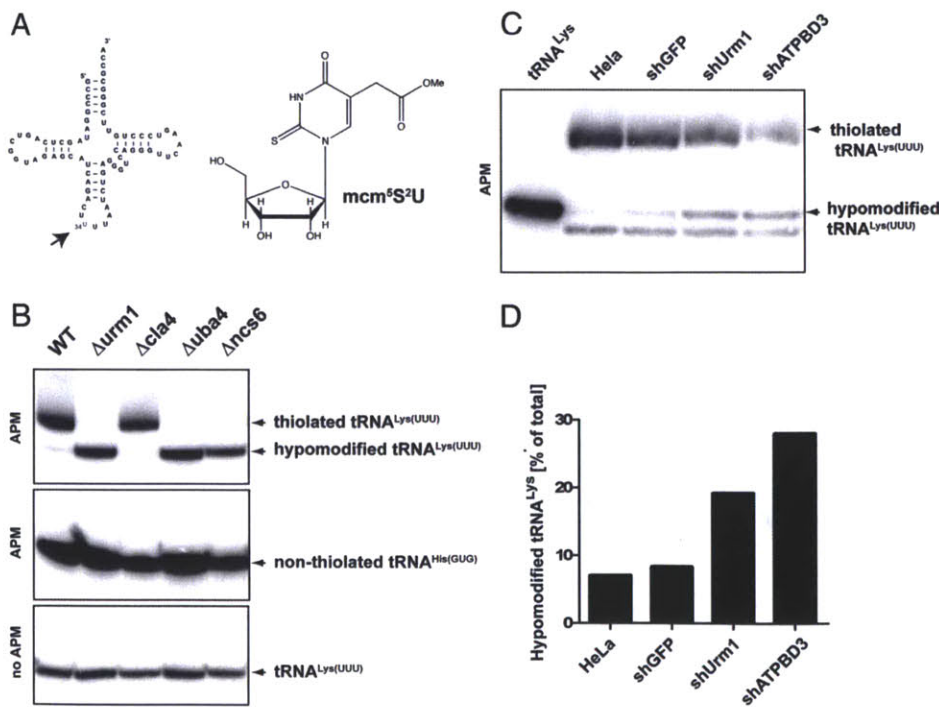

Fig. 4. Urm1 is required for the thiolation of cytosolic tRNAs. (*A*) U-rich anticodons of several cytosolic tRNAs, exemplified by the human tRNA$^{LYS(UUU)}$, contain a modified uracil, mcm^{5}S^{2}U, at position 34. (*B*) Urm1 and Urm1-associated enzymatic activities are required for tRNA thiolation 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 (*Top*) were used to discriminate between thiolated and hypomodified tRNAs, a gel devoid of APM served as control (*Bottom*). The indicated nonthiolated tRNAHis serves as control (*Middle*). Radioactive oligonucleotides with the indicated specificities served as probes. (*C*) Urm1 and AT-PBD3 contribute to tRNA thiolation in human cells. RNA isolated from the indicated HeLa cell lines was analyzed as in *B*, purified unmodified tRNALys$^{(UUU)}$ (5 pmol) served as control. (*D*) Comparison of thiolation levels obtained from a densiometric quantification of the bands seen in *C*.

3 cytosolic tRNAs (19). Moreover, a protein complex homologous to ATPBD3–UPF0432, Ctu1–Ctu2, was recently identified in *Schizosaccharomyces pombe* and likewise implicated in uracil thiolation (18). Genetic evidence suggests that *URM1*, *NCS2* (the UPF0432 homolog), and *NCS6* are linked in budding yeast (9, 22), but the functional connection between Ncs2, Ncs6 and Urm1 remained unclear.

Urm1 is in fact more similar to the prokaryotic sulfur carriers MoaD and ThiS than it is to any bona fide Ub-like protein modifier (Fig. S1). Moreover, Uba4 and MOCS3, the human Uba4 homolog, are more similar to the MoaD-activating enzyme MoeB than to the *E*1 enzymes that participate in the typical *E1–E2–E3* cascades. Uba4/MOCS3 both feature a rhodanese-like domain, a module that is commonly involved in sulfur transfer. Although purified Uba4 displays sulfur transfer activity in vitro, it was unclear whether this activity is relevant in a cellular context (23). All in all, these criteria suggest that the Urm1 pathway is mechanistically closely related to the prokaryotic MoaD and ThiS pathways (1, and references cited therein; 4, 24). Specifically, we propose that Urm1 serves as sulfur donor in the context of tRNA thiolation.

We isolated HA-Urm1 introduced into mammalian cells by transfection and showed that peptides derived from the Urm1 C terminus were present as 2 species, corresponding to unmodified Urm1 and to a C-terminal thiocarboxylate (Fig. 3 *B* and *C*). This observation supports our hypothesis and is an example of a Ub-related modifier that undergoes a thiocarboxylation reaction in human cells. Furthermore, we show that the deletion of *URM1*, *UBA4*, or *NCS6* results in a quantitative loss of thiolation of 3 cytosolic tRNAs in *S. cerevisiae* (Fig. 4*B*). Consistently, the mcm^{5}S^{2}U fingerprint mass was not detectable in nucleoside preparations isolated from *urm1Δ* strains (Fig. S4). In human cells, a reduction in the levels of either Urm1 or ATPBD3 likewise resulted in an increase of hypomodified tRNAs$^{LYS(UUU)}$ (Fig. 4*D*), suggesting a similar, if not identical, function for Urm1 in both organisms. Although UPF0432 associates with ATPBD3, we did not observe covalent adduct formation between Urm-VME and UPF0432 (data not shown). We speculate that UPF0432 might be involved in the Urm1-independent derivatization of the 5-position. The failure to obtain complete suppression of thiolation by Urm1 knockdowns in mammalian cells could indicate that residual Urm1 levels are adequate to sustain the observed tRNA thiolation levels. Based on these results, we assign a conserved sulfur carrier function to Urm1.

How the sulfur is mobilized upstream of Uba4/MOCS3 is not known. The cysteine desulfurase Nsf1, a pyridoxal 5′ phosphate (PLP)-dependent enzyme implicated in tRNA thiolation (25), binds to the RLD domain of MOCS3, where it can generate a persulfidic intermediate in vitro (26). In this scenario, the sulfur flux would start by a Nsf1-catalyzed sulfur transfer from L-cysteine to MOCS3, and MOCS3 would use the sulfur to charge Urm1 to yield a thiocarboxylate. Finally, ATPBD3 would transfer the sulfur from the Urm1 thiocarboxylate to the 2-position of uracil, a suggestion that can only be confirmed by detailed structural analysis.

While this manuscript was in preparation, Nakai *et al.* (27) placed Urm1 in the tRNA thiolation pathway in *S. cerevisiae* and arrived at similar conclusions, although evidence for thiocarboxylate formation on Urm1 was not presented. Consistent with this report and our study, deletions in any of the components that now define the Urm1 pathway (MOCS3, Urm1, ATPBD3 in humans; Uba4, Urm1, Ncs6 in budding yeast), or those that are required for the derivatization of the 5-position of the uridine, confer resistance to the *Kluyveromyces lactis* killer toxin (20, 22, 28, 29). This toxin exerts its effect by cleaving the anticodon loop of tRNAs, and the presence of uridine modifications is a prerequisite for the killer toxin's nuclease activity (30).

As far as the pleiotropic consequences of Urm1 mutations under stress conditions are concerned, many mutations in the Urm1 pathway should affect the modification of at least 3 tRNA species. Because 2-thio modifications have been implicated in translational

fidelity and efficacy (31–33), translational errors and ultimately protein misfolding are the unavoidable outcomes when thiolation is suppressed. As a consequence, the respective mutant strains will display an increased sensitivity against multiple stress conditions, irrespective of a specific function of Urm1 in a given stress response pathway.

How can the sulfur carrier function of Urm1 be linked to the observed cytokinesis defect (Fig. 1)? A similar phenotype was observed in 14-3-3σ knockdown cells, where it was attributed to a defect in translation control at the G$_2$/M transition (34). Dewez *et al.* (18) reported aneuploidy as a phenotypic consequence of the Ctu1 deletion, and mislocalized septa were reported in the context of mutations in the Urm1 pathway in budding and fission yeast (9). Along the same line, inosine modifications have been implicated in G$_1$/S and G$_2$/M transitions in fission yeast (35). We did not observe obvious changes of tRNA thiolation as a function of the cell cycle, neither in synchronized HeLa cells nor in synchronized *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 Urm1 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 Urm1 functions.

Experimental Procedures

Cell Lines, Antibodies, Constructs, and Lentiviral Transduction. *Cells.* HeLa cells were cultured in DMEM containing 10% fetal calf serum and penicillin/streptomycin at 37 °C, 5% CO$_2$.

Antibodies. Human Urm1 was expressed as 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 antiserum 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 Urm1, MOCS3, and ATPBD3 cDNAs. All constructs were cloned into pcDNA3.1$^+$ (Invitrogen) according to standard procedures.

Lentivirus. Human Urm1, 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 was produced according to instructions 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 glycerine, 50 mM NH$_4$Cl, and permeabilized in 0.1% Triton X-100. Fixed and permeabilized cells were blocked in 4% BSA and stained with phalloidin-647 and Hoechst 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 by using a Becton Dickinson FACSCalibur, and cell cycle profiles were generated by using FlowJo 8.5.3 software.

Anti-HA Affinity Purification and MS/MS Analysis. HA-Urm-VME was produced as described for Ub-VME (39). HEK293T cells (10^8) 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 MgCl$_2$, 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 $\times$ g for 15 min at 4 °C). The supernatant was incubated in presence (or absence) of 50 μg of HA-Urm-VME for 30 min at 37 °C, supplemented with anti-HA-agarose (80 μL of slurry 3F10 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 MgCl$_2$, 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 thiocarboxylate detection, samples were digested with endoproteinase Asp-N instead of trypsin.

For immunoprecipitations, one 10-cm dish of subconfluent HEK293T cells was harvested 48 h after transfection and solubilized in 1 mL of ice-cold lysis buffer supplemented with Complete (Roche) and centrifuged (14,000 $\times$g for 10 min at 4 °C). Sixteen microliters of 3F10 beads were added to the supernatant and agitated for 3–4 h at 4 °C, washed 3 times in wash buffer, and eluted by boiling in 30 μL of SDS-sample buffer. Five micrograms of HA-Urm1-VME was added to 1 mL of clarified lysate for small-scale reactions and reacted for 30 min at 37 °C before immunoprecipitation.

In Vitro Transcription/Translation (IVT). IVT reactions were performed by using the TNT system (Promega), according to the manufacturer's instructions. Five micrograms of HA-Urm1-VME was applied to 50 μL of IVT reaction and incubated for 30 min at 30 °C before loading on a gel. A final concentration of 10 mM NEM was applied to the indicated samples before addition of HA-Urm-VME.

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 *HIS3MX6* or the *KANMX6* cassette. All deletions were confirmed by PCR. *cla4Δ* was a

Table 1. Yeast strains and genotypes used in this work

Strain	Relevant genotype
8015 (wild-type) (from A. Amon)	MATa
urm1Δ	MATa *urm1Δ::KANMX6*
ncs6Δ	MATa *ncs6Δ::HIS3MX6*
uba4Δ	MATa *uba4Δ::HIS3MX6*
cla4Δ (from A. Amon)	MATa *cla4Δ::HIS3MX6*

kind gift from the laboratory of Angelika Amon (Massachusetts Institute of Technology, Cambridge, MA).

RNA MS analysis. For MS analysis of yeast tRNA, yeast cultures were grown at 30 °C and harvested in mid log phase. RNA was prepared by using the Qiagen RNA/DNA kit (Qiagen14162) and processed for MS analysis 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) was run on either 8% PAGE gels (Sequagel; National Diagnostics) or on APM gels (8% PAGE gels with addition of 20 μM APM). Gels were transferred to GeneScreen Plus membrane (PerkinElmer), UV-cross-linked, and processed according to standard procedures. DNA probes complementary to the tRNA sequences (either mammalian or yeast) of Lys-tK(UUU), Glu-tE(UUC) or Gln-tQ(UUG) were end-labeled with [γ-^{32}P]ATP (PerkinElmer) by using T4 polynucleotide kinase (NEB).

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Supporting Information
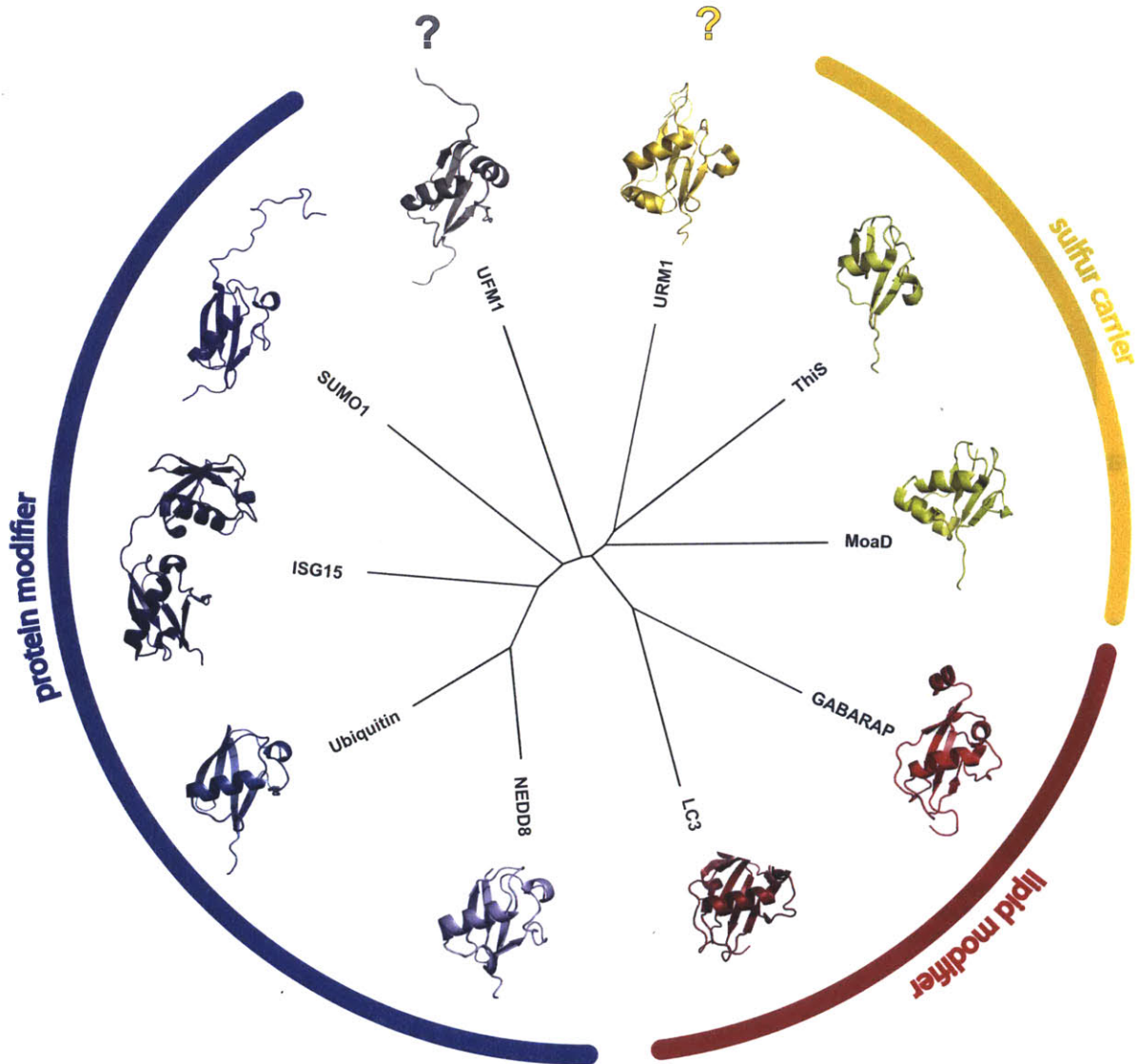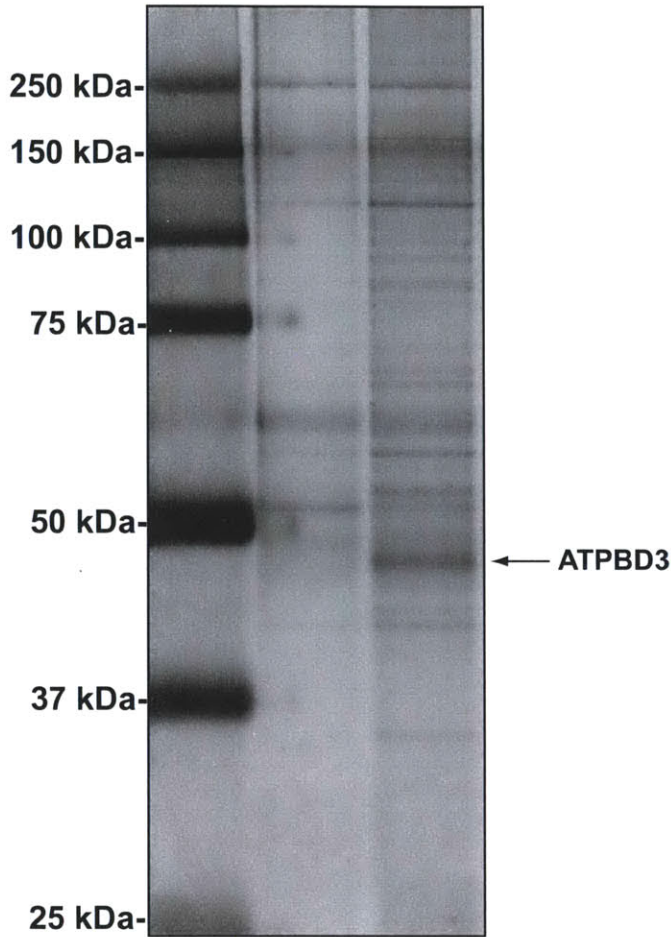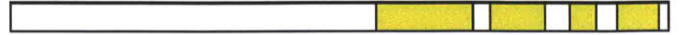
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Fig. S1. Urm1 is related to prokaryotic sulfur donors. Shown is a structural catalog of ubiquitin-related proteins and their prokaryotic ancestors, depicted as cartoon representations by using Pymol. A phylogram representing the sequence relationships between these molecules is shown in the center. The amino acid sequences of the depicted molecules were applied to ClustalW to create a phylogram in Unrooted.

Fig. S2. 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.
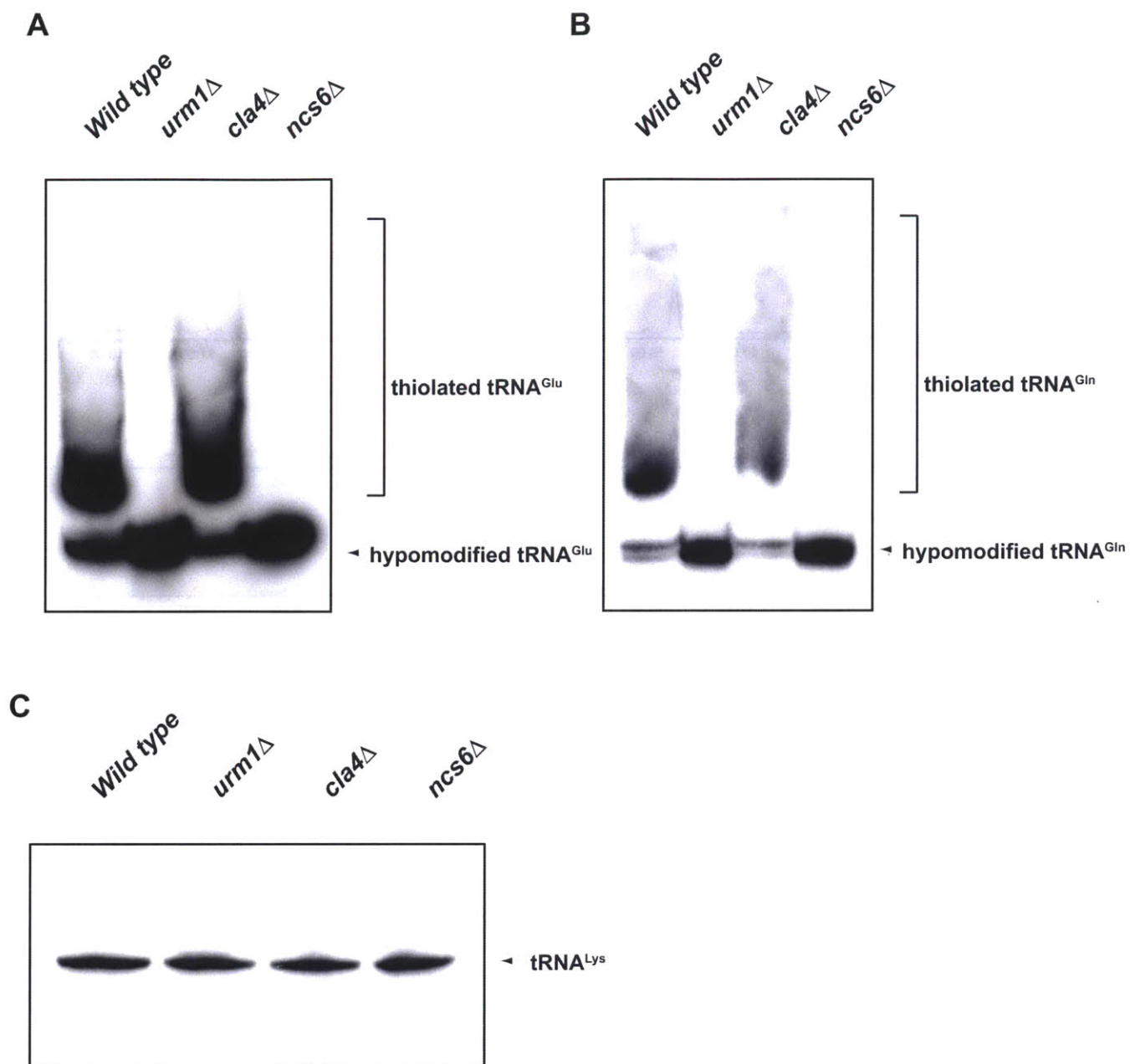
Fig. S3. The thiolation of cytosolic tRNAs is Urm1-dependent in *Saccharomyces cerevisiae*. RNA was isolated from the indicated *S. cerevisiae* strains, resolved by denaturing PAGE, and subjected to Northern blotting. Polyacrylamide gels supplemented with acryloylamino phenyl mercuric chloride (APM) 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.
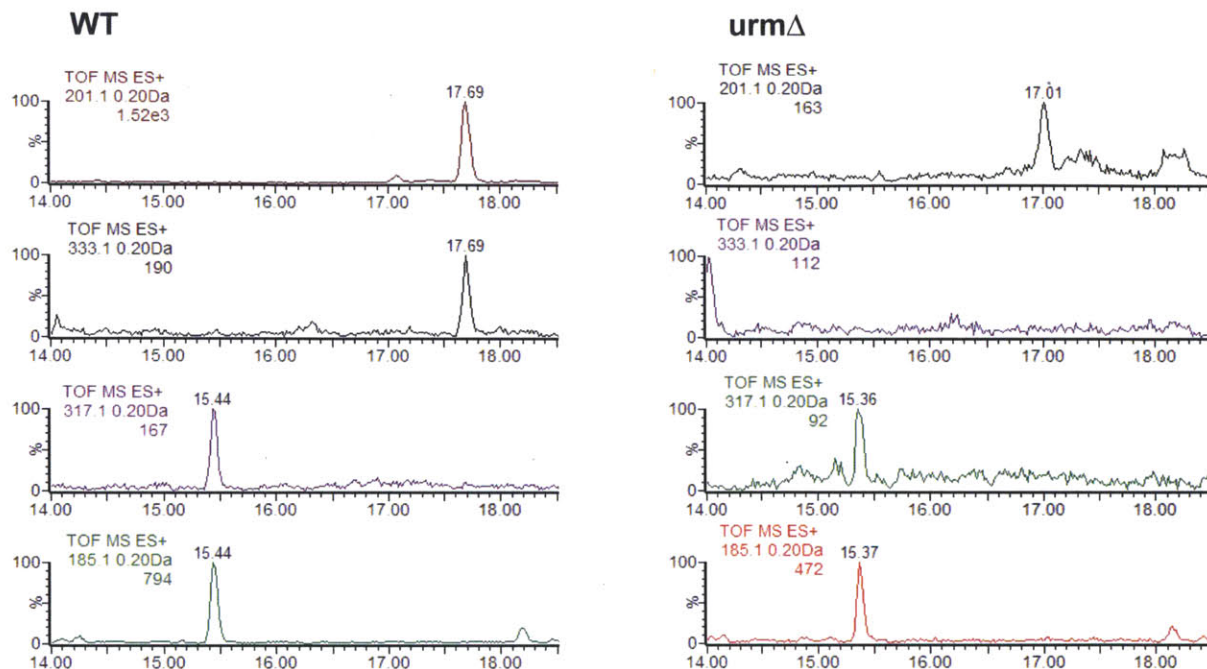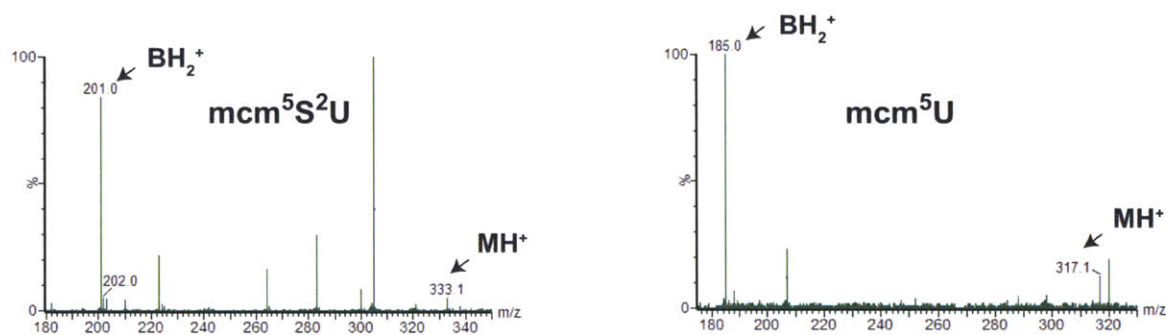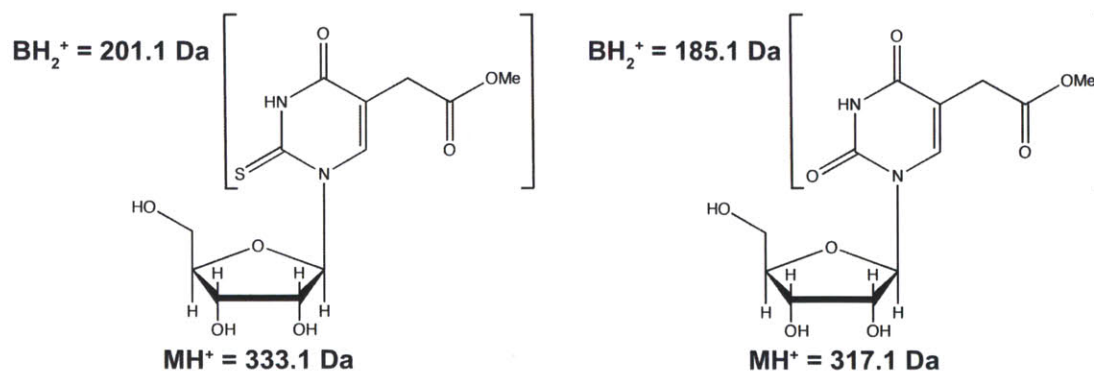
Fig. S4. HPLC-MS analysis of wild-type (WT) and *urm*Δ RNA hydrolysates. (*A*) Ion chromatograms of BH2+ and MH+ ions. RNA was isolated from WT (*Left*) and *urm*Δ strains (*Right*), hydrolyzed, and subjected to HPLC-MS. Axes are time (minutes) (abscissa) vs. relative intensity (ordinate). (*B*) Average spectra across peaks. (*C*) Structure and masses of mcm5U and mcm5S2U as intact ion, and the corresponding BH2+ fragmentation product.