tRNA STRUCTURAL STABILITY AND FUNCTIONALITY ASSOCIATED WITH THE RNA CHAPERONE LA AND POST-TRANSCRIPTIONAL MODIFICATION ENZYME TRM1

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ABSTRACT

RNA molecules have a high propensity in becoming kinetically trapped in misfolded, inactive conformations that have similar thermodynamic stability as the native fold. This is vastly due to the homogeneity in chemical composition of the four nitrogenous bases. To ensure native folding and biological activity, RNA species undergo post-transcriptional modifications (PTMs) while concomitantly associating with RNA-binding proteins. tRNAs are essential noncoding RNAs that are ubiquitously subject to PTMs and in constant interactions with various RNA-binding proteins. La proteins are a class of highly abundant RNA chaperones, characterized as the initial factors that contact pre-tRNAs. La proteins make sequence specific contacts to the UUU-3'OH ends of pre-tRNAs as well as less specific contacts to the core body of tRNAs which is associated with the RNA chaperone activity function. La proteins function redundantly with tRNA PTM enzymes that are predicted to be influential in native pre-tRNA folding and N2, N2-dimethylation of G26 by the methyltransferase Trm1p is an example of such modification enzyme. La deletion in *Saccharomyces cerevisiae* (*S. cerevisiae*) is synthetically lethal when combined with deletion of Trm1p, presumably due to numerous G26 containing pre-tRNAs undergoing degradation due to structural instability. Whether La as an RNA chaperone selectively identifies such misfolded pre-tRNAs from their folded counterparts has not been addressed. In this work, we demonstrate that like *S. cerevisiae,* La and Trm1p are essential for viability in *Schizosaccharomyces pombe* (*S. pombe*). We have identified G26 containing pre-tRNAs that benefit from the presence of Trm1p and/or *S. pombe* La (Sla1p) for their native steady state abundance and charging state. Using *in vivo* and *in vitro* assays we test the affinity of La as an RNA chaperone towards misfolded Trm1p-hypomodified pre-tRNAs and their folded Trm1p-modified counterparts. Our data indicates a nonspecific and transient nature of La in engaging misfolded and folded pre-tRNAs indiscriminately but specifically as processing intermediates. These findings signify the challenges that exist for RNA chaperones in resolving misfolded RNA structures with the inability to directly specify a binding determinant in these misfolded species. We also demonstrate that m_2^2G26 on both mitochondrial and nuclear encoded-tRNAs may play a regulatory role on tRNA functional activity in translation, with the potential to directly regulate global protein expression levels and mitochondrial fitness.

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LIST OF ABREVIATIONS

AC stem	Anticodon stem
ADP	Adenosine diphosphate
Aim23	Altered Inheritance rate of Mitochondria
Ala	Alanine
Arg	Arginine
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
CBP2	Cytochrome B mRNA Processing
CTD	C-terminal domain
CK2	Casein kinase 2
CYT-18	*Neurospora crassa* mitochondrial tyrosyl-tRNA synthetase
Cox2	Cytochrome oxidase subunit II
D-loop	Dihydrouridine loop
EF	Elongation factor
eIF4A	Eukaryotic initiation factor 4A
Eno2p	Enolase 2
FRET	Fluorescence resonance energy transfer
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
GDP	Guanosine diphosphate
GTPase	Guanosine triphosphatase
GTP	Guanosine triphosphate
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLa	Human La
hnRNPs	Heterogeneous nuclear ribonucleoproteins
Hsp70	Heat shock protein 70
IF	Initiation factor
Ifm1	Initiation factor of mitochondria
IRESs	Internal ribosome entry sites
KH	K-homology
La	Lupus autoantigen protein
LAM	La motif
Lhp1	*S. cerevisiae* La homolog
Lys	Lysine
Los1	Loss of suppression
LSU	Large ribosomal subunit
Mdm2	Murine double minute 2
Mef1	Mitochondrial elongation factor
Mitoribosome	Mitochondrial ribosome
mRNA	Messenger RNA
mRNP	Messenger ribonucleoprotein
MSN5	Multicopy suppressor of SNf1 mutation

Mtr10	Mrna TRansport defective
MTS	Mitochondrial targeting sequence
NAD	Nicotinamide adenine dinucleotide
nDNA	Nuclear DNA
NLS	Nuclear localization signal
NMR	Nuclear magnetic resonance
NRE	Nuclear retention element
NTD	N-terminal domain
NTS	Nuclear targeting signal
ORF	Open reading frame
OXPHOS	Oxidative phosphorylation
PAM	Presequence translocase-associated motor
Pre-LysRS	Precursor of lysyl-tRNA synthetase
Pre-mRNA	Precursor messenger RNA
Pre-protein	Precursor protein
Pre-tRNAs	Precursor transfer RNA
Pre-5S rRNA	Precursor 5S ribosomal RNA
Pre-U6 snRNA	Precursor U6 small nuclear RNA
Pol III	Polymerase III
Pol II	Polymerase II
PTM	Post-transcriptional modification
PUS1	Pseudouridylate Synthase 1
Ran	RAs-related nuclear protein
RanGAP	Ran GTPase activating protein
RanGEF	Ran guanine nucleotide exchange factor
RGG	Arginine glycine boxes
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RRF1	Ribosome recycling factor
RRM	RNA recognition motif
RTD	Rapid tRNA decay
SAM	S-adenosylmethionine
SBM	Short basic motif
Sen	tRNA splicing endonuclease subunit
Ser	Serine
SF2	Superfamily 2
SILAC	Stable isotope labeling of amino acids in cell culture
SLa	*S. pombe* La
snoRNA	Small nucleolar RNAs
SS-B	Sjogren's Syndrome Antigen B
SSU	Small ribosomal subunit
Tan1	tRNA acetyltransferase 1
Td	Thymidylate synthase
TIM	Translocase of the inner membrane
Tm	Melting temperature
TMS	tRNA mediated suppression

TPT1	tRNA 2'-phosphotransferase
tRNA	Transfer RNA
tRNA-HydroSeq	tRNA-enriched limited hydrolysis sequencing
TRAMP	Trf4/Air2/Mtr4p polyadenylation
TRL1	tRNA ligase
Trm1	tRNA methyltransferase 1
Trm4	tRNA methyltransferase 4
Trm8	tRNA methyltransferase 8
Trm9	tRNA methyltransferase 9
Trm6/61	tRNA methyltransferase 6/61
Trm44	tRNA methyltransferase 44
TOM	Translocase of the outer membrane
TOPs	Terminal oligopyrimidine tracts
T-loop	TΨC loop/thymine loop
Tuf1	Elongation factor of mitochondria
UP1	hnRNP A1 protein
uORFs	Upstream open reading frames
UTR	Untranslated region
Val	Valine
Xpo-t	Exportin-T
Xrn1	eXoRiboNuclease
7SK snRNA	7SK small nuclear RNA

CHAPTER I: Literature Review

1. The RNA folding problem

RNA molecules are central to many cellular processes and their biological functional role is linked to their three-dimensional complex structures. One of the earliest evidence for RNA structural conformation dominating its biological function was exemplified with tRNAs (1). *In vitro* studies identified multiple conformations for a single tRNA, in which only one conformer was aminoacylated and biologically active in translation (1). While some RNA molecules possess a single functionally active conformation other RNAs such as Riboswitches and RNA thermometers exist in two functionally active conformations that are dependent on cellular conditions (1). Evidence of structural conformation dictating RNA function has been demonstrated for numerous RNA substrates (1–3).

Specifying a biologically active conformation for a single primary sequence of RNA is challenging given the simplicity of the building blocks of RNA. The chemical configuration of RNA consists of a polymer of ribonucleotides that have emerged from the covalent interactions between the four nitrogenous bases and the ribose sugar-phosphate backbone (2, 4). The purine and pyrimidine nitrogenous bases are vastly similar and are distinguished from one another only in the positioning of the carbonyl and amino groups. This results in the slow folding of the RNA and formation of multiple stable secondary structures that are biologically inactive (2, 4). Challenges in identifying a functionally active conformation is recognized as the RNA Folding Problem. Thermodynamic and kinetic resistance define the RNA folding problem. Firstly, RNA molecules develop multiple secondary structures that are thermodynamically stable and in competition with the native conformation (2). The thermodynamic challenge of the RNA folding problem lies within the ability of the biologically active conformation to outcompete the inactive intermediate species in thermodynamic stability. RNA molecules undergo several cycles of folding and unfolding for the search of the active conformation and often become kinetically trapped in long-lived misfolded conformations. The capacity to escape the kinetic folding trap defines the second RNA folding problem (1–3, 5).

Free energy landscapes are used to identify the likelihood of a sequence of RNA to fold into its native conformation based on the relative energy differences of intermediate folds and the kinetic barriers in escaping these entrapments. The kinetic partitioning model recognizes that an

unfolded sequence of RNA can undergo various cycles of folding before the functionally active structure is formed, and in the process generating a population of RNA molecules that transition into the native fold efficiently and those that are slow-folding (6, 7). The kinetic partitioning model has been exemplified for the folding of *Tetrahymena* group I ribozyme using both *in vivo* and *in vitro* studies. *In vitro* folding of *Tetrahymena* group I ribozyme has been shown through Single Molecule FRET (Fluorescence Resonance Energy Transfer) experiments demonstrating that the folding of the ribozyme occurs with ~ 10% of the RNA folding directly into the native conformation, leaving ~ 90% of the ribozyme in search of the native fold, through several cycles of intermediate folds (6, 7). *In vivo* studies in *S. cerevisiae* have shown a similar partitioning pattern of the *Tetrahymena* group I ribozyme into active and misfolded transcripts shortly after synthesis. The escape from degradation relies on the efficient folding of the intron and subsequent splicing of these elements. The processing of the *Tetrahymena* group I intron is shown to be significantly more efficient *in vivo* than *in vitro* predictably due to the larger population of pre-rRNAs that fold into the active state versus the misfolded conformation. The increase in efficiency in folding of *Tetrahymena* intron in yeast in comparison to *in vitro* studies has been linked to the availability of RNA-binding proteins that refold misfolded species (7, 8).

Other examples of *in vivo* and *in vitro* RNA folding studies include splicing of the T4 phage *thymidylate synthase* intron. The catalytic activity of the RNA relies on the correctly folded tertiary structure which is dependent on the efficient splicing of the group I intron. Splicing factors enforce correct folding of the group I intron *in vivo*, allowing for efficient splicing. Conversely, *in vitro* splicing of T4 phage *td* intron is inefficient due to the absence of splicing factors and the accumulation of misfolded, splice-defective conformations (9). Previous findings have also indicated that in addition to splicing factors, the ribosome also enhances folding of group I intron and that translation of the T4 phage *thymidylate synthase* intron relieves misfolding *in vivo* (7, 9). While numerous examples of RNA folding problems have been reported *in vitro*, many of these RNAs fold more efficiently *in vivo* (7). RNA folding is better modulated *in vivo* through a plethora of RNA molecule interacting partners that facilitate RNA folding. *In vivo,* RNA molecules may be engaging small ions, bound by small ligands or associating with various classes of RNA-binding proteins (1). These proteins coordinate RNA folding by decreasing the propensity of misfolding while resolving kinetic and thermodynamic barriers. Some examples of proteins that assist RNA folding include RNA chaperons, RNA helicases, and specific RNA binding proteins (7).

1.1 RNA binding proteins resolve the RNA folding problem

1.1.1 RNA chaperones

RNA molecules are almost invariably associated with one or more proteins *in vivo* with majority, if not all RNA-facilitated reactions involving a protein cofactor. RNA chaperones are a class of RNA-binding proteins that resolve the RNA folding problem (5). These proteins are multifunctional and are essential in numerous cellular processes such as RNA export, ribonucleoprotein (RNP) assembly, regulation of transcription and translation as well as virus replication (7). Ranging from distinct groups of families of proteins, it has been challenging identifying a uniform mechanism that RNA chaperones employ in targeting their substrates. One distinct commonality amongst these proteins is their capacity to engage their targets non-specifically through weak electrostatic interactions in the absence of external energy such as ATP (adenosine triphosphate) hydrolysis (6, 7). In accordance with the kinetic partitioning model, RNA substrates that have a low partitioning factor (high probability of folding incorrectly) are subject to several cycles of remodeling by RNA chaperones to fold into their native functionally active state (6). In the absence of external energy, RNA chaperones assist RNA remodeling by taking advantage of the differences in the free energy between the unfolded, intermediate and native state of the RNA. RNA chaperones are thought to associate with single stranded RNA more readily than double stranded RNA and support unfolding by tightly and transiently engaging these regions (1). This interaction results in the destabilization of the misfolded-intermediate fold as well as the decrease in the free energy state between the intermediate and native fold. In this way, the folding of the RNA into the native conformation is driven by the factor that the formation of the native RNA is more thermodynamically favoured than the chaperone-bound RNA complex (1). This will encourage the release of the RNA chaperone from the newly folded intermediate state into a chaperone-free RNA that is functionally active (Figure 1) (6). Previous studies have indicated the importance in weak and transient interactions between RNA chaperones and their targets. StpA is a recognized RNA chaperone identified in *E. coli* (*Escherichia coli*). Mutations generated in this RNA chaperone that diminish the RNA binding capacity of the protein, displayed better RNA chaperone activity compared to wildtype StpA. This emphasizes the idea that RNA chaperones transiently associate with unfolded RNA species and once the RNA is folded, they must be released onto their next target (7).

The second common element amongst proteins with RNA chaperone activity is the propensity of these proteins possessing highly disordered regions, rich in amino acids that favor disorder over an ordered configuration such as Ala, Arg, Gly, Gln, Ser, Pro, Glu and Lys. These disordered regions are predicted to facilitate the lack of substrate specificity that these proteins possess and models have been proposed in which the disordered regions become ordered upon interaction with RNA targets (7, 10) (Figure 2). While *in vitro* RNA chaperone activity assays have led to the identification of numerous proteins that are accredited as RNA chaperones, it has been nonetheless challenging verifying and studying the RNA chaperone activity of such proteins *in vivo*. The nonspecific nature of these proteins allows for multiple chaperones to facilitate the folding of the same RNA substrate therefore eliminating the opportunity for a visible phenotype in the absence of a specific chaperone (3).

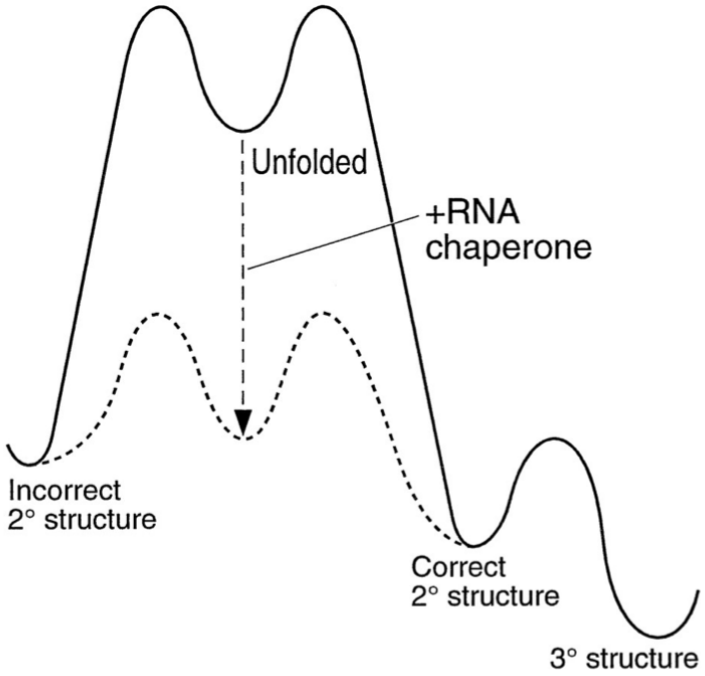


Figure 1. Hypothesized RNA folding model facilitated by RNA chaperones. Folding pathway and free energy barrier schematic illustrating the unfolding and refolding of a misfolded secondary structure of an RNA to a correct secondary and tertiary structure in the presence (dotted line) and absence (solid line) of an RNA chaperone. For simplicity only one incorrect 2° structure is represented. Figure adapted from (2).

1.1.2 Examples of RNA chaperones

Heterogeneous nuclear ribonucleoproteins (hnRNPs)

Heterogeneous nuclear ribonucleoproteins (hnRNPs) are a class of RNA-binding proteins with RNA chaperone activity. hnRNPs, similar to other RNA chaperones have established roles in the cellular system and are ubiquitously active in gene expression with roles in transcription, translation, pre-mRNA processing and stability, RNA localization, export and RNA silencing (11). hnRNPs have 3 established RNA binding motifs: the arginine glycine boxes (RGG), RNA recognition motif (RRM), and K-homology (KH) motif (7, 11). The first evidence that these proteins harbor RNA chaperone activity was observed nearly 35 years ago where it was indicated that a fragment of hnRNP A1 protein (UP1) can renature an inactive tRNA Leucine. The same study also reported RNA chaperone activity of UP1 for renaturation of a misfolded 5S RNA (12). The idea that one protein can assist the renaturation of two distinct RNA substrates that differ in structure, gave rise to the proposition that single stranded regions on these RNAs could be the common factor for the UP1-mediated remodeling (12). Since these findings, further evidence of the RNA chaperone activity of hnRNPs has been reported with other RNA substrates (eg. hammerhead ribozyme) as well as studies demonstrating strand annealing activity of hnRNP A1 for DNA and RNA which has been mapped to the C-terminal fragment of A1 (13, 14).

StpA protein

Another example of a well-recognized RNA chaperone is the *E. coli* derived StpA protein, first identified for its capacity to suppress a mutant of bacteriophage T4 thymidylate-synthase (td) gene that is defective in splicing (15–17). In accordance with the speculative mechanism for an RNA chaperone, StpA recognizes RNA nonspecifically and relies on weak and transient electrostatic interactions to unfold and restructure its bound RNA to a native conformation. An *in vivo* splicing assay has been used that takes advantage of the T4 thymidylate-synthase pre-mRNA inability to fold in the absence of translation to measure the RNA chaperone activity of StpA. The transient interaction between StpA and the T4 thymidylate-synthase gene allowed for the loosening of the tertiary structure and an opportunity for the RNA to search for the correct fold that favors splicing (16, 17). Restructuring and refolding of T4 thymidylate-synthase gene by StpA was possible only if wildtype or mutants that resembled the structural stability of the wildtype gene were used. Interestingly, mutants of the td gene that were structurally destabilized compared to wildtype were

sensitive to StpA as depicted by the decrease in splicing. These findings suggested that the structural stability of the RNA might dictate a positive or non-beneficial interaction between the chaperone and the RNA substrate, at least for what has been revealed for StpA (16, 17).

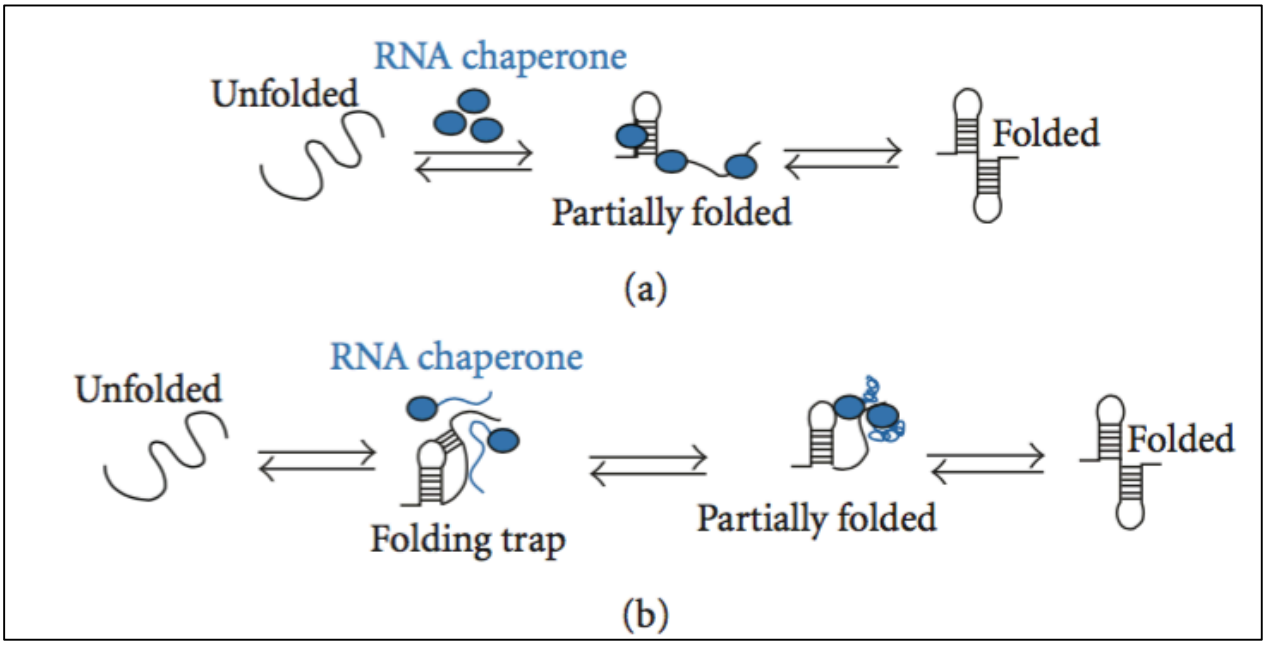


Figure 2. Predicted model for RNA chaperone assisted RNA folding. (A) RNA chaperones (blue) associate with unfolded RNA, prevent misfolding and favor formation of the native structure. (B) Disordered regions in the RNA chaperones interact with a misfolded RNA and in this interaction, the transfer of energy resolves the misfolded RNA and orders the RNA chaperone. Upon folding of the RNA, the chaperone is no longer needed. Figure adapted from (10).

Ribosomal proteins

Protein synthesis relies on the complex assembly of the ribosome. Eubacterial ribosome biogenesis comprises of the assembly of the small and large subunit which consists of the 16S rRNA in a complex with over 20 proteins for the 30S small subunit and the interplay of the 23S rRNA and the 5S rRNA with over 30 ribosomal proteins which constitutes the 50S large subunit (9, 18). Large RNA substrates such as the 23S rRNA have the tendency to become kinetically trapped in inactive conformations (9, 19). Several examples of *E. coli* ribosomal proteins have been reported

harboring RNA chaperone activity. One of the first evidence of RNA chaperone activity was observed with *E. coli* S12 ribosomal protein that was catalytically active in a strand annealing and strand displacement activity *in vitro* in the absence of ATP (19). Other studies showed that ribosome biogenesis can occur efficiently *in vitro* with the presence of the rRNAs and ribosomal protein components, in the absence of added assistance from protein cofactors or RNA chaperones. This confirmed the notion that ribosomal proteins independently harbor RNA chaperone activity and can facilitate ribosome biogenesis (18). *In vitro* trans-splicing assay of the T4 thymidylate-synthase gene was also used to test for the RNA chaperone activity of ribosomal proteins. At 37°C, where trans-splicing is inhibited in the absence of RNA chaperones, the addition of ribosomal protein enhanced splicing (18). Since these findings, other ribosomal proteins from the large subunit have been tested for RNA chaperone activity and of the 34 *E. coli* ribosomal proteins tested, a third of these proteins displayed RNA chaperone activity with the RNA chaperone activity of L1 ribosomal protein being conserved in Archaea, Bacteria and Eukarya (7, 18).

Lupus autoantigen (La) protein

Another well characterized RNA chaperone is the Lupus autoantigen protein (La) which is a ubiquitous RNA binding protein that is highly conserved and essential in higher eukaryotes, but can be deleted in fission and budding yeast (20–24). La proteins associate with both coding and noncoding RNA substrates and are involved in their processing and metabolism (20–24). The best characterized substrates of La are polyuridylate-containing RNAs, which consists of all polymerase III (pol III) transcripts (pre-tRNAs, pre-5S rRNA, pre-U6 snRNA etc.), and some polymerase II (pol II) substrates that terminate in UUU-3'OH such as U1, U2, U3, U4 and U5 snRNAs found in yeast (20, 25). For poly U-tail containing RNA substrates, La association serves to protect these RNA transcripts from 3' exonucleases and assists in their maturation. Aside from poly U-tail containing noncoding RNAs, La has also been shown to engage cellular (Bip and cyclin D1) and viral (poliovirus and Hepatitis C virus) mRNAs, either enhancing or inhibiting translation through mechanisms still under investigation and not in the scope of this review (20, 26–28). Some common features present in the mRNAs that La associates with include, internal ribosome entry sites (IRESs), terminal oligopyrimidine tracts (5'TOPs) and upstream open reading frames (uORFs) (20). The RNA chaperone activity of human La (hLa) has been shown using *in vitro* self-splicing intron assay as well as confirmed with FRET based assays demonstrating strand annealing

and displacement activity for both human and Sla1p (29, 30). This activity has been mapped to the RRM1 and the adjacent α3-helix that is part of a highly conserved N-terminal domain of La proteins. Interestingly, mutants defective in RNA chaperone activity are also incapable of rescuing mutated, misfolded tRNAs *in vivo* as shown using a tRNA mediated suppression (TMS) assay (29).

1.1.3 RNA helicases assist RNA folding

RNA helicases are a well characterized class of RNA binding proteins that resolve the RNA folding problem. All proteins with helicase activity are apportioned to a class of helicase super families 1 through 4 which also includes proteins with DNA helicase activity (7). For the interest of this review, the focus in this section will be on some examples of RNA helicases that belong to superfamily 2 (SF2) such as DEAD-box, DEAH and the DExH RNA helicases (7, 31). The SF2 RNA helicases all possess 'RecA-like' domains that have insertions with sites dedicated to ATP binding and hydrolysis as well as insertions for polynucleotide binding ((31), Figure 3). The two helicase domains are predicted to have an undefined orientation in the absence of ATP or nucleic acid binding and this is particularly noted for the DEAD-box proteins. Biochemical studies have suggested a cooperative relationship between ATP binding and nucleic acid association, specifically for the DEAD-box proteins which rely on nucleic acid binding for ATP hydrolysis (31, 32). Contrary to the DEAD-box proteins, DExH and DEAH proteins are active in ATP hydrolysis in the absence of nucleic acid binding. DEAD-box proteins are involved in many cellular processes such as translation initiation with eIF4A (eukaryotic initiation factor 4A) as an example (32). eIF4A assists in the preparation of the mRNA for the small ribosomal subunit by resolving RNA-duplex structures or by removing proteins that may be bound by the mRNA. Other DEAD-box proteins such as Ded1 and Vasa have also been identified to partake in translation initiation, but their exact roles are still under investigation. Studies in *S. cerevisiae* have identified 15 DEAD-box proteins as well as 3 other RNA helicases that are involved in ribosome biogenesis (32). One possible role for these proteins in ribosome biogenesis is to resolve duplexes that are formed between snoRNA and rRNA, two rRNAs or resolving interactions between RNA-protein

complexes. Aside from the examples mentioned above, DEAD-box proteins are predicted to play essential roles in RNA decay and export, pre-mRNA splicing and transcription (32).

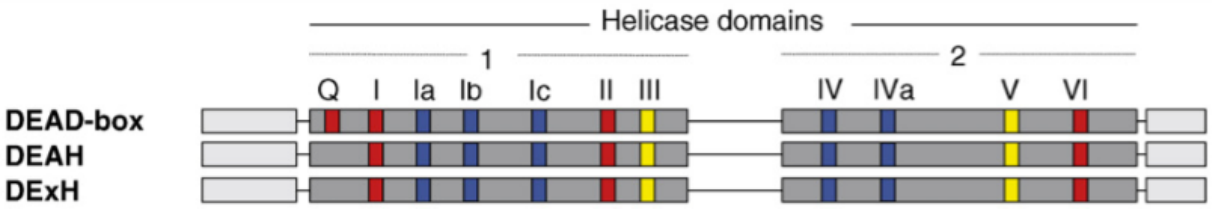


Figure 3. Architecture of the SF2 helicases. DEAD-Box, DEAH and DExH of the SF2 helicases carry two helicase domains that are 'Rec-A like' (dark grey bars) positioned between the N and C-terminal domain (light grey bars). The helicase domains have multiple insertions responsible for the helicase activity of this class of proteins including a polynucleotide binding site and ATPase activity (yellow) and a site of ATP binding and hydrolysis (red). The Nucleic acid binding site is shown in blue. Figure adapted from (31).

1.1.4 Specific RNA binding proteins

Specific RNA cofactors are recognized as RNA-binding proteins that like RNA chaperones resolve the RNA folding problem, but in contrast, display substrate specificity and are committed to one or a defined set of RNA substrates. Specific RNA binding proteins, recognize a conserved structure on their target RNA substrates and similar to RNA chaperones, engage the RNA molecule transiently (and in some cases remain bound to their substrates in the form of a "stabilizer"), resolving misfolded structures and accelerating the search for the native conformation (1, 7). The *Neurospora crassa* CYT-18 protein (tyrosyl- tRNA synthetase) is an example of a specific RNA binding protein. CYT-18 promotes splicing of group I introns by directly contacting and stabilizing either conserved structures or specific sequence elements on the intron (1, 7, 33). CBP2 (yeast mitochondrial protein) is an example of another specific RNA binding protein that promotes splicing of bI5 group I intron. Single molecule FRET studies have indicated that aside from specific interactions between CBP2 and bI5 group I intron, that CBP2 also makes nonspecific contacts to the intron that result in large structural rearrangements on the RNA, reminiscent of RNA chaperone activity (1).

2. La proteins and their nuclear and cytoplasmic targets

The Lupus autoantigen (also known as Sjogren's Syndrome Antigen, SS-B) is a 46kDa protein factor identified as a component of a ribonucleoprotein complex, first recognized as an antigen targeted by autoantibodies in the sera of patients diagnosed with systemic lupus erythematosus and Sjogren's syndrome (34). Although evidence underlying the autoantigenicity of La proteins remains weak, speculations have been made suggesting that its cellular localization and phosphorylation status may be influential (20, 34–36). La proteins are ubiquitous factors, essential in multicellular eukaryotes including *Drosophila* and mice. Studies on La function has been conducted in unicellular eukaryotes *S. pombe* and *S. cerevisiae* where the gene can be deleted without loss of viability (20, 37–39). La proteins associate with a multitude of RNA substrates with nascent pol III transcripts (such as pre-tRNAs, pre-5S rRNA, pre-U6 snRNA, 7SK snRNA, Y RNAs and Alu RNAs) identified as the primary RNA species in La ribonucleoprotein particles. In addition, some pol II transcripts have also been targeted by La and those include U1, U2, U3, U4 and U5 snRNAs in yeast as well as human pre-U1 snRNA (20, 40–42). La proteins preferentially associate with pol III and pol II transcripts by making sequence specific contacts to the polyuridylate tail present at the end of these nascent transcripts. This specific binding mode is entirely dependent on the length of the 3' terminal oligo (U), with human La binding transcripts that terminate in 3 or more terminal oligo (U) residues and *S. pombe* La binding with highest affinity to transcripts with 4 or more terminal uridylate residues (20, 43). These findings coincide with the minimal number of dT residues required for efficient transcription termination by pol III which has been identified to be a minimum of 4 dT residues for human pol III and 5 dTs for *S. pombe* pol III (20, 43, 44). Findings by Huang et al. suggested a link between transcription termination by Rpc11p (subunit of pol III with 3'exonulcease activity) and La dependent processing of nascent pol III transcripts (43). More specifically, the heterogeneity in the length of 3'oligo-U tracts produced at the end of pre-tRNAs was shown to dictate the extent to which processing and maturation of a pre-tRNA would rely on La (43). Two distinct pathways for pre-tRNA processing have been identified in *S. pombe* and *S. cerevisiae*; a La-dependent and a La-independent pathway. Pre-tRNAs processed through the La dependent pathway, associate with La via their 3' oligo-U tracts and in this way are shielded from 3'exonucleases (Rex1p or Rrp6p) (20, 45). La-bound pre-tRNAs are initially subject to 5' leader maturation by RNAse P, followed by

the endonucleolytic processing of the 3' end by RNAse Z which cleaves at the first unpaired nucleotide in the acceptor stem, releasing La from the tRNA with subsequent addition of CCA to the mature 3'end (45). Pre-tRNAs processed via the La-independent pathway (in the absence of La or for those pre-tRNAs that do not engage La) are initially subject to 3' end maturation by an exonuclease (Rex1p or Rrp6p if pre-tRNA is defective and targeted by the nuclear exosome) followed by processing of the 5' leader by RNAse P (20, 43, 45). It should be noted that while La is the best characterized factor involved in pre-tRNA processing, other protein factors have also been shown to impact pre-tRNA metabolism and processing in *S. cerevisiae* such as Lsm proteins (Lsm2p-8p complex) in which their role moderately resembles La function in pre-tRNA processing (42, 45). Synthetic lethality has previously been detected in a *lhp1Δ* strain (La homolog in *S. cerevisiae*) bearing a mutation made to LSM8, suggesting a functional link between these proteins. Furthermore, it has been speculated that in *S. cerevisiae*, Lsm proteins may play a role in the assembly of pre-tRNA/protein complexes such as those described between La and pre-tRNAs (Figure 4) (42, 45).

In addition to the well characterized role of La proteins in the processing and maturation of nascent pol III transcripts such as pre-tRNAs via their oligo (U) tracts, these factors have also been implicated in rescuing structurally impaired pre-tRNAs which can result from DNA mutation, deletion of an essential modification enzyme or errors in transcription. Hypomodified pre-$tRNA_i^{Met}$ at residue A58 is subject to polyadenylation by the poly (A) polymerase Trf4p and subsequently degraded by the 3' exonuclease, Rrp6p. The decay of the hypomodified pre-$tRNA_i^{Met}$ is reversed with overexpression of La (43). Evidence of rescue of a misfolded pre-tRNA by La has also been made for tRNA-Arg^{CCG} in *S. cerevisiae*. In search of mutants in *S. cerevisiae* that cause Lhp1p dependence, tRNA-Arg^{CCG} carrying a mutation in the anticodon stem (G to A) was identified (46). tRNA-Arg^{CCG} is the only tRNA in *S. cerevisiae* that bears a mismatch in the anticodon stem, amplifying the effect of this mutation in weakening of the anticodon stem. Chakshusmathi et al. showed that Lhp1p is required for efficient growth of strains carrying this mutation and that defects in aminoacylation of the mature tRNA-Arg^{CCG} was detected in cells depleted of Lhp1p (46). To determine whether rescue of misfolded pre-tRNAs reported in literature rely on La binding strictly to the oligo (U) tracts of these nascent transcripts or whether La interacts with other sites on pre-tRNAs, Huang and colleagues used an opal suppressor tRNA-Ser^{UGA} with three base pair disruptions (C37:10 U40 U47:6) that uniquely relied on La function for successful suppression of

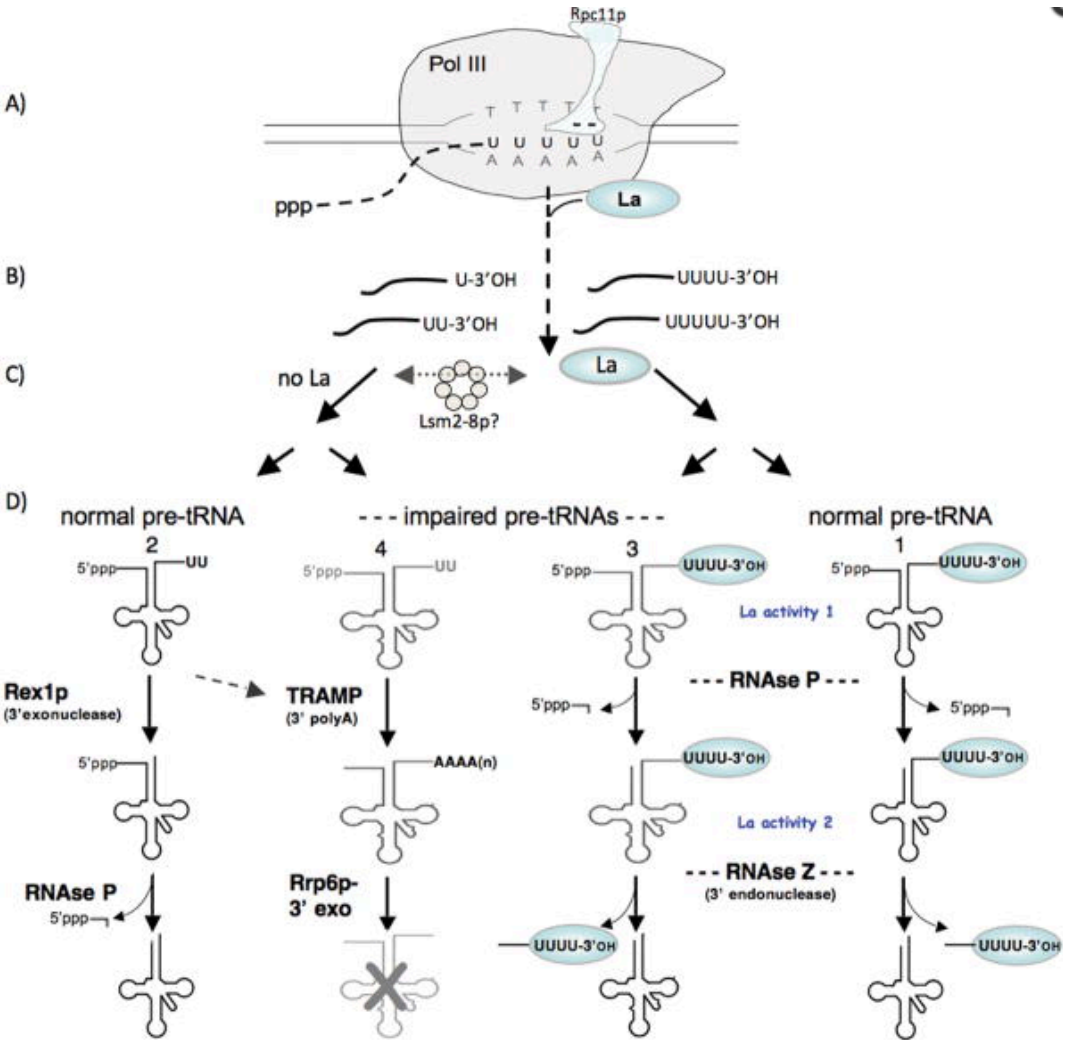


Figure 4. Pre-tRNA processing pathway in the presence and absence of La. (A) The heterogeneous 3'oligo (U) tails generated at end of pre-tRNAs results from pol III pausing at the tRNA gene terminator and the subsequent 3'end cleavage carried out by an acidic hairpin located at the N-terminal domain of Rpc11p (pol III subunit). (B) Nascent pre-tRNAs are segregated based on their 3'oligo (U) tails into the La-dependent (right) or La-independent (left) pathway of processing. (C) Lsm2-8 proteins presumably play a role in early pre-tRNA metabolism and can impact the extent in which pre-tRNAs associate with La, but their role remains unclear. (D) Pre-tRNAs are processed through 1 of 4 pathways depending on La-associated processing and whether the pre-tRNA is deemed defective or is natively structured. Normal pre-tRNAs are subject initially to 5' end processing by RNAse P if bound by La (pathway 1) and then 3' end cleavage by an endonuclease (RNAse Z). This order is reversed in a La independent pathway such that 3' end cleavage is initiated by an exonuclease (Rex1p) and then followed by 5' end maturation by RNAse P (pathway 2). For structurally defective pre-tRNAs, the La-dependent pathway protects these species from degradation by the nuclear exosome (pathway 3), whereas in the La-independent pathway, the defective pre-tRNA is subject to nuclear surveillance involving the TRAMP complex and the nuclear exosome, Rrp6p (pathway 4). Figure adapted from (45).

a nonsense codon in the *ade6-704* gene (47). Their findings showed that the degree to which La-dependent rescue was necessary correlated with the severity of the mutation, with tRNA-Ser^{UGA} U40 U47:6 and tRNA-Ser^{UGA} C37:10 U40 U47:6 relying more strongly on La for suppression than tRNA-Ser^{UGA} C37:10. This study also revealed that, while La-associated 3'poly (U) tail binding was sufficient for rescue of misfolded tRNA-Ser^{UGA} C37:10, that La engaging the main body of the tRNA might play a secondary but equally essential role in rescuing misfolded tRNA-Ser^{UGA} U40 U47:6 and tRNA-Ser^{UGA} C37:10 U40 U47:6. Other studies have followed since, providing evidence in La associating with the main body of tRNAs through a distinct binding mode unique from the poly (U) tail binding mode and this has been linked to the RNA chaperone activity function of La (22, 29, 47, 48).

Aside from the abundant nuclear pol III transcripts that associate with La, there is also evidence of La engaging cellular and viral coding RNAs with La implicated in translational and metabolic regulation of these transcripts (20). In the absence of a poly (U) tail, there are commonalities in regions that La targets in engaging these coding RNAs and some examples include mRNAs that possess either an IRES, 5'TOPS or uORF (20). While La has been recognized as a mRNP, its role in translation initiation has remained vastly unclear due to the opposing effects reported depending on the mRNA that La engages. La facilitates translation initiation for poliovirus mRNAs by making contacts with the IRES as well as enhancing translation initiation for both poliovirus and HIV-1 mRNAs by associating with the 5'UTR region (28, 49). Other studies have shown that La binding IRESs enforce a cap-dependent translation initiation by masking the ribosomal binding site. In this form, La can oppose a positive or a negative regulatory effect on translation initiation depending on the mRNA and the presence or absence of a 5' terminus m^{7}GpppN cap (50). Evidence for a more direct enhancement of translation has also been identified for the coding RNA mdm2. Expression of MDM2 was linked to La associated binding of the 5' UTR of mdm2 mRNA, where overexpression of La enhanced translation and repression was induced in La mutants (51).

2.1 Structure and architecture of La

2.1.1 N-terminal domain

The general architecture of La proteins is highly conserved, particularly the N-terminal domain

(NTD) and to a lesser extent the C-terminal domain (CTD). The NTD comprises of a La motif (LAM) and RRM1 connected via a short linker sequence. Like DNA binding transcription factors, studies in Human La have indicated a winged helix type fold for the LAM (20, 52). The RRM1 takes on the typical $\beta_1\alpha_1\beta_2\beta_3\alpha_2\beta_4$, fold routinely recognized in other RRM containing proteins. NMR-based studies and site specific mutagenesis findings have identified regions with highly conversed basic and aromatic residues in the LAM and RRM1 that were proposed to be sufficient for La targeting poly (U) containing substrates (53, 54). In addition, Teplova et al. have successfully resolved the NTD crystal structure of human La while bound to a short RNA (U1-G2-C3-U4-G5-U6-U7-U8-U9 OH-3') substrate ending in 3' polyuridylate tail giving further insight into the binding mechanism that La utilizes in associating with poly (U) tail containing substrates (55). While anticipated that the β sheet surface of the RRM1 motif or the typical recognition helix of the winged helix fold of the LAM would be essential in La-UUU-3'OH interaction, the co-crystal structure proved otherwise, suggesting the availability of these sites for a second potential La binding mode (55). Intriguingly, the results from the co-crystal structure revealed site specific contacts made to the poly (U) tail by highly conserved residues in the LAM and RRM1. A glutamine (Q20) and a tyrosine (Y23) residue in the LAM were shown to make uridylate specific contacts and stacking on the U-8 ($U_7U_8U_9$-3'OH) of the RNA substrate respectively, while phenylalanine residues (F35 and F55) were involved in base and sugar stacking on U-9, respectively. Contact made by a conserved aspartate (D33) residue to the 2'OH and 3'OH of U-9 enforces La to differentiate between RNA and DNA substrates as well as RNA substrates that have been trimmed by ribonucleases as they terminate with a 3'PO_4 (20, 55). While the LAM and RRM1 individually, weakly bind the RNA substrate, the formation of an alpha helix in the linker segment between the LAM and the RRM1 and subsequent rigidification of this site upon interaction with the poly (U) tail, contributes to the high affinity binding mode of La to poly (U) containing substrates (55). Aside from the contribution of RRM1 in high affinity binding of poly (U) tail containing substrates, studies in hLa have identified the role of RRM1, in particular loop-3 (interlink between β_2 and β_3 strands) in making UUU-3'OH independent contacts (Figure 5B). Previous studies have suggested that loop-3 is responsible for engaging the main body of tRNAs during pre-tRNA processing, in addition to the canonical aromatic residues in RNP-1 and RNP-2 of RRM1 that are typically used in RNA binding (20, 22). To date, structural and mutagenesis studies have summarized La binding to pre-tRNAs through two modes: the UUU-dependent and

UUU-independent binding mode which is facilitated through the high affinity binding of the LAM to the poly (U) tail of pre-tRNAs and secondly, through the loop-3 of RRM1 engaging the main body of pre-tRNAs (also associated with RNA chaperone activity) respectively (20, 22).

2.1.2 C-terminal domain

Contrary to the highly conserved NTD, the CTD of La is more divergent and this is partly linked to the expansion and complexity of this region in multicellular eukaryotes compared to unicellular eukaryotes (Figure 5A) (20, 24). In addition to RRM1, a second RRM has been identified in the CTD of multicellular eukaryotes with its function still under investigation. While studies with the NTD of hLa have revealed binding of the La module to nuclear encoded-poly (U) tail containing substrates, recent findings have elucidated a role for the CTD, specifically RRM2 of hLa in targeting cytoplasmic coding substrates. Distinct from the poly (U) tail binding mode, it was revealed that the LAM and RRM1 in conjunction with RRM2 are responsible for engaging the IRES domain IV of hepatitis C virus (HCV) mRNA (56). This binding mode was dictated through the recognition of structural elements on the RNA, specifically single stranded regions flanked by a double stranded stem loop, independent of the sequence of the RNA. These findings illustrate the plasticity in the binding modes that La utilizes in contacting various RNAs and a potential for La RNA chaperone activity on both nuclear and cytoplasmic targets (56).

In addition to RRM2 that is found in the CTD of higher eukaryotes of La, other elements such as the nuclear retention element (NRE), short basic motif (SBM) and nuclear localization signal (NLS) are also located C-terminal to RRM2 (20). The subcellular localization of La is under the control of the NRE and NLS which is positioned at the end of all La proteins with the exception of La in budding yeast in which the NLS is positioned more central in the protein (20). These elements not only assist with La shuttling but also directly influence the pool of RNA substrates that La engages. Defects in these regions have previously been linked to impaired pre-tRNA processing and maturation (57, 58). Adjacent to the NRE, the SBM motif is located which comprises of roughly 40 amino acids, harboring the site of phosphorylation at Ser-366 for hLa. The SBM serves as an additional RNA binding element that can alter the overall La-RNA interaction depending on the phosphorylation status of the motif by casein kinase II (CK2) at

Ser-366. Phosphorylated La has been mapped to the nucleoplasm, while non-phosphorylated La has been largely located in the cytoplasm. Other sites of phosphorylation have also been identified in various species such as mouse (T-302) and *S. cerevisiae* La (Ser-14, Ser-18, Ser-234), however the lack of conservation in the sites of phosphorylation across species suggests varying requirements for phospho-La (20, 59).

3. Pre-tRNAs: La's preferred nuclear targets

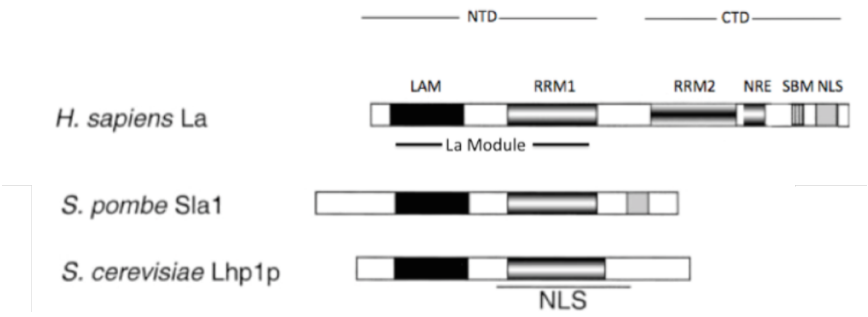
The role of tRNAs as adaptor molecules converting the genetic code to a functional polypeptide sequence has long been established. In addition, tRNAs have been characterized as multifaceted RNA substrates with the capacity to play essential roles in other biological processes such as serving as metabolic sensors in detecting nutritional stress, regulating apoptosis and functioning as signalling molecules in the general amino acid control pathway (GAAC) (60). As these multifunctional transcripts are crucial to the translational machinery, numerous biological processes and cellular survival, they are subject to a surplus of post-transcriptional processing and modifications which regulate the activity of these RNA substrates.

3.1 From transcription to post-transcriptional processing of pre-tRNAs

3.1.1 5' leader and 3' trailer processing

Nuclear tRNAs are products of RNA pol III transcription that are transcribed into their pre-mature state and subject to multiple cycles of processing before they are converted into their mature functional form (45, 61). While transcription of pre-tRNAs and termination of transcription by RNA pol III is highly conserved across species from vertebrates to yeast, some variations exist particularly in the length of oligo (U) generated at the 3'end of pre-tRNAs. Efficient transcription termination by RNA pol III is carried though the identification of an oligo (dT) tract at the 3'end of all transcripts (45). Interestingly, variability in the length of oligo (dT) requirement for transcription termination has been detected that is species specific. Moreover, while an oligo (dT) tract of four or five Ts is sufficient for complete transcription termination in *Homo sapiens*,

A



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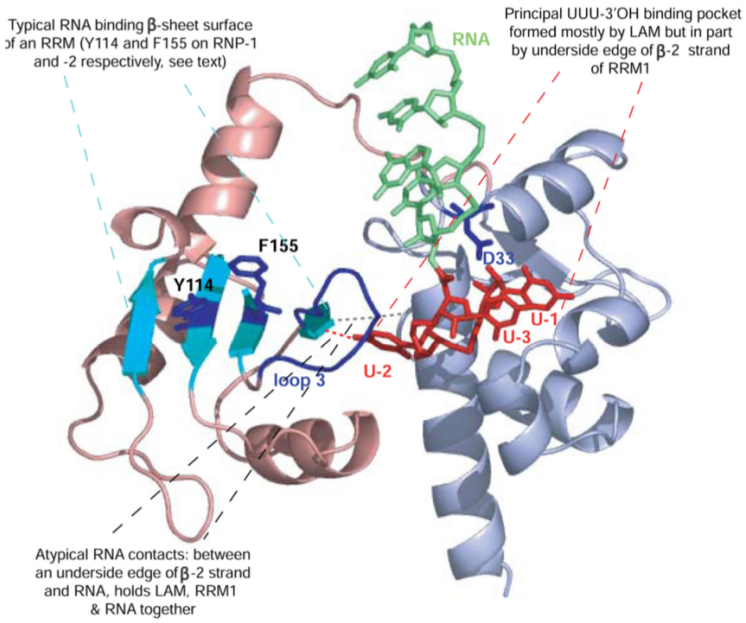

Figure 5. Sequence alignment of La in three eukaryotic species (Human hLa, *S. pombe* Sla1p, and *S. cerevisiae* Lhp1p) and a detailed crystallographic representation of hLa engaging a short poly (U) tail-containing RNA. (A) Sequence conservation in La is maintained in the NTD which comprises of the LAM and the RRM1, followed by an extended and more divergent CTD in higher eukaryotes possessing RNA binding elements such as the SBM as well as NLS and NRE which regulate the subcellular localization of La. (B) The UUU-3'OH dependent binding mode of La is illustrated with contacts made to U-1 and U-3 (shown in red) by primarily the LAM (shown in purple) and assisted by the β-2 strand of RRM1 (shown in turquoise). The UUU-3'OH independent binding mode is facilitated by loop-3 of RRM1 (shown in blue), available to engage the main body of pre-tRNAs and presumably other structured RNA substrates. Other sites in the LAM and RRM1 that assist RNA binding include residue D33 in the LAM and aromatic residues Y114 and F155 in the RRM1 which are essential in binding the 3'OH and 2'OH of terminal U (U-1) and aiding pre-tRNA processing respectively. Figure 5 (A) adapted from (24) and (B) adapted from (20).

S. pombe RNA pol III transcription termination relies on the detection of five to six oligo (dT) tracts, with the length of oligo (dT) required for *S. cerevisiae* RNA pol III termination exceeding six oligo (dT) tracts (43, 45). The variability in the length of oligo (U) generated at the 3'end of pre-tRNAs has been linked to the 3'-5' exoribonuclease activity of RNA pol III subunit, Rpc11p, with defects in this subunit showing increase in the length of oligo (U) tracts (43, 45).

La proteins are one of the first factors that contact pre-tRNAs upon transcription termination and this interaction is highly dependent on the length of 3' oligo (U) tract, with Human La associating with pre-tRNAs carrying 3 or more oligo (U) tract and *S. pombe* La requiring 4 or more oligo (U) tract for stable interaction (43, 45). Pre-tRNA processing can be guided through a La-dependent or La-independent pathway dictating the order of 3' trailer and 5' leader processing (61, 62). La binding to the 3' oligo (U) tail on pre-tRNAs, eliminates the competition between exonuclease (Rex1p or Rrp6p) and endonuclease (RNAse Z) cleavage of the 3' trailer sequence and commits the 3' trailer to endonucleolytic cleavage by RNAse Z (61, 62). In this fashion, the 5' leader is processed initially by the endonuclease RNAse P, while La remains bound to the 3' oligo (U) tract protecting the tRNA from exonucleases. Upon completion of 5' leader processing, RNAse Z cleaves the 3'end of the tRNA at N73 (discriminator base) preparing the 3'end for CCA addition (61). Since La is non-essential in unicellular eukaryotes and pre-tRNAs can be processed via a La-dependent or La-independent pathway, in the absence of La and for pre-tRNAs that do not engage La, the pre-tRNA is first subject to 3'trailer processing by exonucleases, followed by 5' leader processing by RNAse P. In preparation for tRNA aminoacylation, tRNA nucleotidyltransferase contacts 5' and 3' end-matured pre-tRNAs and is responsible for the addition of CCA to the 3' end of pre-tRNAs (61, 62). While nucleotidyltransferase catalysis occurs post-transcriptionally for yeast and vertebrate tRNAs, *E. coli* tRNAs are encoded with a 3' terminal CCA sequence. Nucleotidyltransferase (*CCA1*) is encoded in multiple isoforms which have distinct subcellular localizations. While the nuclear encoded CCA1 isoform is responsible for CCA addition to the 3' terminal end of pre-tRNAs, the cytoplasmic isoform is responsible for 3' end repair (61, 62).

3.1.2 pre-tRNA splicing

Although there is significant divergence amongst species in the presence or absence (0% intronic

regions in *E.coli* tRNAs) or even percentage (~5% in human genome compared to >20% and >50% in yeast and archaeal genome, respectively) of tRNAs that possess intronic regions, splicing of introns is unanimously considered essential for the functional activity of tRNAs (62). While eukaryotic tRNAs often possess intronic regions 3' to the anticodon (between nucleotide 37 and 38), introns have been found in other locations on tRNAs, particularly in archaeal tRNA genes (61, 62). Pre-tRNA splicing in yeast is divided into two major steps and is initiated by cleavage of the tRNA molecule by an endonuclease into two-half molecules at the site in which the exons are segregated by an intronic region, generating a 5' half with a 2'-3'cyclic phosphate and a 3' half with a 5'OH group. In yeast, tRNA splicing is catalyzed by a heterotetramer endonuclease Sen2, Sen34, Sen15 and Sen54, in which Sen2 and Sen34 are conserved between Archaea and humans, while Sen15 and Sen54 are entirely absent in Archaea and only poorly conserved between yeast and vertebrates (61, 62). The second step of tRNA splicing is compartmentalized into four sections and is catalyzed by the yeast protein Trl1. The initial step comprises of the hydrolysis of the 2'-3' cyclic phosphate that was generated in the 5' half of the tRNA molecule into a 2'phosphate by the cyclic phosphodiesterase activity of Tr1l (61, 62). This is then followed by the phosphorylation of the 5'OH group of the 3'half of the tRNA molecule. Subsequently, the 5' phosphate is adenylated and used as the site of ligation for the two halves of the tRNA molecule, leaving the 2' phosphate at the splice site which is lastly transferred to NAD by a phosphotransferase (Tpt1 in yeast) (61, 62) (Figure 6). While a similar mechanism of tRNA splicing has been detected in vertebrates, a second splicing pathway has also been identified in which a vertebrate ligase directly catalyzes the ligation of the two halve tRNA molecules using the 2'-3' cyclic phosphate. Although two pathways of tRNA splicing coexist in vertebrates, it is unclear which method of splicing is primarily utilized for tRNAs (61, 62).

3.2 tRNA post-transcriptional modifications

While differences exist amongst varying species in pre-tRNA processing, one intriguing commonality in all organisms is the abundance of tRNA modification enzymes that uniquely decorate tRNAs with the addition of chemical groups to the ribose sugar/base moieties, catalyzed by deamination, isomerization, glycosylation, thiolation or the addition of methyl groups (63).

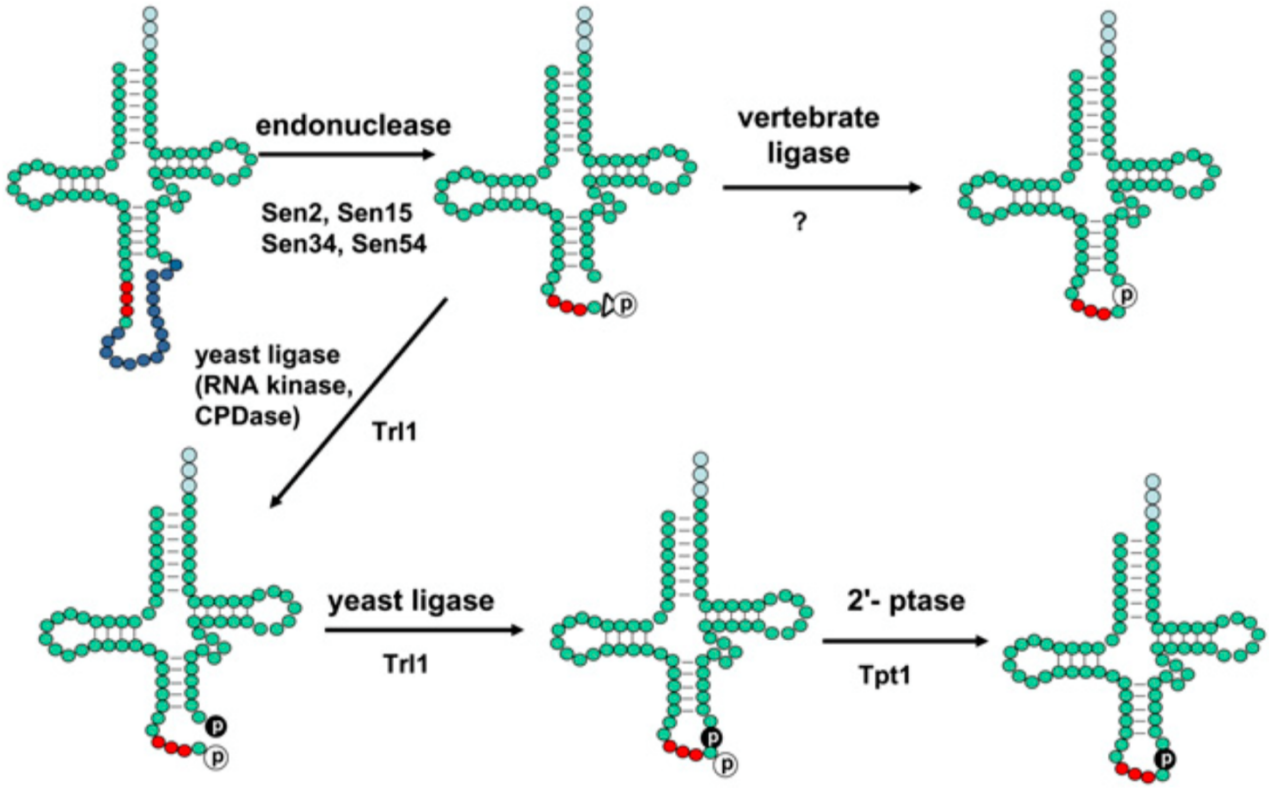

Figure 6. tRNA splicing pathway. tRNA splicing in yeast is initially catalyzed by the Sen complex which is an endonuclease, comprising of Sen2, Sen15, Sen34 and Sen54 which generates two half tRNA molecules through the endonucleolytic cleavage at the junction of the intronic/exonic region generating a 5' half bearing a 2'-3' cyclic phosphate and a 5'-OH created at the 3' half of the tRNA molecule. Introns are illustrated by blue circle and the white circle labeled "p" with a triangle depicts the 5' half with the 2'-3' cyclic phosphate. Yeast ligase Trl1, facilitates the next four steps, by (1) hydrolysis of the 2'-3' cyclic phosphate generating a 2' phosphate (2) phosphorylation and (3) adenylation of the 5'- OH and (4) final ligation of the 3' and 5' half of the tRNA molecule. The 2' phosphate remaining at the splice site is subsequently removed by Tpt1, yeast phosphotransferase. Vertebrates utilize the same mechanism in yeast for splicing but also carry a second splicing machinery in which a vertebrate ligase can directly ligate the 2'-3' cyclic phosphate to the 5'-OH of the 3' half of the tRNA. Adapted from (61).

Previous analysis of 561 sequenced tRNA genes, collected from various organisms (archaea, eubacteria, fungi, animals and plants) has elucidated the universal modification of tRNAs in all phylogenies with a median of 8 modifications per tRNA (61). Although the prevalence of tRNA PTMs has been identified, challenges exist in determining the role of individual modifications on tRNAs. Firstly, not all tRNAs are uniformly modified by the same enzyme, and modification by a particular enzyme can vary not only between different tRNA groups, but also tRNA isoacceptors and even vary between similar tRNA families in different organisms from the same domain of life (63). Modification enzymes have been identified that specifically catalyze the addition of a functional group to a particular nucleoside, while other enzymes have been shown to function more promiscuously (63). Lastly, the over-abundance of PTMs on tRNAs *in vivo* exacerbates the challenges in determining the unique functional role of each modification enzyme as they likely function redundantly with one another in supporting the overall structure and function of these adaptor molecules (61, 63). Extensive research in this field has led to the categorization of tRNA PTMs into two classes: modification enzymes that assist tRNA folding and structural stability and enzymes that directly impact tRNA functional activity and mRNA decoding (61, 63).

3.2.1 Post-transcriptional modifications support tRNA structural stability

The L-shaped three-dimensional structure of tRNAs is maintained through an interplay of tertiary interactions and a plethora of functional chemical groups attached onto the nucleotides by numerous modification enzymes. Decades of research consensually agree upon the role of PTMs on the core body of tRNAs in supporting structural stability, rigidity and flexibility of the cloverleaf structure (61, 63). Example studies have shown that while one modification such as pseudouridylation can enforce rigidification of the tRNA structure, in opposition the presence of a dihydrouridine can confer flexibility. These intriguing findings delineate the role of modification enzymes in cooperatively devising the most suitable tRNA structure dependent on cellular conditions (63). More specifically, previous findings have demonstrated the requirement for flexibility-inducing dihydrouridines in tRNAs from psychrophilic archaea compared to their thermophilic archaea family. Other studies have shown an overall change in modification levels on tRNAs that is related to alterations in growth temperature, reinforcing the idea that tRNA modification can act as a regulatory element, controlling tRNA structural conformation (63). *In*

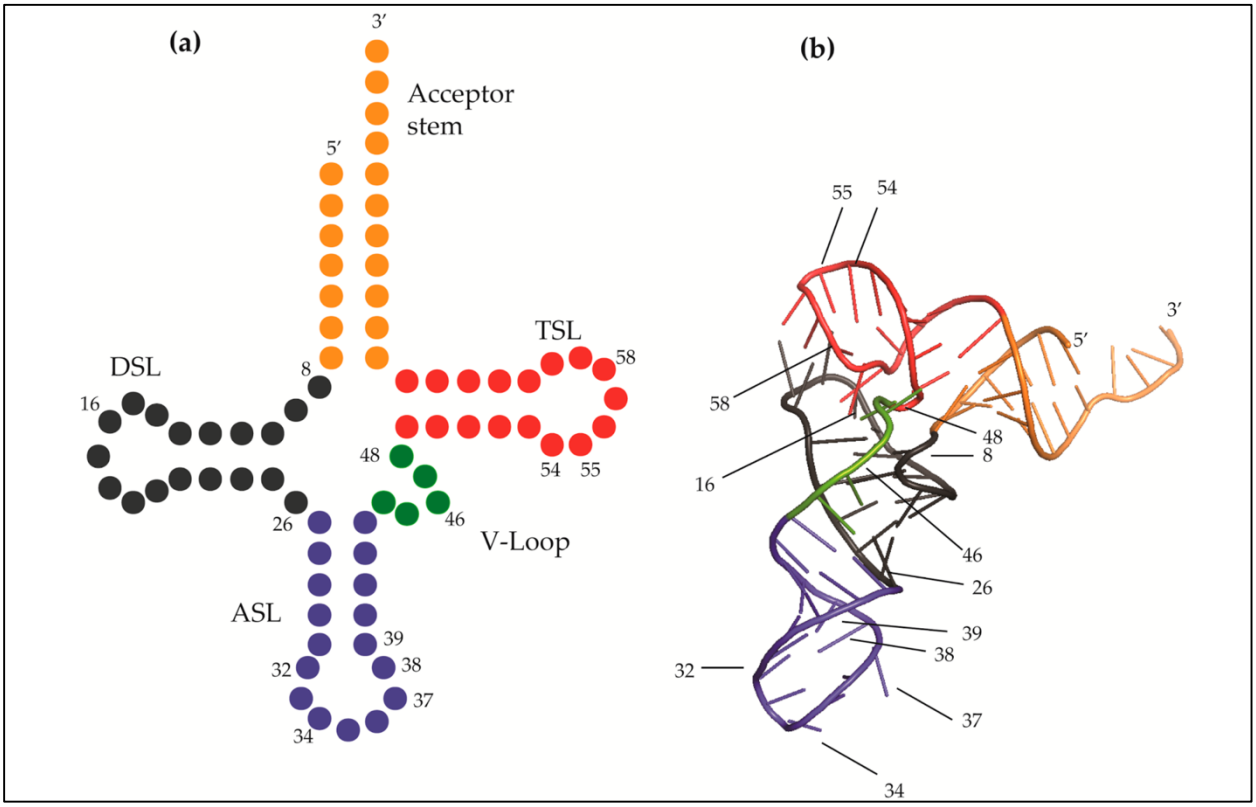

Figure 7. General secondary and tertiary structure of tRNAs. A) Secondary cloverleaf structure of yeast initiator tRNA. Acceptor stem (orange), D-stem/loop (DSL, black), anticodon stem/loop (ASL, purple), variable loop (V-loop, green) and TΨC stem/loop (TSL, red) have been labeled. Some sites of tRNA post-transcriptional modifications have been indicated (e.g. N16, N26, N32 etc.). B) Tertiary L-shaped structure of tRNA. The colors correspond to the respective sites on the cloverleaf, secondary structure. The sites of some PTMs have been indicated (e.g. N26 corresponding to possible site of G26 dimethylation by Trm1p). Figure adapted from (64).

vitro studies, also demonstrate a link between PTMs of tRNAs and structural stability. A natively modified tRNA is structurally more stable and has ~ 5°C higher Tm in comparison to an unmodified tRNA (61).

Investigating the essential role of individual modification enzymes in providing structural stability for tRNAs has proven to be challenging as the deletion of a single modification enzyme does not entail phenotypic growth defects. Studies in yeast have shown the redundant functionality of modification enzymes, as only the combination of modification gene deletions leads to synthetic lethality and only as a result of defects in a particular tRNA and not all tRNAs modified by the enzymes of these deleted genes (63). To exemplify this scenario, yeast cells depleted of Trm8 and Trm4 (catalyze methylation; m^7G46 and m^5C49 respectively) display a phenotypic growth defect and temperature sensitivity which has been linked to the degradation and deacylation of mature tRNA-Val^{AAC}. While other tRNAs such as tRNA-Phe , $tRNA_i$-Met and tRNA-Val^{CAC} are all targets of Trm8 and Trm4, the synthetic lethal phenotype is directly linked to structural defects imposed on tRNA-Val^{AAC} (61, 65, 66). Similar growth defects and temperature sensitivity have also been reported for a combination of other modification enzymes, including Tan1 and Trm44 which catalyze the N-4 acetylcytidine formation (ac^4C12) and 2'O methylation (Um44) on tRNAs, respectively (67, 68). Although, tRNA-Ser^{CGA}, tRNA-Ser^{UGA} and tRNA-Ser^{IGA} are all modified by Tan1 and Trm44, the synthetic lethality has been connected to the degradation of tRNA-Ser^{CGA} and tRNA-Ser^{UGA} (61).

While many modification enzymes have reported to function redundantly with one another and have shown to cumulatively cause structural defects for tRNAs in their absence, other modification enzymes have been reported to function redundantly with the RNA chaperone, La. Anderson et al. have demonstrated that Trm6/61 (Gcd10/Gcd14) which catalyzes the methylation of A58 (m^1A58) on pre-$tRNA_i$-Met and 17 other tRNAs (including elongator pre-tRNA-Met) is essential for the steady state levels of pre-$tRNA_i$-Met such that in *trm6/61-* cells, there was a reduction in mature pre-$tRNA_i$-Met levels as a result of increase in turnover of the initiator tRNA. Intriguingly, this phenotype was reversible with either overexpression of pre-$tRNA_i$-Met or overexpression of LHP1 (*S. cerevisiae* La homolog) (61, 69). In addition to this finding, another methyltransferase (tRNA methyltransferase 1; Trm1) has shown overlapping function with La in supporting tRNA structural stability (70). Trm1p catalyzes the N2, N2-dimethylation of G26 on several tRNAs (71). Trm1p can modify both intron-containing and intron-less tRNAs and its

activity is transcriptionally regulated such that the subcellular localization and targeting of this enzyme is dependent on the transcription of Trm1 with or without an extended 5' N-terminal region. The short transcript without the 5' extended N-terminal region, commits the enzyme to the inner nuclear membrane, while the 5' extended N-terminal region allows for shuttling to the mitochondria (62, 72). Previous studies in *S. cerevisiae* have revealed that the presence of G26 at the junction of the D-stem and the anticodon stem do not constitute as the determining factor for Trm1p modification and that this methyltransferase displays tRNA substrate specificity towards tRNAs that display a favorable 3D structure surrounding G26. These studies identified two determining factors for Trm1p modification on tRNAs: 1) The presence of a GC rich region preceding G26 in the D-stem in the form of C11 pairing with G24 and G10 pairing with C25 and 2) the length of the variable loop in tRNAs facilitating an overall favorable 3D structure such that tRNAs bearing < 5nt in the variable loop are considered unsuitable substrates of Trm1p and were identified as tRNAs that are non-Trm1p targets (eg. tRNA-Gly^{GCC}, Gln^{TTG} and Gln^{CTG}) (71, 73, 74). tRNAs that possess these elements are potential substrates of Trm1p and undergo dimethylation by the enzyme such that the N2 atom of the G26 residue is dimethylated in a reaction assisted by the methyl donor S-adenosylmethionine (SAM) ((73); Figure 7A and B). In addition to these preliminary studies carried two decades ago, more recent findings have added a level of confidence in the substrate specificity that Trm1p displays depending on tRNA identity. More specifically, recent next-generation tRNA sequencing (tRNA-HydroSeq) results, assaying mature tRNA sequences in *S. pombe* identified a list of 27 tRNAs from a total of 36 G26 containing tRNAs to be significantly modified by Trm1p (modification was measured as a factor of misincorporation by reverse transcriptase incorporating a "non-C" nucleotide at position 26 in the presence of m_2^2G26; Figure 7C). These findings are of significant interest as unlike other modification enzymes that display minimal substrate specificity (eg. yeast Pus1 modifies many tRNAs at various positions U1, U26-28, U32 ,U34-36, U65 and U67 and Trm4 which catalyzes methylation of C34, 40, 48,49 on numerous tRNAs), Trm1p not only specifically catalyzes modification exclusively at G26 but also does this in a manner related directly to tRNA identity (61, 71).

Although the evolutionary conservation of the cloverleaf structure of tRNAs has led to the assumption that all tRNAs are confined to this secondary structure, studies have shown that some mitochondrial tRNAs fold into alternate secondary structures, different from what is uniformly observed in cytosolic tRNAs (75). Mitochondrial tRNAs can take on a type 5 and 7 fold, distinct

from the canonical type 6 fold found in cytosolic tRNAs which carry 6 base pairs in the anticodon stem (75). Interestingly, further studies revealed that while these alternate configurations were originally identified for some mitochondrial tRNAs, some cytosolic tRNAs studied in unicellular eukaryotes (eg. tRNA-Lys, Arg and Ala) may also commit to these alternate folds. Cytosolic tRNAs that are subject to folding into mitochondrial type conformations (type 5 in addition to the canonical type 6 fold) all possess a dimethylated G26 (75). Type 6 tRNAs display base pairing between nucleotide 26 in the D-stem and 44 in the anticodon stem, whereas nucleotides 26 and 11 are involved in a base pairing interaction in the mitochondrial type 5-fold tRNAs (75). These pairing rearrangements in cytosolic tRNA folding into type 5 conformation has been speculated to cause conformational instability for the tRNA and label the tRNA aberrant. Further analysis of these cytosolic tRNAs revealed that N2, N2-dimethylation at G26 eliminates the potential for G26 to interact with N11 and invariably commits G26 to base pairing interactions with A, U or G at N44 (with some exceptions C) driving the formation of the canonical type 6 cytosolic tRNA conformation (75).

With plentiful evidence demonstrating the role of Trm1p in supporting tRNA structural stability, the overlapping function of this PTM enzyme with La as an RNA chaperone has further reconfirmed its functionality. While it has been repeatedly shown that La is involved in tRNA biogenesis and pre-tRNA processing and maturation, its' dispensability in unicellular eukaryotes has been puzzling. In search for factors that function redundantly with La, it was shown that LHP1 becomes essential for survival in *trm1-* cells subject to growth under elevated temperatures (70). This finding suggested the presence of one or more essential tRNAs that rely on either the presence of La or Trm1 for structural stability and to potentially divert these essential tRNAs from forming inactive conformations by stabilizing the anticodon stem (70).

In addition to the above mentioned PTMs (Trm1, Trm4, Trm8, Trm6/61, Trm44, Tan1 and Pus1) numerous other PTMs can be exemplified that are beyond the scope of this review that would demonstrate the crucial role of these enzymes and other RNA chaperone-like proteins that are essential in pre-tRNA processing and structural stability. While the cloverleaf structure is highly conserved, minor deviations from this structure would lead to inactive tRNA substrates that are either degraded or remain inactive and inoperative for the translational machinery.

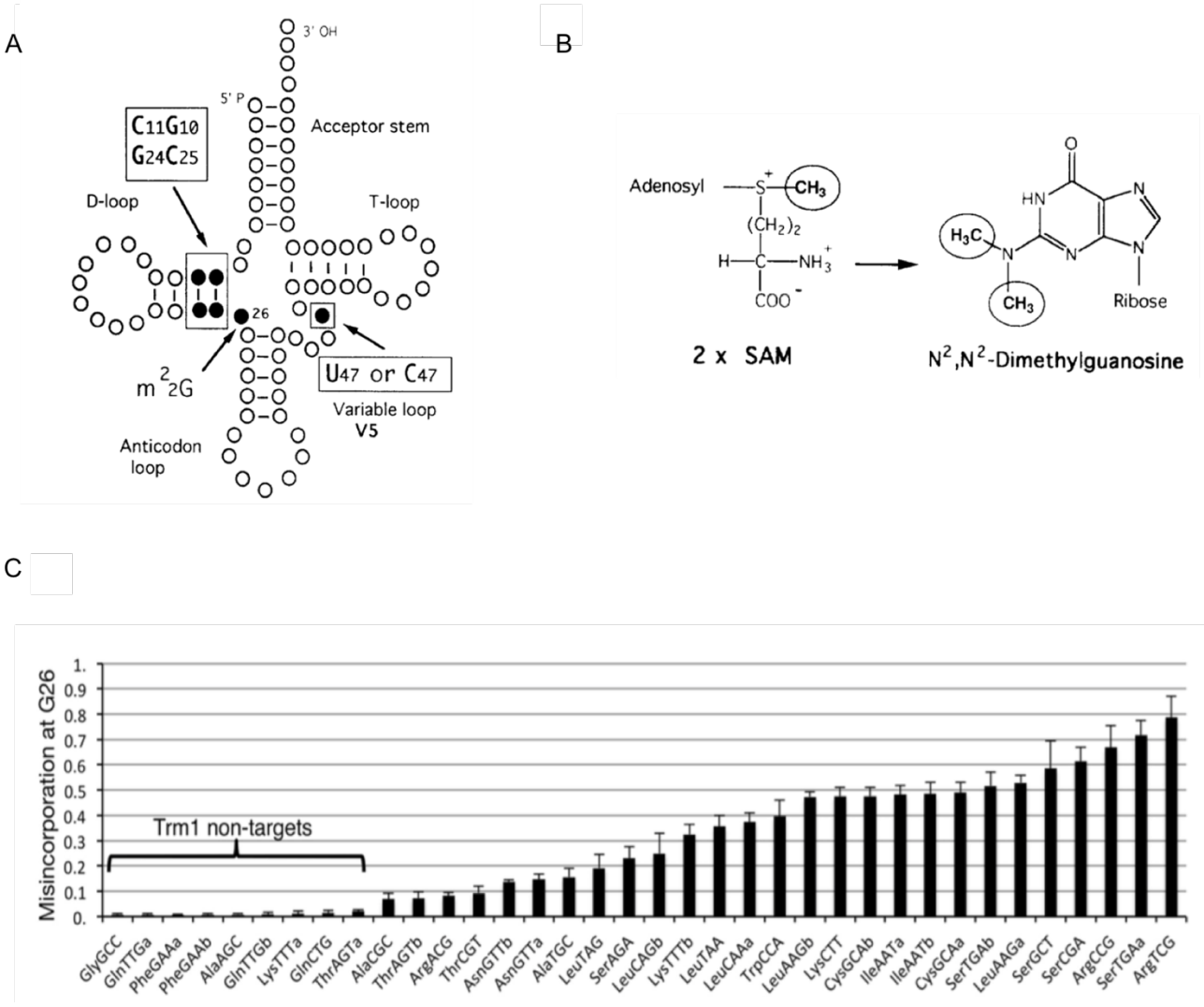

Figure 8. tRNA identity elements associated with specific m_2^2G26 modification by Trm1p. (A) N2, N2-dimethylation of G26 at the junction of the D-stem and the anticodon stem is favored by the presence of a GC rich region preceding G26 (C11:G24 and G10:C25) in conjunction with a variable loop possessing a minimum of 5 nucleotides (increase in the length of variable loop from 4 to 5 with the insertion of either U47 or C47 produced favorable conditions for Trm1p modification of tRNAs). (B) SAM facilitates the transfer of two methyl groups to the N2 atom of G26 in the Trm1p catalyzed formation of m_2^2G26. (C) tRNA-HydroSeq results identified 27 G26 containing *S. pombe* tRNAs as Trm1p targets (measured by the level of misincorporation detected at G26) and 9 G26 containing tRNAs as non-Trm1p targets. Figure 7A&B adapted from (73) and Figure 7C adapted from (71).

3.2.2 Post-transcriptional modifications regulate tRNA activity in mRNA decoding

The translational machinery has evolved to successfully produce elaborate protein sequences with minimal error with the assistance of tRNA molecules involved in decoding of the mRNA transcript. PTM of tRNAs, particularly in the anticodon loop region has been recognized as an elemental factor in avoidance of frame-shift mutations and ribosomal pausing with a post-transcriptionally modified tRNA in the anticodon loop increasing translation fidelity and enhancing codon-anticodon interactions (76). Two sites that are unanimously modified in almost all tRNAs, in all organism is nucleotides at position 34 and 37 in the anticodon loop. Modifications made to N37, block potential Watson-Crick interactions between N37 and N33. This results in a U-shape, open loop conformation in the anticodon loop of tRNAs, an essential conformation in enhancing codon-anticodon interactions and avoidance of a frameshift mutation. N37 on tRNAs is occupied by a purine and depending on the presence of A or G, appropriate modifications are made to the tRNA aiming for the same outcome; inhibition of inappropriate base pairing interactions, avoidance of frameshift mutation and maintaining translation fidelity (63, 76). While various chemical groups have been identified that modify the purine residue at N37 (isopentenyl adenosine i^6A, N-1-methylguanosine m^1G, wybutosine yW, N^6- threonylcarbamoyladenosine t^6A), the most commonly reported modification is the methylation of G37 which is present in tRNAs in all domains of life. This modification is facilitated by the methyl donor SAM and is catalyzed by the methyltransferase Trm5p in Eukaryotes (61, 63). Hypomodification of tRNAs at G37 has been correlated with a frameshift mutation, imposing translational error (63, 77). Prevention of frameshift mutations is not the only purpose of methylation at G37, but rather this PTM also serves as the starting point for subsequent modification at this site such as the formation of a hypermodified tRNA-Phe bearing a wybutosine nucleoside (63, 77). In the presence of an adenine nitrogenous base at position 37, the common PTM identified on tRNAs is the isopentenylation of adenine in the formation of i^6A which mediates translation efficiency (63).

In addition to PTMs made to N37 that ensure in-frame translation of the mRNA transcript, modifications made to N34 in the anticodon loop adds an additional layer of confidence to translation fidelity by rigidifying the anticodon-codon interaction. Numerous PTMs have been previously reported at N34 (methylation, acetylation, pseudouridylation etc.) but the most well

characterized modification is the hypermodification of U34 which includes a cascade of enzymes that cooperatively modify this base (63). U34 is initially modified by the elongator complex for the formation of 5-carboxy-methyluridine (cm^5U) which is a substrate for Trm9 (in conjunction with Trm112) which catalyzes the synthesis of 5-methoxycarbonyl-methyluridine (mcm^5U). Modification at U34 has been noted in several tRNAs including tRNA-Lys^{UUU}, Gln^{UUG} Glu^{UUC}, Arg^{UCU} and Gly^{UCC} (63, 78). For tRNAs-Lys^{UUU}, Gln^{UUG} and Glu^{UUC}, mcm^5U34 is subsequently thiolated to produce mcm^5S^2U34 (78). Quantitative proteomics studies in yeast have revealed that Trm9 modification of U34 is necessary for native expression levels of certain proteins enriched in AGA and GAA codons corresponding to conserved Trm9 tRNAs substrates (tRNA-Arg^{UCU} and Glu^{UUC}). These studies demonstrated that *trm9-* cells showed a general shift in translation of a subset of proteins, with repression of translation noted for the transcripts containing codon clusters of AGA and GAA. Although, not all Trm9 tRNA substrates (tRNAs translating GGA, GGG, AGG and AAA codons) were affected in their decoding capacity in *trm9-* cells, these findings are clear evidence of the role of PTMs in regulating tRNA activity in protein expression *in vivo* (78).

Other recent evidence for the role of PTMs in fine-tuning tRNA activity has also been made with Trm1. TMS assays have been used widely in assaying suppressor tRNA biogenesis and activity (47, 71). Arimbasseri and colleagues have shown that the activity of the opal suppressor tRNA-Ser^{UCA} in suppressing a nonsense codon in the adenine synthetic gene (*ade6-704*) relies on the PTM of the suppressor tRNA by Trm1 (71). Moreover, their studies demonstrated that the overall upregulation of tRNA transcription in *maf1-* cells (highly conserved global repressor of RNA pol III) concomitantly results in antisuppression due to limiting levels of Trm1 and an overabundance of Trm1 substrates such as suppressor tRNA-Ser^{UCA} which rely on Trm1 modification for their activity (71). These intriguing insights suggest a potential role for Trm1, a PTM enzyme that modifies G26 on tRNAs, distant from the anticodon loop in playing a pivotal role in fine-tuning tRNA activity in mRNA decoding, in addition to the already characterized role for Trm1p in supporting tRNA structural stability.

3.3 tRNA turnover

During the entirety of tRNA biogenesis, from the time of transcription to 3' and 5' end processing, splicing and undergoing PTMs, tRNAs are under surveillance for their structural integrity. As these

adaptor molecules play an essential role in mRNA decoding, the cell has evolved to implement mechanisms in which aberrant tRNAs are subject to degradation and prevented from participating in the translational machinery (62). A well characterized example of pre-tRNA degradation has been made with initiator pre-tRNA-Met that is hypomodified at A58 (69, 79). Anderson et al., showed a decrease in the levels of mature $tRNA_i$-Met in *trm6/61* mutants that was attributed specifically to the hypomodification of $tRNA_i$-Met at A58 and its subsequent degradation by the nuclear exosome and the TRAMP complex (Trf4/Air2/Mtr4p polyadenylation) (69, 79). Nuclear degradation of aberrant tRNAs such as hypomodified $tRNA_i$-Met is initiated by the polyadenylation of the tRNA by Trf4 poly (A) polymerase which is mediated through the RNA binding capacity of zinc knuckle RNA-binding proteins Air1p or Air2p (79, 80). Upon polyadenylation of the tRNA by Air1/2p-Trf4p, the RNA helicase Mtr4p locates a 4 - 5 single stranded poly (A) extension at the 3' end and begins unwinding the tRNA. At this stage, the tRNA is exposed to the two exonucleases of the nuclear exosome and is considered a favorable substrate for the 3' exoribonucleases Rrp6p or Rrp44 (45, 80). In addition to hypomodified pre-tRNAs serving as substrates for degradation by the nuclear exosome, inappropriately processed pre-tRNAs are also targeted by the TRAMP complex and the nuclear exosome (61). Pre-tRNAs are safe from 3'-5' exonuclease decay during processing, if they possess a 3' poly (U) tail, long enough in length for efficient La binding. In this way, the pre-tRNA is processed by the endonuclease, RNase Z which is involved in 3' trailer processing and generating a single unpaired nucleotide at the 3' end of the tRNA, an unfit substrate for Trf4p (45, 61).

More recent studies in alternative pathways of tRNA turnover, independent of the TRAMP complex, led to the identification of two 5'-3' exonucleases (*rat1* and *xrn1)* in co-operation with met22 (enzyme in the methionine biosynthesis pathway) to be the elemental factors in degrading structurally defective hypomodified tRNAs (Figure 8) (61, 80). The role of these exonucleases in rapid tRNA decay (RTD) were made clear in *trm4- trm8-* (m^5C49 and m^7G46) as well as *tan1- trm44-* cells (Um44 and ac^4C12) with depleted levels of mature tRNA-Val^{AAC} and tRNA-$Ser^{CGA/UGA}$ respectively (65–67). The specificity of the RTD pathway for structurally defective and unstable tRNAs has been demonstrated by Whipple and colleagues reporting that only tRNA-$Ser^{CGA/UGA}$ is subject to degradation in *tan1- trm44-* cells , even though tRNA-Ser^{IGA} is also a recognized substrate of Tan1 and Trm44 (81). Further investigation revealed that modification by Tan1 and Trm44 increase the structural stability of tRNA-Ser^{CGA}, particularly in the acceptor and T-stem of

the tRNA and in this manner protects the tRNA from decay (81). Conversely, tRNA-Ser^{IGA} is not reliant on Tan1 and Trm44 for structural stability and therefore remains active and protected from RTD in *tan1- trm44-* cells (62). As Xrn1 is located in the cytoplasm and Rat1 is nuclear, the location of degradation of tRNA substrates of the RTD pathway has been puzzling. Since deletion of just Xrn1 or Rat1 only leads to partial rescue of these hypomodified tRNAs, and only the deletion of both Xrn1 and Rat1 in conjunction with Met22 leads to complete stability of these tRNAs, it has been proposed that RTD can target structurally defective tRNAs both in the nucleus and the cytoplasm (62).

3.4 tRNA subcellular localization

3.4.1 pre-tRNA splicing: nuclear or cytoplasmic?

Preliminary studies on tRNA biogenesis viewed precursor-tRNA processing to mature-tRNAs as a simple unidirectional process; transcription by RNA pol III, 3' and 5' end processing, PTMs on nucleosides and splicing, all of which presumably occurred in the nucleus followed by transfer of these tRNAs to the cytoplasm for aminoacylation and participation in the translation machinery (82). Decades of studies in tRNA nuclear-cytoplasmic localization have diverged from this mindset, demonstrating a more complex machinery involved in the subcellular localization of tRNAs, that is highly linked to cellular conditions (82). It is now known that pre-tRNAs are initially subject to 3' and 5' end processing and then further maintained in the nucleus for tRNA splicing in the case of vertebrates or transported to the cytoplasm for splicing in the case of yeast tRNAs. The finding that the yeast splicing machinery does not reside in the nucleus and is rather found in the cytoplasm (ligase and the 2'phosphotransferase) and the outer surface of the mitochondria (endonuclease), in combination with the lack of conservation in the localization of tRNA splicing machinery between yeast and vertebrates led to an expanded interest in tRNA subcellular dynamics.

3.4.2 tRNA nuclear export

To accommodate pre-tRNA splicing in yeast and the transfer of spliced pre-tRNAs in vertebrates

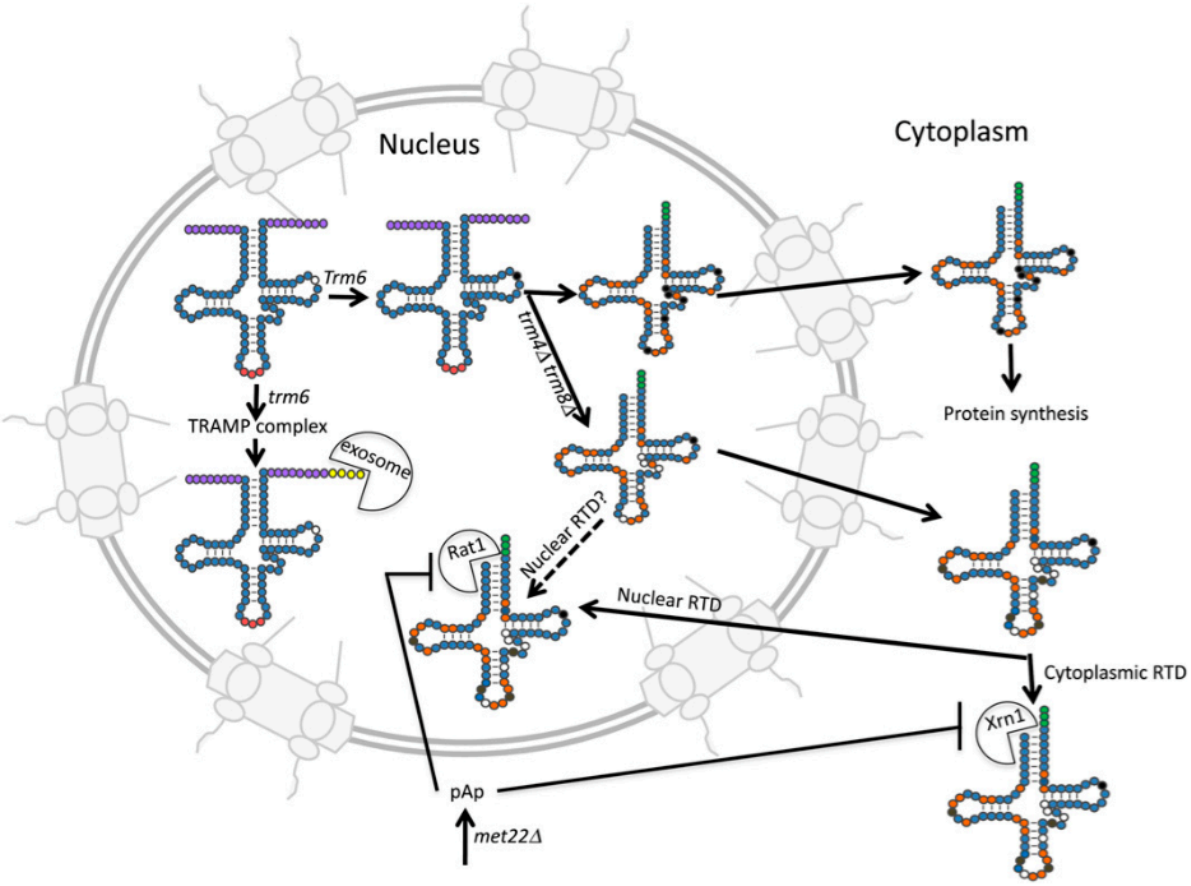

Figure 9. Model for structurally defective and unprocessed tRNAs subject to nuclear and cytoplasmic decay in *S. cerevisiae*. Upon transcription termination, pre-tRNAs undergo processing at the 5' leader and 3' trailer (purple circles) as well as acquiring PTMs on their nucleoside that aid in the structural stability and activity of the tRNA. Poorly processed pre-tRNAs (not shown), or lack of an essential PTM (white circle) such as m^1A58 results in the nuclear decay of the tRNA via initial polyadenylation of the tRNA by the TRAMP complex (yellow circles) and subsequent degradation by 3'-5' exonucleases of the nuclear exosome. Structurally defective tRNA or tRNA lacking more than one PTM (white circles) that is essential for its structural stability (eg. hypomodified C49 and G46 in *trm4- trm8-* cells) is also targeted for decay, both in the nucleus and the cytoplasm through the RTD pathway. Rat1 (nuclear) and Xrn1 (cytoplasmic) are the 5'-3' exonucleases in charge of degradation of these aberrant tRNAs. Rat1 and Xrn1 are inhibited in *met22-* cells as a result of the presence of pAp, a by-product of the methionine biosynthesis pathway. Modified residues in the nucleus and the cytoplasm are shown in orange and brown circles respectively. Green circles depict processed, CCA containing tRNAs, and the anticodon is represented with red circles. Figure adapted from (62).

to the cytoplasm, pre-tRNAs associate with transport receptors (importin-β family member; exportin) that facilitate their export from the nucleus to the cytoplasm in a Ran-GTP dependent manner (83). Ran is a GTPase that is under the control of its regulators that reside in the nucleus (RanGEF; Ran guanine nucleotide exchange factor), and the cytoplasm (RanGAP; Ran GTPase activating protein), controlling whether Ran is bound by GDP or GTP (82, 84). These regulators enforce a Ran-GTP gradient across the two interface such that Ran-GTP levels are high in the nucleus and low in the cytoplasm, in this way allowing for import and export of tRNAs to and from the nucleus to the cytoplasm (82, 84). Pre-tRNA export in budding yeast is facilitated by the exportin Los1 (Xpo-t in fission yeast; exportin-t in vertebrates) that binds the trimeric complex which consists of the 3' and 5' end processed pre-tRNA bound to Ran-GTP in the nucleus, and delivers this complex to the cytoplasm. Once in the cytoplasm, Ran-GAP hydrolyzes Ran-GTP to Ran-GDP which results in the disassembly of the exportin-pre-tRNA complex and the recycling of Los1 for the transport of other tRNAs to the cytoplasm (82, 84). Crystallographic studies of *S. pombe* Xpo-t in association with tRNA and Ran-GTP have reported the specificity of the exportin not only towards intron-containing, 5' and 3' end processed tRNAs but more particularly towards the dihydrouridine (D-loop), TΨC loop and the aminoacyl-stem of a correctly structured tRNA (62, 83). These findings are intriguing in that tRNA export by Xpo-t (and other exportin homologs) inhibits the entry of immature or damaged tRNAs to the cytoplasm, the site of protein synthesis (82). In addition to tRNA export and quality control, the localization of Los1 and its availability for tRNA transport from the nucleus to the cytoplasm is linked to cellular conditions (62). This is exemplified when cells are exposed to unfavorable growth conditions, such as glucose starvation and growth under a non-fermentable carbon source, leading to depletion of tRNA levels in the cytoplasm (62, 83). This was linked to both the downregulation of RNA pol III transcription of tRNAs but also by regulation of tRNA nuclear export by means of relocating Los1 to the cytoplasm where it is unable to facilitate tRNA-nuclear export (62). Similarly, Los1 confinement to the cytoplasm under other stress conditions such as DNA damage has also been reported, again regulating the level of tRNAs in the cytoplasm available for protein synthesis under unfavorable cellular conditions (62).

While Los1 has been implicated in tRNA export, quality control and subcellular trafficking of tRNAs based on cellular conditions, it is found to be non-essential in yeast, in contrast to exportin-t which is essential and serves as the major exporter of tRNAs in vertebrates (62). Another

class of importin-β family of transport receptors in yeast that participates in tRNA nuclear export is Msn5. Like Los1, Msn5 transports tRNAs across the nucleopore using the Ran-dependent pathway of transport (62, 83). Although, *Los1-/ Msn5-* cells display no growth defects, indicating the presence of other undiscovered exportins in tRNA nuclear export in yeast, the overlapping function of Los1 and Msn5 is evident by the increase in the pool of tRNAs in the nucleus in the absence of both exportins (62, 83). However, Msn5 unlike Los1 displays minimal affinity towards intron-containing tRNAs and has been proposed to be responsible for transport of tRNAs that are either intron-less or intron-containing tRNAs that have already been transported to the cytoplasm for splicing and are in the nucleus, aminoacylated and ready for transport back to the cytoplasm (62).

3.4.3 Retrograde pathway: re-import to nucleus and re-export to cytoplasm

The unanticipated finding that the yeast tRNA splicing machinery is cytoplasmic and that nuclear spliced tRNA pools increase in nutritional stress conditions (glucose or phosphate deprivation), the presence of tRNA aminoacylation inhibitors or defects in aminoacylation led to the discovery of tRNA nuclear re-import and re-export; the retrograde pathway (82, 83, 85). While much has yet to be answered for the role and the protein factors involved in the retrograde pathway, based on current data it is widely speculated that the retrograde pathway serves to 1) regulate protein synthesis based on cellular conditions with controlling tRNA levels in the cytoplasm, and 2) as an additional form of quality control, ensuring only mature and correctly structured, functionally active tRNAs access the cytoplasm and participate in translation (83). These propositions have emerged from previous studies reporting, the increase in nuclear pool of tRNAs under nutritional stress (also with defects in aminoacylation) in conjunction with the relocation of mRNAs to P-bodies from polysomes under conditions of glucose starvation (86, 87). The relocation of mRNAs and tRNAs during cellular stress conditions and the re-export of tRNAs to the cytoplasm and concurrent relocation of mRNAs back to polysomes with a replete of glucose have led to the speculations of the retrograde pathway playing a role in regulating protein synthesis based on cellular conditions (82, 83). It should be noted that while these independent findings have led to plausible propositions for role of tRNA retrograde pathway in protein synthesis regulation, further studies have yet to clearly verify this (83).

To address the second plausible role of the retrograde pathway as an additional quality control mechanism, it has been suggested that tRNA processing and modification which primarily occurs in the nucleus is in direct competition with tRNA nuclear export which can lead to inaccuracy in processing of the tRNA and the export of an immature, hypomodified tRNA to the cytoplasm (83). Kramer and colleagues put this theory to test by reporting the accumulation of two types of aberrant tRNAs (5', 3' end-extended, spliced tRNAs and hypomodified tRNAs) in cells with defects in tRNA nuclear import factor (88). In addition to these findings, it has been reported that *mtr10-/xrn1-* cells (mtr10; nuclear import and xrn1; 5'-3' exonuclease) are inviable suggesting that the RTD and the retrograde pathway may function in parallel in removal of aberrant tRNAs from the cytoplasm (88).

Taken all together, the cell employs several mechanisms in ensuring the presence of only mature, functionally active tRNAs in the cytoplasm. The first quality control check of tRNAs is met through the nuclear exosome and the TRAMP complex which degrade hypomodified $tRNA_i$-Met lacking m^1A58 and tRNAs with improperly processed 3' ends (82, 88). Subsequently, Los1 monitors 5', 3'end processed unspliced tRNAs with a correctly structured D-loop, TΨC loop and the aminoacyl stem for tRNA export but displays no particular affinity for tRNAs bearing or lacking nucleoside modifications (82, 88). The third opportunity for tRNA quality control involves the RTD pathway which degrades structurally unstable tRNAs that lack particular modifications and this can occur both in the nucleus and cytoplasm (65, 66). Lastly, and the more recently discovered quality control mechanism that appears to function in parallel with the RTD pathway is the retrograde nuclear import mechanism which recognizes 5', 3'end processed, spliced and hypomodified tRNAs and is responsible for re-import of aberrant tRNAs to the nucleus (88). Although, it is now clear that the retrograde pathway functions in the delivery of aberrant tRNAs to the nucleus, the fate of these tRNAs is still a mystery; they may be subject to tRNA repair or degraded by the TRAMP complex or the nuclear RTD (88).

As the retrograde pathway in tRNA subcellular localization is a more recently discovered phenomenon, there are still debates on factors involved in tRNA nuclear import. Mtr10 is among one of the members of the β-importin family of transport receptors and has been linked to tRNA nuclear import in the retrograde nuclear localization of tRNAs (83, 88). While the essential role of Mtr10 in tRNA nuclear import has been shown with increase in accumulation of aberrant tRNAs in the cytoplasm in *mtr10-* cells and the lethality of the *mtr10-/xrn1-* cells, the mechanism in which

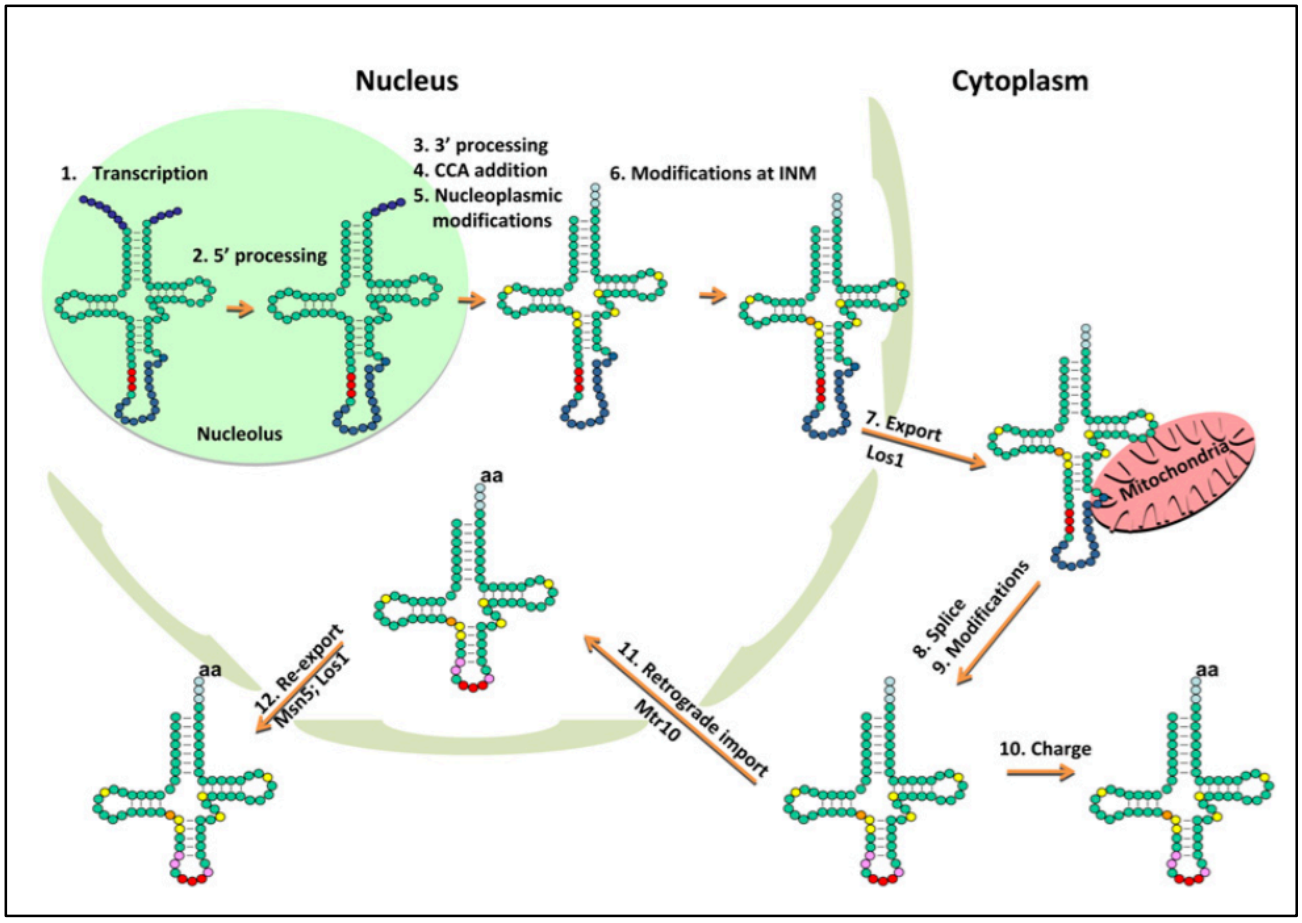

Figure 10. Model for nuclear-cytoplasmic biogenesis and transport of tRNAs in *S. cerevisiae*. The nucleolus hosts the site of tRNA transcription and 5' end processing while the 3' end processing, CCA addition and certain modifications made to the tRNA (orange and yellow circles) are carried out in the nucleus. Intron-containing pre-tRNAs (blue circles) are exported to the cytoplasm and surface of mitochondria by Los1 where the splicing machinery in yeast resides. Following splicing, acquiring cytoplasmic PTMs (pink circles) and aminoacylation, the mature tRNAs are sent to the translation machinery. The retrograde import pathway facilitated by Mtr10 re-imports mature tRNAs to the nucleus. Re-export of mature tRNAs is dependent on cellular conditions (eg. nutritional stress) and facilitated by Msn5 and Los1. Figure adapted from (61).

Mtr10 associates with tRNAs and the mode of transportation is still under investigation with unclear insights as to the Ran-dependent or Ran-independent mode of transportation by Mtr10 (Figure 9) (83, 88).

3.4.4 tRNA import into mitochondrial organelle

The mitochondrion is a double membrane-structured organelle that facilitates oxidative phosphorylation and is widely recognized as the major powerhouse of the cell in all eukaryotes (89). In production of ATP and maintenance of a functional respiratory complex, the mitochondrion is supplied with a large volume of nucleus-encoded proteins that are imported into the mitochondria, in addition to the proteins that are synthesized directly from the mitochondrial genome (83). For the small, but nonetheless essential proteins synthesized in the mitochondria, a complete set of tRNAs facilitating protein synthesis is required. The initial insight into nuclear-encoded tRNA import into the mitochondria was made over 50 years ago by Suyama et al. by noting that the tRNA fractions isolated from the mitochondrial of protozoan *Tetrahymena,* were largely nuclear-encoded tRNAs (90). Since these initial findings, many more claims of nuclear encoded-tRNA import into the mitochondria have been made, varying from the import of a few tRNAs into yeast and human mitochondria organelle to a complete set of nuclear-encoded tRNAs imported into the mitochondria of protists (83). The import of cytosolic tRNAs into the mitochondria have been most clearly studied in *S. cerevisiae* with the import of tRNAs coordinated through two pathways; the co-import and the direct import (90).The better characterized co-import pathway relies on mitochondrial protein import factors and a series of other proteins that engage the tRNA and translocate it from the outer membrane through the inner membrane and lastly deliver the tRNA to the matrix of the mitochondrial membrane, the site of protein synthesis (83). Protein entry into the mitochondrial matrix is directed through two major heteroligomeric protein complexes, TOM (translocase of the outer membrane) and TIM (translocase of the inner membrane) and other protein factors in association with these translocator complexes (90, 91). The heteroligomeric TOM complex comprises of Tom40 as the main component with Tom20, Tom22 and Tom70 functioning as the pre-protein receptors, and a few smaller TOM proteins (90, 91). Generally, nuclear-encoded mitochondrial pre-proteins (precursor proteins) carry a targeting signal (commonly found in the N-terminal but also present internally in the pre-protein or C-terminal) that determines its localization in the subcompartments of the mitochondria (91).

Pre-proteins are initially recognized by Tom20 and Tom22 which direct the translocation of the pre-proteins through the import channel of Tom40 (90, 91). Once the pre-protein has passed through the TOM complex, it is either released in the inter-membrane space or transported to the inner-membrane or the matrix. This is directly related to the type of protein and the targeting signal present on the pre-protein (91). In the interest of this review, the focus will be on pre-proteins destined to the matrix using the TIM complex. The transfer of the pre-protein into the inner-membrane is assisted by the TIM complex which consists of Tim23 as the central component and Tim17, Tim50 and Tim21 as the associating receptors (91). The TOM complex in cooperation with the TIM complex, translocate the pre-protein to the inner-membrane or directly to the matrix by interacting with the presequence translocase-associated motor (PAM) and its central subunit Hsp70 which coordinates the ATP-dependent transport of the pre-proteins to the matrix (90, 91).

While the import of tRNAs into the mitochondrion matrix is now well established, studies in this field are still premature with only a handful of well characterized tRNA import mechanisms reported. The most notable example is the import of tRNA-Lys^{CUU} using the co-import pathway of translocation that has been studied in *S. cerevisiae* (90). The glycolytic enzyme, enolase (Eno2p) specifically recognizes tRNA-Lys^{CUU} and subsequently carries the tRNA to the surface edge of the mitochondria, delivering tRNA-Lys^{CUU} to pre-LysRS (precursor of mitochondrial lysyl-tRNA synthetase) (90, 92, 93). At this stage, Eno2p is released in the outer mitochondrial membrane, associating with a glycolytic multiprotein complex, while the tRNA-Lys^{CUU} -pre-LysRS complex is directed to the mitochondrial matrix through the protein import pathway, mentioned above (Figure 10) (90, 92, 93). Recent *in vitro* FRET experiments have revealed a structural rearrangement noted for tRNA-Lys^{CUU} in association with Eno2p such that the tRNA diverts from the canonical L-shaped configuration while bound by Eno2p and regains a conformation like the tertiary L-shaped conformation when bound by pre-LysRS (90, 94). These new insights give possible reason for the specificity of Eno2p engaging tRNA-Lys^{CUU} instead of its isoacceptor tRNA-Lys^{UUU} as it is plausible that this conformational change may be unique to tRNA-Lys^{CUU} (90, 94). In addition to this structural specificity possibly dictating tRNA-Lys^{CUU}-mitochondrial import, tRNA-Lys^{CUU} must also be aminoacylated to engage pre-LysRS. Furthermore, the role of pre-LysRS is strictly limited to tRNA-Lys^{CUU} mitochondrial import and is incapable of aminoacylating tRNA-Lys^{CUU} , in direct contrast to its capacity in aminoacylation of mitochondrial tRNA-Lys^{UUU} (90, 95). *In vitro* and *in vivo* mutagenesis studies on pre-LysRS have identified the

N-terminal region of the protein responsible for tRNA import and the full-length protein possessing the aminoacylation activity (90, 95). Lastly, the notion that mitochondrial encoded tRNA-Lys^{UUU} can in theory decode both lysine codons, and the idea that tRNA-Lys^{CUU} is only utilized in one cycle of elongation as it cannot be aminoacylated in the mitochondria begs the question of the purpose of tRNA-Lys^{CUU} -mitochondrial import. Recent studies have addressed this counterintuitive phenomenon by revealing the essential role of tRNA-Lys^{CUU} in decoding AAG codons when cells are grown at 37°C in which tRNA-Lys^{UUU} is hypomodified at the wobble uridine and non-functional in decoding AAG codons (90, 96).

The second piece of evidence for cytosolic tRNA import into the yeast mitochondria has been reported with tRNA-Gln^{CUG} and tRNA-Gln^{UUG} (90, 97) . In contrast to the co-import pathway used for tRNA-Lys^{CUU}, the import of tRNA-Gln^{CUG} and tRNA-Gln^{UUG} is through the direct import pathway, eliminating the requirement for the protein import complex, a membrane potential and cytosolic factors. While evidence is missing for the purpose and the exact mechanism of import for cytosolic tRNA-Gln, the localization and import of this tRNA in the mitochondria was illustrated by the capacity of a suppressor cytosolic tRNA-Gln^{CUA} in partially suppressing a nonsense mutation created in cytochrome oxidase subunit II (cox2) verifying the import and the role of this cytosolic tRNA in the mitochondrial translation machinery (90, 97). Albeit, there have been significant improvement in the field of tRNA subcellular localization and specifically translocation to the mitochondria, clarity is still much needed in identifying guidelines for import of nuclear-encoded tRNAs to the mitochondria through the co-import or direct import pathway and factors involved in this shuttling.

4. Mitochondria, the powerhouse of eukaryotic cells

The mitochondria, conserved in almost all eukaryotic cells are well characterized for their essential role in ATP production through oxidative phosphorylation (OXPHOS) and managing other biochemical processes of the cell such as citric acid cycle, pyruvate oxidation and electron transport (89, 98, 99) . These double-membrane bound organelles are also responsible for cellular metabolism, biogenesis of iron-sulfur proteins, regulation of cytosolic calcium levels, heme biosynthesis and apoptosis (89, 99). While the mitochondrion is the host of essential cellular processes, the maintenance of a functional mitochondrion is highly reliant on the elegantly

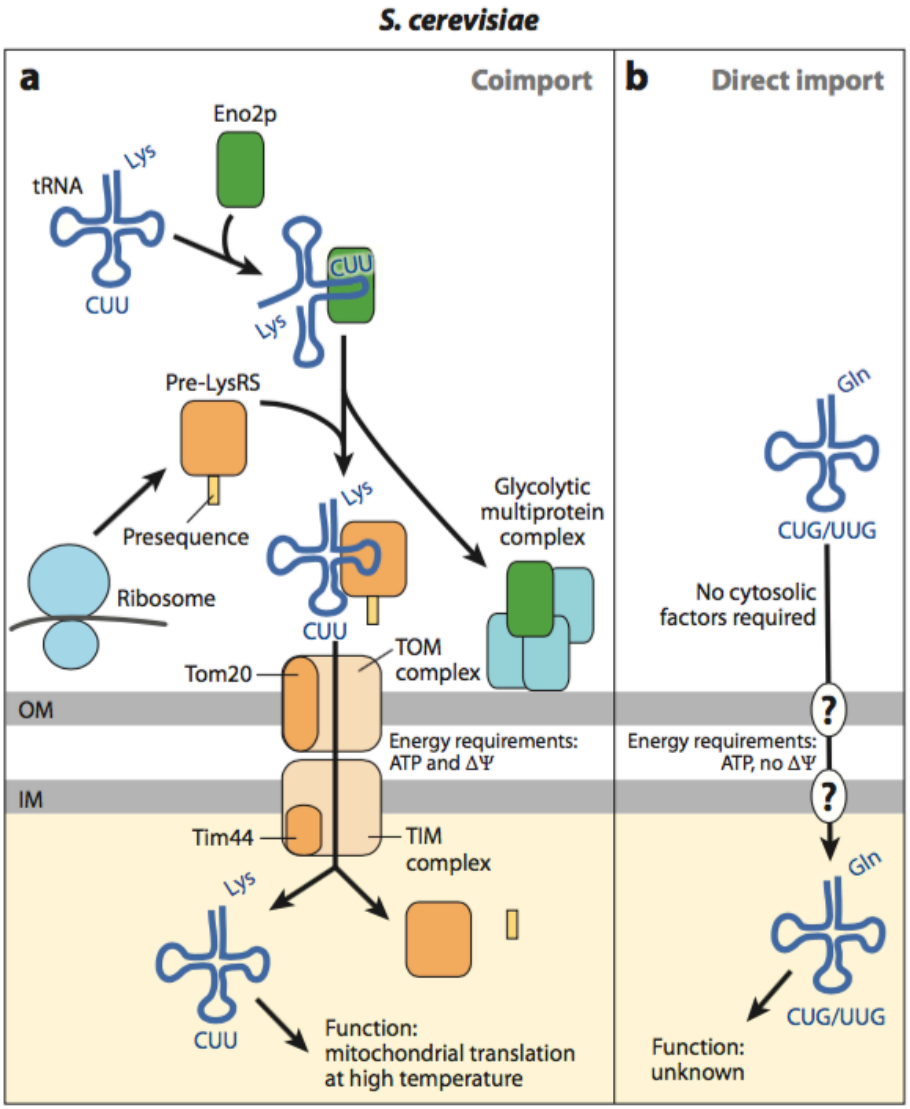

Figure 11. Model for cytosolic-tRNA import into the subcompartments of mitochondria in *S. cerevisiae*. (A) Cytosolic tRNA-Lys^{CUU} is directed to the mitochondrial matrix for use in mRNA decoding using the co-import pathway by initial binding to Eno2p (green) which induces structural changes on the tRNA, deviating from the canonical L-shaped configuration (tertiary conformation not represented here, note secondary structural changes). Pre-LysRS (orange) which is located at the surface of the mitochondria subsequently binds tRNA-Lys^{CUU}, releasing Eno2p which is then free to engage the glycolytic multiprotein complex (blue) found at the surface of outer-membrane of the mitochondria. The pre-LysRS- tRNA-Lys^{CUU} complex is then shuttled through the outer-membrane to the inner-membrane and lastly the matrix of the mitochondria through the protein import pathway; TOM and TIM complex (see text for details). (B) Direct import is used for the translocation of cytosolic tRNA-Gln^{CUG} and tRNA-Gln^{UUG} into the mitochondrial matrix and this is carried out in the absence of assisting cytosolic factors, the protein import complex and a membrane potential through an undefined mechanism. Figure adapted from (90).

complex communications between the nuclear and the mitochondrial genome (89). The region of the inner membrane and the matrix of the mitochondria are protein-rich with proteomics studies in yeast identifying ~1000 mitochondrial proteins of which only 8 (in *S. pombe* and *S. cerevisiae*) of these proteins are encoded by mitochondrial DNA (mtDNA) and a product of mitochondrial translational machinery (98). These express the mitochondrial ribosomal protein of the small subunit (Var1; *S. cerevisiae*) as well as some components of the structural subunits of the five multimeric OXPHOS respiratory complex which includes the respiratory complexes III, cytochrome c oxidase (IV) and ATP synthase (V) (98, 100). Aside from the handful of mitochondrial encoded proteins reported to date, the rest of the mitochondrial proteins residing in the organelle are nuclear encoded, translated in the cytoplasm and subsequently imported into the mitochondria (98). Therefore, the maintenance of an operative respiratory complex and propagation of these semi-autonomous organelles relies on the communications between the nuclear and mitochondrial genome, facilitating in-house mitochondrial translation and the import of cytosolic proteins (89).

4.1 Mitochondrial translation

The expression of the few mitochondrial encoded genes, namely the assembly of the multimeric OXPHOS subunits is dependent on both the import of nuclear-encoded subunits of the respiratory complex and the concomitant translation of the mitochondrial-encoded OXPHOS subunits and their subsequent assembly. To facilitate mitochondrial translation, the genome of this organelle possesses mtDNA encoding 2 ribosomal RNAs and a set of tRNAs (98, 100). The number of tRNAs encoded in the mtDNA is reported to be highly species specific with yeast possessing a set of 25 tRNAs in the mitochondria and only relying on the import of a few cytosolic tRNAs, while conversely the mtDNA of some protists lack tRNA genes and rely on the import of a full set of cytosolic tRNAs (83, 100).The mtDNA also encodes some components of the mitochondrial ribosome (mitoribosome) which like the cytoplasmic ribosome, consists of a small (SSU) and large ribosomal subunit (LSU). The mitochondrial gene expression system follows the universally conserved steps of translation; initiation, elongation and termination (89, 101, 102). Translation initiation begins with mitochondrial initiation factors ($IF2_{mt}$ &$IF3_{mt}$; Ifm1 & Aim23 yeast homologs) associating with the mitoribosome and assembling the initiation complex. While there

are still many unanswered questions regarding the exact initiation mechanism of mitochondrial translation due to differences between mitochondrial mRNAs in comparison with prokaryotic (presence of Shine-Dalgarno sequence) and eukaryotic mRNAs (7-methylguanylate capped), it is currently postulated that the SSU of the mitoribosome initiates translation with the selection of unstructured regions in the 5' end of mRNAs (101). Subsequently the initiation complex identifies the start codon (AUG) and the $IF3_{mt}$ facilitates the correct binding of the mRNA AUG codon to the peptidyl site (P) of SSU (89, 102). $IF2_{mt}$ assists the interaction between fMet-tRNA-Met and SSU. This results in joining of the LSU to SSU and the release of the initiation factors and the completion of translation initiation (101). Elongation is coordinated by mitochondrial elongation factor Tu (nuclear-encoded genes; $EF\text{-}Tu_{mt}$ and Tuf1 yeast homolog) which orchestrates the interaction between the mitoribosome, tRNA and the mitochondrial elongation factor G1 ($EF\text{-}G1_{mt}$, Mef1 yeast homolog) (89, 102). Elongation proceeds with the continuous delivery of charged tRNAs to the growing polypeptide chain until the stop codon (UAA, UAG, AGA or AGG) is encountered by the release factor ($RF1_{mt}$, Mrf1 yeast homolog) which works in conjunction with the recycling factor ($RRF1_{mt}$, Rrf1 yeast homolog) (101, 102). Termination results in the hydrolysis of the ester linkage between the nascent polypeptide chain and the last tRNA at the P-site of SSU and the dissociation of the mitoribosome into SSU and LSU subunits, recycled for future cycles of translation (89, 102). While mitochondrial translational machinery is sufficiently operative in the expression of a set of the OXPHOS subunits, the remainder of the subunits and the majority of the mitochondrial proteins detected in the protein-rich matrix, the outer and inner-membrane are all nuclear-encoded, expressed in the cytoplasm and imported. In addition to the import of OXPHOS nuclear encoded subunits, the mitochondria also significantly rely on the import of other nuclear encoded proteins to support the organelle and examples of these include: proteins supporting the mitochondrial translational machinery, protein folding and transport, turnover and tRNA PTM enzymes (98, 101).

4.2 Import of cytosolic proteins

Nuclear-encoded mitochondrial proteins are synthesized by the cytoplasmic ribosome and are post-translationally transported into the mitochondria as pre-proteins, carrying a mitochondrial targeting sequence (MTS). Mitochondria-destined pre-proteins are categorized into two main

groups: pre-proteins with a cleavable N-terminal MTS and pre-proteins that possess an internal non-cleavable MTS (103, 104). The class of proteins with a cleavable N-terminal MTS are largely destined to either the inner-membrane, inter-membrane or the matrix of the mitochondria (104). The MTS of these pre-proteins often comprise of 10-90 residues, highly enriched in positively charged amino acids such as arginine and lysine residues (103, 104). Previous findings have proposed a model wherein the MTS forms an amphiphilic α-helical structure in which the hydrophobic site associates with the Tom20 subunit of the TOM complex and the positively charged residues interact with Tom22 (103, 104). As a result, the pre-protein is directed towards the main import receptor, Tom40 by the sequential interactions with Tom20, 22 and Tom5. Once the pre-protein is delivered to its destination, the mitochondrial processing peptidase (MPP) removes the MTS from the pre-protein and with the assistance of molecular chaperons (eg. Hsp60) the protein is folded into a functionally active conformation (103–105).

The proteins identified in the outer-membrane largely encompass the second class of nuclear-encoded mitochondrial pre-proteins carrying the internal MTS. While pre-proteins with internal MTS are routinely shuttled to the outer-membrane, many may also be transported to the inner and inter-membrane space. Examples of this class of pre-proteins consists of outer membrane proteins and some hydrophobic carrier pre-proteins of metabolites such as ATP/ADP or phosphate carrier pre-proteins, with no reports indicating any conservation in the sequence or location of the internal signal amongst this class of pre-proteins (104, 106). Cytosolic protein chaperones (eg. Hsp70 & Hsp90) play a large role in the transport of these pre-proteins to ensure minimal aggregation of the hydrophobic carrier proteins and to facilitate their interaction with the Tom70 receptor. Although Tom70 has been recognized as the major site of entry for pre-proteins with internal MTS, with Tom20 and 22 being classified as major entry receptors for pre-proteins with N-terminal MTS, previous studies support the notion that defects in one receptor can be partially compensated by the role of other mitochondrial import receptors (104).

4.3 Trm1: Nuclear and mitochondria targeted tRNA methyltransferase

The ubiquitous and abundant nature of tRNA PTMs has led to decades of research in the role of these functional groups that decorate the clover-leaf structure. PTMs are now known to facilitate

the structural stability of tRNAs in addition to playing an elemental role in mRNA decoding (61, 63). Like nuclear-encoded tRNAs, mitochondrial tRNAs have been postulated to benefit from PTMs particularly for structural stability to even a larger extend than nuclear-encoded tRNAs (101). Mitochondrial tRNAs (mt-tRNAs) are recognized to deviate from the canonical type 6 fold found in cytoplasmic tRNAs, are identified to be generally shorter, and display variability in the D and T-loop and are deficient in some conserved nucleotides that are thought to be essential for the formation of a stable tertiary L-shaped fold (101). Given the role of mitochondrial-encoded tRNAs in the translational machinery of the organelle and their susceptibility in forming non-canonical and aberrant structures, the role of PTMs on these tRNAs should be emphasized.

Trm1, which facilitates N2, N2-dimethylation of G26 is an example of a PTM enzyme that shuttles between the nucleus and the mitochondria for modification of tRNAs. Early studies on Trm1 in *S. cerevisiae* revealed two-in frame start codons, the first positioned at amino acid 1 and the second ATG was found at amino acid 17 (107). This indicated the potential for the formation of two isoforms of the enzyme: Trm1p-I and Trm1p-II. Further investigation revealed that the translation of the two isoforms of Trm1p from either start codon is transcriptionally regulated with the 5'ends of the mRNA being located upstream of either ATG1 (translating Trm1p-I) or positioned between ATG1 and ATG17 (translating the shorter isoform, Trm1p-II) (107). With Trm1p being located strictly in the nuclear periphery and the mitochondria and entirely absent in the cytosolic region of the cell, the early simplistic insights categorized the two Trm1p isoforms into the two compartments, with the shorter Trm1p-II located at the nuclear periphery and the longer extended isoform (Trm1p-II) located in the mitochondria (107). However, it was later discovered that while Trm1p-I is entirely imported into the mitochondria, that Trm1p-II can be found in the nuclear periphery as well as imported into the mitochondria at a level sufficient enough to modify G26-containing tRNAs in the organelle under wildtype conditions (72, 107). These insights led to the findings that the amino-terminus of Trm1p-II, in conjunction with the amino-terminal extension, form a MTS sequence for Trm1p that most efficiently delivers the enzyme to the mitochondria. More specifically the region between amino acids 1-48 serve as the MTS, while amino acids 70-213 are responsible for directing Trm1p to the nucleus with NTS-like (nuclear targeting signal) residues more primarily localized to amino acids 95-102, enriched in lysine residues (72, 107).

5. Summary of literature review

The RNA folding problem, central to this review, emphasizes the barriers for a primary sequence of RNA in specifying a single, functional fold. The kinetic and thermodynamic problems that ensue the RNA folding problem are now known to be largely resolved with the assistance of various classes of RNA-binding proteins. With the focus on tRNAs as RNA candidates that routinely deviate from the canonical type 6 clover-leaf secondary structure, numerous examples of extensive post-transcriptional processing steps have been described that illustrate the level of cellular control devoted to ensuring a final, correctly structured functional tRNA. The role of the RNA chaperone La and PTMs made to the body of tRNAs such as dimethylation of G26 by Trm1p have been extensively reviewed. This demonstrates the influence of both an RNA chaperone and a PTM in specifying a unique functional fold for a tRNA and protecting the tRNA from misfolding and subsequent degradation. In addition, the role of Trm1p (a PTM distant from the anticodon loop) in potentially regulating the mRNA decoding capacity of tRNAs has also been emphasized. With a direct link between structure and function, elucidating the role of RNA chaperones and other PTM enzymes involved in RNA biogenesis and assembly has become central to the interest of researchers.

CHAPTER II: Statement of Purpose

Noncoding RNAs facilitate a myriad of biological processes. The functionality of RNA molecules is tightly linked to their complex tertiary structural conformation (1). The similarities in the structural composition of the nitrogenous bases predispositions RNA molecules in forming non-functional conformations that are thermodynamically as stable as the native, functionally active fold (2, 4). To ensure proper biological activity and native folding, RNA substrates constantly associate with a variety of RNA-binding proteins and concomitantly acquire numerous PTMs on their sugar-ribose moiety during processing (2, 4, 7, 63). RNA chaperones are a class of RNA-binding proteins that nonspecifically and transiently interact with RNA substrates and facilitate their search for the native conformation (1-7). La proteins are highly abundant RNA chaperones that assist processing of all pol III transcripts with pre-tRNAs being their best characterized substrates (20). La proteins make specific contacts to pre-tRNAs via the UUU-3'OH tail and nonspecifically by interacting with the main body of the tRNA which is associated with the RNA chaperone activity function (20, 29, 47, 48). Given the limiting abundance of La proteins relative to the highly abundant UUU-3'OH containing substrates (43), the mechanism in which La differentiates between a misfolded and folded UUU-3'OH containing substrate such as pre-tRNAs has not been addressed. Additionally, tRNAs are highly post-transcriptionally modified with modifications made to the body of tRNAs facilitating structural stability (61–63). m_2^2G26 modification by Trm1p, functions redundantly with La in supporting structural stability of G26-containing tRNAs (70). While Trm1p modification presumably stabilizes the "hinge" region between the anticodon and D-stem, the impact of m_2^2G26 on tRNA functionality has not been investigated (74, 116). In this work, we aimed to elucidate the capacity of La as an RNA chaperone in differentiating a misfolded pre-tRNA from its folded counterpart. Secondly, we have sought out to investigate the impact of m_2^2G26 on tRNA activity in mRNA decoding.

Chapter III: The RNA chaperone La promotes pre-tRNA maturation via indiscriminate binding of both native and misfolded targets

La deletion is synthetically lethal when combined with the loss of modification enzymes that support tRNA structural stability such as Trm1p. In this work, we have assessed the mechanism in

which the RNA chaperone La targets and differentiates a misfolded Trm1p-hypomodified tRNA from its folded, Trm1p-modified counterpart. We have identified *S. pombe* cytosolic G26-containing pre-tRNAs that misfold in *sla1-/trm1-* cells. These candidate tRNAs showed reduced steady state abundance or were not as efficiently aminoacylated as shown in wildtype conditions. Using *in vivo* and *in vitro* assays, we tested for possible differential binding affinity of La towards a misfolded Trm1p-hypomodified tRNA candidate versus its modified, folded state. Our data conclusively demonstrates the non-discriminatory La binding affinity towards a misfolded and folded pre-tRNA. Our findings add clarity to the ambiguous mechanism previously hypothesized for RNA chaperone activity.

Chapter IV: Global protein expression regulated by Trm1p-catalyzed m_2^2G26 modification of mitochondrial and nuclear encoded tRNAs

N2, N2-dimethylation of G26 by Trm1p has been reported for *S. pombe* cytosolic tRNAs in recent tRNA-HydroSeq (71). Trm1p modification efficiency is variable depending on tRNA identity. TMS assay using *S. pombe* suppressor tRNA-Ser^{UCA} shows significant reliance on m_2^2G26 in suppressing a nonsense codon in the *ade6-704* allele (71). In this work, we have sought out to identify a novel role for m_2^2G26 in altering tRNA activity and the impact of this modification on global protein expression levels. Using a SILAC (stable isotope labeling of amino acids in cell culture) proteomics approach we have identified changes in protein expression levels that coincide with Trm1p depletion. We have noted a correlation between the change in expression levels of a subset of polypeptides in *trm1-* cells with the enrichment of CTA codons in these transcripts, likely due to inefficient activity of the corresponding Trm1p-hypomodified tRNA-Leu^{UAG} in decoding these CTA codons. Additionally, we have identified metabolic processes upregulated in *trm1-* cells, particularly those associated with mitochondrial function. We have reported robust presence of m_2^2G26 on specific mt-tRNAs and have further assessed their charging state +/- m_2^2G26. We have also evaluated the expression levels of mitochondrial and cytoplasmic translation products and demonstrate that while the expression of mitochondrial-encoded OXPHOS components is not altered in *trm1-* cells, the overexpression of mitochondrial-targeted Trm1 or its nuclear-bound counterpart displays a decrease in cytoplasmic translation products. Our data alludes to a novel role for Trm1p in modulating cytoplasmic and mitochondrial physiology potentially through altering tRNA functional activity and stability.

CHAPTER III: The RNA chaperone La promotes pre-tRNA maturation via indiscriminate binding of both native and misfolded targets

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The RNA chaperone La promotes pre-tRNA maturation via indiscriminate binding of both native and misfolded targets

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PREFACE

Noncoding RNAs have critical roles in biological processes, and RNA chaperones can promote their folding into the native shape required for their function. La proteins are a class of highly abundant RNA chaperones that contact pre-tRNAs and other RNA polymerase III transcripts via their common UUU-3'OH ends, as well as through less specific contacts associated with RNA chaperone activity. However, whether La proteins preferentially bind misfolded pre-tRNAs or instead engage all pre-tRNA substrates irrespective of their folding status is not known. La deletion in yeast is synthetically lethal when combined with the loss of tRNA modifications predicted to contribute to the native pre-tRNA fold, such as the N2, N2-dimethylation of G26 by the methyltransferase Trm1p. In this work, we identify G26 containing pre-tRNAs that misfold in the absence of Trm1p and/or La (Sla1p) in *S. pombe* cells, then test whether La preferentially associates with such tRNAs *in vitro* and *in vivo*. Our data suggest that La does not discriminate a native from misfolded RNA target, and highlights the potential challenges faced by RNA chaperones in preferentially binding defective substrates.

INTRODUCTION

Nascent RNA transcripts have a significant propensity to become kinetically trapped in non-functional conformations. It has thus been hypothesized that one of the earliest functions of polypeptides would be to help resolve these aberrant structures so that RNAs can better access their functionally relevant, native folds (108). Proteins that promote the native fold have been classified based on their proposed mechanism of action (reviewed in (7)). One such class of factors is the RNA chaperones, which are hypothesized to unwind misfolded structures in the absence of ATP hydrolysis via mechanisms that remain ambiguous (2, 6, 109). By assisting RNA substrates in attaining their native fold, RNA chaperones can rescue defective or misfolded RNA substrates from RNA quality control systems such as nuclear surveillance (47, 69, 79). Though it's been recognized that many noncoding RNAs are susceptible to folding into alternate inactive conformations, mechanisms by which RNA chaperones might discriminate misfolded RNA substrates are not well understood. It has been previously noted that the greater homogeneity of functional groups in RNA relative to amino acids might make for greater challenges in discrimination of fold for RNA chaperones relative to protein chaperones, even if thermodynamic models for misfolded target recognition by RNA chaperones have been proposed (2, 6). For RNA chaperones such as StpA and hnRNP A1, it has been proposed that these preferably engage single stranded regions of RNA that are hypothesized to be more prevalent in misfolded conformations (12, 16, 110). One significant challenge to accessing insights into target recognition is the identification of physiologically relevant, misfolded RNA conformations that the RNA chaperone in question samples *in vivo*. Thus, the issue of functional target discrimination by RNA chaperones has not been explored extensively.

The La and La-related proteins are a conserved class of eukaryotic RNA-binding proteins that have been characterized as RNA chaperones (21, 22, 29, 30, 47). La proteins have important roles in the processing of nascent RNA polymerase (Pol) III transcripts, with pre-tRNAs being their best-characterized substrates (reviewed in (24)). This association relies in part on specific recognition of the uridylate containing trailer sequence (UUU-3'OH) that is found at the end of all Pol III transcripts and some noncoding RNA polymerase II processing intermediates. In yeast, La binding to the UUU-3'OH containing trailer results in pre-tRNA stabilization and accumulation, and commits the pre-tRNA to the La-dependent pathway of processing, in which leader processing

precedes endonucleolytic trailer processing, rather than trailer removal by exonucleases like Rex1p in the absence of La (25, 111).

Structural and biochemical work have indicated that UUU-3'OH dependent La-RNA binding relies on a highly conserved N-terminal domain which comprises a winged-helix like La-motif (LAM) and an adjacent RNA recognition motif (RRM1) that synergize to form the so-called La-module (20, 112). Co-crystal structures of the La module interacting with UUU-3'OH containing RNAs have revealed conserved residues in the LAM involved in recognition of the poly-uridylate sequence (55, 113), and this binding mode is associated with protecting nascent Pol III transcripts from exonuclease digestion (47). However, other non-UUU-3'OH dependent RNA contacts also make a major contribution to La/pre-tRNA binding. La binds short UUU-3'OH containing oligonucleotides approximately 10X less avidly than the same UUU-3'OH motif presented in the context of a full pre-tRNA (22). This is at least in part due to other regions of the RRM1 domain, as well as disordered amino acids C-terminal to this region, that engage the main body of the tRNA and promote the native fold through RNA chaperone activity (22, 29, 47, 48). Mutation of basic amino acids in RRM1 result in decreased pre-tRNA binding without affecting UUU-3'OH binding (22) , and mutation of aromatic amino acids in the RNP motifs of RRM1 normally associated with canonical RRM-associated RNA binding result in defective RNA chaperone activity *in vitro* and degradation of defective (mutated) pre-tRNAs *in vivo* (29, 47). These mutations also result in decreased levels of the stable pre-tRNA species normally associated with the La-dependent pre-tRNA processing pathway (47). Importantly, decreased pre-tRNA accumulation due to RRM1 mutation only correlates with decreased mature tRNA levels if the tRNA is defective (mutated); conversely, wild-type pre-tRNAs show normal mature tRNA levels despite decreased pre-tRNA accumulation in the presence of La-RRM1 mutants. These data led to a model in which La engages all pre-tRNAs regardless of folding status for the benefit of rescuing defective substrates and at the expense of processing efficiency for non-defective pre-tRNAs (47). However, the ability of La to differentially bind any folded versus misfolded substrates within the UUU-3'OH containing RNA target cohort has not previously been tested *in vitro* or *in vivo*. Since La is hypothesized to be limiting relative to RNA Pol III transcript abundance (43), whether La discriminates native from misfolded substrates could play an important role in determining which pre-tRNAs access the La-dependent versus La-independent processing pathways. Attempting to experimentally test whether the affinity of La for a physiologically

relevant, misfolded RNA target differs relative to its natively folded counterpart is a major theme of this work.

Pre-tRNAs are among the most highly post-transcriptionally modified classes of RNA species, with a median of 8 modified nucleosides per mature transcript (114). Several cases have been reported in which the absence of an essential modification has resulted in the degradation of select tRNAs. Mature tRNAs lacking particular modifications can be degraded at elevated temperatures via the rapid tRNA decay pathway (65, 115), or hypomodified pre-tRNAs can be degraded in the nucleus by nuclear surveillance. One such example is the methylation of A58 (m^1A58) by Trm6/61 on pre-$tRNA_i^{Met}$ in *S. cerevisiae*; absence of this modification can result in degradation of pre-$tRNA_i^{Met}$ via the TRAMP complex and nuclear exosome (79), but this can be rescued by overexpression of the La homolog Lhp1p (69). Similarly, deletion of Lhp1p is synthetically lethal with tRNA mutations shown to affect their folding (46) or with deletion of other tRNA modification enzymes (70, 116), consistent with La functioning redundantly with elements that stabilize native pre-tRNA structure.

One tRNA modification associated with La function is the N2,N2-dimethylation of G26 containing tRNAs by the methyltransferase Trm1p (70), a tRNA modification enzyme that localizes to the nuclear membrane (117). N2,N2-dimethylation of G26 has been hypothesized to stabilize tRNA structure in the "hinge" region between the coaxially stacked anticodon- and D-stems , as well as prioritize the native fold of cytoplasmic tRNA anticodon stem loops over aberrant structures occasionally observed in native mitochondrial tRNAs (75). Deletion of Trm1 is synthetically lethal with deletion of Lhp1 when *S. cerevisiae* is grown at elevated temperature (70), and N2,N2-dimethylated guanosine containing tRNAs are immunoprecipitated by La protein (118), suggesting that G26 modification occurs during the window of La-pre-tRNA occupancy. Taken together, these data suggest that La binds the nascent pre-tRNA transcript and promotes its stability and/or the native fold until this is reinforced by methylation of G26 by Trm1p. Thus, the study of La binding to Trm1p modified versus unmodified pre-tRNAs represents an opportunity to compare the affinity of an RNA chaperone to a natively folded versus misfolded substrate in which the misfolded conformation is physiologically relevant and is sampled by the RNA chaperone *in vivo*.

Previous work suggests that modification at G26 by Trm1p and La RNA chaperone activity function redundantly in stabilizing tRNA structure (70). However, which tRNAs might misfold

in the absence of dimethylation at G26, or whether La can discriminate any folded versus misfolded substrate, has not previously been tested. In this work, we have attempted to directly test whether La differentiates misfolded G26 unmodified versus Trm1p modified pre-tRNAs both *in vitro* and in *S. pombe* cells. We demonstrate that similar to *S. cerevisiae*, deletion of *S. pombe* La (Sla1p) and Trm1p is synthetically lethal at elevated temperatures. We then further show that Sla1p dependent suppression of lethality relies on amino acids associated with La-dependent RNA chaperone activity, consistent with Trm1p and La functioning redundantly to stabilize pre-tRNA structure. Using lead acetate chemical probing, we identify tRNA-Ser^{UGA} as a tRNA whose fold changes on the basis of Trm1 modification, and further demonstrate its impaired charging in a *sla1-/trm1-* strain, consistent with misfolding *in vivo*. We also identify cohorts of G26 containing tRNAs whose steady-state abundance is depleted in *trm1-*, *sla1-* and *sla1-/trm1-* cells, consistent with Trm1p functioning in the stabilization of certain tRNA species. Using pulldown of endogenous La-pre-tRNA complexes formed *in vivo* and gel-shift analysis of La-pre-tRNA complexes *in vitro*, we then test for the capacity of La to differentially bind pre-tRNAs with respect to their modification status at G26. In sum, our results suggest that La binds natively folded tRNAs and tRNAs that likely misfold due to hypomodification of G26 indiscriminately. Finally, we further demonstrate that human La also binds a fully modified tRNA substrate indiscriminately from its unmodified, T7 transcribed counterpart. Our data suggest that La is poorly capable of differentiating substrates whose folding and accumulation rely on the modification of G26 by Trm1p and the RNA chaperone activity of La, consistent with a model in which alternate tRNA folds may not form a primary binding determinant for La proteins. We propose that these RNA chaperones are directed to their substrates more by features related to the processing stage of their targets and less by their folding status, in an analogous manner to the variant protein molecular chaperones calnexin and calreticulin. Thus, our work adds an interesting new facet with respect to our understanding of how RNA chaperones can engage their targets.

MATERIAL AND METHODS

Northern analyses

Cells were grown in YES at 32ºC and then shifted to minimal media for ten hours prior to harvest. Total RNA was extracted and purified from yeast cells and Northern analysis was performed as described (22). Values represent the mean fold abundance relative to the respective tRNA in the wild-type strain over a minimum of three independent growths/total RNA extractions. These were normalized to U5 snRNA as a loading control and presented relative to the respective tRNA abundance in the WT strain. Significant differences in tRNA abundance between mutant strains and the wild-type strain were assessed by performing a one-way ANOVA to compare means across all treatments, followed by a Tukey posthoc test with alpha set to 0.05 (*, Figure 4B-D) or 0.01 (**). DNA probes were made for indicated tRNAs by 5' end labeling of the oligonucleotides using T4 polynucleotide kinase and ^{32}P-γ-ATP. Probes used for Northern analysis were all complementary to the TψC-loop of their respective targets. Probe sequences are provided in Supplementary Table 3.

Analysis of Aminoacyl-tRNA charging

Cells were grown in YES at 32ºC and then shifted to minimal media for ten hours prior to harvest. Total RNA was extracted under acidic conditions using Trizol reagent (Invitrogen). For acid-Northern, half of the WT total RNA was deacylated with 0.2 M Tris pH 9.0 for 2 hours at 37°C and then ethanol precipitated and stored in 10 mM sodium acetate pH 5.0. All samples were fractionated on a 15% polyacrylamide acid (0.1 M sodium acetate pH 5.0) denaturing gel (vertical Gel Electrophoresis System V16-2, Apogee) and transferred onto a GeneScreen Plus transfer membrane (PerkinElmer) using the iBlot Gel Transfer system (Invitrogen). Periodate oxidation/β elimination based analysis of charging levels was performed as described (119). Briefly, total RNA was extracted under acidic conditions and half of the total RNA was deacylated as for acid-Northern. Both the deacylated and untreated total RNA were subjected to periodate oxidation (5 mM NaIO4, 50 mM NaOAc pH 5.0, 60 min at 37°C). The reaction was stopped with the addition of 50 mM glucose (0°C for 90 min) and ethanol precipitation. The RNA samples were subject to

β-elimination by resuspending the total RNA samples in 1 ml of 1M lysine pH 8.0 and incubating 60 min at 45°C. All samples were then treated with 500 µl of 0.4 M Tris pH 9.0 (37°C, 30 min) for deacylation. RNA samples were treated with phenol chloroform and ethanol precipitated. Fraction charged was quantitated for each tRNA (N=1) as the ratio of upper to lower bands relative to the wild-type strain.

Electromobility shift assays (EMSAs)

Radiolabelled pre-tRNAs were generated by T7 transcription in the presence of ^{32}P-α-ATP (Ambion Megascript) using T7 promoter containing DNA templates generated by annealing and PCR amplification of respective pre-tRNA sequences. Radiolabeled pre-tRNAs were purified using PAGE extraction. EMSAs were performed as described (22). Briefly, 3000 cpm (approximately 0.1 nM) of pre-tRNA was incubated with various concentrations of Sla1p in a 20 µl reaction containing 20 mM Tris pH 7.6, 100 mM or 300 mM KCl and 5 mM β-mercaptoethanol. Pre-tRNAs were initially slow-cooled from 95°C to room temperature and then incubated with protein at 30ºC or 37°C for 20 minutes. Complexes were resolved on 10% (w/v) polyacrylamide nondenaturing gels at 4°C at 100V. Supershifts were treated as supplementary binding events to the primary binding event, and binding curves were fit using a non-linear specific binding curve fitting program (GraphPad, Prism; Non-linear regression used to assess all EMSA analyses).

***In vitro* modification of pre-tRNA transcripts by Trm1p**

Trm1p was cloned from *S. pombe* genomic DNA into the KpnI/EcoRI site of pET30a and purified by nickel affinity chromatography using standard methods. Trm1p modification on pre-tRNAs was carried out in a 30 µl reaction containing 33.33 mM Tris-HCl pH 7.6, 0.083 mM EDTA, 8.3 mM $MgCl_2$, 33.3 mM NH_4Cl, 0.83 mM DTT, and 83.3 mM S-Adenosyl methionine (SAM). Reactions were incubated at 32°C for 3 hours and purified using phenol chloroform extraction. Charging efficiencies were determined by performing modification reactions in the presence of 14C-SAM (Perkin-Elmer) followed by TCA precipitation of modified RNA, filtration through Whatman GF/C glass microfiber filters and scintillation counting. Reverse transcriptase primer

extension for detection of dimethylated Trm1p was performed using SuperScript III (Invitrogen) and standard methods.

In vitro chemical probing assay with lead acetate

Pre-tRNA Ser^{UGA} was generated by T7 transcription (Ambion Megascript) and then treated with Calf Intestinal Alkaline Phosphatase (CIP) (NEB) at 37°C for 30 minutes followed by phenol/chloroform extraction and ethanol precipitation. Pre-tRNA Ser^{UGA} was 5' end labeled using T4 polynucleotide kinase and ^{32}P-γ-ATP and purified using a denaturing gel. *In vitro* modification of pre-tRNA Ser^{UGA} transcript by Trm1p was carried out as above in the presence and absence of S-Adenosyl methionine (SAM). Chemical probing reactions were completed by the addition of 1 µl of 1 mg/ml of yeast RNA and 1X Ambion structure buffer (10 mM Tris pH 7, 0.1 M KCl, 10 mM $MgCl_2$) to heat-renatured pre-tRNA Ser^{UGA} in a total volume of 8 µl. Pre-tRNA samples were treated with a final concentration of 5 mM lead acetate with incubation at 37°C for 2 minutes, compared to mock treated unmodified controls. The reaction was stopped with the addition of loading buffer and incubation on ice for 5 minutes.

Harvesting yeast cells and immunoprecipitation of Sla1p-PrA pre-tRNA complexes

Integration of the Protein A tag into the *sla1+* chromosomal locus was performed and validated by sequencing and western blot as described (120). Yeast strains were grown in YES at 30°C or 37°C to OD_{600} ~ 0.8-1.0, harvested and subjected to cryogenic disruption in Retsch PM100 planetary ball mill as described (120). Immunoprecipitation was carried out on 1 g of powder with magnetic Dynabeads (Invitrogen) conjugated with Rabbit IgG (MP-Biomedicals) as described (120). Lysate and wash buffer contained 50mM NaCl, 0.5% Triton X, 0.1% Tween-20, 20 mM Hepes pH 7.4, 55 mM KOAc, 1 mM β-mercaptoethanol, 0.2 mM PMSF and 1:100 Protease Inhibitor Cocktail (HALT, Pierce). Complexes were treated with proteinase K (Sigma) and RNA was purified using phenol chloroform extraction.

cDNA synthesis and quantitative real time PCR analyses

RNA extracted from immunoprecipitation assays and was quantified by TaqMan qPCR. Primers and probes were from IDT. Reverse transcription on the purified RNA was done using the qScript™ cDNA Synthesis kit (Quanta Biosciences). Elution cDNA was used at 5 ng and total cDNA at 10 ng for pre-tRNA and U5 snRNA analyses. Forward primers and probes used for qPCR provided in Supplementary Table 4. Forward primer (IDT) and PerfeCta® Universal PCR primer (Quanta Biosciences) were used at 0.4 μM and probes (IDT) were used at 0.2 μM. The cycling conditions were as follows: an initial 95°C for 3 minutes, followed by 40 cycles of 95°C for 3 sec, 60°C for 20 sec for annealing and extension. qPCR results were evaluated using the ΔΔCt method and pulldown efficiency between inputs and elutions were assessed using U5 snRNA as the endogenous control. Comparison of enrichment within elution samples was performed by measuring the fold enrichment of the query pre-tRNA Ct versus the geometric mean Ct of three Trm1p non-modified pre-tRNAs (IleUAU, AlaCGC and TyrGUA) as described (121). Fold enrichments were calculated from the Ct values for each pre-tRNA performed as triplicate technical replicates; histograms of the means of these triplicate values were assembled from values obtained from two independent pulldowns each obtained from a minimum of two independent growths for each strain and temperature.

RESULTS

La RNA chaperone activity is required to rescue growth of *sla1-/trm1-* cells at elevated temperature

Mutations to the canonical RNA binding surface of RRM1 impair La RNA chaperone activity *in vitro* and La dependent rescue of defective (mutated) pre-tRNAs in *S. pombe* cells (29). Previous work in *S. cerevisiae* has demonstrated functional overlap of the dimethylation of G26 (N2, N2-dimethylguanosine) by the methyltransferase Trm1p and the La homolog Lhp1p, as a *trm1Δ/lhp1Δ* strain displays a temperature sensitive lethal phenotype (70). To compare previous insights on La dependent RNA chaperone activity with La function on hypomodified pre-tRNAs in a homologous system, we generated a *sla1-/trm1-* strain of *S. pombe* and compared its growth to the respective single deletion mutants at 32ºC versus 37ºC (Figure 1A&B). We observed that growth of the *sla1-/trm1-* strain was slightly impaired compared to the *sla1-, trm1-* or wild-type strain at 32°C, and that transition to 37°C was lethal in the double mutant, similar to *S. cerevisiae*. Interestingly, the synthetic lethality at 37ºC was seen only when cells were grown in minimal media (EMM), with no synthetic lethality observed when cells were grown in rich media. This is consistent with previous work describing slow growth of *sla1-* strains under tRNA-associated stresses that accompany growth in media that use NH_4 as a source of fixed nitrogen (122). Previous work has indicated that impairing nuclear surveillance by deletion of the nuclear exosome subunit Rrp6p can rescue tRNA-mediated suppression associated with a defective (mutated) pre-tRNA (47). We also tested a *sla1-/trm1-/rrp6-* triple mutant in case this might suppress the synthetic lethality of the *sla1-/trm1-* strain. Instead of a rescue, however, we observed that this strain grew worse than the *sla1-/trm1-* double mutant, as it was unable to grow on minimal media even at 32ºC.

To determine whether the RNA chaperone/RRM1 associated binding mode was important for La dependent rescue of the *sla1-/trm1-* strain, we transformed this strain with plasmids expressing wild-type human La (hLa) or Sla1p, as well as mutant hLa or Sla1p carrying mutations to the previously characterized RNP aromatic residues of RRM1 important for RNA chaperone function (hLa Y114A/F155A or Sla1 Y157A/F201A), or a vector control (Figure 1C). While all transformants grew at lower temperature, only the wild-type hLa or Sla1p transformants were able to rescue robust growth at 37ºC, and the RRM1 mutants displayed poor rescue more similar to the

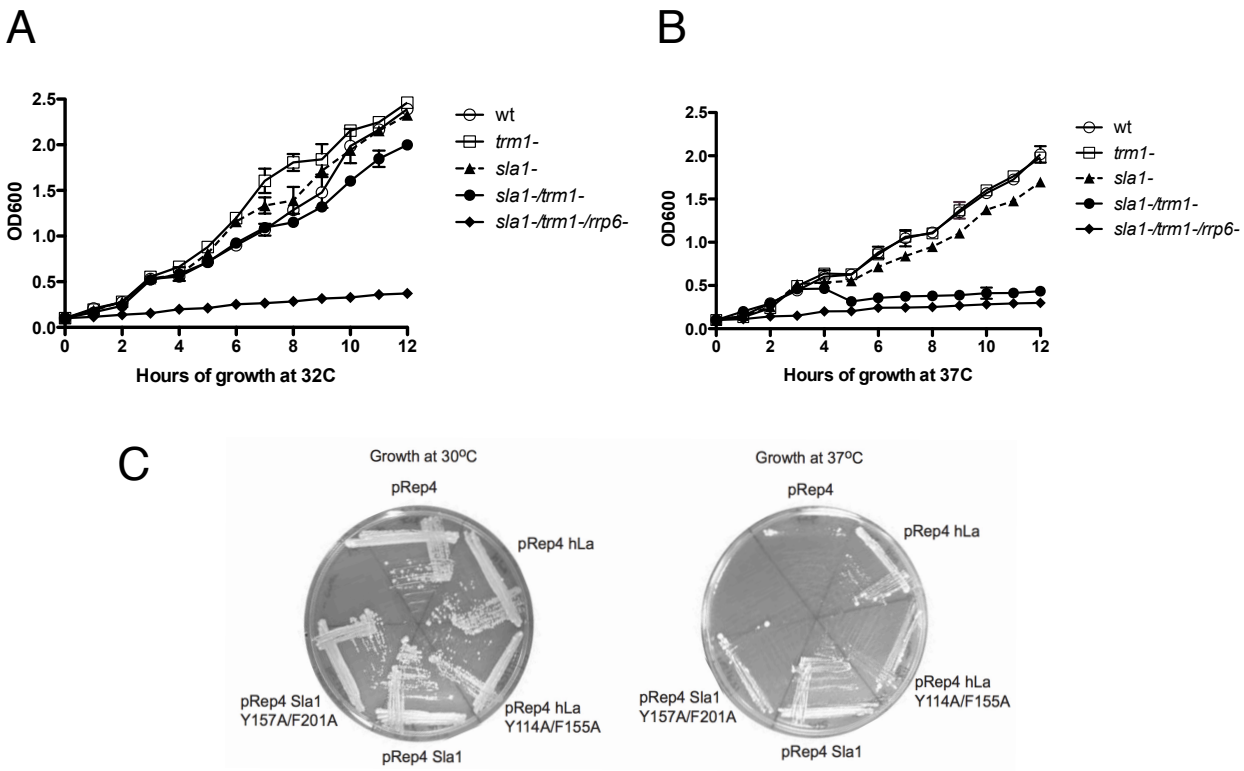

Figure 1. Synthetic lethality of *sla1-/trm1-* cells relies on La RNA chaperone activity. Indicated yeast strains were grown in liquid EMM media with essential supplements at 32°C (A) and 37°C (B) for 12 hours with absorbance collected at OD_{600}. Growth of the *sla1-/trm1-* was slowed at 32ºC and inhibited at 37°C, while *sla1-/trm1-/rrp6-* did not grow at either temperature. (C) La RNA chaperone mutants are defective in rescuing growth of the *sla1-/trm1-* cells at 37°C. *sla1-/trm1-* cells were transformed with empty pRep4 or pRep4 containing hLa, hLa Y114A/F155A, Sla1 or Sla1 Y157A/F201A (47).

vector control. We therefore hypothesize that the RNA chaperone activity of La proteins is important for the rescue of pre-tRNAs that misfold due to lack of modification at G26, and that tRNA misfolding due to lack of modification at G26 is sensitive to both temperature and the tRNA-associated stress previously linked to growth in minimal media.

Identification of candidate misfolded tRNAs in *trm1-* and/or *sla1-* strains of *S. pombe*

In most native tRNAs, G26 extends the anticodon stem by making a non-Watson-Crick interaction with N44 (whose identity is biased against "C") in the so-called "hinge" region, generating a six base-pair long ("Type 6") anticodon stem (including the non-Watson-Crick 26-44 base pair). Dimethylation of G26 has been previously hypothesized to stabilize these non-Watson crick G26-N44 interactions over particular folds previously observed for some mitochondrial tRNAs (75). For example, depending on a compatible sequence in the D-stem, lack of dimethylation of G26 could result in an alternate Watson-Crick pairing of G26 with C11 and a resulting non-native 5 base-pair long anticodon stem ("Type 5"). Alternatively, an unmodified G26 could form a Watson-Crick pair with a rare C44 and promote a seven base-pair long anticodon stem ("Type 7") should nucleotides 25 and 45 be compatible and available for pairing. We examined the sequences of G26 containing tRNAs in *S. pombe* in an attempt to ascertain which tRNAs may be particularly prone to taking these misfolded forms (Supplementary Table 1). We also took into consideration which G26 containing tRNAs are actually modified at G26 by Trm1, based on published results obtained by next-generation tRNA sequencing (tRNA-HydroSeq, (71)). Surprisingly, we found that contrary to the case in *S. cerevisiae* (i.e. tRNA-Lys^{CUU}), humans (i.e. tRNA-Asn^{GUU}) and other investigated species, there was not a single G26 containing tRNA in *S. pombe* (with the exception of a single allele for tRNA-Asn^{GUU}) that was predicted to be capable of forming the Type 5 fold, due to resultant mismatches in the remodeled D-stem (Supplemental Table 1). Among the G26 containing tRNAs, there was only a single tRNA species (tRNA-Gly^{GCC}) containing a C at position 44, but interestingly, tRNA-Gly^{GCC} is not a Trm1 target in *S. pombe* (71), consistent with a complete lack of GC base pairs in the Trm1p D-stem recognition element of tRNA-Gly^{GCC}. Together, these data suggest that prevention of the aberrant Type 5 and Type 7 folds may not be as critical a consideration for tRNA misfolding in *S. pombe* tRNAs compared to other species.

Previous work has identified particular tRNAs that appear to be sensitive to impairment of La function in yeast. In *S. cerevisiae*, tRNA-Arg^{CCG} has been linked to La associated rescue and is hypothesized to have a fragile anticodon stem due to a UC mismatch (46, 70). We therefore also considered the possibility that fragile anticodon stems might sensitize tRNAs to loss of La and/or Trm1. Since La function has also been linked to pseudouridylation of U38 and U39 by Pus3p (70), we also looked for the presence of uridines at these positions in G26 containing tRNAs (Supplementary Table 1). We noted that unlike in *S. cerevisiae,* the anticodon stem of tRNA-Arg^{CCG} does not have a mismatch in *S. pombe*. We did note, however, that uniquely among Trm1 targets in *S. pombe*, tRNA-Ser$^{CGA/UGA}$ has only a single G-C base pair in its anticodon stem, with all other Trm1 targets containing between 2 and 4 G-C base-pairs. tRNA-Ser^{CGA} and tRNA-Ser^{UGA} have also been previously linked to Trm1 function: they have been previously noted to undergo rapid tRNA decay in the context of a trm1Δ/trm4Δ strain in *S. cerevisiae* (115), and hypomodification of G26 in suppressor tRNA-Ser^{UCA} (an anticodon variant of tRNA-Ser^{UGA}) results in compromised tRNA-mediated suppression in *S. pombe* (71).

To experimentally identify candidate tRNAs that might misfold causing functional impairment, we assessed the amino acid charging levels of several G26 containing tRNAs in wild type, *sla1-*, *trm1-*, and *sla1-/trm1-* strains, as it has been demonstrated that tRNA misfolding can result in lower charging levels by aminoacyl-tRNA synthetases (46, 123, 124). In order to assess tRNA charging levels we performed acid-Northern (125) (Figure 2A) or β–elimination (126) (Figure 2B) of tRNAs isolated from cells grown in minimal media. As previously noted, we were particularly interested in investigating the charging efficiency of tRNA-Ser$^{CGA/UGA}$, tRNA-Arg^{CCG}, as well as other tRNAs predicted to have less stable anticodon stems. We observed a drop in charging efficiency (~25%) for tRNA-Ser$^{CGA/UGA}$ in the *trm1-* and *trm1-/sla1-* strains, but no drop in charging efficiency for tRNA-Arg^{CCG} or for other tested tRNAs containing two GC base pairs in their anticodon stems (tRNA-Leu^{AAG}, tRNA-Lys^{CUU}, tRNA-Ser^{AGA}, tRNA-Ile^{AAU}, tRNA-Trp^{CCA}, tRNA-Arg^{UCG}) or an anticodon stem mismatch (tRNA-Leu^{UAA}). To test the importance of growth conditions on tRNA charging, we also tested for the charging of several tRNAs from cells grown in rich media (Supplementary Figure 1). We found that tRNA-Ser$^{UGA/CGA}$ again had the greatest decrease in charging level among the tRNAs tested, with most tRNAs showing no charging defect in the mutant strains, although the degree of impaired charging was slighter than observed in minimal media. These results identify tRNA-Ser$^{UGA/CGA}$ as a tRNA whose fold is likely influenced

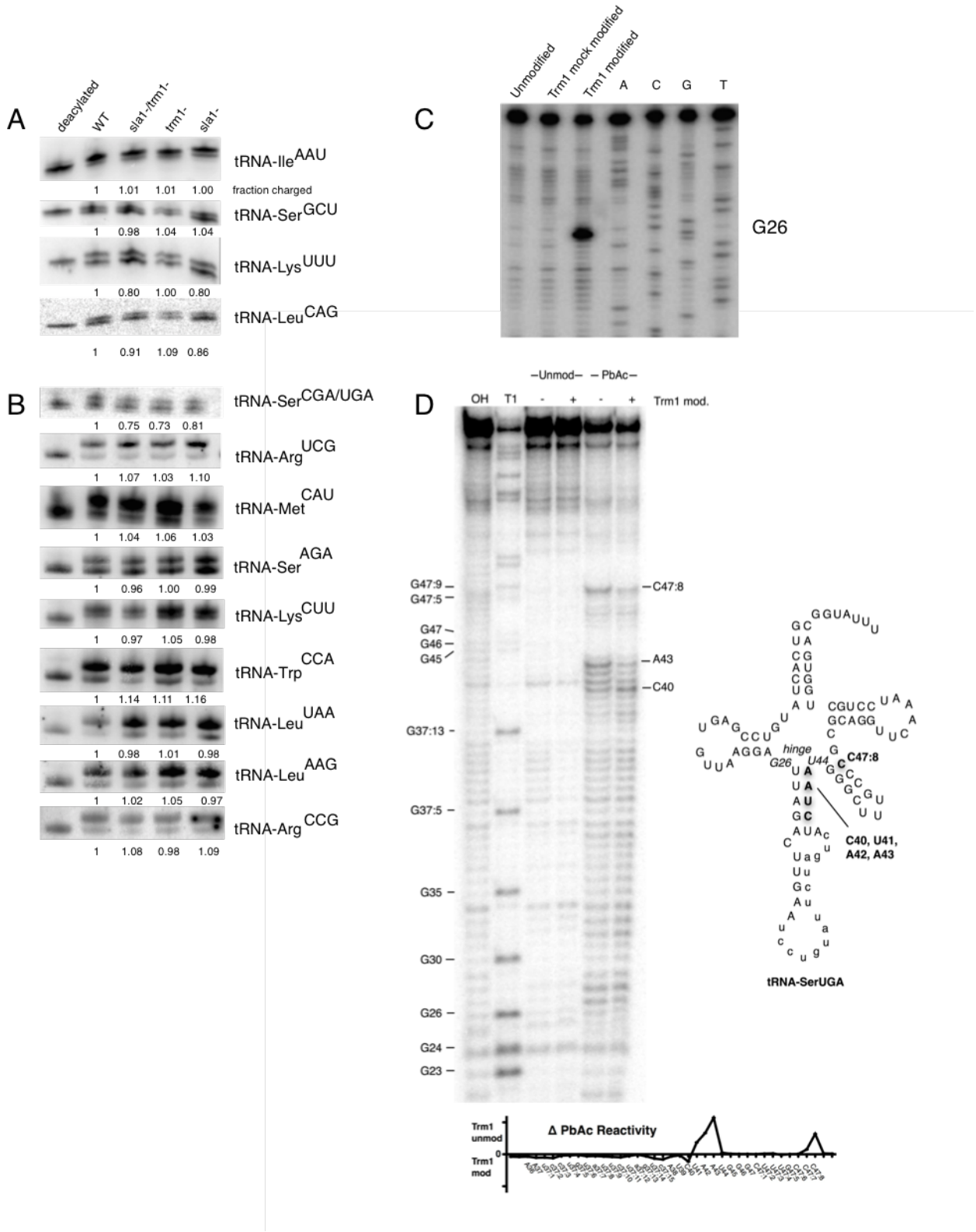
A
deacylated
WT
sla1-/trm1-
trm1-
sla1-
tRNA-Ile AAU
1 1.01 1.01 1.00
fraction charged
tRNA-Ser GCU
1 0.98 1.04 1.04
tRNA-Lys UUU
1 0.80 1.00 0.80
tRNA-Leu CAG
1 0.91 1.09 0.86
B
tRNA-Ser CGA/UGA
1 0.75 0.73 0.81
tRNA-Arg UCG
1 1.07 1.03 1.10
tRNA-Met CAU
1 1.04 1.06 1.03
tRNA-Ser AGA
1 0.96 1.00 0.99
tRNA-Lys CUU
1 0.97 1.05 0.98
tRNA-Trp CCA
1 1.14 1.11 1.16
tRNA-Leu UAA
1 0.98 1.01 0.98
tRNA-Leu AAG
1 1.02 1.05 0.97
tRNA-Arg CCG
1 1.08 0.98 1.09
C
Unmodified
Trm1 mock modified
Trm1 modified
A C G T
G26
D
−Unmod− − PbAc−
OH T1 - + - + Trm1 mod.
G47:9
G47:5
G47
G46
G45
G37:13
G37:5
G35
G30
G26
G24
G23
C47:8
A43
C40
Trm1 unmod
Trm1 mod
Δ PbAc Reactivity
hinge
G26
U44
C47:8
C40, U41, A42, A43
tRNA-SerUGA

Figure 2.

Figure 2. Lack of Trm1p modification at G26 results in likely misfolding of tRNA-Ser$^{CGA/UGA}$. Acid-Northern (A) and periodate oxidation/β-elimination (B) to assay charging levels of various tRNA species indicates a Sla1p/Trm1p associated tRNA charging defect for tRNA-Ser$^{CGA/UGA}$. (C) Reverse transcriptase primer extension of recombinant Trm1p modified tRNA-Phe^{GAA} confirms specific modification of G26 *in vitro*. (D) Left: Lead acetate chemical probing indicates altered structure in the anticodon stem loop and variable arm of pre-tRNA-Ser^{UGA} after *in vitro* modification with Trm1p. Right: Secondary structure of tRNA-Ser^{UGA}. Nucleotides with altered chemical reactivity to lead acetate probing +/- Trm1p modification indicated in bold. Bottom: quantitated differential lead acetate reactivity +/- Trm1p modification.

by dimethylation of G26 *in vivo*, consistent with previous work in *S. pombe* and *S. cerevisiae* linking this modification and tRNA-Ser$^{UGA/CGA}$ function (71, 115).

Lack of G26 dimethylation causes conformational changes in tRNA-Ser^{UGA} *in vitro*

Based on our results demonstrating impaired charging of tRNA-Ser$^{CGA/UGA}$ *in vivo*, we attempted to ascertain whether dimethylation of G26 might alter the structure of pre-tRNA-Ser^{UGA} as measured by chemical probing *in vitro*. In order to obtain G26 modified tRNAs, we cloned and purified recombinant Trm1p from *S. pombe* and used this to modify a T7-transcribed pre-tRNA-Ser^{UGA} sequence. We observed between 80 to 100% transcript modification of pre-tRNA-Ser^{UGA} as measured by use of 14C S-adenosylmethionine as the methyl donor, followed by TCA precipitation/filter binding and quantitation by liquid scintillation (data not shown). As the *in vitro* modification of pre-tRNAs by Trm1p from *S. pombe* has not previously been reported, we confirmed the specificity of our cloned enzyme by taking advantage of the propensity of Trm1p G26 dimethylation to cause an N-1 reverse transcriptase stop (127), and confirmed that a T7 pre-tRNA transcript was modified exclusively at the G26 nucleotide by our recombinant *S. pombe* Trm1p *in vitro* (Figure 2C). As measured by primer extension, our Trm1p dimethylated our T7 transcript to approximately 60% (ratio of G26 band to run off primer extension product), although this likely an underestimate as reverse transcriptase (Superscript III) can read through modified G26 through misincorporation of non-C nucleotides (71).

We then investigated the structure of pre-tRNA-Ser^{UGA} in the absence or presence of dimethylation at G26 by lead acetate chemical probing, as this method has previously been demonstrated to be highly sensitive to changes in secondary and tertiary structures in tRNA substrates (128). Upon Trm1p modification we observed a significant decrease in Pb^{2+} based cleavage in the A-U rich anticodon stretch (U41, A42 and A43) immediately adjacent to the hinge region of pre-tRNA-Ser^{UGA}, and a concomitant slight increase in reactivity at C40 (Figure 2D). Notably, these nucleotides are next to the U44 nucleotide that base pairs with G26 in pre-tRNA-Ser^{UGA}, and modification at G26 has been previously hypothesized to stabilize tRNA structure in this region (73, 75, 129). Furthermore, the accessibility of this region to chemical probing has been demonstrated to change upon *S. cerevisiae* La (Lhp1p) binding to pre-tRNA-Arg^{CCG} (46). Interestingly, we also observed a G26 modification dependent decrease in reactivity near the

beginning of the variable loop, close to the hinge, around C47:8. It is notable that both the C40/U41/A42/A43 and C47:8 regions lie at or near nucleotides whose mutation (C40U; C47:6U) results in defective tRNA suppression for tRNA-Ser^{UCA} (an anticodon mutant derived from tRNA-Ser^{UGA}) in *S. pombe* (130). While the exact nature of structural changes observed cannot be inferred from these results, they are strongly indicative that dimethylation at G26 can influence the stability of the hinge region of tRNA-Ser^{UGA} with consequent effects on tRNA function *in vivo*. Since tRNA-Ser$^{UGA/CGA}$ has only a single GC base pair in the anticodon stem, it is thus possible that the stability of the anticodon stem loop may be linked to La and Trm1 function, consistent with other work in *S. cerevisiae* (46). We did not notice any trend in tRNA fold or stability (see below) correlating with the possibility of pseudouridylation at positions 38 and 39 (Supplementary Table 1).

Trm1 promotes tRNA-mediated suppression of tRNA-Ser^{UCA}

In order to further investigate the importance of modification of G26 in the native folding of pre-tRNA-Ser^{UGA}, we tested for the capacity of Trm1p dependent dimethylation to promote tRNA-mediated suppression in a red-white assay that relies on the activity of tRNA-Ser^{UCA}, a suppressor-tRNA derivative of tRNA-Ser^{UGA} (Figure 3). We compared the overexpression of Trm1p (pRep4-Trm1) to a vector alone control (pRep4) in both a *sla1-/trm1+* (Fig. 3A) and a *sla1+/trm1-* (Fig. 3B) background for the original tRNA-Ser^{UCA} as well as two other tRNA alleles (tRNA-Ser^{UCA} C40U & tRNA-Ser^{UCA} C40U C47:6U) that carry additional mutations that have been previously demonstrated to increasingly rely on Sla1p for their function in the assay (47). We observed that overexpression of Trm1p resulted in enhanced tRNA-mediated suppression relative to the vector control for all three tRNA alleles in the *sla1-/trm1+* background, as well as for the tRNA-Ser^{UCA} C40U & tRNA-Ser^{UCA} C40U C47:6U alleles in the *sla1+/trm1-* background (the tRNA-Ser^{UCA} allele demonstrated no red pigment accumulation in this background and thus could not be assessed in this way), similar to previous results also testing for the function of Trm1p in tRNA-mediated suppression in *S. pombe (71)*. Together, these data further support impaired function for a tRNA-Ser^{UGA} derived tRNA in the absence of dimethylation at G26, and are consistent with our other data indicative of a tRNA-Ser^{UGA} folding defect that is rescued by Trm1p.

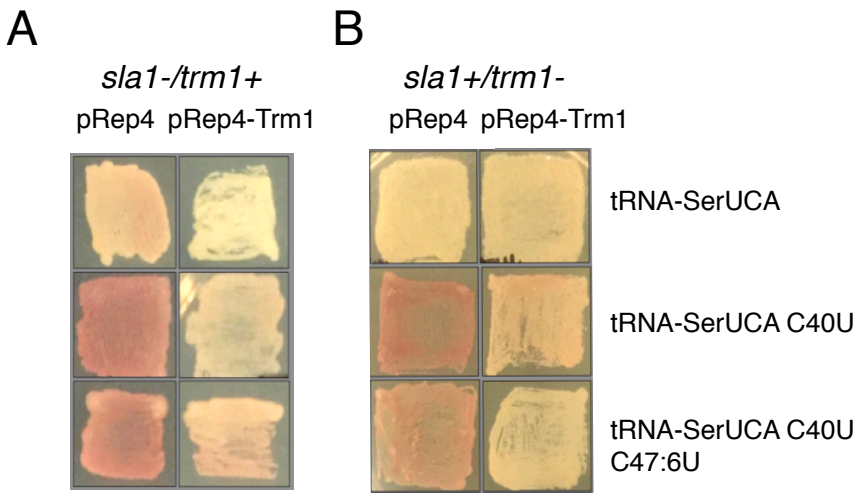

Figure 3. Dependence of tRNA mediated suppression on dimethylation at G26 by Trm1p. (A) tRNA mediated suppression of tRNA-SerUCA, tRNA-SerUCA C40U and tRNA-SerUCA C40U/C47:6U in a *sla1-/trm1+* strain background in the absence (pRep4) or presence (pRep4-Trm1) of overexpressed Trm1p. (B) Same as for A, but in a *sla1+/trm1-* background.

Sla1 and Trm1 promote the accumulation of selective sets of G26-containing tRNAs

We hypothesized that misfolding of tRNAs in *sla1-/trm1-* cells may alternatively result in their degradation, analogous to the demonstrated dependence of other tRNAs and pre-tRNAs on their modification status to protect these from rapid tRNA decay and nuclear surveillance. We therefore used Northern blots to quantify steady state levels of G26 containing tRNAs in *S. pombe* from *sla1-*, *trm1-*, and *sla1-/trm1-* strains relative to amounts in the wild-type parent strain, ten hours after a shift to minimal media (Figure 4). To normalize for differences in loading, we also probed our blots for U5 snRNA. As an internal control, we also measured steady state levels for a subset of tRNAs lacking a guanosine at position 26 (tRNA-Ile^{UAU}, tRNA-Gly^{CCC}, tRNA-Met^{CAU}, and tRNA-His^{GUG}) and for which deletion of Trm1p should thus not affect their relative abundance. Some representative Northerns are provided in Figure 4A. Certain tRNAs were more sensitive to deletion of Trm1p than others, for example, tRNA-Ser^{AGA} & tRNA-Arg^{ACG} accumulated to lower levels in the *trm1-* and *trm1-/sla1-* strains compared to the wild-type and *sla1-* strains (compare lanes 2 & 4 to lanes 1 & 3, Fig. 4A). We summarized the levels of all tRNAs sampled in the *trm1-*

strain normalized to a loading control (mature U5 snRNA) and relative to wild-type levels in Figure 4B. We observed that the levels of some tRNAs dropped slightly in *the trm1-* strain, and noted that the identities of these seemed to correlate with those tRNAs known to be modified by Trm1p (Trm1p modified tRNAs represented by white bars, Figure 4B-D; black bars represent tRNAs lacking G26 or known to not be modified at G26 in *S. pombe* as measured by RNA-seq (71)). Other tRNAs, for example tRNA-Phe^{GAA} and tRNA-Ile^{AAU}, constituted a non-overlapping set that were more sensitive to deletion of Sla1p (compare lanes 3&4 to lanes 1&2, Fig 4A; summary data for *sla1-* strain provided in Figure 4C). Examination of the *sla1-/trm1-* double mutant strain were indicative of more substantial decreases in several tRNAs, some to the degree to which they became statistically significant (P<= 0.05 or 0.01), thus identifying which tRNAs were most dependent on Sla1p and Trm1p for their steady state abundance (Figure 4D). Interestingly, we observed that the fold decrease for tRNAs in the *sla1-/trm1-* strain correlated positively with the product of the fold decrease of the same tRNAs in the *trm1-* and *sla1-* single mutant strains (Figure 4E), suggesting that the degree of depletion of tRNAs in the *sla1-/trm1-* double mutant is indeed due to the additive effect of the two single mutations impairing a related process. These experiments provided us with a subset of tRNAs for further analysis whose abundance is most sensitive to lack of modification at G26 in the absence of Trm1p.

Sla1p does not differentially engage hypomodified pre-tRNAs *in vivo*

Our analysis of tRNA charging, tRNA-mediated suppression and tRNA abundance in our mutant strains identified candidate pre-tRNAs that La may preferentially engage should La display affinity for potentially misfolded RNA substrates. In order to sample Sla1p/pre-tRNA complexes formed *in vivo,* we integrated a C-terminal 4X protein A (PrA) tag into the *S. pombe sla1* chromosomal locus in *trm1+* and *trm1-* strains and confirmed its integration by sequencing and Western blot (Figure 5A). To confirm the activity of our endogenously tagged Sla1-PrA protein, we compared the ability of our Sla1-PrA strain to promote tRNA-mediated suppression relative to *sla1+* and *sla1-* strains using a previously characterized, Sla1p dependent red/white assay (47) and showed that Sla1-PrA was active in the assay, similar to the WT *sla1+* strain (Figure 5B). We pulled down native Sla1p-PrA ribonucleoprotein complexes using a rapid pulldown protocol optimized for retention of endogenous particles (120), and pre-tRNA enrichment was determined by

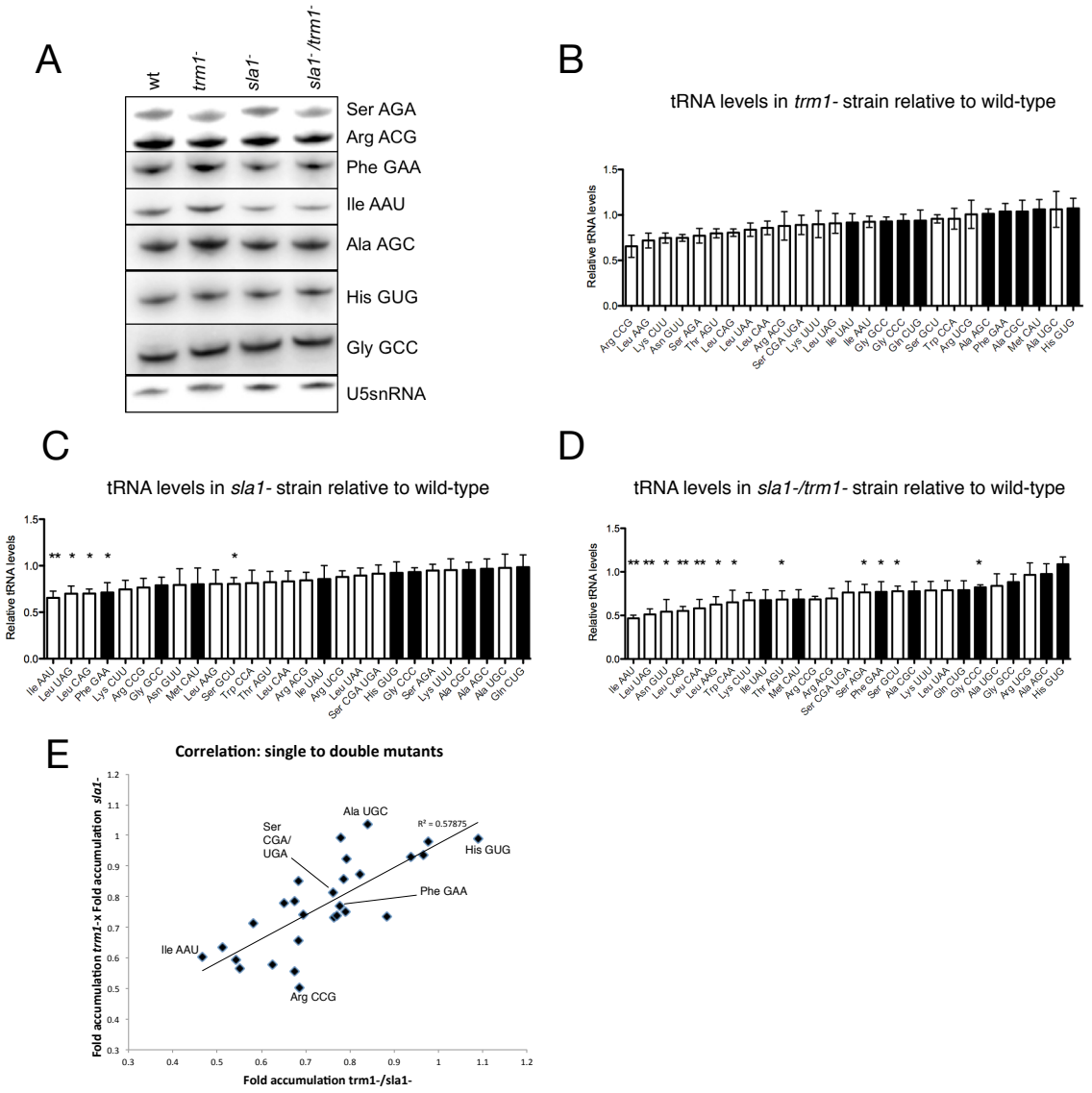

Figure 4. Variability in relative tRNA abundance in *sla1-/trm1-* cells. (A) Northern blot of total RNA extracted from indicated strains probed for various tRNAs or the U5 snRNA. (B) Relative abundance of G26-containing and non-G26 containing tRNAs in *trm1-* cells, normalized to tRNA levels in the wild type strain as well as the U5 snRNA as the endogenous loading control. Note that in *S. pombe*, the mature tRNA-Ser^{CGA} and tRNA-Ser^{UGA} species differ by a single nucleotide in the anticodon loop, and so our probe for Northern blot is expected to react with both. White bars: G26 modified tRNAs; black bars: tRNAs lacking a "G" at position 26 or lacking modification at G26 by Trm1p (71). Error bars: SEM. (C) Relative abundance of G26-containing and non-G26 containing tRNAs in *sla1-* cells. Significant drops in tRNA abundance relative to the wild-type strain are marked with * (P<= 0.05) or ** (P<=0.01). (D) Relative abundance of G26-containing and non-G26 containing tRNAs in *sla1-/trm1-* cells. (E) Correlation between the product of the tRNA levels in the *trm1-* and *sla1-* strains versus the levels of the respective tRNAs in the *sla1-/trm1-* strain. All tRNAs are represented; some tRNAs are annotated as reference points.

quantitative real-time PCR using tRNA specific exonuclease hydrolysis probes (i.e. 'TaqMan') and mature U5 snRNA as a normalization control. To ensure we sampled nuclear pre-tRNA La targets and not mature tRNAs in our analyses, we took advantage of the order of tRNA processing in yeast. Pre-tRNAs are transcribed by RNA polymerase III, are occasionally capped (131), and engage La through both their terminal UUU-3'OH and other contacts to the tRNA, which affects the order of tRNA processing events (25). When tRNAs are bound by La, 5' processing precedes 3' trailer processing before tRNAs are exported to the cytoplasm and spliced, should they have an intron (122, 132), after which, under some conditions, they can re-enter the nucleus via tRNA import (85, 133, 134). We ensured we sampled nascent pre-tRNAs in our work by limiting our qPCR analysis to intron containing pre-tRNAs using intron specific forward primers This design also ensured that all primers/probes would anneal downstream of G26, so that modification of this residue (if any) would not interfere with cDNA synthesis (see Supplementary Figure 2).

To determine whether Sla1p might discriminate pre-tRNAs that may be misfolded as a result of the absence of G26 N2, N2-dimethylation, we focused on several G26 containing pre-tRNAs that we demonstrated as having impaired charging *in vivo*/misfolding *in vitro* (pre-tRNA-Ser^{UGA} & Ser^{CGA}; Figure 5C) or decreased steady state stability (pre-tRNA-Lys^{CUU}, Leu^{CAA}, Leu^{UAG}, Leu^{CAG}, Arg^{CCG}, Ser^{GCU}) (Figure 5D; mean differences in pulldowns and associated P-values provided in Supplementary Table 2). In order to be able to compare relative pulldown of candidate misfolded pre-tRNAs across the *trm1+* and *trm1-* strains, we normalized the levels of these candidate G26-containing pre-tRNAs to the mean pulldown values of three pre-tRNAs whose folding and binding to La would not be influenced by Trm1p activity, by virtue of not possessing a G at position 26 (pre-tRNA-Ile^{UAU}, pre-tRNA-Tyr^{GUA}), or by virtue of being poorly modified by Trm1p at G26 as previously determined by RNA-Seq and Northern blot (pre-tRNA-Ala^{CGC}; (71)). We found that Sla1p association with pre-tRNAs in the presence or absence of G26 modification did not vary significantly for any of the candidate pre-tRNAs tested, with observed fold enrichments in *trm1-* versus *trm1+* strains on the order of two-fold or less, and with no differences in pulldown efficiency reaching statistical significance (P=0.05, Supplementary Table 2). This was in contrast to a highly substantial enrichment of each candidate pre-tRNA relative to a non-La target, the U5 snRNA, validating the effectiveness of the pulldown and quantitation (Figure 5C, D). Similarly, pulldown of candidate pre-tRNAs from the *trm1-* strain grown at 37ºC versus 30ºC also did not indicate preferential engagement of candidate pre-tRNAs at higher temperature. In

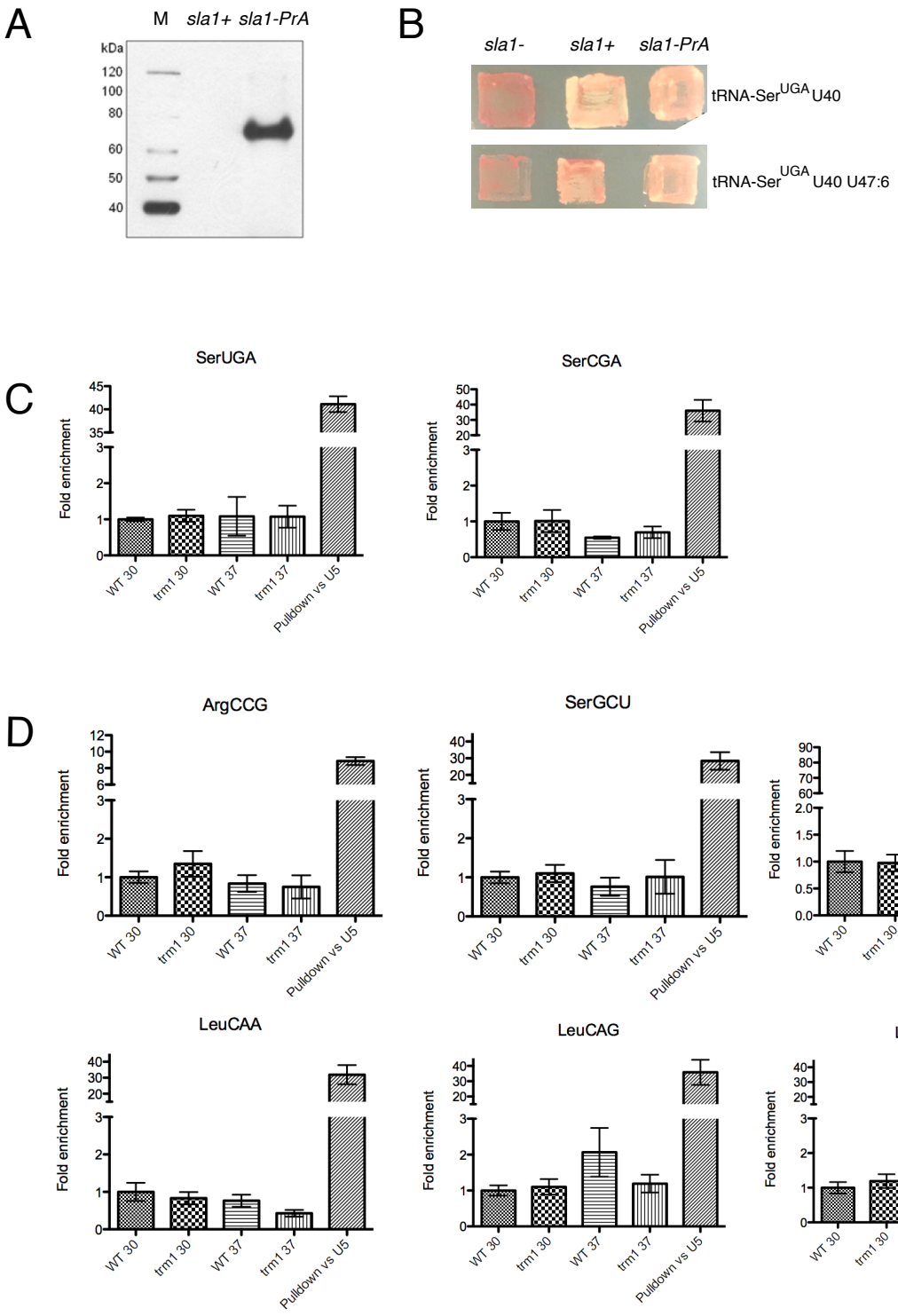
A
M
sla1+
sla1-PrA
kDa
120
100
80
60
50
40
B
sla1-
sla1+
sla1-PrA
tRNA-Ser^UGA U40
tRNA-Ser^UGA U40 U47:6
C
SerUGA
SerCGA
Fold enrichment
WT 30
trm1 30
WT 37
trm1 37
Pulldown vs U5
D
ArgCCG
SerGCU
LysCUU
LeuCAA
LeuCAG
LeuUAG

Figure 5.

Figure 5. Association of pre-tRNAs with Sla1p-PrA +/- G26 modification in *S. pombe* cells. Endogenous Sla1p-PrA/pre-tRNA complexes from *trm1-* and *trm1+* cells grown at 30ºC versus 37ºC were immunoprecipitated and levels of candidate pre-tRNAs were quantitated by 'TaqMan' quantitative real-time PCR (qPCR). (A) Western blot confirming the expected size and expression of the Sla1-PrA fusion protein relative to the parent *sla1+* strain. (B) Endogenous Sla1p containing an integrated Protein-A tag at the C-terminus (sla1-PrA) is comparably active to wild-type Sla1p as determined by a tRNA mediated suppression assay using two different defective suppressor tRNA alleles (47). (C) Analysis of fold pulldown levels of candidate misfolded pre-tRNAs (as measured by impaired charging and/or chemical probing, Figure 2) compared to an internal control, the geometric mean pulldown level of three pre-tRNAs not modified by Trm1p (pre-tRNA-Ile^{UAU}, pre-tRNA-Tyr^{GUA} and pre-tRNA-Ala^{CGC}) in the same elution samples. As a reference for robust pulldown of La targets versus non-targets, fold pulldown of indicated pre-tRNA in elution samples versus total RNA samples (wild-type strain) is provided relative to the La non-target U5 snRNA (right hand side, histograms). (D): same as for (C) but for candidate pre-tRNAs identified as having decreased accumulation in *sla1-/trm1-* strains (see Figure 4).

order to ensure that our results were not confounded by rearrangement of complexes during the pulldown procedure, we also repeated the analysis but in the presence of formaldehyde crosslink prior to harvest. These experiments were also indicative of a lack of substantial differences in affinity, similar to the minus crosslink cohort (on the order of 2X or less; Supplementary Table 2). Together, these data are consistent with a lack of discrimination of Sla1p binding to pre-tRNAs according to their G26 modification status *in vivo*.

Sla1p does not differentially engage Trm1p modified pre-tRNAs *in vitro*

To test Sla1p binding to Trm1p modified versus unmodified pre-tRNAs more directly, we tested Sla1p affinity for G26 modified versus unmodified pre-tRNA transcripts *in vitro* by electromobility shift assay (EMSA; Figure 6A). We performed EMSA analysis for tRNA-Ser^{UGA}, whose charging was defective in the *sla1-/trm1-* strain and whose structure was altered +/- G26 modification *in vitro*, as well as two pre-tRNA sequences corresponding to tRNAs with decreased abundance in the *sla1-/trm1-* strain (tRNA-Leu^{UAG} and tRNA-Leu^{CAG}). For these experiments, we first formed complexes at the temperature which should favour misfolding (37ºC) and at 100 mM KCl. For each of these substrates, we observed minimal (~ 2X or less) differences in affinity, similar to the trends we observed *in vivo*. Since misfolding of RNAs *in vitro* has been hypothesized to be temperature and salt dependent, we also compared complex formation between 30ºC and 37ºC and between 100 mM and 300 mM KCl and still observed no modification dependent changes in affinity (Supplementary Figures 3-5). Thus, our *in vitro* and *in vivo* data are consistent with Sla1p having similar affinity for pre-tRNAs regardless of the presence or absence of N2,N2-modification at G26, despite our biochemical and genetic data confirming the functional overlap of this modification with the importance of La in promoting productive pre-tRNA processing.

As a final proof of principle, and to compare the conservation of La binding to pre-tRNAs between yeast and humans, we compared the affinity of human La (hLa) for a fully modified, mature tRNA-Phe^{GAA} (purified from *S. cerevisiae*; Sigma-Aldrich) with its completely unmodified, T7 transcribed counterpart (Figure 6B). While this mature tRNA should not normally be a substrate for La proteins *in vivo* by virtue of nuclear pre-tRNA trailer processing and 3'CCA addition, we reasoned that the UUU-3'OH independent La-tRNA binding mode should still be sensitive to tRNA modification induced structural changes should these be important for La target

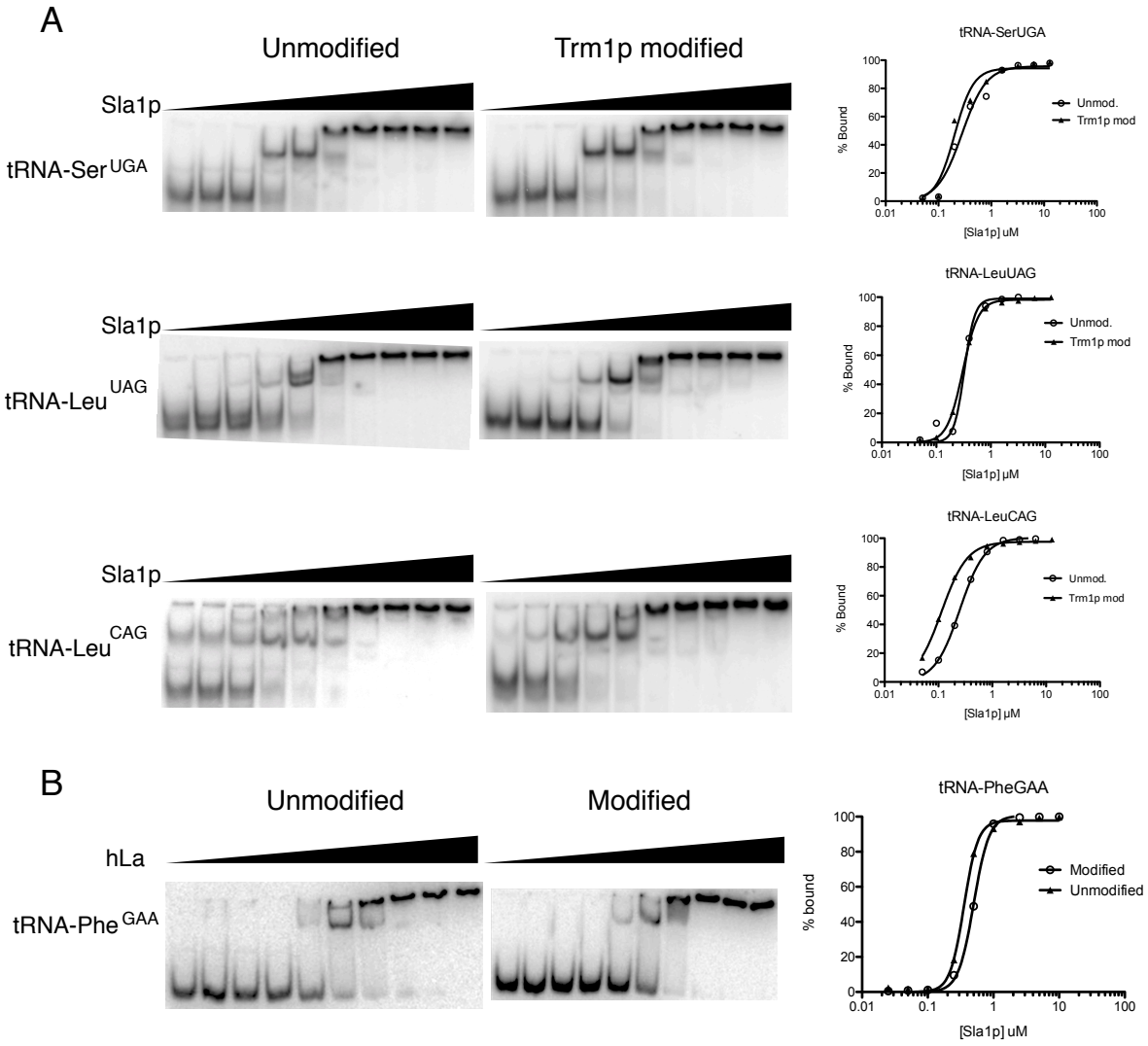

Figure 6. Association of Sla1p and hLa with modified or unmodified pre-tRNAs *in vitro*. (A): EMSA of Sla1p with T7 transcripts corresponding to candidate pre-tRNAs identified as having impaired charging/*in vitro* misfolding (tRNA-Ser^{UGA}) or impaired accumulation (tRNA-Leu^{UAG} & tRNA-Leu^{CAG}) in the absence (unmodified) or presence (Trm1p modified) of G26 modification. (B) EMSA of hLa with a T7 transcript corresponding to mature *S. cerevisiae* tRNA-Phe^{GAA} or its fully modified counterpart purified from *S. cerevisiae* cells.

discrimination. Similar to our earlier results, we observed less than a 2X difference in affinity between the fully modified tRNA and the corresponding *S. cerevisiae* tRNA-Phe^{GAA} T7 transcript, consistent with the specific fold imposed via tRNA modification not being an important determinant for La engagement, at least for the folds assumed by our tRNA substrates under the conditions of our analysis *in vitro*.

DISCUSSION

In this work, we have attempted to shed light on how La proteins may discriminate targets on the basis of fold by examining pre-tRNAs, their best-characterized substrates. Proteins that enhance the folding of RNAs into their native conformation have been apportioned into a variety of classes (RNA chaperones, RNA cofactors and RNA helicases) based on their proposed mechanisms of action (7, 135). Unlike RNA cofactors, RNA chaperones can display activity across a variety of targets and do not need to remain associated with their remodeled RNA targets for these to retain their function. Thus, RNA chaperones are hypothesized to bind RNA relatively non-specifically (2, 6, 7). Some RNA chaperones have been hypothesized to have a preference for binding single stranded regions of RNA (12, 16, 110), and models for RNA chaperone binding have been proposed in which the free energy of binding to unfolded substrates varies from that for folded substrates (2, 6). However, it is not clear how generally these principles should be applied across the wide breadth of proteins with RNA chaperone activity, especially ones like La, in which a non-specific RNA chaperone-associated RNA binding mode is juxtaposed with its sequence specific UUU-3'OH dependent RNA binding mode (22, 29, 48).

La proteins engage pre-tRNAs via at least two distinguishable binding modes: the UUU-3'OH dependent trailer binding mode associated with the La motif, and a UUU-3'OH independent mode associated with the canonical RNA binding surface of the RRM1 domain and with RNA chaperone function, with both modes contributing additively to maximize pre-tRNA binding affinity (22, 29, 55, 113, 136). Disordered regions C-terminal to RRM1 have also been implicated in RNA chaperone-like functions, and have also been proposed to contribute to La-RNA target affinity (48, 56, 128, 137). While the importance of the UUU-3'OH motif in La recruitment is well-established (136, 138), the question of whether other binding determinants influence La selection of targets within the UUU-3'OH containing cohort has not been tested previously. This issue may be of importance in light of the observation that the maturation of defective suppressor tRNAs benefits from additional, plasmid encoded Sla1p in *sla1+* cells (43), suggesting that for at least some La targets, endogenous levels of La protein are limiting, and that UUU-3'OH containing RNAs could compete for La binding.

Using *in vivo* pulldown assays of endogenous complexes as well as *in vitro* EMSAs, we have tested whether La differentially binds hypomodified pre-tRNA substrates. While it is

challenging to demonstrate direct evidence for misfolded conformations of precursor tRNAs *in vivo*, there are several lines of evidence consistent with a lack of modification of G26 causing folding defects for at least some Trm1 targets: a) we demonstrate that the synthetic lethality associated with the *trm1-/sla1-* strain is poorly rescued using mutants of La previously demonstrated to be defective in RNA chaperone activity but not UUU-3'OH end binding (47) ; b) we show that tRNA-Ser$^{UGA/CGA}$ is charged to a lesser extent *in vivo* in a *sla1-/trm1-* strain, similar to other work showing impaired charging by aminoacyl-tRNA synthetases on misfolded tRNAs; c) we demonstrate that lack of Trm1p modification at G26 results in defective tRNA mediated suppression *in vivo* in *S. pombe*, for a suppressor tRNA (tRNA-Ser^{UCA}) derived from tRNA-Ser^{UGA}; d) we show that the anticodon stem loop hinge region of a T7 transcript of pre-tRNA-Ser^{UGA} shows differential reactivity to lead acetate based cleavage in the presence versus absence of Trm1p modification, which to our knowledge is the first experimental result supporting the importance of this modification on the stability of tRNA anticodon stems (75) , and e) we show that several tRNAs accumulate to lower steady state levels in *trm1-* and *trm1-/sla1-* strains relative to wild-type strains.

We did not observe substantially different binding to modified versus unmodified pre-tRNA substrates *in vitro* or *in vivo,* suggesting that Sla1p does not differentiate a potential tRNA misfold associated with G26 hypomodification, despite the apparent link between Sla1p and Trm1p function in cellular growth. This is, to our knowledge, the first demonstrated example of an RNA chaperone being unable to discriminate a native from a physiologically relevant misfolded target. Thus our work is reminiscent of some previously proposed models for RNA chaperones in which the challenges associated with discriminating native from misfolded RNA conformations due to the relative homogeneity of functional groups within nucleotides have been highlighted (2, 6, 7). A significant caveat in the interpretation of our results is that we cannot account for differential La binding to every possible misfolded pre-tRNA conformation. Studies on the absence of other tRNA modifications or tRNA anticodon stem loop mutants have also been linked to La function (46, 69, 116, 130), and these may present misfolded conformations that are indeed preferentially engaged by La. Nevertheless, for at least the misfolded RNA species used in our study, our data are consistent with previous work suggesting that La engages pre-tRNAs irrespective of their specific fold for the benefit of misfolded species and at the expense of processing efficiency of correctly folded pre-tRNAs (47). Consistent with this, we show that La

shows marginal differences in affinity between a fully modified mature tRNA substrate and its modification lacking, *in vitro* synthesized counterpart.

In the absence of discrimination on the basis of fold, we hypothesize that the specific targeting of RRM1 associated La RNA chaperone activity to nascent transcripts thus relies on the UUU-3'OH dependent binding mode and the coupled progression of tRNA processing events. In this context, the La motif (which we have previously demonstrated to lack RNA chaperone activity *in vitro*; (29)) may be viewed as an accessory domain for RNA refolding (similar to analogous domains in some RNA helicases), as we have hypothesized previously (29); (Figure 7). Thus, the time between nascent transcript binding and trailer processing provides La with a fixed window of opportunity to assist the pre-tRNA in reaching the native state, irrespective of whether or not the pre-tRNA has assumed an early misfold that necessitates La dependent rescue. In this scheme, a pre-tRNA that misfolds will benefit from the window of La engagement, however, this comes at the cost of decreased processing efficiency for pre-tRNAs that may have assumed a native fold even in the absence of La binding. La RNPs contain many post-transcriptionally modified nucleotides found in tRNAs (118), thus one function of this window may be to provide an opportunity for such modifications to reinforce the native fold before La dissociates upon trailer (i.e. UUU-3'OH) removal (22). In this light, the engagement of La proteins to its RNA targets would seem more akin to the ER resident protein chaperones calnexin and calreticulin, which are hypothesized to recognize a specific N-linked glycosylation motif associated with an intermediate stage of lumenal protein maturation (reviewed in (139)), rather than the exposed hydrophobic regions more directly associated with misfolds that are recognized by classical protein chaperones.

We observed that the *sla1-/trm1-* double mutant was synthetically lethal when grown at higher temperature, similar to *S. cerevisiae* (70). Notably, this synthetic lethality could be fully rescued with overexpression of *S. pombe* or human La but not with mutants of these previously demonstrated to be defective in RNA chaperone function (22, 29, 47). Previous work has demonstrated that La RNA chaperone activity associated with the canonical RNA binding surface of RRM1 can rescue defective suppressor tRNAs in a red-white assay. This work builds on this result, pointing specifically to the importance of La-associated RNA chaperone activity in rescuing viability associated with the misfolding of endogenous pre-tRNAs. We hypothesized that this synthetic lethality could be due to degradation of particularly sensitive pre-tRNAs, as previous work has demonstrated that deletion of the nuclear exosome subunit *rrp6+* can rescue the

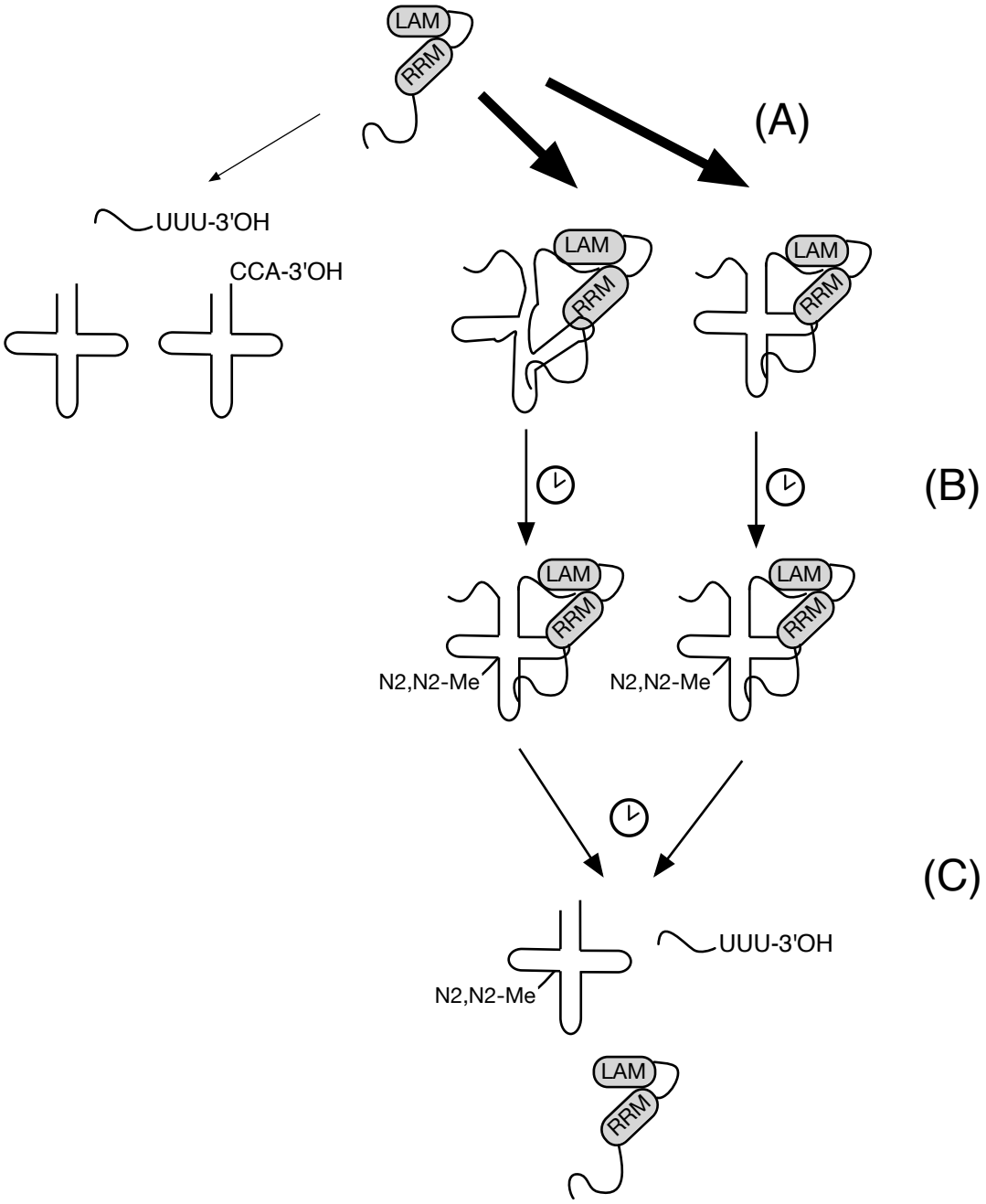

Figure 7. Model for La engagement of folded versus unfolded pre-tRNAs. (A) The RNA chaperone La preferentially engages pre-tRNAs via their UUU-3'OH containing trailers (25, 55, 113, 136) as well as through contacts to the main body of the pre-tRNA (22, 47), but without discriminating folded from misfolded pre-tRNA substrates (this work). (B) During the window of La occupancy, misfolded pre-tRNAs acquire the native fold through post-transcriptional modification by Trm1 (70, 118) and/or La RNA chaperone activity (22, 26, 29, 30, 46, 47). La affinity for the pre-tRNA does not change as the native fold is acquired. Thus this window benefits the misfolded pre-tRNA but not the pre-tRNA that did not benefit from La dependent rescue. (C) La remains with the pre-tRNA until trailer cleavage and La dissociation (22, 25).

degradation and tRNA-mediated suppression associated with suppressor tRNAs that have been mutated such that they are predicted to misfold. We indeed noted decreases in steady state levels of several tRNAs in the context of a *sla1-/trm1-* strain, as well as a decrease in the charging of tRNA-Ser$^{CGA/UGA}$. Instead of a rescue, we observed an exacerbated lethal phenotype when combining the *sla1-/trm1-* deletion with *rrp6-* deletion, as the *sla1-/trm1-/rrp6-* triple mutant was unable to grow on minimal media even at 32ºC. Since the nuclear exosome is predominantly associated with the degradation of defective substrates, this result may be consistent with the synthetic lethality of the *sla1-/trm1-* strain being due to the persistence of a misfolded Trm1p substrate (or substrates) acting in a dominant negative fashion, although the specific basis for this phenotype is at this point still unresolved. In this sense, La and Trm1p could co-operate with the nuclear surveillance machinery to ensure that defective pre-tRNAs do not enter the pool of mature tRNAs, rather than to ensure such tRNAs are not degraded below unsustainable levels.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY DATA

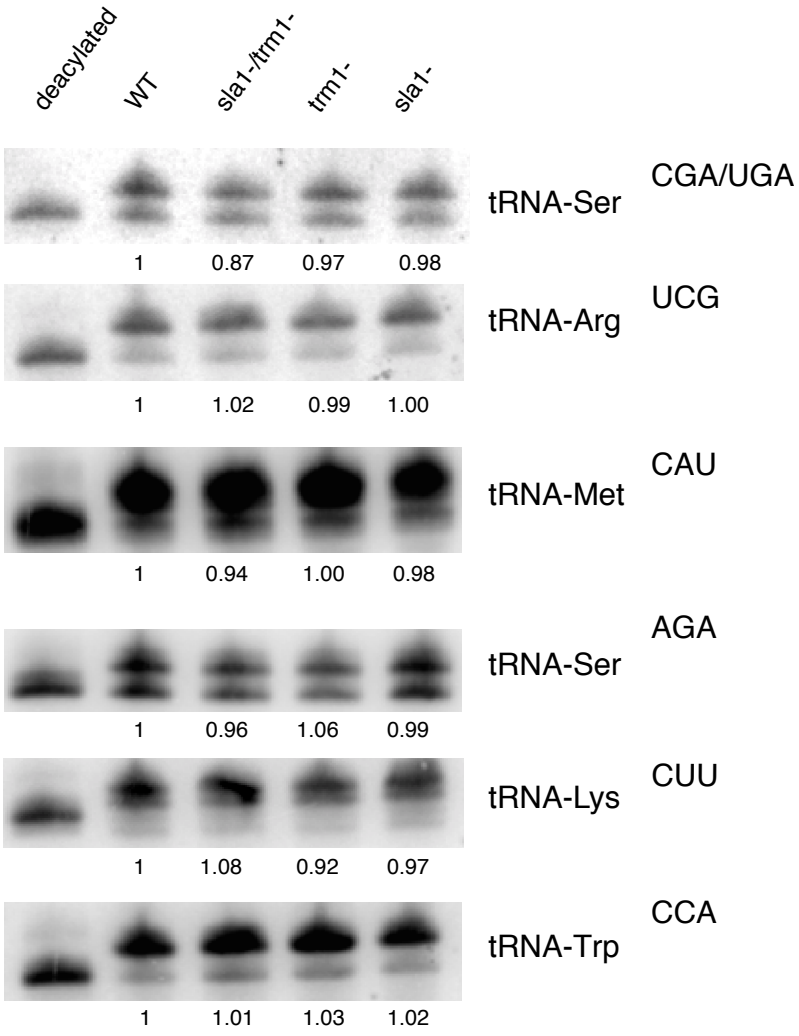

Figure S1. Beta-elimination measurement of tRNA charging levels for candidate tRNAs grown in rich media (YES).

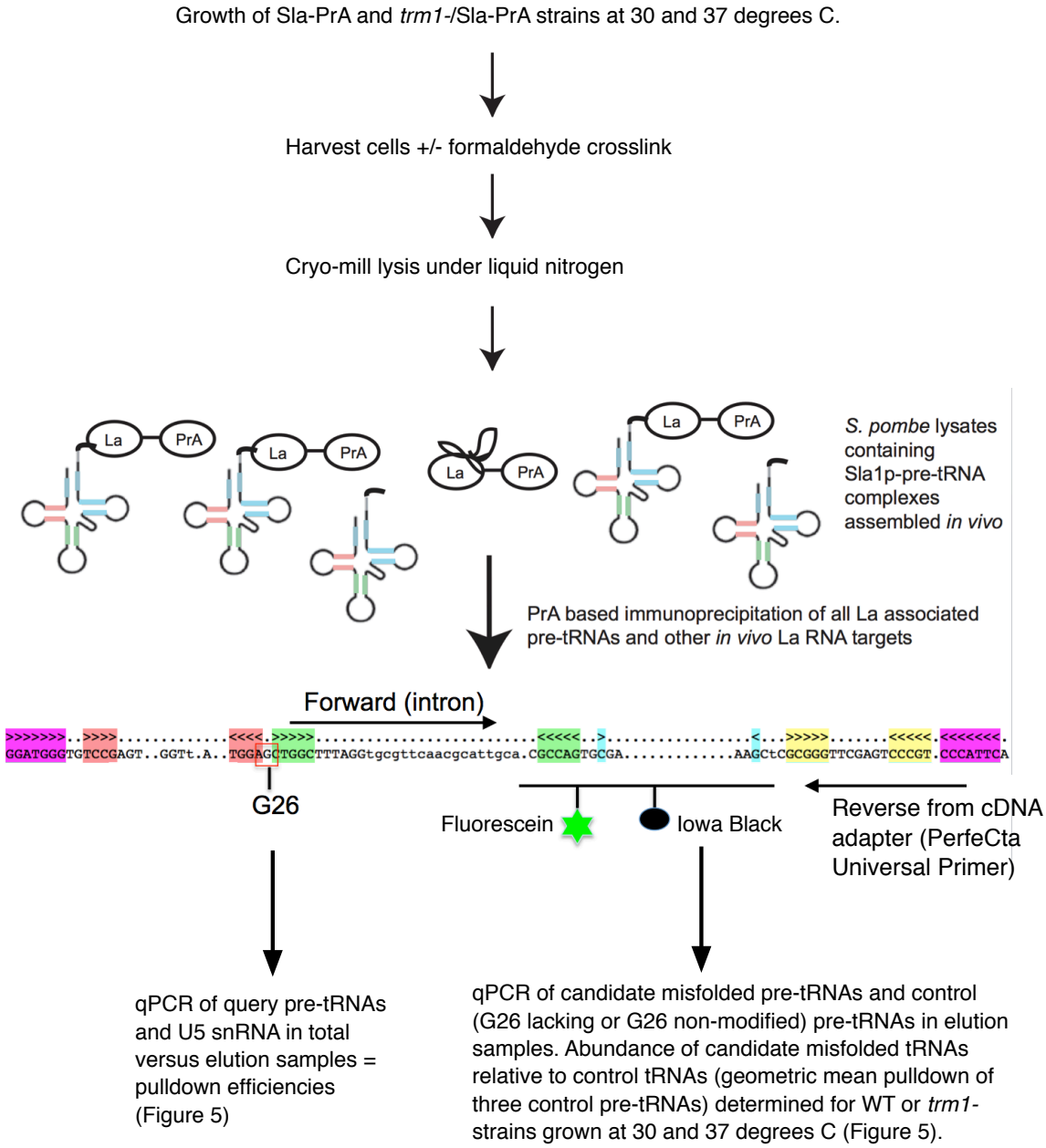

Figure S2. Purification and quantitation scheme for pulldown of endogenous Sla-PrA/pre-tRNA complexes. Sample qPCR detection demonstrated (pre-tRNA Leu TAG); relative position of probes held constant for all tested pre-tRNAs.

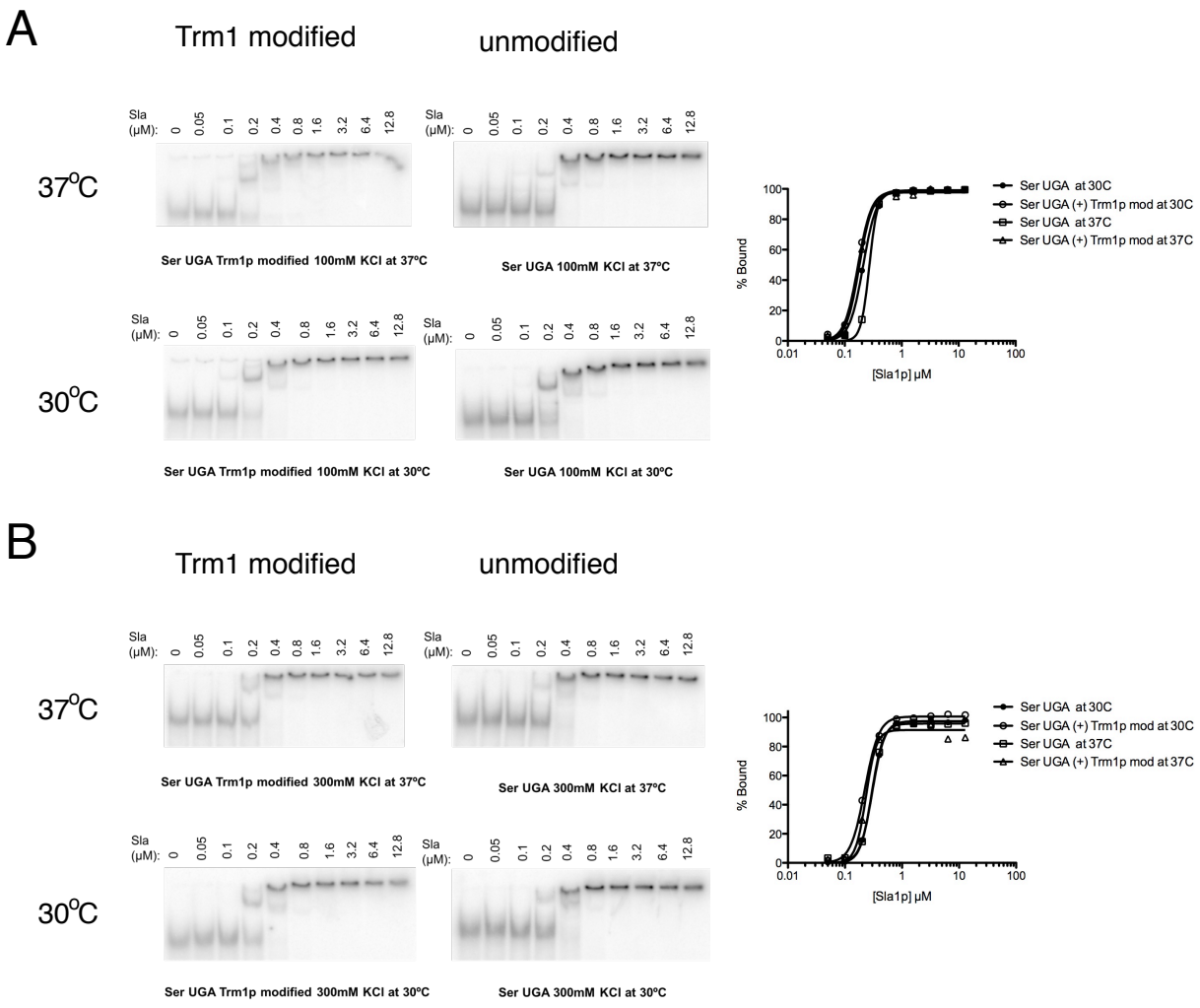

Figure S3. Affinity of Sla1p for tRNA-Ser^{UGA} is not influenced by its modification by Trm1p irrespective of salt (A:100mM KCl vs B:300mM KCl) or temperature (incubation at 30°C vs 37°C).

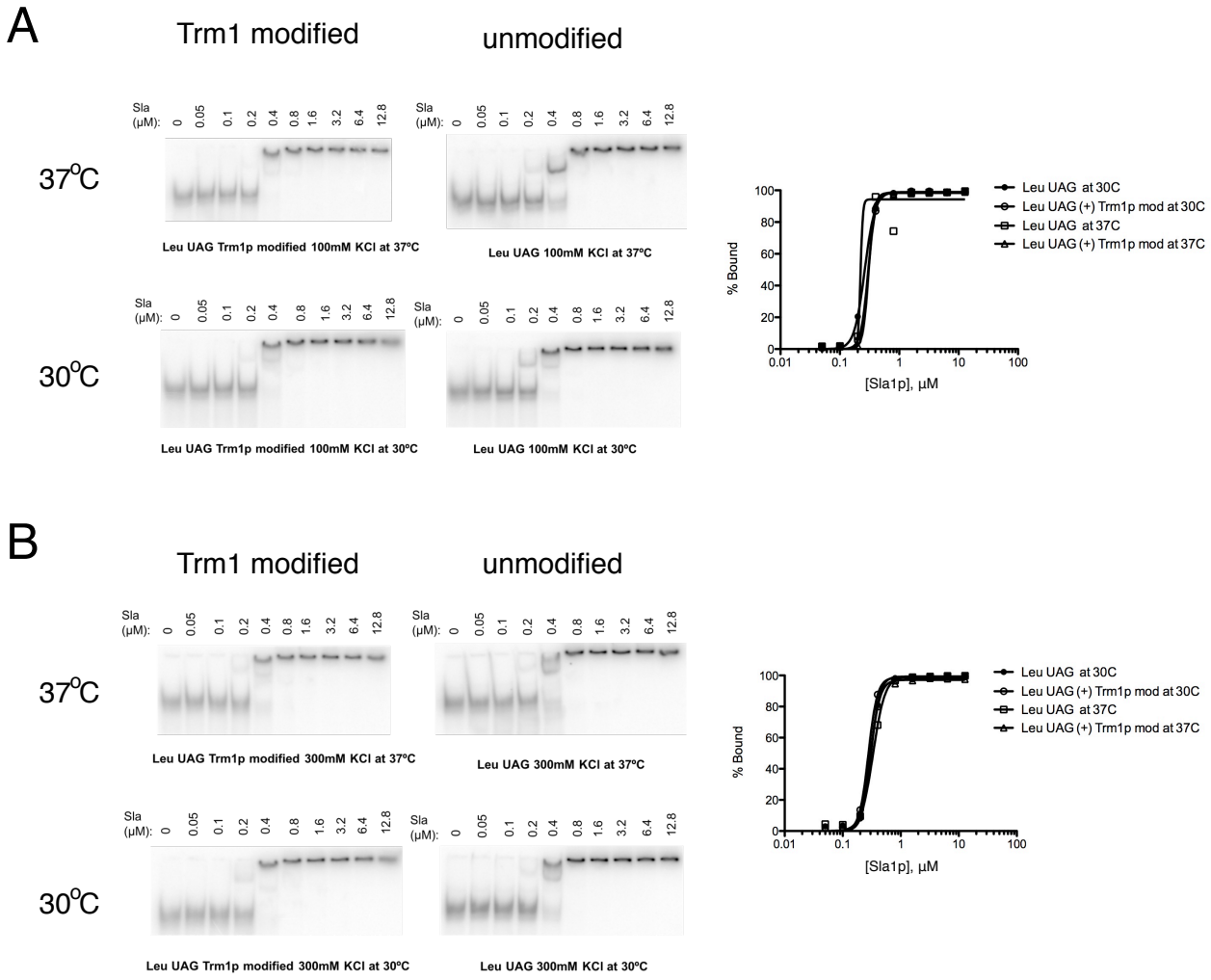

Figure S4. Affinity of Sla1p for tRNA-Leu^{UAG} is not influenced by its modification by Trm1p irrespective of salt (A:100mM KCl vs B:300mM KCl) or temperature (incubation at 30°C vs 37°C).

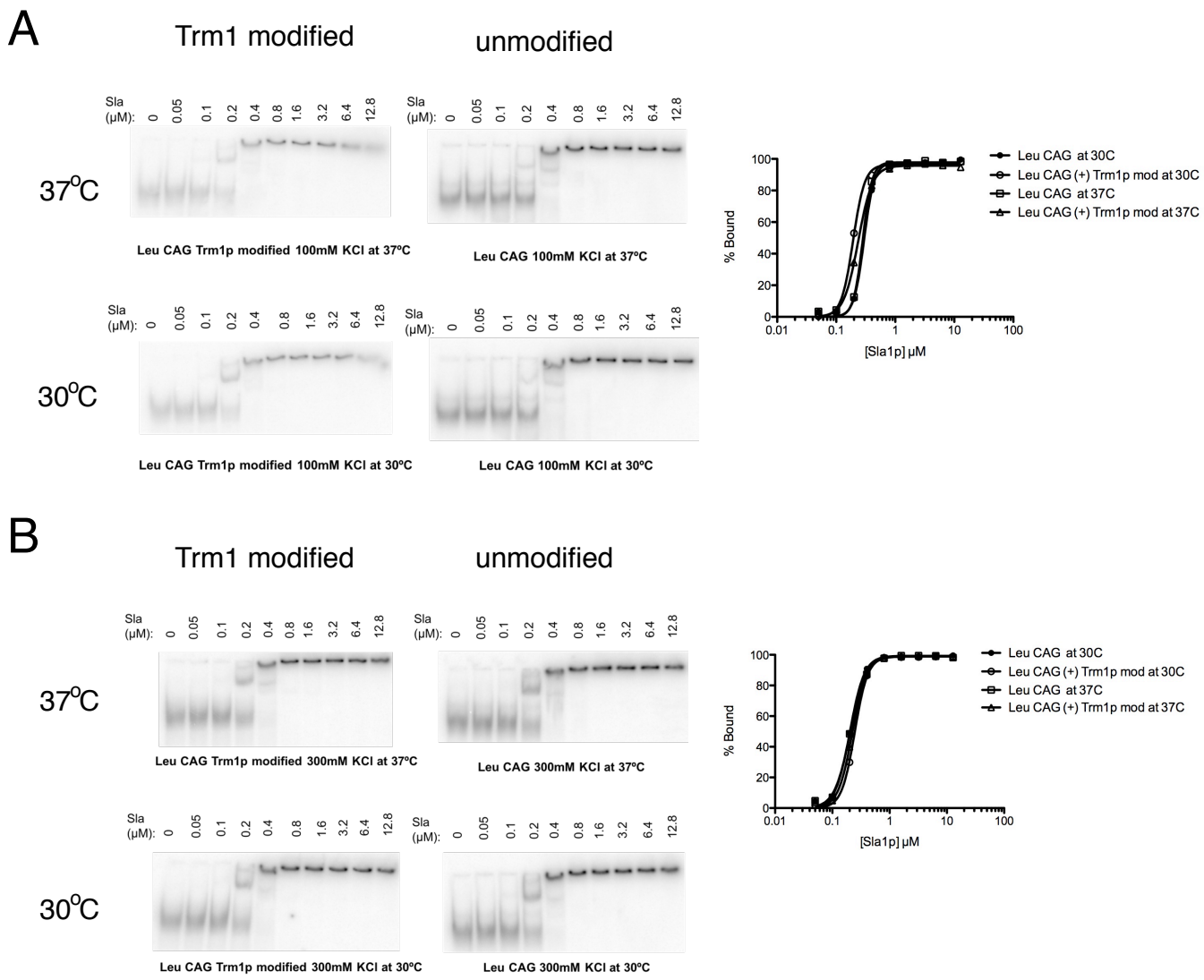

Figure S5. Affinity of Sla1p for tRNA-Leu^{CAG} is not influenced by its modification by Trm1p irrespective of salt (A:100mM KCl vs B:300mM KCl) or temperature (incubation at 30°C vs 37°C).

Table S1. Architecture of *S. pombe* tRNAs of interest in this study. Modification at G26 from Arimbasseri et al., 2015 *PLoS Genetics* 11, e1005671. *: a single allele of tRNA Asn GUU (Chromosome 2, nt 4210126) is predicted to be able to form a Type-5 misfold, albeit with a G-U wobble. Seq 11-13/24-26 at this locus: CGU/GCG. **: tRNA Leu UAA has an A-C mismatch in the anticodon stem.

	G26?	G26 Modified?	C11?	Seq: 11-13/ 24-26	C44?	GC's in AC stem	U@38/39
Arg CCG	Yes	Yes	Yes	C**C**U/G**C**G	No	2/5	C38/G39
Leu AAG	Yes	Yes	Yes	C**CG**/**GA**G	No	2/5	C38/G39
Lys CUU	Yes	Yes	Yes	C**U**C/G**C**G	No	2/5	A38/A39
Asn GUU	Yes	Yes	Yes	C**A**U/G**C**G*	No	3/5	A38/**U39**
Ser AGA	Yes	Yes	Yes	CU**G**/**G**GG	No	2/5	**U38/U39**
Thr AGU	Yes	Yes	Yes	C**U**C/G**C**G	No	3/5	A38/G39
Leu CAG	Yes	Yes	Yes	C**CG**/**GC**G	No	3/5	**U38**/G39
Leu UAA	Yes	Yes	Yes	CC**G**/**G**GG	No	3/5**	C38/C39
Leu CAA	Yes	Yes	Yes	C**CG**/**GC**G	No	3/5	**U38/U39**
Arg ACG	Yes	Yes	Yes	C**C**C/G**C**G	No	2/5	A38/**U39**
Ser CGA/UGA	Yes	Yes	Yes	C**C**G/G**A**G	No	1/5	A38**/U39**
Lys UUU	Yes	Yes	Yes	C**U**C/G**C**G	No	4/5	A38/A39
Leu UAG	Yes	Yes	Yes	C**CG**/**GA**G	No	4/5	G38**/U39**
Ile UAU	No						
Ile AAU	Yes	Yes	No	UGU/AUG	No	2/5	A38/G39
Gly GCC	Yes	No	No	U**U**U/A**U**G	Yes	2/5	**U38/**C39
Gly CCC	No						
Gln CUG	Yes	No	No	UGU/ACG	No	2/5	**U38/U39**
Ser GCU	Yes	Yes	Yes	C**C**G/G**C**G	No	3/5	A38/**U39**
Trp CCA	Yes	Yes	Yes	C**U**C/G**U**G	No	2/5	A38**/U39**
Arg UCG	Yes	Yes	Yes	C**C**U/G**C**G	No	2/5	C38/A39
Ala AGC	Yes	No	No	UGU/ACG	No	3/5	**U38/**G39
Phe GAA	Yes	No	No	UGU/AUG	No	2/5	A38/**U39**
Ala CGC	Yes	No	No	U**U**U/A**C**G	No	1/5	**U38/U39**
Met CAU	No						
Ala UGC	Yes	Yes	No	UG**C**/**A**UG	No	4/5	**U38/**G39
His GUG	No						

Table S2. Relative pulldown efficiencies of Sla1-PrA with various pre-tRNAs

Minus Crosslink (see Figure 5)

Fold enrichment in Sla1p-PrA elutions								
pre-tRNA	*trm1-* 30/ WT 30	P-value	WT 37/ WT 30	P-value	*trm1-* 37/WT 37	P-value	*trm1-* 37/*trm1-* 30	P-value
LysCUU	0.976	0.925	0.913	0.453	0.815	0.571	0.762	0.807
LeuCAG	1.098	0.715	2.065	0.180	0.578	0.270	1.086	0.779
LeuUAG	1.191	0.581	1.914	0.535	0.493	0.076	0.792	0.273
LeuCAA	0.831	0.579	0.763	0.439	0.563	0.110	0.517	0.064
Arg CCG	1.347	0.376	1.744	0.728	1.069	0.509	1.384	0.768
Ser GCU	1.100	0.892	0.761	0.686	1.331	0.948	0.921	0.681
Ser CGA	1.009	0.982	0.547	0.156	1.271	0.429	0.689	0.413
Ser UGA	1.099	0.410	2.176	0.410	0.493	0.437	0.977	0.944

Plus formaldehyde crosslink:

Fold enrichment, tRNA pulldown by Sla1p in trm1- strain versus WT strain plus crosslinking								
	trm1-/WT at 37 degrees				trm1-/WT at 30 degrees			
	Trial 1	Trial 2	average	stdev	Trial 1	Trial 2	average	stdev
LysCUU	0.986	0.778	0.882	0.147	0.763	0.836	0.800	0.052
LeuCAG	0.999	0.794	0.897	0.145	0.517	0.501	0.509	0.012
LeuUAG	1.265	0.701	0.983	0.399	0.423	0.621	0.522	0.140
LeuCAA	0.752	0.602	0.677	0.106	0.646	1.436	1.041	0.559
Arg CCG	1.716	1.513	1.614	0.144	0.852	1.242	1.047	0.275
Ser GCU	0.650	0.851	0.751	0.142	0.450	0.749	0.600	0.211
Ser CGA	1.904	1.667	1.785	0.168	0.514	0.854	0.684	0.241
Ser UGA	0.637	0.686	0.662	0.035	0.457	0.661	0.559	0.144

Table S3. Probes used for Northern Blots

tRNA	Northern probe
Ala AGC	5'-TGG ACA AGC CAG AAC TCG AAT CTG GGA C-3'
Ala CGC	5'-GGA GAT GCC GGG AAT CGA ACC C-3'
Ala UGC	5'-TGG ACA CGC TGG GGA TTG AAC CCA GGA CGA-3'
Arg ACG	5'- CGA TCC CGC CAG GAG TCG AAC CTG GAA T-3'
Arg CCG	5'- CGG CGA GAT TTG CAC TCG CGA TC-3'
Arg UCG	5'- CAG TGT GAC AGG ACT CGA ACC TGC AG-3'
Asn GUU	5'-CGG TCA GGG AGG GAC TCG AAC CCT CGA-3'
Phe GAA	5'- TGT CAC AAA CCG GGA TCG AAC CGA TGA-3'
Gln CUG	5'- AGG TCG TAC TGG GAA TCG AAC CCA GGT-3'
Gly GCC	5'- TGC TTT GGC CGG GAA TCG AAC CCG G-3'
Gly CCC	5'- TGC ATC AGC CGG GAG TCG AAC-3'
His GUG	5'- TGC CCA CAC CAG GAA TCG AAC CTG GGT-3'
Ile AAU	5'- TGG TCA CAG CCG GGT TCG AAC CGG CGA CAT-3'
Ile UAU	5'- TGC TCC CGG CGA GGA TCG AAC TCG CGA T-3'
Leu AAG	5'-AAG CTC GTG GGT TCG AGT CCC ACC CCT TTC A-3'
Leu CAA	5'- TGA CCA GTG AGG GAT TCG AAC CCT CGC A-3'
Leu CAG	5'- CGA AGG TGG GGT TCG AAC CCA CGC-3'
Leu UAA	5'- TGC GGC CAG AGA GGT TCG AAC TCT-3'
Leu UAG	5'-ATG GGA CGG GAC TCG AAC CCG CGA G-3'
Lys CUU	5'- CTC CCA AGG CGA GAC TCG AAC TCG CAA-3'
Lys UUU	5'- CTC CCA CTG CGA GAT TCG AAC T-3'
Met CAU	5'- TTG CGC GGC CAG GTT TCG ATC CTG GGA-3'
Ser AGA	5'- GCA GGT TCA AAT CCT GCT GGT GTC G-3'
Ser CGA/UGA	5'- GTC ACC AGC AGG ATT TGA ACC TGC GCG G-3'
Ser GCU	5'- CGA CAA CGG CAG GAT TCG AAC CTG C-3'
Thr AGU	5'- TGC TCC AGC AGT GAC TCG AAC ACT G-3'
Trp CCA	5'- TGA CCC CTA AGT GAC TTG AAC ACT TGA-3'
U5 snRNA	5' GCA CAC CTT ACA AAC GGC TGT TTC TG 3'
For Beta-elimination blot to assay tRNA charging:	
Ser CGA/UGA	5' GGG CAA AGC CCA TTA GAT 3'

Table S4. Primers and probes used for qPCR analysis

tRNA	Forward primer	Probe	Reverse Primer
Ala CGC	5'-TCG CAT AGT TCG CAC CTT-3'	5'-/56-FAM/ CGG GTT CGA/ZEN/TTC CCG GCA TCT /3IABkFQ/-3'	5'-AAA AAT AGT TTC TAA CAT TGG-3'
Arg CCG	5'-GGA GAC TCC AGG TTC GA-3'	5'-/56-FAM/CGA GTG CAA/ZEN/ATC TCG CCG TGG TC/3IABkFQ/-3'	PerfeCta Universal PCR primer (Quanta Biosciences)
Leu CAA	5'- AAG TAA TAC TTG GAC GAT AGT CTT G-3'	5'-/56-FAM/AGA TGC CAG /ZEN/GGT TCG AAT CCC TCA CT/3IABkFQ/-3'	PerfeCta Universal PCR primer (Quanta Biosciences)
Leu CAG	5'-CTA GCT TCA GGT GAA AGC A-3'	5'-/56-FAM/ TGC TAG TCT /ZEN/ACG TAT GTG GGC GTG /3IABkFQ/-3'	PerfeCta Universal PCR primer (Quanta Biosciences)
Leu UAG	5'- TGG CTT TAG GTG CGT TC-3'	5'-/56-FAM/ TGA ATG GGA/ZEN/CGG GAC TCG AAC C/3IABkFQ/-3'	PerfeCta Universal PCR primer (Quanta Biosciences)
Leu UAA	5'- CAG ATT TAA GAG GCT TCT GC-3'	5'-/56-FAM/ TCG AAC CTC/ZEN/TCT GGC CGC A/3IABkFQ/-3'	PerfeCta Universal PCR primer (Quanta Biosciences)
Lys CUU	5'-TCT GAC TCT TAT GAT GGT AAT CAG A-3'	5'-/56-FAM/ CGA GTT CGA/ZEN/GTC TCG CCT TGG G /3IABkFQ/-3'	PerfeCta Universal PCR primer (Quanta Biosciences)
Ile UAU	5'-TGA TAC TCC CCT TAG GAG GTG-3'	5'-/56-FAM/ ACG TGG AAA/ZEN/TCG CGA GTT CGA TCC T/3IABkFQ/-3'	PerfeCta Universal PCR primer (Quanta Biosciences)
Ser GCU	5'- TGC TAT TCT GTA GCC CAG C-3'	5'-/56-FAM/CTT TGG GAG/ZEN/ CGC AGG TT GAA TCC /3IABkFQ/-3'	PerfeCta Universal PCR primer (Quanta Biosciences)
Ser CGA	5'-GAA TTC CTA CAT TCG TGG CAT C-3'	5'- /56-FAM/CA GGT TCA A/ZEN/A TCC TGC TGG TGA CG/3IABkFQ/ -3'	PerfeCta Universal PCR primer (Quanta Biosciences)
Ser UGA	5'- TAG ACT TGA ATC CTG TAT TCT AGT CA -3'	Same as SerCGA	PerfeCta Universal PCR primer (Quanta Biosciences)
Tyr GTA	5'- CCG GCT GTA ATT ATA AAG ATA CC-3'	5'-/56-FAM/TTG GTC GCT/ZEN/AGT TCG ATT CTG GCT C/3IABkFQ/-3'	PerfeCta Universal PCR primer (Quanta Biosciences)
U5 snRNA	5'-TGC ACA CAC ATT TCA GCA TAA TC-3'	5'- /5HEX/AG CTA ACG T/ZEN/A TCT GTT TCT TGC CTT /3IABkFQ/ -3'	5'-ACA AAC GGC TGT TTC TGG TA-3'

CHAPTER IV: Global protein expression regulated by Trm1p-catalyzed m_2^2G26 modification of mitochondrial and nuclear encoded tRNAs

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Global protein expression regulated by Trm1p catalyzed m_2^2G26 modification of mitochondrial and nuclear encoded tRNAs

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PREFACE

Post-transcriptional modification of tRNAs is associated with improvements in structural stability and regulation of tRNA activity in mRNA decoding. Defects in tRNA post-transcriptional modification have been linked to human diseases, dysregulation in the mitochondrial organelle and defects in synthesis and function of the OXPHOS complex. N2, N2-dimethylation of G26 by the methyltransferase Trm1p facilitates stabilization of tRNA hinge region at the junction of the D-stem and anticodon stem. *S. pombe trm1-* cells display reduced steady-state levels as well as impaired charging for specific Trm1p-cytoplasmic tRNA substrates. To assess the functional role of m_2^2G26 in modulating tRNA activity, we performed stable isotope labeling with amino acids in cell culture (SILAC) in *trm1- S. pombe* cells. Our data are indicative of changes in protein expression levels that are linked to Trm1p abundance in both the mitochondrial and nuclear organelles. We have evidence of alterations in protein expression levels in *trm1-* cells correlated with the enrichment of CTA codons in the sequences of these proteins, potentially due to changes in the decoding activity of Trm1p-hypomodified tRNA-Leu^{UAG}. Our data also suggests mitochondrial functional irregularity. *S. pombe* Trm1 (similar to *S. cerevisiae*) has two possible initiator AUGs (M1 and M24) separated by a mitochondrial targeting sequence. We have identified mitochondrial G26 containing tRNAs that are robustly modified by Trm1p and have evaluated their charging state. We also demonstrate that overexpression of M1 Trm1p (mitochondrial targeted) and not M24 Trm1p (cytoplasmic) results in growth impairment under both fermentable and non-fermentable carbon source. Our findings infer alterations in mitochondrial function that is regulated by Trm1p modification of mitochondrial targets.

INTRODUCTION

Structural flexibility in RNA transcripts contributes to their propensity in acquiring non-native, dysfunctional conformations and their entrapment in thermodynamically stable aberrant folds (2, 5, 140). Post-transcriptional modifications made to the sugar-ribose moiety of RNAs surmounts the tendency of nascent transcripts in forming dysfunctional conformations and commits the secondary and tertiary structure of RNAs to the biologically active, favored conformer (141). PTMs are essential in supporting structural stability and are also influential in various cellular processes including gene expression regulation, facilitating decoding of mRNA transcripts and maintaining RNA homeostasis (142).

tRNAs are the most highly modified RNA transcripts in all organisms with on average more than 15% of nucleosides of yeast nuclear encoded tRNAs carrying a functional group on A, G, C or U (62, 114). PTMs on tRNAs have been classified into two basic groups: PTMs that are in close proximity or positioned in the anticodon loop that regulate gene expression by enhancing codon-anticodon interactions as well as maintaining a correct reading frame and PTMs on the body of the tRNA that facilitate the overall structural stability (63, 114). An example of the former is Trm9 (tRNA methyltransferase 9) which catalyzes the last step in synthesis of 5-methylcarboxymethyl-2-thiouridine (mcm5S2U) on tRNA wobble nucleoside with the addition of a methyl group (143). Quantitative proteomics studies assaying changes in expression levels of thousands of proteins in a *trm9-* compared to *trm9+* strain in *S. cerevisiae* identified a correlation between two Trm9 tRNA substrates (tRNA-Arg^{UCU} and Glu^{UUC}) and the regulated translation of genes enriched with their corresponding codon pairs (AGA and GAA) (78). Hypomodification of the wobble nucleoside on tRNA-Arg^{UCU} and tRNA-Glu^{UUC} has also been linked to translational stalling with ribosomal pausing in regions enriched with AGA and GAA codons (78). Wobble uridine modification of tRNA Arg and Glu by Trm9 has been shown to enhance the translation of transcripts expressing DNA damage response elements (143). These findings demonstrate a mechanism in which cellular protein expression levels are regulated through selective PTM of tRNAs (78, 144).

The second class of PTM enzymes that decorate the tRNA body predominantly facilitate tRNA structural stability. An example of such is the N2, N2-dimethylation of G26 on tRNAs by the methyltransferase Trm1p. Studies in *S. cerevisiae* have alluded to a stabilized hinge region and

anticodon stem in the presence of m_2^2G26 (70). Knockout of Trm1 has been associated with a synthetic lethal phenotype in *S. cerevisiae* (also conserved in *S. pombe*; refer to Chapter III) when combined with the RNA chaperone La, recapitulating the role of Trm1 in supporting tRNA structural stability (70). The depletion of cytoplasmic tRNAs (tRNA-Arg^{CCG}, Leu^{AAG}, Lys^{CUU} etc.) as well as impaired tRNA charging (tRNA-$Ser^{CGA/UGA}$) contribute to the synthetic lethality in *trm1-/sla1-* (*S. pombe* La; S*la1*) cells (refer to Chapter III). An extension to these findings, Arimbasseri et al. have reported a list of 27 G26-containing *S. pombe* cytoplasmic tRNAs (from the total of 36) that are differentially modified by Trm1 (under wildtype conditions), demonstrating the tRNA substrate specificity of the enzyme (71). While some tRNAs are highly modified by Trm1 such as tRNA-Arg^{UCG}, Arg^{CCG}, Ser^{UGA} and Ser^{CGA} others with a G26 are poorly modified (Gly^{GCC}, Gln^{UUG} , Phe^{GAA} etc.) and are classified as non-Trm1 targets (71). Additionally, *S. pombe* suppressor tRNA-Ser^{UCA} has been identified as a Trm1-specific substrate with a requirement for m_2^2G26 for functionality in a tRNA mediated suppression (TMS) assay. Antisuppression of a nonsense codon in the *ade6-704* allele has been observed in the presence of a Trm1-hypomodified suppressor tRNA-Ser^{UCA} (71).

Impaired tRNA processing and defects in PTM enzymes has previously been linked to human diseases, particularly malfunctions of the mitochondrial organelle (145, 146). Expression of the mitochondrial genome (mtDNA) is facilitated through the highly-regulated communications between the nuclear and mitochondrial organelle. Over 100 nuclear encoded genes are expressed in the cytoplasm and imported to the mitochondria just to facilitate expression of mtDNA (100). These include ribosomal proteins, aminoacyl-tRNA synthetases and proteins involved in translation initiation, elongation and termination (100). The OXPHOS system is comprised of five multimeric complexes, in which most of the subunits of the OXPHOS complex are nuclear-encoded and only a total of 7 subunits (in *S. pombe* and *S. cerevisiae*) are embedded in the mtDNA and expressed in the mitochondria (100, 145). Mitochondrial tRNAs, similar to cytosolic tRNAs rely on PTMs for their structural stability and functionality (101). Defects in the respiratory chain complex, insufficient energy production and lactic acidosis have been linked to mutations in TRMT5 (tRNA methyltransferase 5) and the resulting hypomodification of mt-tRNA-Leu^{CUN} at G37 (145). Additionally, decrease in mitochondrial protein translation and associated mitochondrial myopathy, encephalopathy and lactic acidosis have been connected to a mutated mt-tRNA-Leu^{UUR} (R being A or G) that results in hypomodification of its wobble uridine (147).

The list of defective tRNA processing enzymes and tRNA modifiers associated with mitochondrial diseases has been on a rise with other examples of impaired modifiers reported in mitochondrial-related dysfunction including PUS1, TRMU and TRIT1 (145).

Several recent insights have directed our attention towards identifying a novel role for Trm1p in global regulation of protein expression. Firstly, Trm1p modification is necessary for the native steady state levels of a select set of tRNAs while concomitantly supporting the wildtype charging state of other tRNAs (Chapter III). Secondly, tRNA-HydroSeq results indicate tRNA substrate specificity of Trm1p with significant variations in dimethylation efficiency detected depending on tRNA identity (71). Additionally, m_2^2G26 modification efficiency by Trm1p is altered depending on growth conditions independent of Trm1p protein levels, indicating that the activity of the methyltransferase may be regulated post-translationally (71). Lastly, m_2^2G26 is necessary for the functionality of *S. pombe* suppressor tRNA-Ser^{UCA} in TMS assay suggestive of Trm1p contributing to functional activity of its target tRNA substrates (71). Given the substrate specificity nature of Trm1, its capacity to support tRNA structural stability while maintaining native tRNA charging state as well as its role in stimulating suppressor tRNA-Ser^{UCA} activity in TMS assay, it is probable that alterations in Trm1p modification efficiency of G26 containing-tRNAs may result in altered tRNA activity with subsequent changes in global expression patterns.

To assess the capacity of Trm1 in fine-tuning tRNA activity upon modification, we have used a SILAC-based quantitative proteomics approach in *trm1+* and *trm1-* strains of *S. pombe*. We have identified alterations in expression levels of a cohort of polypeptides that are specifically enriched in CTA codons, potentially associated with decrease in the decoding activity of hypomodified tRNA-Leu^{UAG} as well as changes in expression levels of polypeptides linked to metabolic processes involved in mitochondrial function. Trm1 is a nuclear encoded tRNA modifier that resides both in the nuclear periphery and the mitochondrial organelle. Studies in *S. cerevisiae* have identified two isoforms of Trm1; Trm1p-I and Trm1p-II (72, 107). The expression of either isoform is transcriptionally regulated with two possible initiator AUGs that are connected via a mitochondrial targeting sequence (72, 107). Similar to *S. cerevisiae*, expression of *S. pombe* Trm1 is predictably transcriptionally regulated with the potential for the expression of two isoforms depending on two initiator AUGs (M1 and M24) which are separated by a MTS. Our SILAC data are indicative of Trm1-mediated alterations in protein expression with significant upregulation of eIF3d (eukaryotic translation initiation factor 3d; Moe1 in *S. pombe*) reported. We have confirmed

robust modification of specific G26 containing mt-tRNAs by Trm1p and demonstrate that overexpression of Trm1p-I isoform (M1 AUG; mitochondrial) but not Trm1p-II (M24 AUG; nuclear) results in impaired growth on both fermentable and non-fermentable media. Our data also indicates that *trm1-* cells with and without overexpression of mitochondrial targeted Trm1 do not impact expression of components of the OXPHOS complex including Cox1, 2, 3 (cytochrome c oxidase subunits 1, 2 and 3) and Atp6, 8, 9 (ATPase subunit 6, 8, 9) as well as RpS3 (mito-ribosomal protein of the small ribosomal subunit). Our SILAC data, the growth defective phenotype associated specifically with M1-Trm1p and the northern blot analyses indicate a novel role for Trm1p in regulating mitochondrial physiology.

MATERIAL AND METHODS

PHA26 northern analyses

S. pombe cells were cultured in EMM (Edinburgh minimal media) supplemented with 225mg/L of adenine, leucine, histidine and lysine, grown at 30ºC to an OD_{600} (optical density measured at 600nm) of ~ 0.4 - 0.6 and harvested. Total RNA was extracted from cells and northern analysis was performed following methods as described (22). DNA probes were made for tRNAs of interest by 5'end labeling of oligonucleotides using T4 polynucleotide kinase and ^{32}P-γ-ATP. DNA probes used in northern analysis for detection of modification at G26 for the PHA26 (positive hybridization in the absence of G26 modification) assay were made complementary to the D-stem/loop and anticodon stem. For an internal control, probes were designed to specifically hybridize to the TψC-loop of each tRNA. U5 snRNA was also used as a loading control. List of probe sequences are provided in Supplementary Table 1.

Analysis of Aminoacyl-tRNA charging

Cells were grown in the same manner for PHA26 northern analysis, but were harvested under acidic conditions with washing of the cells with 0.3M sodium acetate and 1mM EDTA at pH 5.0. Total RNA extraction was performed under acidic condition using Trizol reagent (Invitrogen). For a control sample representing migration of a deacylated sample, half of the wt + pREP4 and *trm1*- (+) pREP4 total RNA was deacylated with 0.2M Tris pH 9.0 for 2 hours at 37°C, ethanol precipitated and stored in 10mM sodium acetate pH 5.0. For better separation of charged and uncharged species of tRNAs, periodate oxidation/β-elimination method was used for preparation of total RNA as described (119). Both the wt (+) pREP4 and *trm1*- (+) pREP4 deacylated total RNA and the untreated total RNA samples were incubated with 5mM $NaIO_4$, 50mM NaOAc pH 5.0, 60 min at 37°C. The periodate oxidation reaction was stopped with the addition of 50mM glucose and incubation at 0°C for 90 min followed by ethanol precipitation. Total RNA samples were resuspended in 1ml of 1M lysine pH 8.0 and incubated for 60 min at 45°C. Lastly, all samples were deacylated with 500µl of 0.4M Tris pH 9.0 at 37°C for 30 min and extracted with phenol chloroform and ethanol precipitated. Total RNA was separated on 8% polyacrylamide denaturing

DNA sequencing gel and the relevant region of the gel was isolated and transferred onto a GeneScreen Plus transfer membrane (PerkinElmer) using the iBlot Gel Transfer system (Invitrogen). DNA probes were prepared as stated above. Probes were made complementary to the TψC-loop of each tRNA. List of probe sequences are provided in Supplementary Table 1.

SILAC labeling and whole cell lysate preparation

S. pombe strain FY7455 (h+ leu1-32 ura4 his7 lys1) was purchased from NBRP-Yeast (National BioResource project). FY7455 is a lysine auxotroph used as the parent strain for generating a *trm1-* strain that is *lys1-* using yeast mating and tetrad dissection techniques as described (148). All strains used for SILAC experiments were lysine auxotrophs and grown in EMM supplemented with 225mg/L of adenine, leucine and histidine and 75mg/L of either normal lysine (Bioshop) or [$^{13}C_6$, $^{15}N_2$] L-lysine (Cambridge Isotope Laboratories), grown at 30ºC to an OD_{600} of ~ 0.6. To ensure efficient labeling of proteins with [$^{13}C_6$, $^{15}N_2$] L-lysine, heavy labeled cells were harvested separately and washed with ice cold distilled water and lysed as described below. For all other test experiments, equal amount of heavy-labeled SILAC cells were mixed 1:1 with normal lysine grown cells and lysed. Cell pellets were resuspended in 100µl of lysis buffer (20mM HEPES pH 7.6, 200mM KOAc, 1mM EDTA, 0.1mM PMSF and 1X Protease inhibitor cocktail) and 0.3g of acid-washed glass beads (Sigma-Aldrich) was added. Cells were homogenized using a bead beater for 1 minute at 4ºC and subsequently centrifuged at 14,000 rpm for 10 minutes at 4ºC to remove cell debris. The pellet was washed once more with 100µl of lysis buffer and centrifuged again at 14,000 rpm for 10 minutes at 4ºC. The lysates were pooled and centrifuged at 14,000 rpm for 30 minutes at 4ºC. Bradford assay was performed for quantification purposes and 15µg of lysate was isolated, lyophilized and sent for Trypsin digestion and mass spectrometric analysis (IRCM Proteomics Discovery Platform).

Western blot analysis

Lysine auxotrophic cells used for SILAC-LC-MS/MS analysis were also used for western blot assay. Cells were cultured in EMM supplemented with 225mg/L of adenine, leucine, histidine and lysine, grown at 30ºC to an OD_{600} of ~ 0.4 - 0.6, harvested and lysed as described above. Lysates

were resolved on 12% SDS gel and transferred onto a nitrocellulose membrane (Life Technologies). The membrane was blocked for 1 hour at room temperature with TST (0.01M Tris pH 7.4, 0.15M NaCl, 0.075% Tween-20) containing 0.5% non-fat dry milk. This was followed by probing with 6xHis Monoclonal Antibody (6xHis mAb 1:10,000; Clontech) in TST + 0.5% non-fat dry milk, for 1 hour at room temperature for detection of 6xHis tagged Trm1. Following washes, secondary anti-mouse IgG HRP-linked antibody (Cell Signaling) was prepared in the same buffer as above (1:5000) and incubated with the membrane for 1 hour at room temperature. The blot was washed twice for a period of 10 minutes with TST and once with TSM (0.1 M Tris pH 9.0, 0.1M NaCl, 5mM $MgCl_2$) for 5 minutes. The same buffers and conditions as above were used for subsequent probing of the blot with Moe1. The antibody was kindly shared with our lab by Dr. Eric Chang (Baylor College of Medicine). Moe1 primary anti-rabbit antibody was used at 1:100,00000 dilution and detected using anti-rabbit IgG HRP-linked antibody (1:10,000; Cell Signaling). For loading control, anti-beta Actin antibody (Abcam) was prepared (4:10,000) in TBST (1X TBS +0.1% Tween-20) + 5% non-fat dry milk. All subsequent washes and preparation of secondary antibody, anti-mouse IgG HRP-linked antibody (1:5000) were done in TBST.

In vivo labeling of mitochondrial translation products

S. pombe cells were initially grown in EMM (-) ura + 2 % glucose at 30°C. The preculture was subsequently inoculated into fresh EMM (-) ura media containing 0.1% glucose and 2% galactose and grown at 30°C for ~ 6 generations to a final OD_{600} not exceeding 1 x 10^7 cells/ml. An equivalent of 1.5 OD (~2.5 x 10^7 cells) was transferred to a microtube and cells were pelleted by centrifugation at 12, 000g for 30s. Cell pellets were washed with 500µl of reaction buffer (40mM potassium phosphate pH 6, 2% galactose, 0.1% glucose) and centrifuged at 12, 000g for 30s. Pellets were resuspended in 500µl of reaction buffer containing 10mg/ml of cycloheximide and incubated for 15 minutes. After incubation with cycloheximide, 5µl of [^{35}S]-methionine was directly added to the cells, mixed thoroughly and incubated for 30 minutes. The cell suspension was then pelleted by centrifugation at 10, 000g for 1 minute and the pellet was resuspended in 75µl of solubilization buffer (1.8M NaOH, 1M β-mercaptoethanol, 0.01 phenyl-methyl sulfonyl fluoride (PMSF)) and mixed. The solubilization buffer was then diluted with the addition of 500µl of H_2O and all proteins were precipitated with the addition of 575µl of 50% TCA with incubation on ice for 5

minutes. The precipitated proteins were spun down at 10, 000g for 5 minutes and the pellet was washed twice with 575µl of acetone and centrifuged as above. The precipitated proteins were resuspended in 25µl of gel sample buffer (2%SDS, 10% glycerol, 60mM Tris-HCl pH 6.8, 2.5% β-mercaptoethanol, 0.02% bromophenol blue) and separated on a 17.5% SDS gel and subsequently transferred to a nitrocellulose membrane (Life Technologies) using common wet Western blotting technique and exposed to a phosphor screen overnight. Protocol was adapted from (100) with some minor modifications stated above.

RESULTS

SILAC proteomics identifies alterations in global protein expression associated with Trm1p-tRNA methyltransferase

Chemical modifications made to tRNA nucleosides are essential in regulating tRNA structure and function (62, 63, 114). Trm1p is a well characterized methyltransferase that selectively dimethylates Guanine at position 26 on many tRNAs (61, 62, 149). Dimethylation by Trm1p has been linked to stabilization of the hinge region at the junction of the anticodon and D-stem, contributing to the native steady-state abundance of specific tRNAs and their wildtype charging state (Chapter III) (73, 129). To date, Trm1 modification of G26 containing tRNAs has been studied with a direct focus on tRNA structural stability with less emphasis on the role of this methyltransferase in regulating tRNA activity in translation. However, recent evidence has alluded to m_2^2G26 enhancing the functional activity of *S. pombe* suppressor tRNA-Ser^{UCA} in decoding a nonsense codon in the *ade6-704* allele (71). Since Trm1p is a selective methyltransferase, modifying its preferred tRNA substrates with high efficiency and only at a single site (G26) (71), it is conceivable that Trm1p may be a candidate tRNA methyltransferase that specifically regulates tRNA activity in translation, altering protein expression levels by varying tRNA abundance, charging state and functionality in mRNA decoding. To characterize a potentially novel role for Trm1p in selective regulation of the activity of G26 containing tRNAs in protein biosynthesis, we have used a SILAC-based approach to assay changes in protein abundance in *trm1-* cells (relative to *trm1+*) in *S. pombe*. Alterations in protein abundance in *trm1-* cells is identified by comparison of the [$^{13}C_6$, $^{15}N_2$] L-lysine labeled proteins (*trm1-)* to normal lysine containing proteins (*trm1+*). As a control and to ensure complete incorporation of heavy lysine, separate samples of the [$^{13}C_6$, $^{15}N_2$] L-lysine labeled proteins were assayed by LC-MS/MS indicating near complete incorporation of the heavy labeled lysine, validating the functionality of the assay. Our SILAC LC-MS/MS data has been quantified by comparison of the changes in abundance of proteins in the *trm1-* relative to *trm1+* cells (abundance ratio, using two biological replicates) with an output of a cohort of proteins whose abundance is either upregulated or downregulated in *trm1-* cells (Supplemental Table 2). Using this cohort of proteins with variable expression levels under the

A

Loss of Trm1p associated with reduced expression of proteins enriched in CTA codons in *trm1-* cells

tRNA of interest	Codon	Parameter Estimate	Standard Error	t Value	P-value
Ser UGA	TCA	0.015	0.014	1.06	0.290
Ser CGA	TCG	0.005	0.013	0.37	0.712
Leu CAG	CTG	-0.007	0.012	-0.56	0.577
Leu UAG	CTA	-0.033	0.012	-2.61	0.009
Leu CAA	TTG	0.012	0.025	0.49	0.622
Lys CUU	AAG	-0.023	0.032	-0.72	0.469
Gly CCC	GGG	0.008	0.012	0.67	0.502
Ile UAU	ATA	-0.021	0.013	-1.58	0.114
Tyr GUA	TAC	-0.019	0.015	-1.20	0.230

B

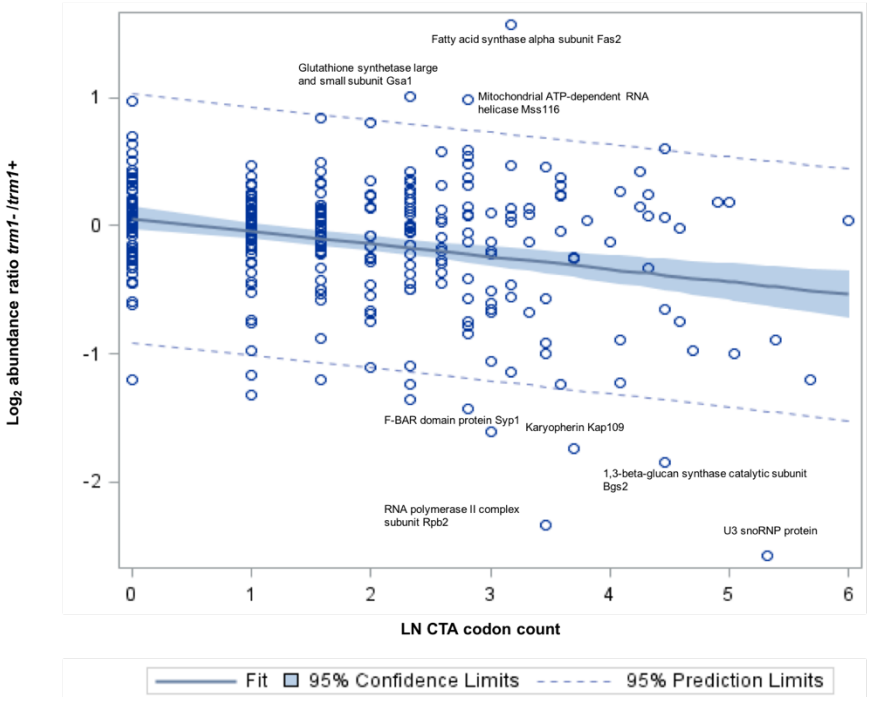


Figure 1.

C

GO biological processes upregulated in *trm1*- cells

Go Terms	Description	Fold Enrichment	p value
GO:0044281	small molecule metabolic process	5.35	8.89E-13
GO:0019752	carboxylic acid metabolic process	7.29	1.59E-10
GO:0043436	oxoacid metabolic process	7.19	2.07E-10
GO:0006082	organic acid metabolic process	7.17	2.20E-10
GO:0044711	single-organism biosynthetic process	5.19	2.22E-09
GO:0044710	single-organism metabolic process	3.59	9.25E-09
GO:0008652	cellular amino acid biosynthetic process	13.45	1.25E-08
GO:1901566	organonitrogen compound biosynthetic process	3.78	7.25E-08
GO:1901607	alpha-amino acid biosynthetic process	13.51	8.38E-08
GO:1901576	organic substance biosynthetic process	2.93	8.40E-08
GO:0016053	organic acid biosynthetic process	9.56	1.55E-07
GO:0046394	carboxylic acid biosynthetic process	9.56	1.55E-07
GO:0009058	biosynthetic process	2.86	1.65E-07
GO:0006520	cellular amino acid metabolic process	8.35	1.91E-07
GO:1901605	alpha-amino acid metabolic process	10.24	3.49E-07
GO:0044283	small molecule biosynthetic process	6.8	8.00E-07
GO:0044249	cellular biosynthetic process	2.74	3.70E-06
GO:1901564	organonitrogen compound metabolic process	2.42	3.99E-05
GO:0071704	organic substance metabolic process	1.73	2.65E-04
GO:0008152	metabolic process	1.68	7.14E-04
GO:0044237	cellular metabolic process	1.7	1.04E-03
GO:0043648	dicarboxylic acid metabolic process	11.38	1.91E-02
GO:0009064	glutamine family amino acid metabolic process	13.48	4.71E-02

Figure 1. Trm1p-associated changes in global protein expression. (A) Codons of interest were counted in polypeptides identified from the SILAC proteomics data to determine which codons are more highly represented in polypeptides with varying expression in *trm1*- cells compared to *trm1*+ cells. Proteins generally downregulated in *trm1*- cells compared to *trm1*+ cells were more highly enriched in CTA codons. The reduction of protein expression as a function of CTA codon enrichment was determined using multiple regression. The significance of the parameter estimates (ie. multiple regression coefficients) was assessed using t-tests and their associated P-values. Control codons corresponding to non-Trm1p tRNA substrates include: GGG, ATA, TAC (B) Linear regression for CTA codon counts in all proteins identified in the two trials of SILAC proteomics with LN (natural log of CTA codon count +1) codon counts on the x-axis and log2 of abundance ratio of *trm1*- to *trm1*+ cells on the y-axis. Some examples of proteins that were most downregulated and three of the most upregulated have been reported. (C) GO enrichment analysis of polypeptides upregulated in the *trm1*- cells demonstrate a link between overrepresentation of metabolic processes and absence of Trm1p.

trm1- background, we searched for enrichment of codons of interest (TCA, TCG, CTG, CTA, TTG and AAG), based on our previous findings of cytoplasmic tRNAs that most likely rely on Trm1p modification for structural stability and native charging state as well as using the list of cytoplasmic *S. pombe* tRNAs reported by Arimbasseri and colleagues as preferred Trm1p-tRNA substrates (Chapter III, (71)). As a control, we also searched for enrichment of codons (GGG, ATA and TAC) in proteins with variable expression levels in *trm1-* cells that correspond to tRNAs that are either non-Trm1p targets as reported (71) or lack G26 and cannot be modified by Trm1p (Figure 1A). Amongst the proteins that displayed variability in expression levels in *trm1-* cells and codons scanned in these polypeptides, we observed an overrepresentation of CTA codons supported by statistical significance (Figure 1A and B). This analysis has also been completed using the candidate and control codons and evaluating the enrichment of these codons in proteins with altered expression in *trm1-* cells, normalized to the total number of codons in the open reading frame (ORF) (Figure S1; CTA codon enrichment in this analysis may also be associated with reduced protein expression with the caveat of the P-value). As previously reported, tRNA PTMs enhance tRNA structural stability and influence the decoding capacity of tRNAs (62, 78). tRNA-Leu^{UAG} has previously been characterized as a Trm1p-tRNA target (71) and we have demonstrated that the absence of Trm1p modification results in the decrease in accumulation of mature tRNA-Leu^{UAG} levels (Chapter III). Therefore, it may be probable that hypomodified tRNA-Leu^{UAG} is less efficiently capable of decoding transcripts enriched in CTA codons amounting to the alterations in the expression levels of CTA enriched proteins.

Although defects in tRNA-Leu^{UAG} decoding activity can reason the changes observed in the expression of CTA enriched proteins, this alteration in protein expression detected in *trm1-* cells only accounts for ~ 5% of the variability, suggesting that other unanticipated factors may play a role in the observed alterations in the protein abundance. Gene Ontology (GO) enrichment analysis of the upregulated proteins in *trm1-* cells (Figure 1C) show an interesting enhancement in expression of proteins associated with metabolic processes particularly ones that correlate with mitochondrial activity (eg. carboxylic acid metabolic processes, small molecule metabolic processes). These findings were intriguing since Trm1p is a methyltransferase that has a dual targeting sequence and can potentially regulate both cellular and mitochondrial gene expression and physiology.

Expression of Moe1 altered as a function of Trm1p levels

Stable isotope labeling of polypeptides in *trm1-* cells combined with LC-MS/MS identified changes in protein expression and metabolic processes that link to Trm1p abundance. In particular, GO analysis of these polypeptides identified a correlation between Trm1p and metabolic processes associated with mitochondrial physiology. Aside from the SILAC-LC-MS/MS results reported in Figure 1 (Supplemental Table 2), a subsequent trial showed substantial upregulation in expression of Moe1p (*S. pombe* homolog of eIF3d) in *trm1-* cells (Supplemental Table 3). Amidst all eIFs, the most complex and largest translation initiation factor is eIF3 which comprises of 11 subunits in *S. pombe* (150). eIF3 assists in the assembly of the 40S ribosome on the mRNA transcript and initiates the recruitment of other eIFs that facilitate translation initiation through AUG recognition and mRNA binding (150). Alterations in abundance of eIFs is a regulatory mechanism utilized by the cell to control the expression of specific mRNAs depending on cellular conditions, with variations in abundance of specific subunits of eIFs previously linked to human cancers (150). Moe1p and in its interacting partner Intp6 (eIF3e) are specifically responsible for the translation of mRNAs encoding components of the mitochondrial electron transport chain (ETC) (150). Metabolic homeostasis is under the control of eIF3 with cells deleted of Moe1 and Intp6 subject to respiratory defects, hypersensitivity to stress conditions and a metabolic switch to glycolysis with the concomitant strong reliance on glucose uptake (150). Given that our SILAC-LC-MS/MS data (Figure 1C) suggest a potential role for Trm1p-mediated function in mitochondrial physiology, with the upregulation of mitochondrial associated metabolic processes in *trm1-* cells as well as a subsequent, independent trial of SILAC-LC-MS/MS data showing significant increase in Moe1 levels in *trm1-* cells, we found interest in replicating this SILAC-LC-MS/MS data using an alternate method. We used western blot analysis to assess the changes in abundance of Moe1, conditional to the varying levels of Trm1 using the *trm1+* and *trm1-* cells with the presence and absence of plasmid encoded Trm1 (pREP4 and pREP4-Trm1) (Figure 2A). While the western blot analysis did not clearly identify an upregulation of Moe1 in the *trm1-* cells relative to *trm1+* cells, we demonstrate, that similar to the SILAC-LC-MS/MS data, a possible link between Moe1 abundance and trm1 abundance may exist given that overexpression of Trm1 (in both *trm1-* and *trm1+* cells) results in significant downregulation of Moe1 levels (Figure 2A). To assess more comprehensively the connection between the abundance of Trm1 and expression of Moe1 we took

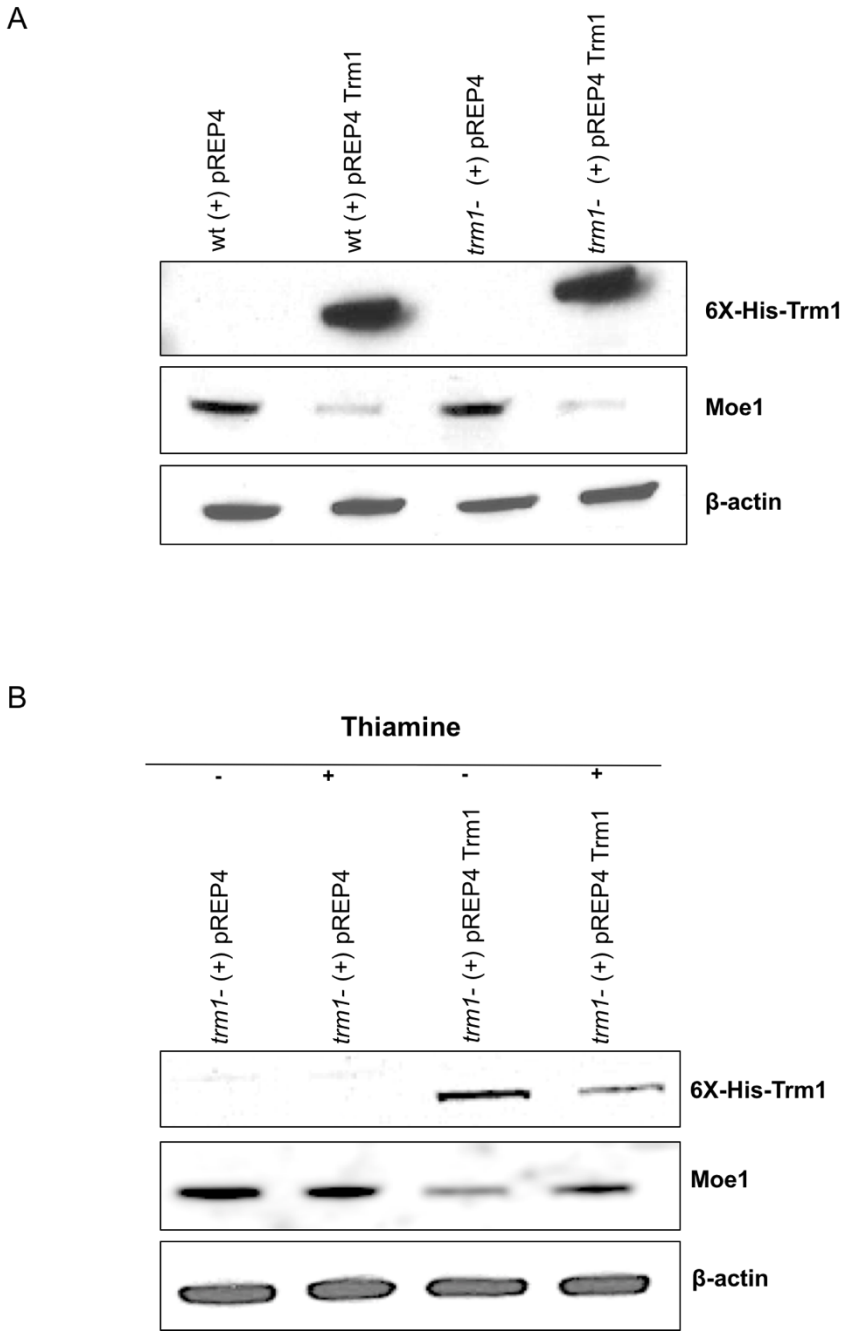
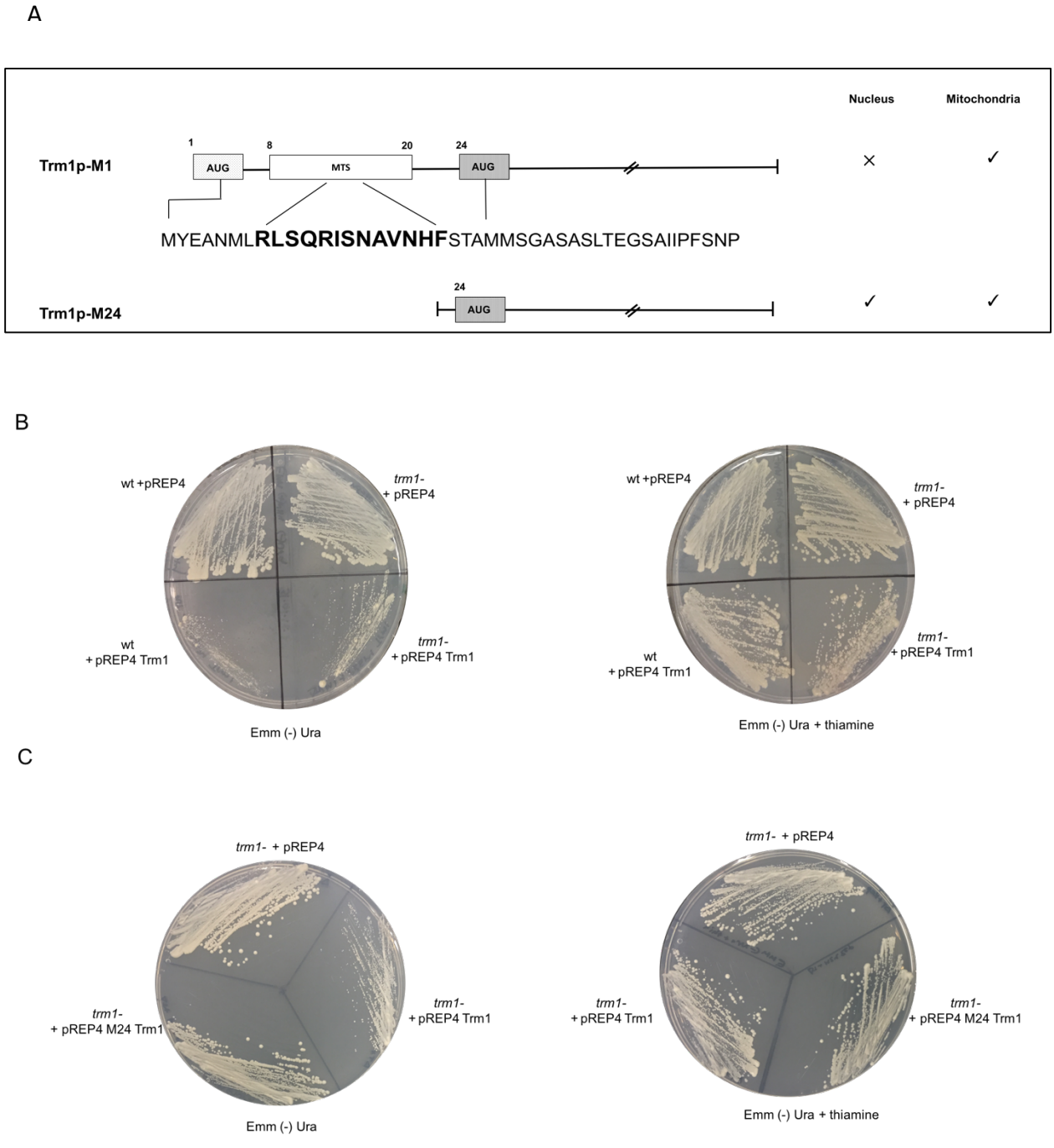


Figure 2. Moe1 expression regulated with Trm1 abundance. (A) Western blot analysis of lysates prepared from *trm1*+ and *trm1*- cells, probing for 6X-His tag for plasmid encoded Trm1, probing for Moe1, and β-actin as a loading control. Expression of Moe1 is variable with the abundance of Trm1. (B) The correlation between Moe1 expression and Trm1 abundance reconfirmed through partial repression of the nmt1 promoter with addition of 15μM thiamine.

advantage of the nmt1 promoter in pREP4 plasmid that is under the control of thiamine repressor. Partial repression of the nmt1 promotor in the presence of thiamine results in the decrease in expression levels of Trm1 and an increase in abundance of Moe1 compared to the unrepressed promoter and overexpression of Trm1 (Figure 2B). The SILAC-LC-MS/MS data and the western blot analysis, collaboratively suggest a Trm1-mediated mechanism regulating Moe1 abundance with potential for subsequent alterations in the translation of mRNAs that encode components of ETC and metabolic processes associated with mitochondrial function.

Overexpression of M1-Trm1 results in growth impairment and sensitivity to non-fermentable carbon source

The *in vivo* SILAC-Mass spectrometric data combined with western blot analysis show an apparent link between Trm1 abundance and changes in metabolic processes linked to mitochondrial function. Intriguingly, during these experimentations we observed a growth defective phenotype that was detectable in both *trm1+* and *trm1-* cells overexpressing Trm1. Partial repression of nmt1 promoter with addition of thiamine rescued the growth impairment (Figure 3B). Earlier studies in *S. cerevisiae* have characterized Trm1 as a PTM enzyme that shuttles between the nuclear periphery and the mitochondria using a N-terminal MTS (72, 107, 149). Similarly, *S. pombe* Trm1 has two in-frame AUGs (M1 and M24) separated by a MTS (Figure 3A). While both isoforms of Trm1 have the capacity to travel to the mitochondria, M1-Trm1 is predicted to reside primarily in the mitochondria while M24-Trm1 is hypothesized to be much less efficiently imported into the mitochondria. Given the proteomics and western blot data indicating a correlation between Trm1 expression and alterations in metabolic processes and Moe1 levels, we anticipated that the growth impairment observed with overexpression of M1-Trm1 may be due to abnormalities in mitochondrial function. As a result, we generated a second construct of *S. pombe* Trm1, pREP4 M24-Trm1 in which Trm1 in this construct is predicted to be less efficiently and minimally transported to the mitochondria to evaluate the role of both isoforms in this growth impairment. Interestingly, we noted a specific growth impairment associated with M1-Trm1 and not M24-Trm1 in *trm1-* cells, further validating the Trm1p-associated alterations in mitochondrial function (Figure 3C). Consistent with previous findings indicating a direct role of Moe1 in the synthesis of the ETC components, and the reliance of *moe1-* cells to glucose for growth, we next checked the


A
Nucleus
Mitochondria
Trm1p-M1
1
8
20
24
AUG
MTS
AUG
×
✓
MYEANMLRLSQRISNAVNHFSTAMMSGASASLTEGSAIIPFSNP
Trm1p-M24
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AUG
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wt +pREP4
trm1-
+ pREP4
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C
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trm1-
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Emm (-) Ura + thiamine

Figure 3.

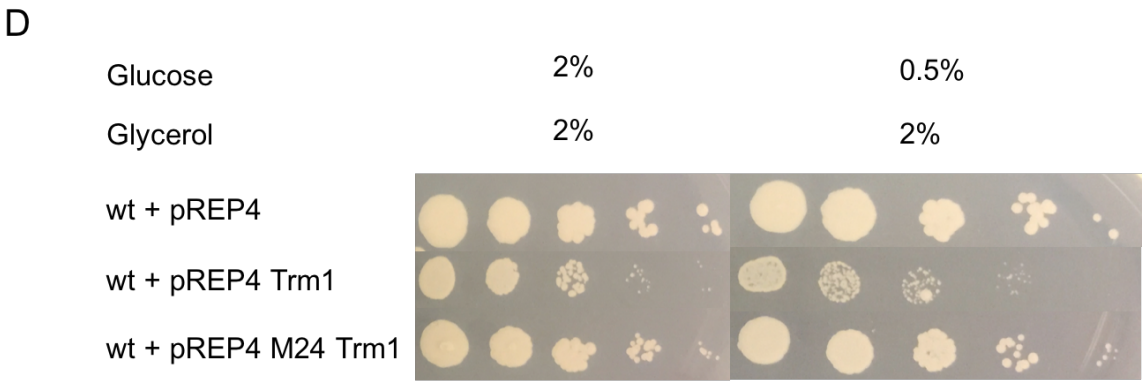


Figure 3. Overexpression of mitochondrial targeted Trm1 induces growth sensitivity in fermentable and non-fermentable media. (A) Schematic of the localization of the two isoforms of Trm1 (Trm1p-M1 and Trm1-M24) based on the two in-frame initiator methionine residues as well as the predicted site of the MTS. (B) Growth of *trm1*+ and *trm1*- cells with empty pREP4 or pREP4 Trm1 on minimal media with and without thiamine was tested. Overexpression of Trm1p-M1 results in growth impairment in both *trm1*+ and *trm1*- cells (left image). Partial repression of nmt1 promoter with the addition of 15 μM thiamine rescues growth for both *trm1*+ and *trm1*- cells with plasmid encoded Trm1 (right image). (C) Overexpression of Trm1p-M1 in *trm1*- cells impairs growth relative to *trm1*- and *trm1*- cells overexpressing Trm1p-M24 (left image). Partial repression of the pREP4 plasmid with 15 μM thiamine improves growth conditions for *trm1*- cells overexpressing Trm1p-M1 (right image). (D) Sensitivity to non-fermentable carbon source (glycerol) was tested for *trm1*+ cells with plasmid encoded Trm1p-M1 and Trm1p-M24 and empty plasmid as control. Cells were plated with 5-fold serial dilutions on minimal media containing 2% glycerol with 2% glucose or 2% glycerol with 0.5% glucose. Growth is more limiting in *trm1*+ cells with overexpression of M1-Trm1p (compared to M24-Trm1p or plasmid only) on plates where glycerol is the major carbon source.

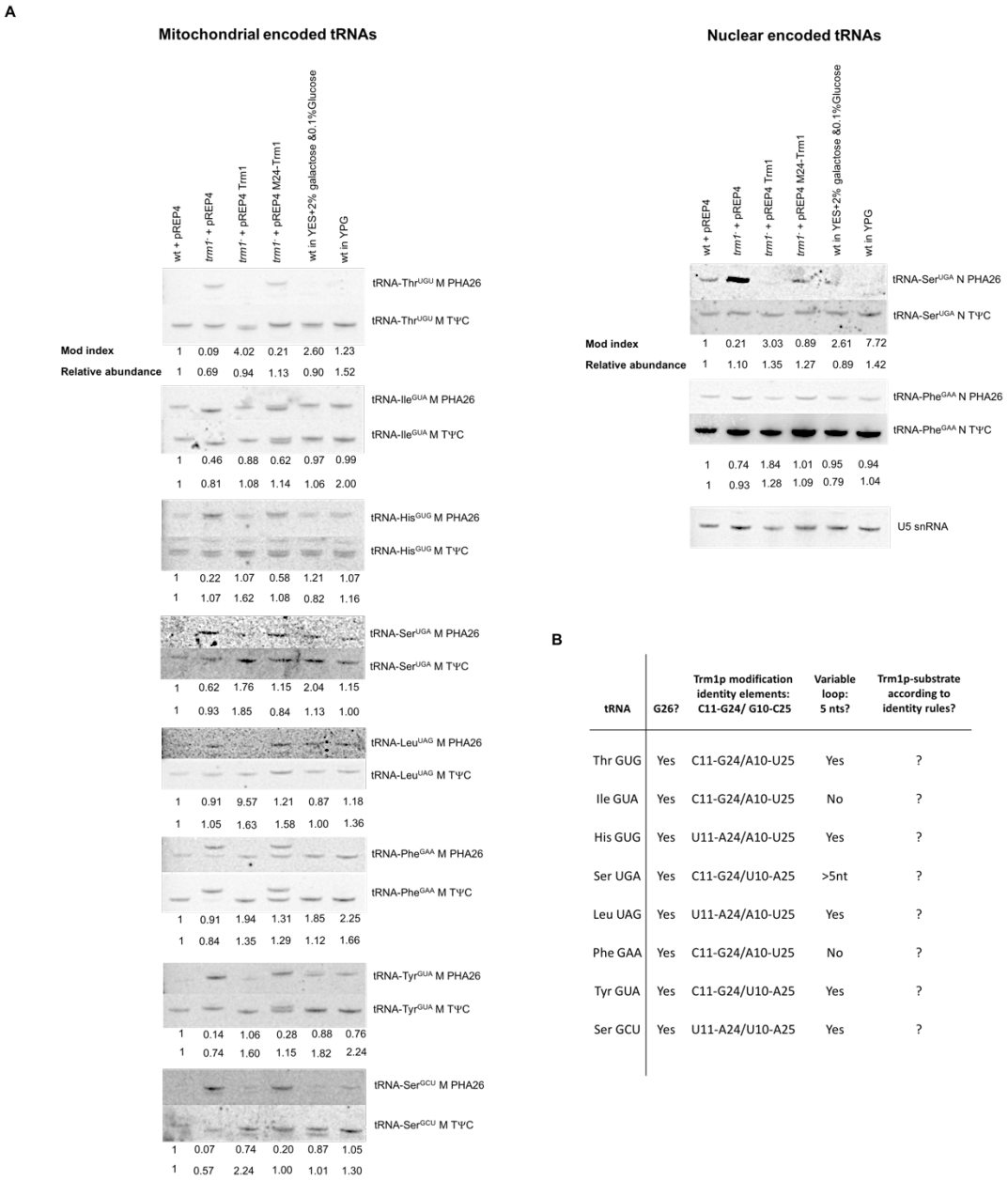
growth sensitivity of *trm1+* cells with and without overexpression of Trm1 (M1 and M24) on media with 2% glycerol as a non-fermentable carbon source supplemented with either 0.5% or 2% glucose. Growth of *trm1+* cells with plasmid encoded M1-Trm1 relative to *trm1+* cells with empty plasmid or M24-Trm1 was limiting, particularly when glycerol was the major carbon source for growth (Figure 3D).

Trm1 catalyzes the synthesis of m_2^2G26 on nuclear and mitochondrial encoded tRNAs

N2, N2-dimethylation of G26 of *S. pombe* cytoplasmic tRNAs has been reported through tRNA Hydro-Seq results (71). These findings demonstrated the tRNA-substrate specificity of Trm1p with certain G26-containing tRNAs more robustly modified than others. TMS assay with *S. pombe* suppressor tRNA-Ser^{UCA} demonstrated the importance of m_2^2G26 in controlling tRNA functionality with antisuppression detected in *trm1-* cells (71). Trm1 is located in the nuclear periphery but can be imported into the mitochondria for G26 dimethylation of mt-tRNAs. Our SILAC data and the growth sensitivity of M1-Trm1 and not M24-Trm1 cells to glycerol (and in fermentable media) may be suggestive of alterations in mitochondrial function that is modulated through Trm1p modification of mt-tRNAs. To examine the modification status of G26 on mt-tRNAs, we used a northern blot probing method previously introduced by Arimbasseri and colleagues referred to as PHA26 assay (positive hybridization in the absence of G26 modification) (71). This method relies on the use of a probe that robustly anneals to the D-stem/loop and AC (anticodon) stem only in the absence of dimethylated G26. As an internal control, a probe that anneals to the TΨC stem was used that would hybridize efficiently to the tRNA, irrespective of the modification status of G26. From all the *S. pombe* mt-tRNAs only 8 are G26-containing (Thr^{UGU}, Ile^{GUA}, His^{GUG}, Ser^{UGA}, Leu^{UAG}, Phe^{GAA}, Tyr^{GUA} and Ser^{GCU}) and could serve as potential tRNA substrates of Trm1p. Dimethylation of G26 on nuclear-encoded tRNAs by Trm1p is efficiently catalyzed in the presence of a consensus sequence preceding G26 in the D-stem such that G10 pairs with C25 and C11 pairs with G24. Additionally, the overall 3D structure of the tRNA is also an important determinant for Trm1p modification with tRNAs possessing 5nt in the variable loop characterized as preferred Trm1p-tRNA substrates (74). While these Trm1p-identity elements have been deduced for nuclear-encoded tRNAs, we wondered whether mitochondrial encoded G26-containing tRNAs possess these Trm1p-identity elements and are modified by mitochondrial targeted-Trm1p.

Strikingly, none of the G26-containing mt-tRNAs carry the GC rich consensus sequence in the D-stem and in fact all of the G26-containing tRNAs carry at least one A: U base pair in the D-stem (Figure 4 B). Moreover, the length of the variable loop also differs amongst these tRNAs, therefore making it challenging in predicting which G26-containing mt-tRNAs may be targets of Trm1p based on the previously characterized identity rules for Trm1p modification. It is likely that mt-tRNAs possess other identity elements for Trm1p modification that differ from those identified for nuclear-encoded tRNAs, possibly linked with the alternate Type 5 and Type 7 folds that they can take on. We have used the PHA26 assay to determine the modification status of eight G26-containing mt-tRNAs. Our data indicates a set of mt-tRNAs that are modified by Trm1p under wildtype conditions and more substantially in *trm1-* cells with plasmid containing M1-Trm1 (tRNA-Thr^{UGU}, Phe^{GAA}, Ser^{UGA} and Leu^{UAG}) and other tRNAs that are just as efficiently modified both under the wildtype condition as well as *trm1-* cells overexpressing M1-Trm1 (tRNA-His^{GUG}, Ile^{GUA} and Tyr^{GUA}) (Figure 4A). We also note that overexpression of M24-Trm1 (which can shuttle to mitochondria but less efficiently than M1-Trm1) does not increase G26 modification status of mt-tRNAs relative to wt or *trm1-* cells with plasmid encoded M1-Trm1, reconfirming the specific role of M1-Trm1 in dimethylation of G26-containing mt-tRNAs and not M24-Trm1 which is more prominently localized to the nucleus. As proof of the functionality of the PHA26 assay for correct detection of m^2_2G26 in mt-tRNAs, we used two control nuclear-encoded tRNAs (tRNA-Ser^{UGA} N and Phe^{GAA} N) to examine (Figure 4A). Previous tRNA Hydro-Seq data have identified the robust G26 dimethylation of tRNA-Ser^{UGA} N, while tRNA-Phe^{GAA} N was reported as an unfavorable Trm1p-target (71). Our PHA26 assay report the same observation, validating the functionality of the PHA26 northern method.

Interestingly, in our PHA26 northern assay we observed two tRNA species in *trm1-* cells as well as *trm1-* cells carrying plasmid encoded M24-Trm1 for tRNA-Phe^{GAA}. To ensure that the two species of tRNA-Phe^{GAA} detected are based on a conformational change and not associated with the cleavage of a portion of the tRNA in the absence of Trm1p modification, we designed specific probes that would anneal to the 5' leader, 3' CCA and acceptor, D-stem/loop region (1 to 25) (Supplementary Figure 3) in addition to the two other probes used for the PHA26 northern blot assay (TΨC stem and PHA26). In this manner, we confirmed that the two species of tRNA-Phe^{GAA} detected is likely due to the presence of two conformations of the tRNA in which one conformation



tRNA	G26?	Trm1p modification identity elements: C11-G24/ G10-C25	Variable loop: 5 nts?	Trm1p-substrate according to identity rules?
Thr GUG	Yes	C11-G24/A10-U25	Yes	?
Ile GUA	Yes	C11-G24/A10-U25	No	?
His GUG	Yes	U11-A24/A10-U25	Yes	?
Ser UGA	Yes	C11-G24/U10-A25	>5nt	?
Leu UAG	Yes	U11-A24/A10-U25	Yes	?
Phe GAA	Yes	C11-G24/A10-U25	No	?
Tyr GUA	Yes	C11-G24/U10-A25	Yes	?
Ser GCU	Yes	U11-A24/U10-A25	Yes	?

Figure 4. Trm1p selectively modifies G26-containing mt-tRNAs. (A)PHA26 northern blot assay demonstrates substrate specificity of Trm1p and m^2_2G26 levels for eight mt-tRNAs in *trm1-* and *trm1+* cells with M1-Trm1 and M24-Trm1 overexpression. Modification status of G26 also evaluated in *trm1+* cells grown in YES supplemented with glycerol or 2% galactose and 0.1% glucose. Control nuclear encoded tRNAs also tested in PHA26 assay. U5snRNA was used as an unrelated RNA control. Modification index (Mod index) is calculated by quantitation of the TΨC/PHA26 probe signal, normalized to *trm1+* cells (wt + pREP4). Relative steady state abundance is calculated by normalization of the TΨC probe signal for each tRNA to U5snRNA and wildtype tRNA abundance. (B) Identity elements for Trm1p-modification of nuclear-encoded tRNAs used to predict G26 modification status of mt-tRNAs tested in the PHA26 probing assay.

is more dominant upon Trm1p modification (Figure 4A: tRNA-Phe^{GAA} PHA26 blot lanes 1- 4, bottom band as modified and top band as unmodified tRNA-Phe^{GAA}). Similar observations were also made for other tRNAs in *trm1-* cells carrying plasmid encoded M24-Trm1 such as tRNA-His^{GUG}, Ile^{GUA}, Tyr^{GUA} and Ser^{GCU}, again likely due to the presence of both Trm1p-hypomodified and m_2^2G26 modified tRNA species that have altered mobility even in denaturing polyacrylamide gels due to tRNA conformational rearrangements associated with m_2^2G26. In addition to identifying G26-containing mt-tRNAs that are Trm1p targets, we also noted changes in the relative abundance of tRNAs +/- m_2^2G26. The relative abundance of tRNAs under *trm1-* cells with and without overexpression of M1-Trm1 or M24-Trm1 was evaluated using the TΨC stem-probed blots, normalized to U5 snRNA and wild type tRNA abundance (Figure 4A; relative abundance). While some tRNAs showed decreased steady state abundance in *trm1-* cells relative to wild type conditions (tRNA-Thr^{UGU}, Ile^{GUA}, Tyr^{GUA}, Ser^{GCU}), all tRNAs were more robustly abundant in *trm1-* cells overexpressing M1-Trm1.

Given the growth impairment detected in *trm1-* and *trm1+* cells with M1-Trm1 in the presence of glycerol, we tested for the modification status of the eight G26 containing mt-tRNAs under *trm1+* cells in the presence of rich media (YES) supplemented with glycerol (YPG) as well as growth under rich media supplemented with 2% galactose and 0.1% glucose (galactose is used a carbon source that does not cause inhibitory effects on mitochondrial protein expression) (100). This assay was primarily utilized to check the modification status of G26 under a stress-related condition such as growth under a non-fermentable carbon source. From the mt-tRNAs assayed in the PHA26 northern blots, we did not observe significant alterations in m_2^2G26 levels for *trm1+* cells grown in YPG, or YES + galactose with the exception of tRNA-Thr^{UGU}, Leu^{UAG} and Phe^{GAA} that showed relatively higher m_2^2G26 levels compared to *trm1+* cells (wt +pREP4) (Figure 4 A; compare lanes 1 to 5 and 6). While we identified some alterations in m_2^2G26 levels in cells grown under a non-fermentable carbon source, there are other conditions to be tested (eg. growth under oxidative stress) that may also cause variability in Trm1p modification efficiency.

Trm1p-associated alterations in aminoacylation level of mt-tRNAs

Dimethylation of G26 by Trm1p has been characterized as a crucial factor in supporting cytoplasmic tRNA structural stability particularly in the hinge region, the junction between the

A

tRNA	G26?	C11?	Seq:11-13/24-26	C44?	GC's in AC stem
Thr GUG	Yes	Yes	CUC/GUG	No	3/5
Ile GUA	Yes	Yes	CUU/GUG	No	3/5
His GUG	Yes	No	UUC/AUG	No	1/5
Ser UGA	Yes	Yes	CCG/GAG	No	1/5
Leu UAG	Yes	No	UGA/AUG	No	1/5
Phe GAA	Yes	Yes	CUU/GUG	No	2/5
Tyr GUA	Yes	No	CUU/GAG	No	2/5
Ser GCU	Yes	No	UCU/AAG	No	2/5

B

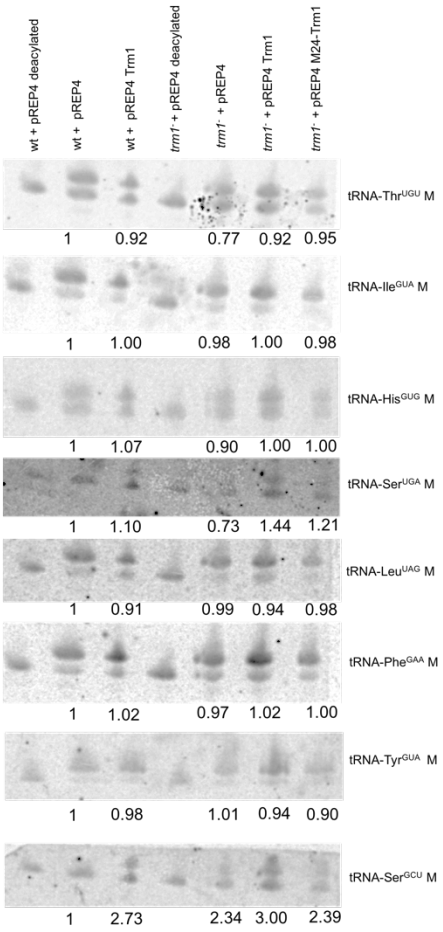


Figure 5. *S. pombe* mt-tRNA sequence identity of the D-stem and AC stem influencing tRNA architecture and the m_2^2G26 associated alterations in tRNA aminoacylation. (A) List of G26 containing mt-tRNAs and identity elements surrounding G26 demonstrating the likelihood of these tRNAs conforming to the canonical Type 6 fold. (B) Periodate oxidation/β-elimination Northern blot analysis used to identify charging variations in G26-containing mt-tRNAs associated with Trm1p.

D-stem and anticodon stem (Chapter III) (73, 129). N2, N2-dimethylation of G26 results in non-Watson-Crick interactions between G26 and N44 (N = A, U or G) enforcing a six base-pair long anticodon stem, generating the Type 6 fold that is commonly found in cytosolic tRNAs (75). Conversely, mt-tRNAs have previously been characterized for their susceptibility in forming conformations distinct from the canonical Type 6 fold which has been associated with G26 Trm1p-hypomodification (75). In the absence of m_2^2G26 and the corresponding steric hindrance introduced by the dimethyl groups, unmodified G26 is accessible to form Watson-Crick interactions with C11 in the D-stem. This results in an extended base-pairing interaction in the D-stem and shortening of the anticodon stem to 5 base pairs, characterized as the Type 5 fold (75). Alternatively, some mt-tRNAs can take on a Type 7 fold with 7 base-pair extended anticodon-stem, given the opportunity for base-pairing interactions between N25 and N45, facilitating Watson-Crick pairing between an unmodified G26 and C44 (75). Since mt-tRNAs have high propensity in deviating from the Type 6 fold and have greater flexibility in taking on alternate conformations particularly in the absence of m_2^2G26, we anticipated identifying a *S. pombe* G26 containing mt-tRNA that carries the characteristic elements for taking on a Type 5 or Type 7 fold. Interestingly, all eight G26-containing mitochondrial encoded *S. pombe* tRNAs (Thr^{UGU}, Ile^{GUA}, His^{GUG}, Ser^{UGA}, Leu^{UAG}, Phe^{GAA}, Tyr^{GUA} and Ser^{GCU}) lack the identity elements for folding into the Type 5 or Type 7 associated mt-tRNA conformation (Figure 5A). Nonetheless, the PHA26 northern blot analysis robustly illustrates the differential Trm1p affinity for G26 modification depending on mt-tRNA identity. We reasoned that this specificity may depict an unidentified role for m_2^2G26 for those candidate tRNAs. To assess tRNA functionality, particularly tRNA activity in protein biosynthesis we examined the charging state of these mt-tRNAs in the presence and absence of M1 and M24 Trm1 under *trm1+* and *trm1-* cells. Surprisingly, we did not observe any charging deficiency associated with a hypomodified G26 under the *trm1-* background (except for tRNA-Thr^{UGU} and tRNA-Ser^{UGA}) for any of the tRNAs, but noted an overall increase in charging state for tRNA-Ser^{UGA} and Ser^{GCU} with overexpression of M1-Trm1 (Figure 5B). These results may be suggestive of a limiting supply of Trm1p for modification of certain G26 containing mt-tRNAs, with overexpression of Trm1p directing more efficient aminoacylation of specific tRNAs such as tRNA-Ser^{UGA} and tRNA-Ser^{GCU}.

Assaying changes in expression of mitochondrial encoded proteins associated with modification status of G26 in mt-tRNAs

The semi-autonomous mitochondrial organelle, hosts the site of protein biosynthesis for several subunits of the OXPHOS respiratory complex (100). In addition to the nuclear encoded (> 100) genes that are destined to the mitochondria to facilitate mitochondrial translation, the mt-DNA also encodes several tRNAs and ribosomal RNA that function in mRNA decoding (100). PTM of mt-tRNAs has previously been demonstrated to play a crucial role in tRNA functionality with hypomodification resulting in mitochondrial dysfunction and respiratory defects (145). This has been directly demonstrated with hypomodification of mt-tRNA-Leu^{CUN} in cells bearing a mutated non-functional TRMT5 (145, 147). Hypomodification at G37 of mt-tRNA-Leu^{CUN} has been associated with irregularities in mitochondrial translation corresponding to the inactive mt-tRNA-Leu^{CUN} with subsequent mitochondrial related disorders (145). Since we have noted a change in expression of proteins related to metabolic processes linked to mitochondrial function in *trm1-* cells, a growth defective phenotype associated with M1-Trm1 and we have shown that mt-tRNAs are differentially modified by Trm1p, we reasoned for investigation of the role of Trm1p in regulating mitochondrial protein synthesis machinery. To assess mitochondrial translation products under *trm1+* and *trm1-* cells with overexpression of M1-Trm1 and M24-Trm1, we have used an *in vivo* technique that incorporates [^{35}S]-methionine into newly synthesized mitochondrial proteins, while inhibiting the cytoplasmic expression system by introducing cycloheximide (100). Using this technique, we checked the expression levels of components of the OXPHOS complex including Cox 1, 2, 3 and Atp 6, 8 and 9. Although, our PHA26 northern assay indicates differential modification efficiency of G26 containing mt-tRNAs, in combination with the growth phenotypes detected with overexpression of M1-Trm1 in *trm1+* and *trm1-* cells being symptomatic of mitochondrial dysfunction, we did not identify any differential expression of the OXPHOS mitochondrial encoded subunits under the conditions tested (Figure 6). However, we did observe a decrease in cytoplasmic translation products ((-) cycloheximide; Figure 6 Lanes 5-8) in *trm1-* cells with plasmid encoded M1-Trm1 and M24-Trm1 suggestive of alterations in cytoplasmic protein biosynthesis associated with overexpression of Trm1p, in addition to the SILAC findings of changes in polypeptide abundance related to *trm1-* cells.

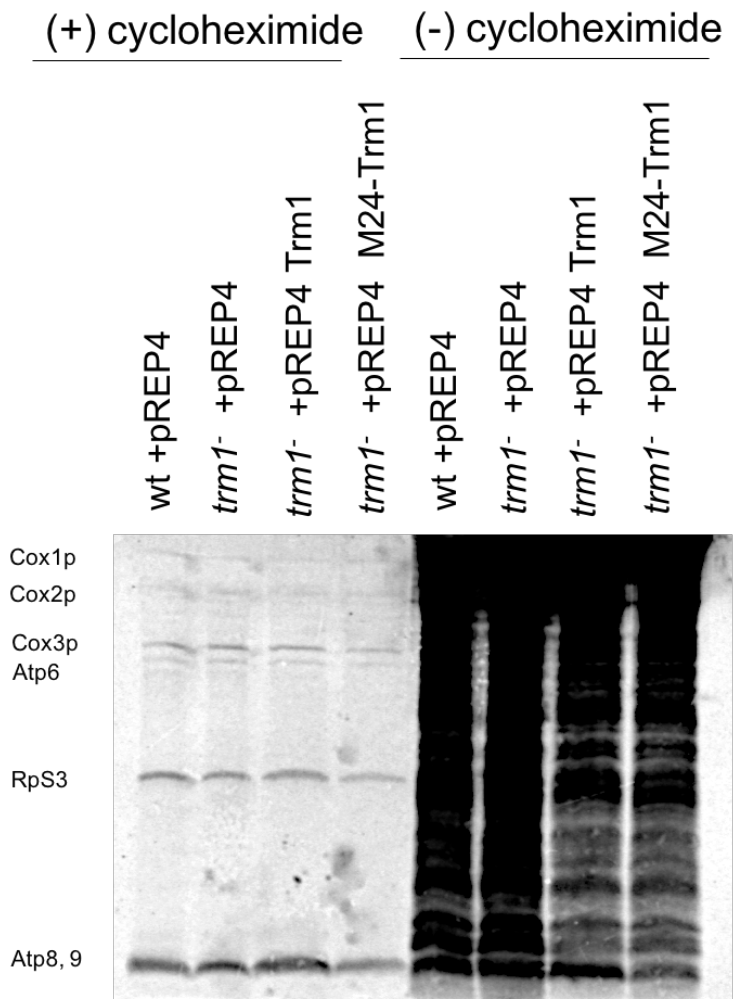


Figure 6. Examining mitochondrial translation products in *S. pombe*. Expression levels of newly synthesized mitochondrial proteins (Cox1p, Cox2p, Cox3p, Atp6, RpS3 and Atp8 and 9) under *trm1+* and *trm1-* conditions overexpressing M1 and M24 Trm1 determined using 35-S methionine pulse-chase of mitochondrial translation products with inhibition of cytoplasmic translation with the addition of cycloheximide. As a control, cytoplasmic protein synthesis was also evaluated for the same strains with elimination of cycloheximide.

DISCUSSION

The overabundance of chemical groups modifying tRNA nucleosides and their evolutionary conservation implicates PTMs with fine-tuning tRNA activity and supports the premise of these factors regulating protein expression (61–63, 114, 151). While modifications in the anticodon loop, or near this region have long been characterized as essential in the mRNA decoding capacity of tRNAs, PTMs on the body of tRNAs have been apportioned to supporting structural stability (61–63). An example of the latter is Trm1p, a methyltransferase well recognized for its functionality in promoting native tRNA folding (70, 75, 129). N2, N2-dimethylation of G26 on cytoplasmic tRNAs has previously been linked to native steady state abundance of tRNAs, preservation of wildtype charging state (Chapter III) as well as enhancing the decoding activity of suppressor tRNA-Ser^{UCA} in TMS assay (71). Additionally, Trm1p contains both a nuclear and mitochondrial targeting sequence that allows for subcellular localization of the enzyme in both the nucleus and mitochondria, facilitating modification of both nuclear and mitochondrial encoded tRNAs (72, 107, 149). This may be particularly important for mt-tRNAs as they have previously been characterized in forming alternate, non-canonical folds which are avoided in the presence of m^2_2G26 (75).

To gain greater insight into this multifaceted methyltransferase and possibly unravel novel roles for Trm1p-associated regulation of tRNA activity in mRNA decoding, we sought after potential changes in global protein expression associated with Trm1p. Stable isotope labeling of polypeptides in *trm1-* cells combined with LC-MS/MS analysis led to the identification of several proteins whose expression levels were altered in *trm1-* cells. We observed a pattern of CTA codon enrichment in the sequences of proteins that showed the most variation in expression levels in *trm1-* cells. While we have yet to validate the association of these data with defects in G26-hypomodifed tRNA-Leu^{UAG} decoding capacity, it is probable that misfolding of the anticodon stem, depletion of mature tRNA-Leu^{UAG} levels or impaired tRNA aminoacylation in *trm1-* cells may play a significant role in these findings. This type of modification-based regulatory control on tRNA decoding activity has previously been reported for enzymes that modify the anticodon loop region (78), with minimal evidence of tRNA body modifiers playing a regulatory role in translation. This may be evidence for a novel mechanism in which Trm1p fine-tunes tRNA function in protein expression.

While downregulation of various polypeptides associated with Trm1p depletion may be reasoned as above, these data only account for approximately 5% of the variability detected in changes in protein expression. The GO analysis of the two trials of the SILAC-LC-MS/MS data as well an additional independent trial of SILAC-LC-MS/MS implicate a role for Trm1p in modulating mitochondrial function, either through an indirect mechanism by regulating Moe1 levels which specifically translate mRNAs encoding components of the ETC chain and other metabolism-associated mRNAs, or through an independent unidentified mechanism. To gain confidence in the SILAC-LC-MS/MS data demonstrating upregulation of Moe1 in *trm1-* cells, we checked for changes in Moe1 abundance in *trm1-* and *trm1+* cells in the presence and absence of plasmid encoded Trm1 using western blot analysis (Figure 2). In line with the SILAC-proteomics data, we observed a correlation between Moe1 abundance and Trm1p levels, with overexpression of Trm1p resulting in downregulation of Moe1. The SILAC-proteomics data demonstrating changes in abundance of polypeptides associated with mitochondrial metabolic processes, in combination with western blot data suggest a Trm1p-function in alerting mitochondrial physiology.

In search of a novel role for Trm1p in regulating tRNA activity, we identified an unexpected, reproducible, defective growth phenotype associated specifically with M1-Trm1 overexpression in both *trm1-* and *trm1+* cells. Repression of the nmt1 promoter of pREP4 with thiamine circumvented the growth impairment validating the impact of Trm1p abundance in this growth defect (Figure 3B and C). These findings were intriguing because Trm1p has dual catalytic activity. M1-Trm1 is more prominently targeted to the mitochondria compared to M24-Trm1 (primarily found in the inner nuclear membrane). This growth impairment associated with M1-Trm1 is suggestive of a specific Trm1p-mediated mitochondrial irregularity, possibly due to dysfunctions in components of ETC with the downregulation of Moe1 in the presence of upregulated M1-Trm1 levels. These predictions justify further investigations.

Since Trm1p has a dual targeting sequence and can catalyze m_2^2G26 on both cytoplasmic and mt-tRNAs, and we noted a possible mitochondrial related abnormality with Trm1p levels, we searched for the presence of m_2^2G26 on eight G26 containing mt-tRNAs in *trm1-* and *trm1+* cells overexpressing M1-Trm1 and M24-Trm1. We identified the presence of m_2^2G26 on several mt-tRNAs in wildtype cells and further demonstrate that overexpression of M1-Trm1 results in enhancement of m_2^2G26 modification on select mt-tRNAs (tRNA-Thr^{UGU}, Ser^{UGA}, Leu^{UAG} and

Phe^{GAA}) more than others. Given the robust detection of m_2^2G26 for specific mt-tRNAs, we were interested in identifying possible changes in the aminoacylation levels of these Trm1p tRNA targets, should the m_2^2G26 status of the tRNA impact the charging efficiency by tRNA aminoacyl synthetases. While obvious defects in charging levels were not identified for the majority of the G26-containing mt-tRNAs, we did note a decrease in the charging state of mt-tRNAs-Thr^{UGU} and Ser^{UGA} in *trm1-* cells and conversely a robust increase in the charging state of mt-tRNAs-Thr^{UGU}, Ser^{UGA} as well as Ser^{GCU} with overexpression of M1-Trm1. Given these differences in Trm1p affinity for mt-tRNA G26 modification we anticipated possible alterations in expression levels of mitochondrial encoded OXPHOS subunits, should m_2^2G26 impact the decoding activity of these mt-tRNAs. Surprisingly, we did not find any changes in expression levels of the mitochondrial encoded OXPHOS subunits in *trm1-* and *trm1+* cells overexpressing M1-Trm1 and M24-Trm1. These findings are unexpected since overexpression of specifically M1-Trm1 results in a reproducible defective growth phenotype and we have identified specificity of M1-Trm1 for mt-tRNA G26 modification as well as an increase in charged state of tRNA-Ser^{UGA} and tRNA-Ser^{GCU} under conditions where M1-Trm1 is more highly expressed. While changes in expression levels of the OXPHOS subunits were not observed under the condition tested, our findings suggest a malfunction in the mitochondria associated with elevated levels of M1-Trm1.

In this work, we have attempted to identify a possibly novel role for the methyltransferase Trm1p in fine-tuning tRNA decoding activity in addition to the already well characterized role of this enzyme in enhancing tRNA structural stability. Unexpectedly, our data has alluded to a Trm1p-modulated potential alterations in mt-tRNA activity that may regulate mitochondrial function and fitness. This is not unprecedented, given previous reports of several human disorders linked to mutations in mt-tRNAs, tRNA processing enzymes and PTM enzymes. Defects in OXPHOS complex, inadequate energy production and lactic acidosis have been correlated with impaired mt-tRNA PTM enzymes (TRMT5, PUS1 and TRMU) (145–147). Although, the exact role of M1-Trm1 with respect to mt-tRNA functionality has not been directly elucidated here, our findings indicate an uncharacterized role for this methyltransferase in modulating mitochondrial physiology.

SUPPLEMNTARY DATA

Table S1. Probes used for Northern blots

tRNA	Northern probe
Phe GAA M TUC	5’ - TGC TCT GAA ATG GAA TCG AAC C - 3’
Phe GAA M PHA26	5’ - CAA TAC TGC ACT TTA ACC G - 3’
Phe GAA N TUC	5’ - TGT CAC AAA CCG GGA TCG AAC CGA TGA - 3’
Phe GAA N PHA26	5’ - CAG TCT GTC ATG CTC C - 3’
Ser UGA M TUC	5’ - CGA AAG GGG CAG GAT TC - 3’
Ser UGA M PHA26	5’- CTC AAA TAT CGC TCC TTA AA - 3’
Ser UGA N TUC	5’- GGG CAA AGC CCA TTA GAT - 3’
Ser UGA N PHA26	5’ - TTC AAG TCT AAC TCC TTA ACC - 3’
His GUG M TUC	5’- GAT TAC TTG GAA TCG AAC CAA G - 3’
His GUG M PHA26	5’- ATA AGC ATT CTA CCA TTG AAT TAC -3’
Ile GAU M TUC	5’- GTT CTT ATT GGA ATC GAA CCA ATA - 3’
Ile GAU M PHA26	5’- GTC TGC ACT CTG ACC TTT TA - 3’
Leu UAG M TUC	5’- GAT GGA AGG ATT CGA ACC TTC - 3’
Leu UAG M PHA26	5’- GTA AAC ATG TTT ACC AAT TTC AT - 3’
Thr UGU M TUC	5’ - GCT AAT TAG AGG AAT TGA ACC TC - 3’
Thr UGU M PHA26	5’ - GTA CGC ACT CTA CCA ATT - 3’
Tyr GUA M TUC	5’- GGA GAG CAG GAA TCG AAC CTG C- 3’
Tyr GUA M PHA26	5’- CTC ACC TCT TAA AAC AA CA - 3’
Cys CGA M TUC	5’- AGA TAA TGA TGA GAA TTG AAC TCA TAT ATC AAA- 3’
Cys CGA M PHA26	5’- TTC AAT TTC AGA CCA CTG AA- 3’
Ser GCU M TUC	5’- GGA AAA AGT GAG ACT CGA ACT CAC AAG - 3’
Ser GCU M PHA26	5’- GTA TCC TTC TTA CCA ATT AGA AC - 3’
U5 snRNA	5’- GCA CAC CTT ACA AAC GGC TGT TTC TG - 3’

Table S2. List of upregulated and downregulated proteins identified in *trm1-* relative to *trm1+* cells using SILAC proteomics (N=2)

Upregulated in *trm1-* cells	Avg. Log_2 Abundance Ratio: (*trm1-*) / (*trm1+*)
aspartate semialdehyde dehydrogenase	0.202175
acetyl-CoA ligase	0.20393
dihydroxy-acid dehydratase	0.2046
type I ribosomal protein arginine N-methyltransferase Rmt3	0.208425
ribonucleoside reductase large subunit Cdc22	0.21089
translation elongation factor eIF5A	0.213255
histidinol dehydrogenase His2	0.21397
60S ribosomal protein L4	0.217485
pyridoxal 5'-phosphate synthase subunit PDX1	0.22407
40S ribosomal protein S2	0.227485
phosphoglycerate mutase family	0.23532
alcohol dehydrogenase Adh4	0.237825
pentafunctional aromatic polypeptide Aro1	0.23796
alditol NADP+ 1-oxidoreductase activity	0.240655
F1-ATPase alpha subunit	0.25501
40S ribosomal protein S12	0.25875
IMP 5'-nucleotidase Isn1	0.258905
phosphoribosylformylglycinamidine synthase Ade3	0.26057
4-nitrophenylphosphatase	0.27076
RQC complex ubiquitin-protein ligase E3 Rkr1	0.27305
flavin dependent monooxygenase	0.27494
phosphoribosylamine-glycine ligase/phosphoribosylformylglycinamidine cyclo-ligase	0.31102
chaperonin-containing T-complex beta subunit Cct2	0.312635
homocitrate synthase	0.32011
mitochondrial and cytoplasmic alanine-tRNA ligase Ala1	0.321425
argininosuccinate synthase Arg12	0.33496
asparagine synthetase	0.33901
sepiapterin reductase	0.339285
pyruvate carboxylase Pyr1	0.345125
glyoxylate reductase	0.35704
imidazoleglycerol-phosphate synthase His4	0.359405
anthranilate synthase component II, multifunctional enzyme Trp1	0.36698
MCM complex subunit Mcm4/Cdc21	0.374185
Stm1 homolog Oga1	0.37592
aconitate hydratase Aco1	0.379905
glutamate-ammonia ligase Gln1	0.38662
cyclophilin family peptidyl-prolyl cis-trans isomerase Cyp2	0.39161
V-type ATPase V1 domain, subunit A	0.39212
short chain dehydrogenase	0.41333
Importin subunit beta-3	0.418335
translation elongation factor EF-1 alpha Ef1a-a	0.462535
homoaconitate hydratase Lys2	0.46791
signal recognition particle subunit	0.476855
N-acetyl-gamma-glutamyl-phosphate reductase/acetylglutamate kinase	0.482255
60S ribosomal protein L14	0.51726
trehalose-6-phosphate phosphatase Tpp1	0.575395
malate dehydrogenase Mdh1	0.592275
chorein homolog Vps1302	0.60674
ribonucleotide reductase small subunit Suc22	0.95077
pyruvate decarboxylase	0.96924
fatty acid synthase alpha subunit Fas2	1.571115

Downregulated in *trm1-* cells	Avg. Log_2 Abundance Ratio: (*trm1-*) / (*trm1+*)
RNA polymerase II complex subunit Rpb2	-2.33438
19S proteasome regulatory subunit Rpn501	-1.205025
DNA-directed RNA polymerase I complex large subunit Nuc1	-1.14303
translation elongation regulator Gcn1	-1.00152
protein disulfide isomerase	-0.969325
U5 snRNP complex subunit Brr2	-0.88826
translation initiation factor eIF3a	-0.6625
C-1-tetrahydrofolatesynthase/methylenetetrahydrofolatedehydrogenase/methylenetetrahydrofolatecyclohydrolase/formyltetrahydrofolatesynthetaseAde9	-0.65179
COPII-coated vesicle component Sec31	-0.550945
coatomer alpha subunit Cop1	-0.45575
iron responsive transcriptional regulator, peptidase family	-0.42193
gamma-glutamyl phosphate reductase Pro1	-0.375365
alpha glucosidase I Gls1	-0.36183
mitochondrial and cytoplasmic glycine-tRNA ligase Grs1	-0.32997
coatomer beta subunit	-0.177315
homoserine O-acetyltransferase	-0.16609
ribosomal-ubiquitin fusion protein Ubi1	-0.152895
protein disulfide isomerase	-0.1519
heat shock protein, ribosome associated molecular chaperone Sks2	-0.146165
tubulin alpha 2	-0.13872
ornithine transaminase Car2	-0.13164
cysteine synthase	-0.12677
cytosolic thioredoxin Trx1	-0.09213
fructose-bisphosphate aldolase Fba1	-0.066855

Table S3. List of upregulated and downregulated proteins identified in *trm1-* relative to *trm1+* cells using SILAC proteomics (independent trial, N=1)

Upregulated in *trm1-*	Log_2 Abundance Ratio: (*trm1-*) / (*trm1+*)
Uncharacterized oxidoreductase	0.509
ENTH domain-containing protein	0.511
F-actin-capping protein subunit beta	0.528
54S ribosomal protein L23, mitochondrial	0.544
Tubulin beta chain	0.564
F-actin-capping protein subunit alpha	0.604
Uncharacterized protein C23H3.15c	0.635
Protein vip1	0.64
ATPase family gene 2 protein	1.594
Uncharacterized protein C63.14	0.672
Ribonucleoside-diphosphate reductase small chain	0.708
Protein sds23/moc1	0.753
Superoxide dismutase [Mn], mitochondrial	0.812
L-azetidine-2-carboxylic acid acetyltransferase	0.827
Elongator complex protein 1	0.837
UBA domain-containing protein 7	0.930
Antisense-enhancing sequence 1	0.988
Protein lsd90	1.01
Malate dehydrogenase, mitochondrial	1.02
RNA-binding protein rnc1	1.05
Uncharacterized glutaminase C222.08c	1.07
Uricase OS=Schizosaccharomyces pombe	1.26
Eukaryotic translation initiation factor 3 subunit D	4.96

Downregulated in *trm1-*	Log_2 Abundance Ratio: (*trm1-*) / (*trm1+*)
Eukaryotic translation initiation factor 3 subunit A	-3.30
Zuotin OS=Schizosaccharomyces pombe	-2.32
Nucleoporin nup124 OS=Schizosaccharomyces pombe	-1.46
Serine/threonine-protein kinase shk1/pak1	-1.33
Uncharacterized RNA-binding protein C126.11c	-1.15
Probable proteasome subunit alpha type-2	-1.09
Large subunit GTPase 1	-0.92
Isoleucine--tRNA ligase, cytoplasmic	-0.76
ATPase synthesis protein 25, mitochondrial	-0.758
Orotidine 5'-phosphate decarboxylase	-0.601
Histidinol dehydrogenase	-0.591
Leukotriene A-4 hydrolase homolog	-0.591
Valine--tRNA ligase	-0.575
40 kDa peptidyl-prolyl cis-trans isomerase	-0.571
Bifunctional purine biosynthetic protein ADE1	-0.552
Phosphoglycerate kinase	-0.529
Probable phosphoribosylformylglycinamidine synthase	-0.526
Heat shock protein homolog pss1	-0.516
Transcription factor gaf1	-0.510

A

Alterations in protein expression associated with *trm1-* cells assessed using the candidate and control codons normalized to the proportion of codons in the open reading frame

tRNA of interest	Codon	Parameter Estimate	Standard Error	t Value	P-value
Ser UGA	TCA	2.58	4	0.64	0.52
Ser CGA	TCG	3.38	6.44	0.52	0.6
Leu CAG	CTG	-5.66	5.99	-0.95	0.345
Leu UAG	CTA	-8.96	6.48	-1.38	0.167
Leu CAA	TTG	0.209	1.93	0.11	0.914
Lys CUU	AAG	-0.376	0.945	-0.4	0.691
Gly CCC	GGG	1.58	8.26	0.19	0.8483
Ile UAU	ATA	-5.53	4.94	-1.12	0.2651
Tyr GUA	TAC	-2.19	2.22	-0.98	0.3255

B

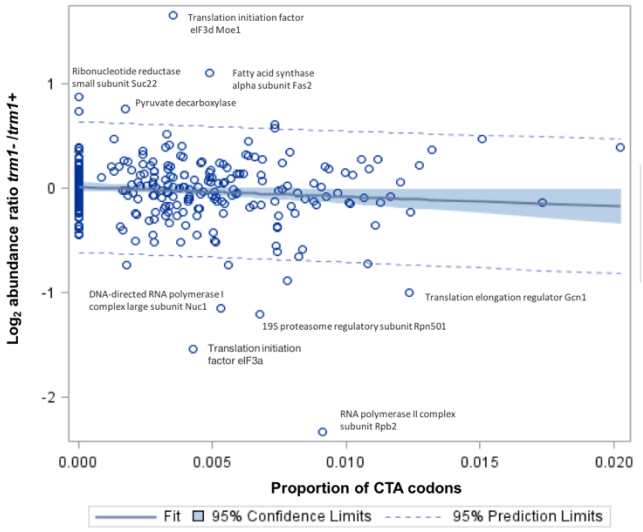


Figure S1. Assessing alterations in protein abundance in *trm1-* cells associated with enrichment of candidate codons corresponding to Trm1p-tRNA targets. A) Multiple regression coefficients (parameter estimates) were used to determine whether various candidate codons can account for reduced protein expression. This analysis controls for ORF size by using the proportion of candidate codons in each ORF. While reduced protein expression can be associated with CTA codon enrichment (according to the parameter estimates value), these findings are not significant as demonstrated by the P-value. B) Abundance ratio of *trm1-* vs. *trm1+* cells have been plotted against CTA codon proportions. Some examples of proteins upregulated and those downregulated have been reported. Control codons included in this analysis are: GGG, ATA, TAC which correspond to non-Trm1p tRNA substrates, as they are non-G26 containing tRNAs.

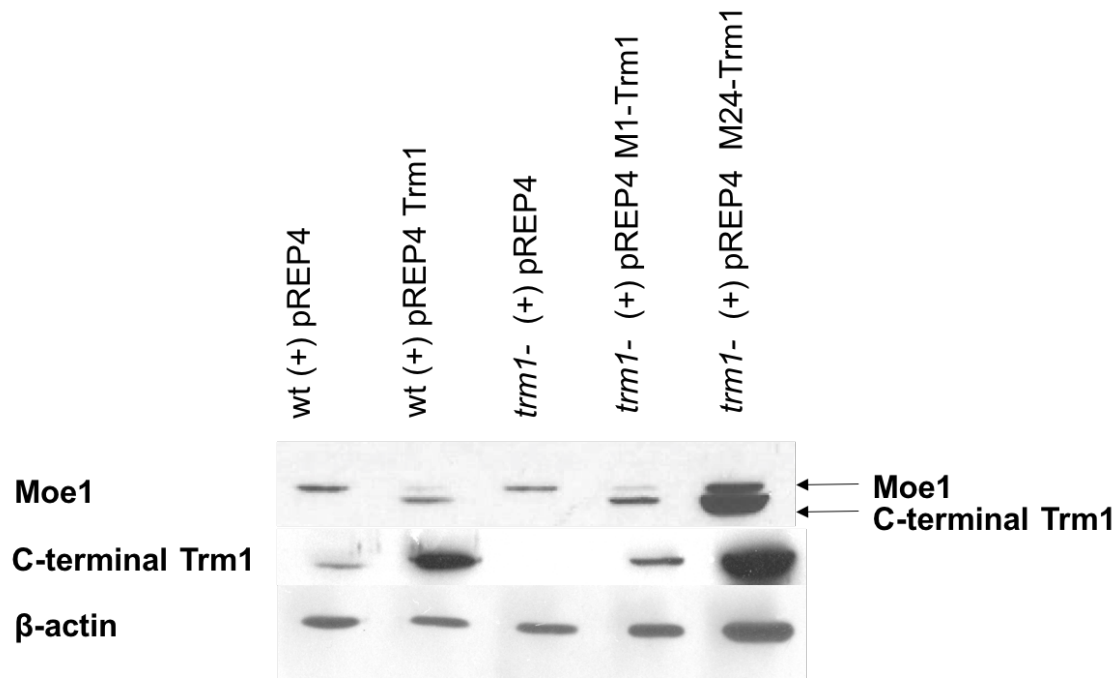


Figure S2. Western blot analysis used to evaluate the levels of Moe1, and endogenous Trm1. The abundance of Moe1 and endogenous level of Trm1 was assessed under *trm1*+ and *trm1*- cells with and without overexpression of M1 and M24-Trm1p. Beta-actin was used as the loading control.

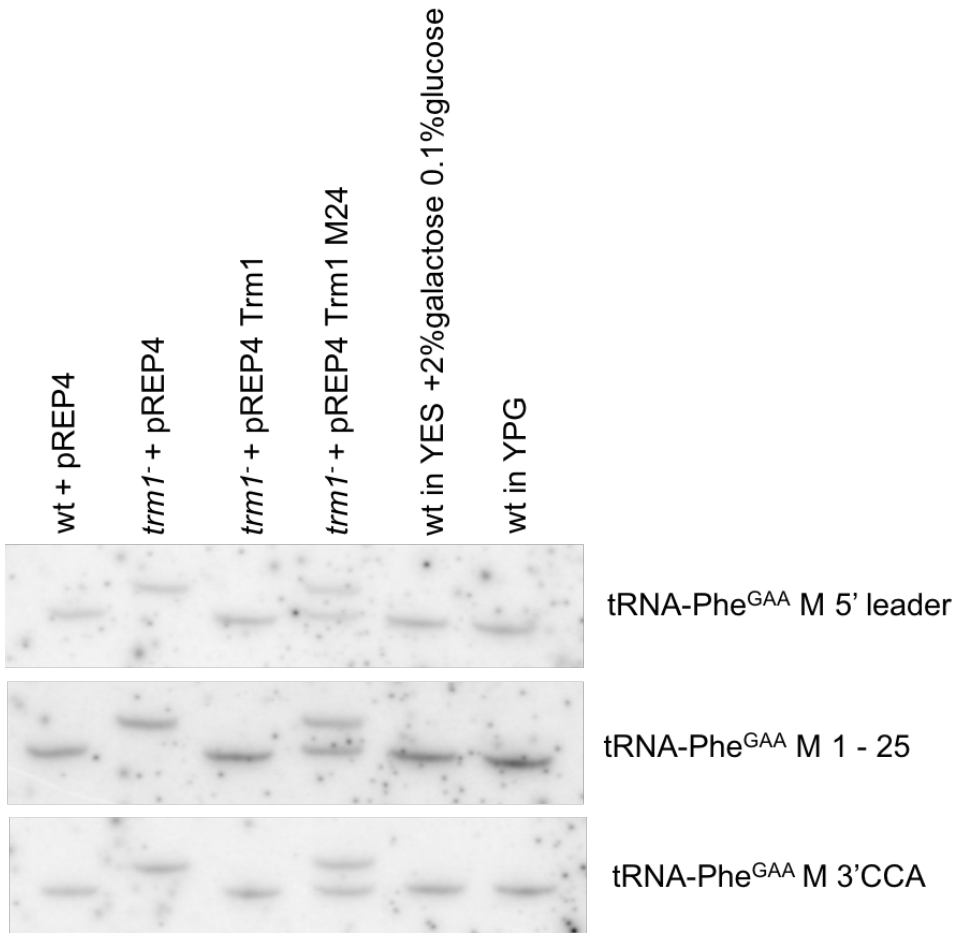


Figure S3. Northern blots of mitochondrial encoded tRNA phenylalanine. In addition to the PHA26 and TΨC probe used in Figure 4 to assess G26 modification status of the tRNA by Trm1p, other probes annealing to additional sites on the tRNA such as the 5' leader, 1 to 25 (anneals to the acceptor stem and D-stem loop) and 3' CCA were used to investigate the two tRNA-Phe^{GAA} species detected under *trm1-* and *trm1-* (+) pREP4 M24 Trm1 cells.

CHAPTER V: Summary and Future Directions

SUMMARY OF DISSERTATION

RNA molecules are virtually essential in all cellular processes and their functionality is tightly regulated by their complex three-dimensional conformation (2, 3). All RNA species have significant propensity in developing alternate non-functional conformations. This structural promiscuity stems from the lack of chemical complexity in the building blocks of RNA giving rise to a multitude of stable non-native RNA conformations from a single primary sequence (2, 4). Interactions with RNA binding proteins and PTM of RNA nucleosides directs RNA molecules towards the formation of the native, biologically active conformation (2, 4, 7, 63). Proteins with RNA chaperone activity are predicted to resolve misfolded RNA conformations and promote an accelerated escape from kinetic folding traps through transient and weak electrostatic interactions (1-7). The mechanistic insights of RNA chaperone activity and target discrimination has remained largely ambiguous because of the overlapping function and the non-specific nature of RNA chaperones and challenges in identifying a physiologically relevant RNA target that an RNA chaperone of interest assays *in vivo*.

La proteins are RNA chaperones that associate with coding and noncoding RNAs with pre-tRNAs being their best characterized substrates (20). In this work, we have attempted to compare the affinity of the RNA chaperone La in targeting a misfolded pre-tRNA relative to its folded counterpart. N2, N2-dimethylation of G26 containing tRNAs by the methyltransferase Trm1p has been predicted to function redundantly with La in tRNA structural stability in *S. cerevisiae* (70). As in *S. cerevisiae*, we have demonstrated that the deletion of *S. pombe* La in combination with Trm1p results in synthetic lethality at elevated temperatures. The lethality can be rescued with overexpression of full-length La but not hLa or Sla1 mutants defective in RNA chaperone activity, recapitulating the notion that Trm1p and La redundantly stabilize pre-tRNA structure. Using lead acetate chemical probing and Northern blot analyses we identified tRNA-Ser^{UGA} as a candidate tRNA that undergoes structural changes in the absence of Trm1p modification and has a reduced charged state in *sla1-/trm1-* cells. In addition to tRNA-Ser^{UGA}, we have identified a set of G26 containing tRNAs that have reduced steady-state abundance in *trm1-*, *sla1-* and *sla1-/trm1-* cells. Using this cohort of structurally vulnerable G26 containing pre-tRNAs, we have assessed the target

discrimination capacity of La as an RNA chaperone in differentiating between a misfolded Trm1p-hypomodified pre-tRNA from its Trm1p-modified, folded state. Our *in vivo* and *in vitro* data indicate an indiscriminate binding affinity of La for both native and G26 hypomodified misfolded pre-tRNAs. These findings are consistent with previous data demonstrating that the primary binding determinant of La proteins is the UUU-3'OH trailer sequence found at the ends of all nascent polymerase III transcripts (20, 29, 47) . La binding defective and non-defective pre-tRNAs occurs indiscriminately as La engages both species initially via the UUU-3'OH trailer sequence, with the RNA chaperone activity function of La concurrently resolving misfolded structures. While other RNA chaperones (StpA and hnRNP A1) have previously shown preference towards single stranded regions of RNA that are predicted to be more prevalent in misfolded conformations (7), La proteins are targeted to their RNA substrates by identifying features on their targets that validates them as processing intermediates rather than discriminating based on their folded state. Although La target discrimination deviates from other RNA chaperones, La proteins function comparably to protein molecular chaperones calnexin and calreticulin (139). RNA chaperones are a heterogeneous class of RNA binding proteins with no similarity identified in fold, sequence or structure amongst this class of proteins with the single commonality that they resolve misfolded RNA structures (7). Consistent with this, it is challenging to generalize a universal mechanism in which all RNA chaperones utilize to target misfolded RNA substrates. In this work, we have demonstrated that the RNA chaperone La specifically targets pre-mature poly (U) containing RNA substrates and subsequently utilizes this window of opportunity to enforce correct RNA folding using its non-specific RNA chaperone activity function. In this time frame, RNA molecules concurrently acquire PTMs on their nucleosides further supporting their native structural conformation. Our data has shed some clarity to the previously predicted mechanism of target discrimination by RNA chaperones.

Post-transcriptional modification of tRNAs is universal, signifying their essential role in tRNA functionality. Modifications on the body of tRNAs has been associated with structural stability, while modifications made to the anticodon loop regulate translation fidelity and rate (62, 63, 114). Trm1p modification of G26-containing tRNAs supports native tRNA folding and a normal charged state (Chapter III). Additionally m_2^2G26 regulates suppressor tRNA-Ser^{UCA} activity in decoding a nonsense codon in the *ade6-704* allele (71). To assess the capacity of a PTM enzyme distant from the anticodon loop in regulating tRNA decoding activity, we used a SILAC-

based quantitative proteomics approach to investigate changes in protein abundance in *trm1-* cells relative to *trm1+* cells in *S. pombe*. Our data suggests possible changes in tRNA-Leu^{UAG} decoding activity, likely associated with Trm1p-hypomodification of the tRNA in *trm1-* cells that results in alterations in expression levels of a set of polypeptides whose sequences are enriched with CTA codons. We have also noted alterations in abundance of metabolic processes, associated with mitochondrial physiology in *trm1-* cells suggestive of a Trm1p-mediated mitochondrial functional regulation. Consistent with this, we have observed a specific growth defective phenotype associated with overexpression of mitochondrial targeted Trm1p in both *trm1-* and *trm1+* cells. Additionally, we have identified the G26 modification status of eight G26 containing mt-tRNAs and demonstrate the substrate specificity of Trm1p towards these tRNAs given the variability detected in the m^2_2G26 levels based on tRNA identity. While we did not observe defects in charging state of G26-containing mt-tRNAs, we did note that Trm1p levels under *trm1+* conditions may be limiting for certain tRNAs (tRNA-Ser^{UGA} and tRNA-Ser^{GCU}) and overexpression of M1-Trm1p may enhance the charging state of these mt-tRNAs. Since defects in PTM of mt-tRNAs has previously been linked to mitochondrial dysfunction (145, 147), we examined the expression levels of mitochondrial translation products, particularly the mitochondrial encoded OXPHOS subunits under *trm1-* and *trm1+* cells (with and without M1 and M24-Trm1 overexpression) and report no clear changes in the expression levels of these subunits. Although, the exact role of Trm1p modulating mitochondrial fitness has not been directly revealed, we have reason to be believe that there exists an unidentified mechanism in which Trm1p alters mt-tRNA activity, regulating mitochondrial physiology.

FUTURE DIRECTIONS AND CONCLUSIONS

Cellular survival and gene expression is vigorously controlled at various stages, with the universal nucleoside modification of tRNAs playing a crucial role in this process. It is consensually agreed upon that PTM of tRNA nucleosides in the anticodon loop regulate protein biosynthesis by stabilizing codon-anticodon interactions, minimizing frame-shift mutations, increasing codon-bias or alternatively weakening the codon-anticodon interaction to allow near-cognate codon decoding (61–63, 151). In addition to the anticodon nucleoside modifications that regulate gene expression

directly, PTMs made to the tRNA body enhance structural stability and regulate tRNA functionality. In this work, we have demonstrated that N2, N2-dimethylation of G26 supports the structural stability and the native charged state of a subset of cytoplasmic tRNAs. Although distant from the anticodon loop, m^2_2G26 has also been linked to regulating decoding activity of suppressor tRNA-Ser^{UCA} (71). We have attempted to examine the role of m^2_2G26 in tRNA functionality in protein biosynthesis more globally by assessing alterations in protein abundance that are related to Trm1p levels using SILAC-LC-MS/MS analysis. Our preliminary data indicate a correlation between the alteration in expression levels of a subset of polypeptides in *trm1-* cells and the enrichment of CTA codons in these transcripts. This is likely a reflection of the inefficient translation of these codons by the G26 hypomodified tRNA-Leu^{UAG}. This pattern of variation in protein expression levels associated with tRNA hypomodification has previously been reported with Trm9 in which transcripts enriched with AGA and GAA codons were downregulated as a result of Trm9-hypomodified tRNA-Arg^{UCU} and Glu^{UUC} (78). Our data, consistent with previous work with other tRNA PTM enzymes demonstrates the role of tRNA PTMs in regulating protein biosynthesis. While tRNA-Leu^{UAG} has previously been identified as a Trm1p target (71), and there is evidence of this tRNA showing reduced steady-state abundance in *trm1-* cells (Chapter III), there is still significant gaps in knowledge for the reasoning behind the necessity of m^2_2G26 in the efficient decoding capacity of tRNA-Leu^{UAG}. In addition to depletion of mature tRNA-Leu^{UAG} levels in *trm1-* cells (possibly due to misfolding and subsequent degradation) it is probable that any alterations in tRNA-Leu^{UAG} structural conformation due to the absence of m^2_2G26 in *trm1-* cells may also impact the charging state of this tRNA, resulting in variable expression levels of proteins enriched with CTA codons. Moreover, examining the charging state of tRNA-Leu^{UAG}, as well as using chemical probing assays to evaluate the structural conformation of this tRNA +/- m^2_2G26 may give reasoning for the changes in expression levels of CTA enriched proteins in *trm1-* cells.

tRNA processing enzymes and chemical modification of tRNA nucleosides has not only been implicated as crucial regulatory factors in nuclear gene expression but has also been associated with maintenance of mitochondrial biology (146). Mitochondrial tRNA mutations, tRNA-hypomodification and alterations in tRNA folding and abundance have all been linked to two well-recognized mitochondrial-associated diseases; mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) and myoclonic epilepsy with ragged red fibers

(MERRF) (146). Alterations in mt-tRNA functionality results in malfunctions in the mitochondria, largely due to a general decrease in mitochondrial protein synthesis of the OXPHOS complex (145–147). *S. pombe* Trm1 (similar to *S. cerevisiae* Trm1) is predictably, transcriptionally regulated with two in-frame AUG codons, resulting in the formation of two isoforms of Trm1p; a mitochondrial and a nuclear localized enzyme. While presumably both isoforms are present under normal conditions, there is little known regarding the abundance and the catalytic activity of each isoform. In this work, we have observed a growth defective phenotype that is specifically associated with the overexpression of mitochondrial-targeted Trm1 (M1-Trm1, not M24-Trm1) suggestive of a specific, unidentified role for this methyltransferase in modulating mitochondrial fitness. Although, our current findings lack clarity in the exact functionality of this methyltransferase in mitochondrial physiology, experimentation in identifying cellular stress cues that regulate transcriptional start site of Trm1p will be informative. Additionally, SILAC-LC-MS/MS analysis of *trm1-* cells overexpressing M1-Trm1 and M24-Trm1 may give more comprehensive insights into the proteins that are upregulated or downregulated uniquely in *trm1-* cells overexpressing M1-Trm1 that may be causing the growth impairment.

Since mt-tRNA modification and folding status has previously been linked to mitochondrial related disorders due to downregulation or defects in the OXPHOS complex (145–147), we reasoned that the growth impairment detected with overexpression of M1-Trm1 may be due to alterations in expression levels of the OXPHOS complex. Our *in vivo* labeling of mitochondrial translation products demonstrated a lack of variation in the abundance of the mitochondrial-encoded OXPHOS subunits +/- M1-Trm1. Although, we have shown minimal alterations in the abundance of these proteins under the strains tested, we are unaware of their functional, enzymatic activity in the growth defective, plasmid encoded M1-Trm1 strain. PTM of tRNAs is used as a regulatory mechanism by the cell to control tRNA activity in protein translation, with hypomodification potentially decreasing tRNA activity, and modification or hypermodification, upregulating the activity of tRNAs in protein translation. It may be probable that the overexpression of M1-Trm1 results in m_2^2G26 modification of a mt-tRNA that is considered a non-Trm1p target under wildtype conditions. This unusual modification status on the tRNA with overexpression of M1-Trm1 may result in misrecognition of the tRNA by a non-cognate tRNA aminoacyl synthetase. Error in tRNA aminoacylation has previously been linked to decrease in translation fidelity and synthesis of mutant proteins (152). While we did not observe

alterations in the abundance of mitochondrial-encoded OXPHOS subunits, it is plausible that cells overexpressing M1-Trm1 possess tRNAs that are misacylated, or functionally more active compared to wildtype conditions, causing their misuse in mitochondrial translation, producing mutant, inactive OXPHOS subunits. It would be informative to check the correct aminoacylation of mt-tRNAs under *trm1-* and *trm1+* cells with plasmid encoded M1-Trm1 compared to *trm1+* cells. Additionally, assessing mitochondrial fitness by evaluating oxygen consumption, ATP levels and activity of the OXPHOS enzymes (153) in *trm1-* and *trm1+* cells with plasmid encoded M1-Trm1 may give insight into the growth defective phenotype observed with these cells.

Our work has alluded to a novel role for Trm1p modification of both cytoplasmic and mitochondrial encoded tRNAs. While it was previously assumed that modifications made to the tRNA body play an ancillary role in tRNA functionality, our data demonstrates an unexpected role of m_2^2G26, distant from the anticodon loop in modulating nuclear and mitochondrial-encoded tRNA activity in translation, as well as potentially serving as a communicator link between the nuclear and mitochondrial genome.

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