2008-07-11

Genetic Analysis of the Role of SmpB in Establishing the Reading Frame on tmRNA

Talina Christensen Watts
Brigham Young University - Provo

GENETIC ANALYSIS OF THE ROLE OF SmpB IN DETERMINING FRAME ON tmRNA

by

Talina C. Watts

A thesis submitted to the faculty of

Brigham Young University

in partial fulfillment of the requirements for the degree of

Master of Science

Department of Chemistry and Biochemistry

Brigham Young University

August 2008

BRIGHAM YOUNG UNIVERSITY

GRADUATE COMMITTEE APPROVAL

of a thesis submitted by

Talina C. Watts

This thesis has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory.

Date	Allen Buskirk, Chair
Date	Barry Willardson
Date	David Belnap
Date	Joel Griffitts

BRIGHAM YOUNG UNIVERSITY

As chair of the candidate's graduate committee, I have read the thesis of Talina C. Watts in its final form and have found that (1) its format, citations, and bibliographical style are consistent and acceptable and fulfill university and department style requirements; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the graduate committee and is ready for submission to the university library.

Date	Allen Buskirk Chair, Graduate Committee

Accepted for the Department

Date	Paul B. Farnsworth Department Chair

Accepted for the College

Date	Thomas W. Sederberg, Associate Dean College of Physical & Mathematical Sciences

ABSTRACT

GENETIC ANALYSIS OF THE ROLE OF SmpB IN

DETERMINING FRAME ON tmRNA

Talina C. Watts

Department of Chemistry and Biochemistry

Master of Science

Ribosomes translate the genetic information encoded by mRNA into proteins. Defective mRNAs can cause stalling of translating ribosomes. The molecule tmRNA (transfer-messenger RNA) rescues stalled ribosomes in eubacteria. Together with its protein partner SmpB, tmRNA mimics a tRNA by entering the ribosomal A site and linking an alanine residue to the growing polypeptide chain. The ribosome then abandons the defective mRNA template and resumes translation on tmRNA, adding ten more amino acids to the nascent polypeptide. As a result of tmRNA action, stalled ribosomes are released and recycled, the defective mRNA is destroyed, and the aborted

protein product is tagged for destruction by proteases. It is unknown how the ribosome correctly chooses the position on tmRNA to resume translation. Previous studies implicate the sequence UAGUC found immediately upstream of the first codon in the tmRNA open reading frame. These nucleotides are highly conserved in natural tmRNA sequences. Mutations in this area cause loss of tmRNA function and improper frame choice. Using a genetic selection that ties the life of *E. coli* cells to the function of tmRNA, we have identified several SmpB mutants that rescue an inactive tmRNA in which this upstream sequence was altered. This links SmpB to the function of these key tmRNA nucleotides. We show that our SmpB mutants affect frame choice using an *in vivo* assay for tagging in the various frames. We conclude that SmpB plays a role in setting the reading frame on tmRNA.

ACKNOWLEDGMENTS

I wish to express my deep gratitude to all those who contributed to this work. I am particularly appreciative of my advisor, Dr. Allen Buskirk, for his support and direction in this research and in writing this thesis. I am grateful to my fellow lab members for all of their help, especially DeAnna Jones and David Healey who contributed directly to this work. A special thanks to Hilary Johnson for her work on the figures for this thesis. I am grateful to my family and above all my wonderful husband, David, for all of his love and unfailing support of this dream. Thank you everyone. I couldn't have done this without you.

TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS

A site	aminoacyl or acceptor site in the ribosome
aa-tRNA	aminoacyl-tRNA
cfu	colony forming unit
CIP	calf intestinal phosphatase
E site	exit site of the ribosome
GTP	guanosine triphosphate
KanR	kanamycin resistance protein
mRNA	messenger RNA
ORF	open reading frame
P site	peptidyl site of the ribosome
QCh	QuickChange PCR
SmpB	small protein B
ssrA	small stable RNA, name of tmRNA gene
TLD	tRNA-like domain
tmRNA	transfer-messenger RNA
tRNA	transfer RNA
WT	wild-type

Chapter 1: INTRODUCTION

Ribosomes and Translation

The process of translating genetic information from nucleotide sequences (mRNA) into amino acid sequences (proteins) takes place on the ribosome. The ribosome is a complex of both RNA and proteins; bacterial ribosomes contain approximately 65% rRNA and 35% protein by weight. Bacterial ribosomes are classified as 70S ribosomes based on their sedimentation coefficient. They are composed of a small subunit (30S) and a large subunit (50S). In E. coli, the 30S subunit is composed of the 16S rRNA and 21 different proteins while the 50S contains 36 total proteins and is composed of both the 5S and 23S rRNAs. The rRNA components form the structural core of the ribosome while the proteins are secondary elements, binding to the surface of the structure. Early structures of the ribosome reveal that there are no proteins within 18 Å of the active site[1].

The two ribosomal subunits fit together to form a channel through which the mRNA passes as translation occurs. The ribosome has three tRNA binding sites designated as the A (aminoacyl or acceptor) site, which accepts the correct

aminoacylated-tRNA complex into the ribosome; the P (peptidyl) site, where the tRNA bound to the nascent polypeptide is bound; and the E (exit) site, where the deacylated tRNA moves before it departs the ribosome.

Translation can be broken down into three separate steps: initiation, elongation, and termination (Figure 1). Initiation of prokaryotic translation begins with the 30S subunit and is catalyzed by three initiation factors: IF1, IF2, and IF3. IF3 helps dissociate the 70S ribosome at the end of a round of translation and then remains bound to the 30S subunit in the area that will become the E site. This binding blocks the small subunit from reassociating with the large subunit prematurely. IF1 binds to the portion of the ribosome that will become the A site to prevent tRNAs from binding until initiation is complete. IF2 is a GTPase (a protein that binds and hydrolyzes guanosine 5′-triphosphate or GTP) that facilitates the association of the charged, initiator tRNA (fMet-$tRNA^{fMet}$) with the small subunit and prevents the association of other charged tRNAs. IF2 binds to IF1 and reaches from the A site into the P site to contact fMet-$tRNA^{fMet}$, which helps position the initiator tRNA in the P site[2]. With all three initiation factors bound, the small subunit binds to the mRNA and the initiator tRNA.

The binding of the mRNA to the small subunit involves base pairing between the Shine-Dalgarno sequence upstream of the mRNA start codon and its complementary sequence at the 3′ end of the 16S rRNA[2]. This base pairing positions the mRNA start codon in the P site. Binding of fMet-$tRNA^{fMet}$ to the small subunit is facilitated by its interaction with IF2 bound to GTP and then by base pairing between the anticodon and

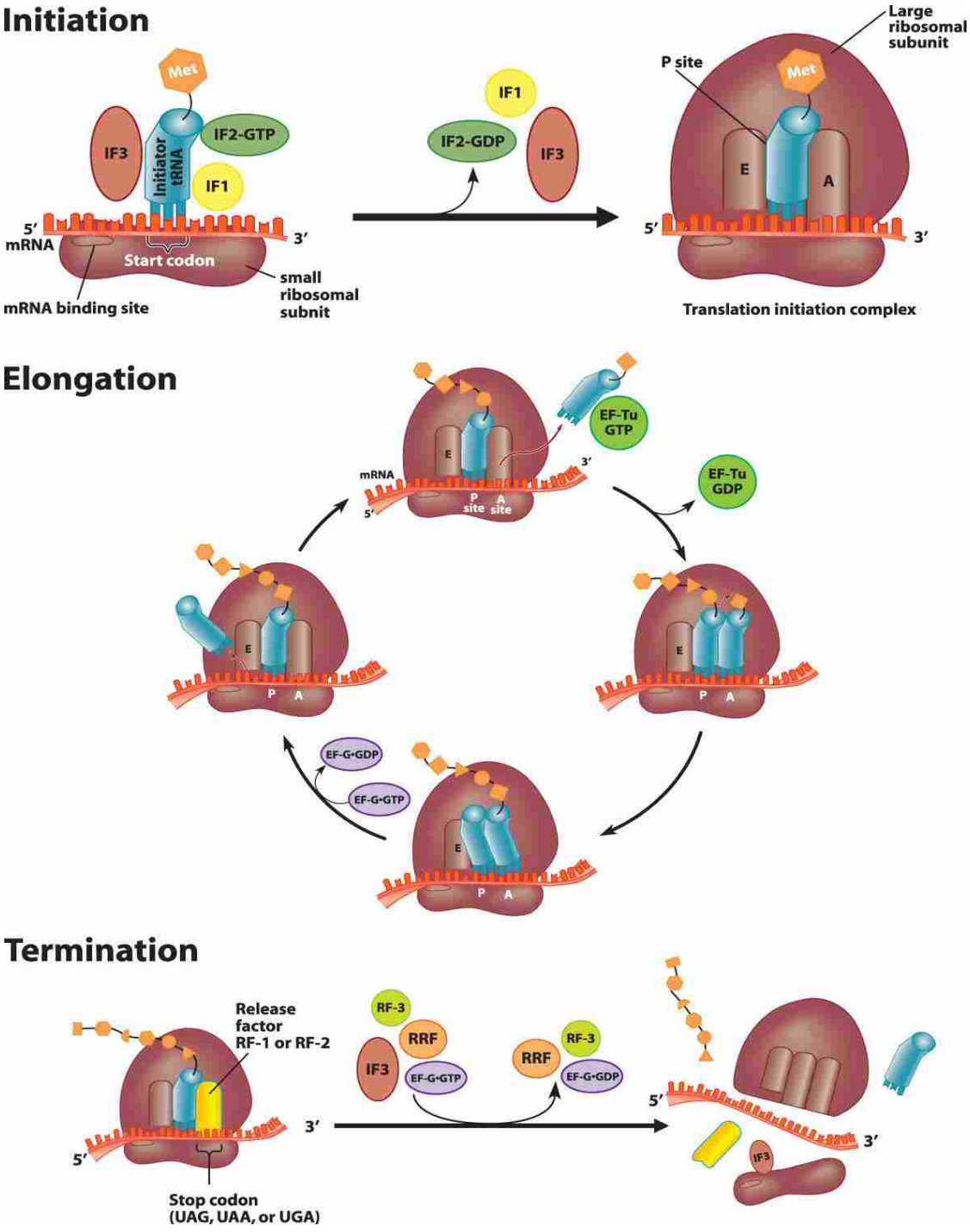


Figure 1: Model of Prokaryotic Translation

the start codon of the mRNA[2].

The last step of initiation is the association of the large subunit to create a function 70S initiation complex. When the start codon and fMet-tRNA^{fMet} base-pair, a conformational change in the small subunit occurs which results in the release of IF3. The large subunit is then free to bind to the small subunit and this binding stimulates the GTPase activity of IF2. The GTP bound to IF2 is hydrolyzed and both IF2 and IF1 are released from the ribosome[2]. The result of initiation is the formation of an intact 70S ribosome with the start codon and initiator tRNA in the P site and a codon in the A site. The ribosome–mRNA complex is ready to accept a charged tRNA and begin elongation.

Once the ribosome is assembled with the charged tRNA in the P site, protein synthesis can begin. First, the correct aminoacyl-tRNA (aa-tRNA) must be escorted to the ribosome by elongation factor EF-Tu[3]. After a tRNA is aminoacylated, EF-Tu binds to the 3′ end, shielding the coupled amino acid and preventing peptide bond formation until EF-Tu releases the tRNA in the A site. Like the initiation factor IF2, EF-Tu binds and hydrolyzes GTP. EF-Tu can only bind aminoacyl-tRNAs when it is associated with GTP. This aa-tRNA–EF-Tu•GTP complex then binds to the A site of the 70S ribosome. The EF-Tu GTPase is activated only when it associates with the same domain of the 50S subunit that activates IF2, known as the factor-binding center[3]. EF-Tu interacts with the factor-binding domain only after the tRNA enters the A site and a correct codon-anticodon match is made. The GTPase is activated and rapid GTP hydrolysis is

followed by release of EF-Tu●GDP and accommodation of the tRNA acceptor arm into the peptidyl transferase center[4].

The fidelity of amino acid incorporation is very high, with an error rate between 10^{-3} and 10^{-4}. The basis for the selection of the correct aminoacyl-tRNA is the base pairing between the tRNA and the A site codon but the energy difference between a perfect match and that of a near match cannot explain this level of accuracy. How does the ribosome manage such a low error rate?

There are at least three mechanisms which contribute to the ribosomes fidelity. One involves two adjacent adenines in the 16S which are located in the A site. These residues form hydrogen bonds with the minor groove of each correct base pair of the anticodon and the first two bases of the A site codon. These interactions result in a lower rate of dissociation from the ribosome for correctly paired tRNAs compared to incorrectly paired tRNAs[5].

The second mechanism that helps to ensure correct codon:anticodon pairing involves the GTPase activity of EF-Tu, described above[6, 7]. GTP hydrolysis is highly sensitive to correct codon:anticodon interactions. A single mismatch incorrectly positions EF-Tu, reducing its ability to interact with the factor binding center of the ribosome which leads to a decrease in EF-Tu GTPase activity. Only once GTP hydrolysis occurs can EF-Tu be released to expose the amino acid coupled to the A site tRNA.

The third mechanism is a type of proofreading that occurs after EF-Tu is released. Mismatched tRNAs are more likely to dissociate from the ribosome before the peptidyl transferase reaction occurs. When a tRNA first enters the A site, bound by EF-Tu, the 3′ end of the tRNA is distant from the active site where peptide bonds are formed. After EF-Tu is released, the tRNA needs to rotate into the peptidyl transferase center in a process called accommodation. During accommodation, the 3′ end of the tRNA moves almost 70 Å. Incorrectly paired tRNAs usually dissociate from the ribosome during accommodation[7]. It is hypothesized that the rotation of the tRNA places a strain on the codon:anticodon interaction and only a correctly paired anticodon can maintain the interaction[5].

Once proper accommodation has occurred and the charged tRNA is correctly positioned in the peptidyl transferase center, peptide bond formation takes place. This reaction is catalyzed by RNA, specifically the 23S rRNA of the 50S subunit[8]. Base pairing between the 23S rRNA and the CCA ends of the tRNAs in the A and P sites help to properly position the substrates for this reaction. The ribosome catalyzes the formation of a peptide bond between the amino acid bound to the A site tRNA and the peptide bound to the P site tRNA, transferring the polypeptide onto the tRNA in the A site. The tRNA in the P site is now deacetylated and the growing polypeptide chain is linked to the tRNA in the A site. Before another round of elongation can occur, the deacetylated tRNA must move into the E site and the A site tRNA must move into the P site. At the same time, the mRNA must move three nucleotides to position the next

codon in the A site. These movements are coordinated by the ribosome and are referred to as translocation.

The first stage of translocation is coupled to the peptidyl transferase reaction. After the peptide bond is formed, the A site tRNA is bound to the polypeptide, which is positioned near the P site, while the tRNA is still bound to the codon in the A site. Likewise, the P site tRNA is no longer bound to the polypeptide but is still bound to the codon in the P site[9]. This results in what is referred to as a "hybrid state." The tRNA 3′ ends have shifted into a new location but their anticodon ends are still in their pre-peptidyl transfer position. The second stage of translocation requires elongation factor EF-G[9]. EF-G binds the ribosome only when it is associated with GTP. The partial translocation that occurs after the peptidyl transferase reaction uncovers a binding site for EF-G located in the large subunit portion of the A site. When EF-G•GTP binds it contacts the factor-binding center of the large subunit and stimulates the GTPase activity of EF-G. GTP is hydrolyzed which results in a conformational change of EF-G. EF-G can now interact with the small subunit and trigger translocation of the A site tRNA. Once translocation is complete, the affinity for EF-G is dramatically decreased and EF-G•GDP is released. The former A site tRNA is now located in the P site, the P site tRNA in the E site, and the mRNA has moved exactly three nucleotides[9]. The ribosome is now ready for the next round of elongation. This continues until the last amino acid of the sequence is added.

The cycles of tRNA binding, peptide bond formation and translocation continues until a stop codon enters the ribosomal A site[10]. There are three stop codons: UAG ("amber"), UAA ("ochre"), and UGA ("opal"). Rather than being recognized by a tRNA, these codons are recognized by one of two proteins known as release factors: RF1 and RF2. RF1 recognizes the stop codon UAG and RF2 recognizes UGA. Both factors can recognize UAA. It is these release factors that trigger hydrolysis of the peptide chain from the tRNA in the P site[10]. Once hydrolysis has occurred, the release factor in the A site needs to be removed from the ribosome. This is accomplished by a third release factor, RF3. This factor is also a GTP-binding protein but unlike other GTP-binding proteins in translation, RF3 has a higher affinity for GDP than GTP, so free RF3 is usually in the GDP-bound form. After RF1 or RF2 stimulates hydrolysis of the polypeptide, a conformational change occurs which results in RF3 exchanging its GDP for a GTP. RF3•GTP has a very high affinity for the ribosome and displaces the RF1 or RF2 from the ribosome. RF3 can then associate with the factor binding center of the ribosome, hydrolyzing the bound GTP to a GDP. Because the RF1 or RF2 is no longer present, the RF3•GDP has a much lower affinity for the ribosome and it is released.

After the release of the completed polypeptide and the release factors, the ribosome is still bound to the mRNA and the tRNAs in the P and E sites. These must be removed and the two ribosomal subunits separated before a new round of translation can occur. These events are referred to as ribosome recycling. In prokaryotes, ribosome recycling requires the ribosome recycling factor (RRF), EF-G, and IF3[10]. RRF binds to the

now vacant A site and recruits EF-G•GTP. This stimulates events similar to EF-G function in translocation. EF-G stimulates the release of the deacylated-tRNAs in the P and E site, though the exact mechanism of this release is still unknown. Once the tRNAs are removed, RRF and EF-G are released as well along with the mRNA. The exact function of IF3 in this process is unclear but it is required for the separation of the two subunits and the resulting products are an IF3-bound small ribosomal subunit and a free large subunit[10]. The released ribosome can now participate in a new round of protein synthesis.

Ribosome Stalling

Normally, a stop codon is required for release of the ribosome from an mRNA. But what happens to a ribosome that initiates translation on an mRNA fragment lacking a proper stop codon in the right frame? Such mRNAs are the result of incomplete transcription or nuclease action. A ribosome can successfully initiate translation on such a fragment and translation will continue until it reaches the 3′ end of the message. The ribosome then stalls at the end of such mRNAs because there is no stop codon to signal termination and ribosome release. This stalling leads to three unwanted consequences for the cell. First, multiple ribosome stalled on a defective mRNA causes a depletion in the pool of ribosomes available for translation and thus a loss of translational efficiency. Second, the defective mRNA will continue to cause additional rounds of unproductive

translation until the mRNA is degraded. Third, the abnormal protein product that results from translation of the defective mRNA can be toxic for the cell.

It was initially observed that tmRNA rescues ribosomes stalled at the very 3′-end of truncated mRNAs lacking an in-frame stop codon. Later studies showed that tagging and rescue by tmRNA also occurs at ribosomes paused at sense or termination codons for extended periods because the cognate aa-tRNA or release factor is scarce, or when translation pauses for other reasons[11-15]. Though these circumstances seem distinct from one another, it is now known that mRNA cleavage occurs after ribosome stalling, converting paused complexes, which are capable of resuming normal translation, into complexes stalled at or near the 3′ end of the mRNA[16-19]. There are two types of mRNA cleavage that occurs with pausing. The first cleaves 10-20 bases downstream of the A site codon, near the position where the mRNA is no longer protected by the ribosome. The second type actually occurs within the A site of the ribosome. It is unknown why pausing can lead to A site cleavage in some instances and 3′ boundary cleavage in others. However, pausing-dependent mRNA cleavage and a vacant A site appear to be important for the recognition of stalled ribosomes by SmpB-tmRNA complexes[20].

Cells need a mechanism to rescue stalled ribosomes. To accomplish this, bacteria have evolved a unique translational quality control system to address all three of the aforementioned concerns. This mechanism, involving a specialized RNA molecule, tmRNA, and its protein binding partner, SmpB, is known as *trans*-translation.

tmRNA and *trans*-translation

History and Discovery of tmRNA

tmRNA is a unique RNA molecule which functions as both a tRNA and an mRNA. It was first discovered in 1978 as a small, stable RNA in *E. coli* with unknown function. It was shown to be present in cells at approximately one tenth the molar abundance of ribosomal RNA[21]. The gene encoding tmRNA was designated *ssrA* for small, stable RNA, also known as 10Sa RNA[22]. Once the sequence and structure were determined, similarities were observed between tmRNA and tRNA. In 1994 it was discovered that the 5′ and 3′ ends of tmRNA fold into a structure similar to the structure of tRNA, especially that of *E. coli* $tRNA^{Ala}$. Komine *et al.* showed that purified SsrA RNA is charged with alanine by alanyl-tRNA synthetase in vitro[23].

In 1989 an internal open reading frame (ORF) was identified in the *ssrA* gene but evidence that it was actively translated was not found until 1995. Tu *et al.* observed that a foreign protein overexpressed in *E. coli* resulted in a small population of protein product with the same C terminal modification[24]. These truncated protein products all contained the same C terminal sequence: AANDENYALAA. The last 10 amino acids of this sequence are encoded by the ORF in tmRNA. They observed that this sequence was not added to the protein in *ssrA* deletion strains.

The tag sequence encoded by tmRNA is similar to other, known, protease recognition sequences and in 1996, it was found that the protease Tsp recognized and degraded tmRNA-tagged proteins[25]. This suggests that a ribosome stalled on an mRNA switches translation from the defective mRNA to the internal ORF of tmRNA, adding the tmRNA tag to the C-terminus of the polypeptide. This led to a model where tmRNA acts as part of a quality control system for protein synthesis. This hypothesis was tested by making nonstop mRNAs (mRNAs lacking a stop codon in the proper frame) and expressing them in *E. coli* cells with and without tmRNA[11]. In cells lacking tmRNA, the mRNA was translated into untagged, stable proteins. In cells with active tmRNA, the protein product was tagged with the sequence AANDENYALAA and was quickly degraded[11]. This process of switching translation from the defective mRNA to the tmRNA is called *trans*-translation.

tmRNA is totally conserved in eubacteria, present even in species with limited genomes such as *Mycoplasma genitalium* (482 genes), and is found in both Gram-negative and Gram-positive species. This suggests that the biological role of tmRNA is important. In some species, such as *E. coli*, SsrA-defective strains are still viable, though they do present some specific phenotypes including temperature sensitivity, inability to add tag to proteins derived from defective mRNAs, and failure to support growth of *λimm*P22 hybrid phage[23, 26].

Mechanism of *trans*-translation

The *trans*-translation system, consisting of tmRNA and its protein binding partner, small protein B (SmpB), rescues stalled ribosomes by switching translation from the defective mRNA to the ORF of tmRNA. tmRNA has two domains: the tRNA-like domain (TLD) and an open reading frame (ORF). The TLD is composed of the 5′ and 3′ ends of the tmRNA and adopts a structure similar to that of canonical tRNAs (Figure 2)[23, 27, 28]. It has an acceptor arm, a T loop, and a D loop. The anticodon loop is replaced by a connector region which contains the ORF and four pseudoknots.

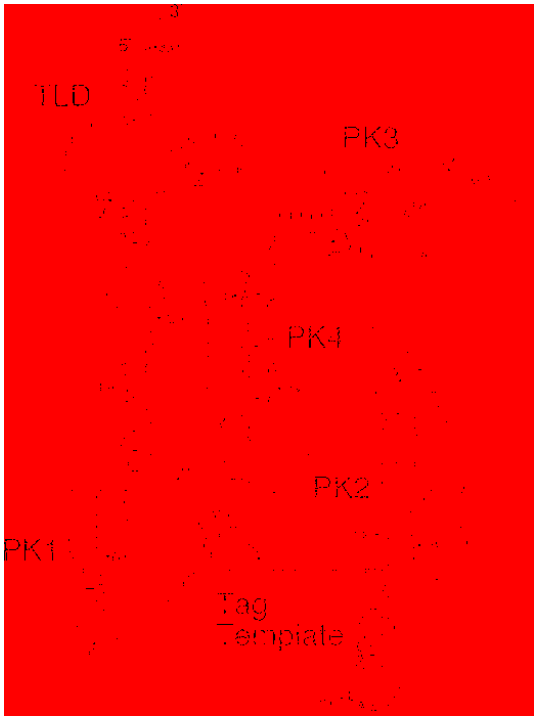


Figure 2: Secondary structure of *E. coli* tmRNA. The tRNA-like-domain (TLD) is composed of both the 3′ and 5′ ends of the tmRNA. There are four pseudoknots (PK1-4) and a tag template between PK1 and PK2. The resume codon and stop codon are marked with boxes (Adapted from Tanner *et al.*, 2006)[29]

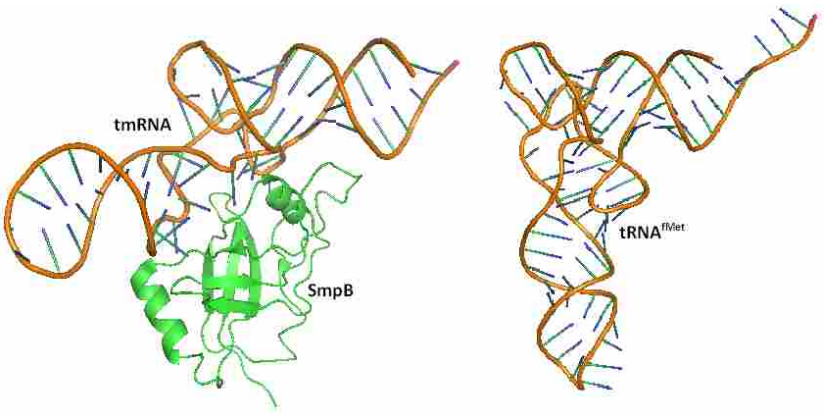


Figure 3: Structure of TLD of tmRNA in complex with SmpB alongside *T. thermophilus* tRNA^{fMet}. tmRNA and tRNA are in orange and the SmpB protein is in green[30, 31].

Alanyl-tRNA synthetase charges tmRNA already bound to SmpB with an alanine. EF-Tu●GTP binds to the tRNA-like domain of Ala-tmRNA and delivers it to the ribosome, just as it delivers canonical tRNAs[32-34]. The Ala-tmRNA–SmpB●EF-Tu●GTP complex enters the A site of the stalled ribosome and is accommodated, independent of any codon:anticodon interaction. How is the tmRNA-SmpB complex able to stimulate GTPase activity of EF-Tu when tmRNA lacks an anticodon arm and there is no mRNA codon present in the A site? The SmpB tail appears to be the key player in stimulating ribosome-dependent GTPase activity of EF-Tu. Based on mutational assays and structural studies, it appears that the C-terminal tail of SmpB may interact with the ribosomal decoding center and mimic the anticodon arm of a canonical tRNA and compensate for the lack of codon:anticodon pairing needed for initiation (Figure 3)[35-38]. Once SmpB triggers GTPase activity and the EF-TU bound GTP is hydrolyzed, the

nascent peptide in the P site is transferred to the Ala-tmRNA in the A site. As long as the nascent peptide is attached to the P site tRNA, the original mRNA template remains stably bound to the ribosome[39]. Once transpeptidation occurs, EF-G catalyzed translocation moves the peptidyl-tmRNA into the P site and the original mRNA quickly dissociates.

The mRNA that is released upon translocation of the tmRNA into the P site is quickly degraded in a tmRNA- and SmpB-dependent manner. How does tmRNA facilitate nonstop mRNA decay? RNase R is the most likely candidate. Previously it was found to associate with a multicomponent protein–RNA complex that included tmRNA and SmpB. There is evidence that RNase R activity is necessary for the degradation of aberrant mRNAs in *E. coli*[40]. It is unknown how tmRNA ORF recruits RNase R to the defective mRNA or when precisely RNase R engages the defective transcript but it is believed to be at an early stage of *trans*-translation, most likely before the defective mRNA is fully expelled from the ribosome.

One of the most interesting questions about *trans*-translation is how does the ribosome resume translation on the tmRNA tag template and how does it select the appropriate codon to resume translation on? As will be discussed later, this process depends on neither an initiator tRNA nor a base-pairing interaction with the ribosome, like the Shine-Dalgarno sequence on bacterial mRNA. Thus, the resume-codon selection for *trans*-translation is very different from start-codon selection during normal translation. There is significant experimental data that implicates the six bases

immediately upstream of the resume codon as being important determinants of the resume-codon selection[41, 42].

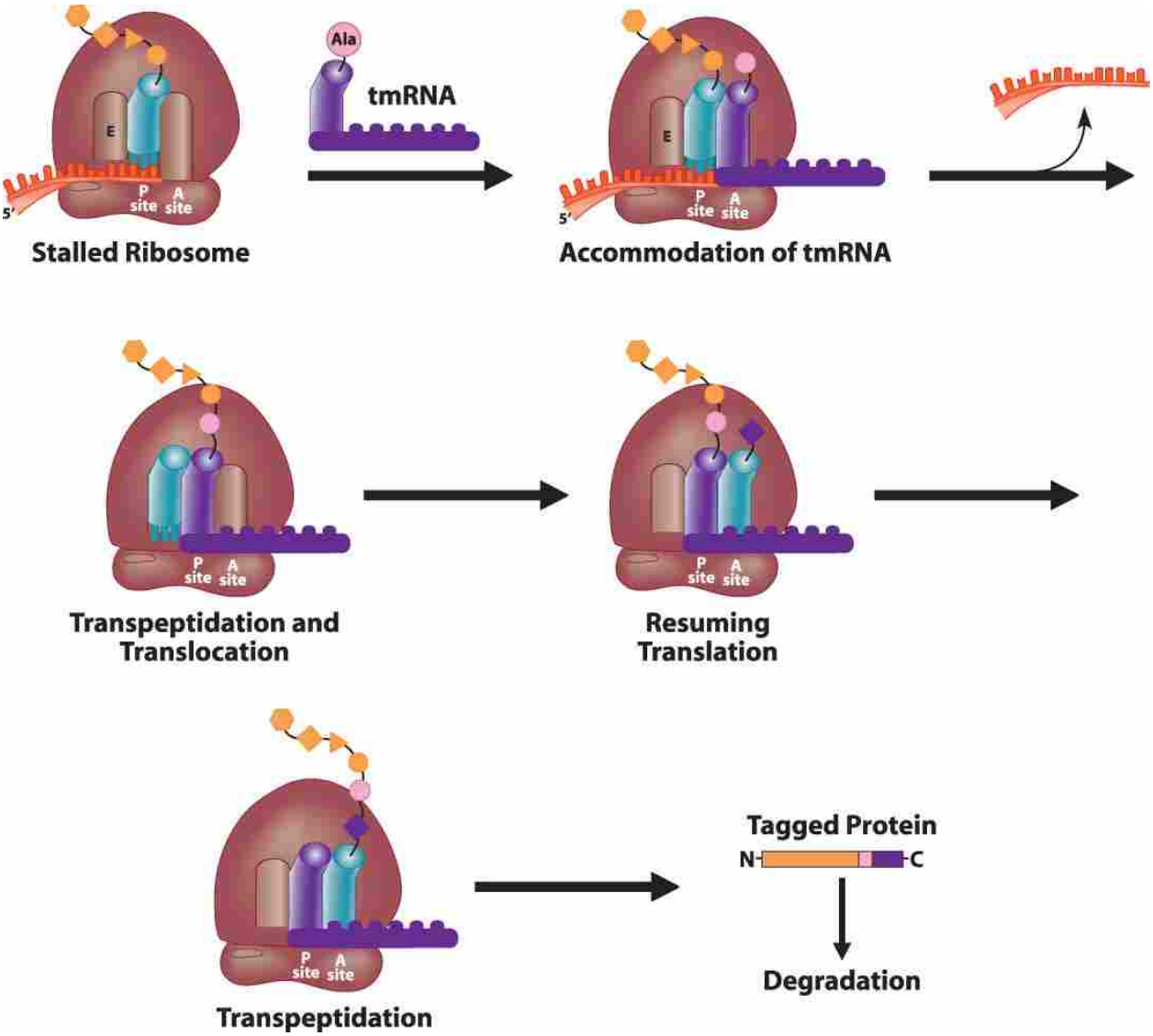


Figure 4: Model of *trans*-translation

After the peptidyl-tmRNA is situated in the P site and the correct resume codon is determined, the ribosome resumes translation on the tmRNA tag template, marking the nascent polypeptide with an 11-amino acid degradation tag: AANDENYALAA in *E. coli*. Normal termination occurs once the ribosome reaches the tmRNA-encoded stop codon, allowing the ribosome to be recycled back into the cellular pool. The tagged peptide is released from the ribosome and is recognized and degraded by specific cellular proteases. So far, the periplasmic energy-independent protease Tsp and the energy-dependent proteases ClpXP, ClpAP and FtsH have been shown to degrade tmRNA-tagged peptides by recognition of the tag sequence[17, 25, 43]. ClpXP and ClpAP were both shown to degrade tmRNA-tagged peptides *in vitro* but the action of ClpAP *in vivo* is not as great as that of ClpXP[17]. This is because of the adaptor protein, SspB.

SspB binds to the portion of the tmRNA-tag that ClpAP recognizes and tethers ClpXP to the protein substrate. In this way, SspB enhances degradation by ClpXP while blocking recognition and degradation by ClpAP[44]. SspB is known to associate with ribosomes[45]. SspB could associate with tmRNA-tagged proteins as they are released from the ribosome and help recruit ClpXP to them for degradation. This model explains why proteins that are tmRNA-tagged are degraded so quickly[17]. The tmRNA tag sequence is highly conserved. The consensus of the N-terminal region of the tag is AANDN. In *E. coli*, the first four residues are recognized by SspB while the first two are important for recognition by ClpAP. The C-terminal area of the tag is conserved as YALAA. The Y is part of the SspB recognition motif and the last four residues are

important for ClpX and/or ClpA recognition. The high conservation of residues in the tag suggests that there is constant selective pressure for degradation of proteins tagged by tmRNA.

Finally, how does the SmpB-tmRNA complex recognize stalled ribosomes? The precise "signal" or conformational state that would distinguish a stalled ribosome from a translating ribosome is unknown. The cellular levels of tmRNA and SmpB are only 5–10% of the total amount of ribosomes so it is unlikely that SmpB and/or tmRNA preassociate with ribosomes, waiting for them to stall. tmRNA-mediated tagging occurs when a ribosome stalls at the 3′ end of an mRNA.

Stalled ribosomes experience high levels of tmRNA rescue and tagging provided there are no more than six bases following the P site codon[39, 46]. This suggests that ribosomes stalled at internal codons are not recognized by SmpB-tmRNA unless mRNA truncation occurs as described previously. Why is mRNA truncation necessary for SmpB-tmRNA recognition of stalled ribosomes? According to structures of early stages of *trans*-translation, the site where mRNA enters the ribosome overlaps the area where the ORF of tmRNA sits upon tmRNA A site entry. This steric interference explains why ribosomes with excess mRNA downstream of the P site are poor substrates for tmRNA. In stalled ribosomes the mRNA entrance tunnel is void of mRNA. This could cause conformational changes of the ribosome which would identify the ribosome as stalled.

SmpB

Originally, all understanding of tmRNA function was derived from the tRNA-like properties of tmRNA and the structure and sequence of the proteins tagged by tmRNA. It was unknown if any other cellular factors were required in addition to tmRNA. In 1999, Karzai and coworkers discovered another player in *trans*-translation. They found that insertion mutations in *smpB* (Small protein B), a gene just upstream of *ssrA,* prevented plating of bacteriophage P22, the same phenotype originally reported for SsrA defective strains[26]. Deletion of the *smpB* gene in *E. coli* prevents tmRNA-mediated peptide tagging.

All known biological activities of tmRNA require SmpB[46]. SmpB is a small, basic RNA-binding protein that is only 160 amino acids in *E. coli*. It is composed of an antiparallel β-barrel core, three helices and an unstructured C-terminal tail[47, 48]. Early on it was shown that SmpB binds to tmRNA in the tRNA-like domain with high affinity and great specificity. SmpB protects tmRNA from degradation *in vivo* and enhances the aminoacylation of tmRNA by alanyl-tRNA synthetase[33, 49, 50]. The presence of SmpB is essential for tmRNA-mediated peptide tagging, including the recognition of and association with stalled ribosome and the subsequent accommodation into the ribosomal A site[33, 51].

Since the discovery of SmpB and its crucial role in *trans*-translation, there has been much debate over how SmpB recognizes stalled ribosomes, how it recruits tmRNA to the ribosomes and how many SmpB proteins are involved. Structural studies of SmpB

reveal several pockets of conserved residues that make up RNA-binding domains and biochemical studies have shown that multiple SmpB molecules can bind to a single tmRNA[47, 52, 53]. This data was in conflict with several structural experiments and biochemical studies, including optical biosensor and melting curve analysis, that suggest a single bound copy of SmpB[36, 52, 54, 55]. Additionally, several biochemical and structural studies have shown two or more SmpB proteins bound to the ribosome prior to A site accommodation[20, 39, 56]. In 2006, Frank and coworkers published a cryo-EM structure of the tmRNA-ribosome entry complex in both the pre- and post-accommodated states of tmRNA. They showed that there were actually two SmpB proteins bound to the tmRNA on the ribosome; one at the decoding center on the 30S subunit and the other positioned near the GTPase-associated center (GAC) on the 50S subunit. This contrasted with an earlier cryo-EM structure depicting a single SmpB molecule bound to the D-loop region of the TLD[57].

Another point of uncertainty in *trans*-translation is the actual order of events for ribosome recognition and tmRNA binding. Does SmpB bind to a ribosome first and recruit tmRNA or is a preformed complex of SmpB•tmRNA necessary for recognition and binding of stalled ribosomes? The latter model is well supported by studies that show the high affinity of SmpB for tmRNA. Cellular levels of SmpB are dependent on levels of tmRNA and vice versa, which suggest that the two are found in a complex, resistant to cellular proteases and nucleases. However, there have been *in vitro* studies that show strong SmpB/ribosome binding and *in vivo* data that show SmpB/ribosome

interactions in the absence of tmRNA[20]. This supports the proposal that free SmpB pre-binds the stalled ribosome to recruit tmRNA for ribosome rescue.

Recently, Sundermeier and Karzai published a study that addressed both of the previous questions[58]. They found that SmpB did not co-purify with ribosomes in a high stringency isolation protocol in the absence of tmRNA. They determined that the discrepancy between their data and earlier work was due to lower stringencies employed in previous studies. In order for SmpB to pre-bind the ribosome in a biological setting, it would have to either have greater affinity for the ribosome over tmRNA or it would have to be present at concentrations exceeding tmRNA levels. SmpB and tmRNA are present at roughly a 1:1 ratio in *E. coli* cells, which also points to a single SmpB protein bound to each tmRNA in the cell[46, 59]. They were able to show *in vivo* that SmpB only binds stalled ribosomes in the presence of tmRNA. If SmpB did pre-bind ribosomes to recruit tmRNA, the level of tmRNA in the cell should not affect the amount of SmpB bound to stalled ribosomes. Finally, under normal conditions, the amount of stalled ribosomes in the cell is a very small fraction of the total number of ribosomes. Normal ribosomes are present at 10-20-fold over SmpB and tmRNA levels in the cell. Because of this, pre-binding of SmpB to ribosomes would be unlikely as it would sequester the SmpB on normal, translating ribosomes as there is no evidence that free SmpB binds stalled ribosomes preferentially. All of this data supports a model where the SmpB•tmRNA complex forms first, is aminoacylated by AlaRS, bound by EF-Tu and this complex then binds to stalled ribosomes.

In light of the canonical model of translation, tmRNA-SmpB entrance into stalled ribosomes to act as a tRNA and then as an mRNA template poses several questions. One of these questions is how does tmRNA activate the ribosomal decoding site when it lacks an anticodon and there is no mRNA in the A site? Several of the early structures of the tmRNA pre-accommodation stage show SmpB positioned in the A site in such a way that the unstructured C terminal tail region is located near the ribosomal decoding center[36]. It was soon discovered that truncating the C terminal tail resulted in inactive SmpB and tmRNA[38, 60]. Furthermore, mutations of specific tail residues resulted in decreased tagging. Sundermeier and coworkers truncated the tail at various lengths. Their results show that deletion of I154 and M155 is highly deleterious to the proteins ability to support tmRNA-mediated endogenous tagging. Yet none of these truncated or mutated SmpB proteins are defective in binding tmRNA *in vivo* or *in vitro* or in their ability to promote association of tmRNA to stalled 70S ribosomes. This implies that SmpB possesses a previously unknown function that must occur after tmRNA–SmpB complex formation and association with a stalled ribosome but before transfer of the polypeptide to the tmRNA-linked alanine in the A site[37]. These results indicate that SmpB is involved in the peptidyl transfer reaction at the A site of the ribosome in addition to delivering tmRNA to the A site.

As previously mentioned, in canonical translation, accommodation and transpeptidation are accomplished when a cognate codon-anticodon pair is present in the A site and EF-Tu hydrolyzes GTP. This is necessary before the ribosome can

catalyze the formation of the peptide bond that links the amino acid in the A site to the peptide bound to the P site tRNA. tmRNA lacks the traditional anticodon stem loop and so accommodation of the tmRNA must proceed in a different way. From the previous reports on the SmpB tail function, it was proposed that SmpB could play a role in GTPase activation, either directly or indirectly, with the positively charged C terminus interacting with the ribosomal decoding center. This theory was further explored by Shimizu *et al.* They used an *in vitro* system to measure the amount of EF-Tu-dependent GTP hydrolysis with an *in vitro trans*-translation system[35]. Using a tmRNA mutant composed of only the TLD, they detected GTP hydrolysis only in the presence of both SmpB and EF-Tu and by using only the TLD domain of tmRNA, they were able to determine that the template tag is not necessary for accommodation as has been suggested[61]. A SmpB lacking the last seven amino acids of the C-terminal tail was unable to stimulate the transfer of the tmRNA-linked alanine. These results demonstrate a vital role for SmpB, and more specifically the SmpB tail, in the ribosome-dependent GTPase activity of EF-Tu and the peptidyl transfer reaction independent of any codon:anticodon interaction.

In addition to stabilizing tmRNA against degradation, recognizing stalled ribosomes, and allowing tmRNA accommodation into the A site by stimulating the GTPase activity of EF-Tu, several studies have suggested a possible role for SmpB in setting the frame on the ORF of tmRNA[52, 62].

Originally SmpB was only implicated in the initiation of *trans*-translation. It was thought that the function of SmpB was to recognize stalled ribosomes and deliver tmRNA to the A site. Early structures of the pre-accommodation stage showed tmRNA and SmpB in the A site[48, 57]. Shpanchenko and co-workers were later able to study various steps of *trans*-translation by blocking translation at different positions and they determined that a SmpB molecule remains bound to the ribosome•tmRNA complex throughout all of the steps of *trans*-translation[63]. Later studies show SmpB binding sites in both the A site and the P site[64]. This demonstrated that SmpB has roles beyond delivery of tmRNA to the stalled ribosome.

Setting the Frame

The ribosome's ability to correctly determine the start codon of a gene is essential for accurate translation. During initiation, the start codon (AUG) is positioned by the Shine-Dalgarno sequence and placed in the P site by the anticodon of a specific initiator tRNA (fMet-tRNA^{Met}). This tRNA is responsible for selecting the correct frame for translation. In the case of tmRNA, however, there is no specific resume codon between species such as AUG and the resume codon is determined in the A site before cognate tRNA pairing occurs. So the question arises, how does tmRNA determine the correct reading frame when the ribosome abandons the template mRNA and resumes translation on the tmRNA ORF?

Originally, it was thought that tmRNA was a highly structured RNA and that distant structural elements positioned the resume codon in the A site[41], particularly the four conserved pseudoknots that connect the two ends of the TLD. Nameki *et al.* explored this theory by replacing the four pseudoknots with single-stranded RNA and interchanging the sequences with one another[65]. Replacement of PK3 and PK4 with single-stranded RNA or interchanging PK3 and PK4 showed little effect on transpeptidation and alanine incorporation onto the P site peptide. The ability was retained in PK2 single-stranded mutants and replacement mutants as well, though at about half of wild-type efficiency. Replacement of PK1 with single-stranded RNA resulted in very low alanine incorporation so PK1 was the only pseudoknot assumed to be essential for tmRNA activity. Still, all four of these replacement mutants retain proper frame choice and only incorporate the "0 frame" amino acid, alanine. Arginine and threonine, which are coded in the respective alternative frames, are not incorporated in any of the four pseudoknot mutations, indicating that none of these structural elements, including PK1, act as a structural element to set the tag initiation point[65]. Furthermore, a later report by Tanner *et al.* demonstrated that PK1 acted in a purely structural role[29]. Mutations in PK1 that retain the pseudoknot structure or replacement of PK1 with stable hairpins yielded tmRNA mutants with nearly wild-type activity levels.

An obvious candidate for positioning the resume codon correctly in the A site is the resume codon itself, though the resume codon sequence appears to be unimportant.

Though most natural tmRNAs use alanine as the resume residue, mutants with insertions/deletions in and around the resume codon show that tmRNA is able to use a variety of amino acids as the first encoded residue in the tag without an effect on frame [41, 42]. Early work in looking at proper frame-setting implicated the region between pseudoknot 1 and the resume codon as responsible for precise resume codon selection[41]. The sequence of this region is AAAAAAUAGUC, where the underlined adenine is universally conserved (Figure 5) and has been shown to be essential for protein tagging by tmRNA.

Figure 5: Sequence logo displaying the consensus sequence of the upstream region of tmRNA. It includes all 555 known tmRNA sequences. Sequence logo generated by WebLogo[66]. Weblogo generates graphical representations of patterns using a multiple sequence alignment. The overall height of each stack indicates the sequence conservation at that position measured in bits. The number of bits is maximum sequence conservation at that point and is calculated by taking the $\log_2$ of the number of distinct symbols for the given sequence type. So for DNA/RNA, there are four different nucleotides so the number of bits = $\log_2$ (4) =2. For protein sequences, since there are 20 amino acids, the number of bits = $\log_2$ (20) = 4.32. The height of each symbol indicates the relative frequency of each nucleic or amino acid at that position (Adapted from Miller *et al.*, 2008)[67].

Mutations in this upstream region (residues 84-90 in *E.* coli) have been shown to affect frame choice, with several mutations causing the tag to be translated in the incorrect frame[42]. Mutations at A86 resulted in a sharp decrease in tagging efficiency and mutations of the nucleotides surrounding A86 caused a shift in the resume codon, usually to the –1 frame. The mutation of U85 to an adenine caused the greatest –1 shift. They explored the possibility that this upstream region bound to the ribosome in such a way as to set the correct resume codon in the A site. It looked possible since tmRNA residues 86-94 or 85-90 could make nine or six base pairs with 16S rRNA residues 1470-1478 or 1481-1486 respectively. Unusual and/or unstable base pairing could explain some of the shifts that the mutations caused but not all of them[42]. Looking at other species revealed poor conservation of these interactions and altering the rRNA sequence of 1470-1481 showed no effect on tmRNA function[68]. It appears unlikely that the upstream region of tmRNA binds to the ribosome to set the translational frame which suggests that it binds to a trans-acting factor to position the resume codon correctly.

An alternative theory for correct frame-setting is the involvement of the three tmRNA bases immediately upstream of the resume codon (the –1 triplet). If this codon interacted with the decoding center in the A site prior to accommodation, then when tmRNA was moved into the P site, the resume codon would be correctly set in the A site. Lim and Garber proposed a model where the –1 triplet adopts a conformation similar to a cognate codon:anticodon pair[61]. Canonical codon:anticodon base-pairs have a non-deformed A-form structure of the sugar-phosphate backbone. They propose that

the single-stranded –1 triplet is sufficient to assume the A-form conformation recognized by the decoding center. When the tmRNA is translocated to the P site, the –1 triplet moves along with it, placing the resume codon in the A site. From this hypothesis, Lim and Garber proposed a set of "rules" that would define allowed and forbidden –1 triplet sequences, based on the conformation that they adopt. They support their theory by showing that all of the natural –1 triplets fit their guidelines for allowable –1 triplet sequences[61].

Recent work has shown data conflicting with the –1 triplet hypothesis. Miller and coworkers tested tmRNA mutants with all 64 possible –1 triplets in two separate assays in *E. coli*[67]. The first is an *in vivo* selection that ties the life of the cell to the function of tmRNA. They found that many of the –1 triplet mutations were inactive, but their data does not match the predictions made with the –1 triplet hypothesis. This refuted the –1 triplet hypothesis. Interestingly, several –1 triplet mutations that did not survive in the *in vivo* selection were able to support wild-type levels of *λimmP22 c2-dis* bacteriophage plaque formation, which indicates functional tmRNA and successful ribosome release. Testing for frame selection showed that one of the –1 triplet mutations, UGU, tagged in the –1 frame. Other tmRNA mutations tested previously have also shown to have an effect on frame. For example, the upstream mutations U85A and A86C show –1 and +1 frameshifting, respectively[42]. These results once again point toward the upstream region of tmRNA for frame-setting.

All of these findings suggest that A86 is an important determinant for frame choice on the tmRNA template. It is the most highly conserved residue in the upstream region. It has already been shown that the upstream region is unlikely to bind to the ribosome and the frame misrecognition results could be explained by a separate ligand binding to A86 to establish the frame by placing the resume codon into the A site.

The question remains, what is the A86-binding ligand? One controversial possibility is the ribosomal protein S1. S1 helps with translational initiation on many mRNAs and has been shown to bind regions of tmRNA, including PK2, PK3 and the upstream sequence where it was shown to crosslink to U85[69]. Structural studies have shown a structural change in the template sequence in the presence or absence of S1. When S1 is absent, the sequence is more structured and this may suggest that S1 binds to this section of tmRNA and unwinds the tag template[57, 70]. In one study, S1 was shown to be dispensable for tmRNA entry into the A site and for the transpeptidation reaction but S1 was required for the mRNA-like function of tmRNA[71]. Yet other studies show that S1 mutants that inhibit mRNA translation have little to no effect on tmRNA tagging *in vivo*[72]. Many gram positive bacteria lack an S1 orthologue but contain tmRNA and *in vitro* studies with purified components do not require S1 for active *trans*-translation[20, 39, 50]. Moreover, the addition of S1 to an S1-free, cell-free system did not affect *trans*-translation efficiency[73]. S1 is an RNA-binding protein with six RNA-binding domains that can bind to a spectrum of pseudoknots and single-stranded RNA[74] so the binding

interactions observed between S1 and the tmRNA upstream sequence are unlikely to be functionally relevant.

Another candidate for the A86 binding ligand is the SmpB protein. Based on the location of SmpB in the ribosome after translocation occurs, it could be the trans-acting factor that binds the upstream sequence of tmRNA. SmpB binding has been shown to affect the accessibility of the upstream sequence to nucleases in probing assays, indicating that during *trans*-translation, SmpB is bound at or near PK1 and may play a role in resume codon selection[52]. A functional link between SmpB and the upstream region was detected by Konno *et al.* through chemical modification protection assays. Footprinting assays were performed using DMS (methylates A), KE (modifies G), and CMCT (modifies U). Chemical modification of tmRNA was performed in the presence and absence of SmpB protein. This showed protection at U85 in the presence of SmpB. Mutations that caused –1 or +1 frameshifting also shifted the position of protection by –1 and +1, respectively[62]. This shows strong evidence that an interaction between the body of SmpB in the P site and the upstream region of tmRNA sets the correct frame for *trans*-translation of the tmRNA ORF, though a specific interaction was not determined. These results are interesting because this proposed SmpB-tmRNA interaction was not observed in earlier studies including structural probing[49], UV-induced crosslinking[53], and hydroxyl-radical cleavage assays[64]. Such an interaction between SmpB and the upstream region of tmRNA could orient the template sequence in such a way as to position the resume codon in the A site.

From all of the reports given it seems that the upstream region of tmRNA is binding to some ligand which sets the frame for the template tag. It appears that this interaction does not take place between the tmRNA and the ribosome[42, 68] and the most likely candidate for a *trans*-acting ligand is the SmpB protein bound to tmRNA in the P site. There have been several *in vitro* reports of SmpB binding to the upstream region[52, 62] and the C-terminal domain of SmpB in the P site is well positioned to interact with tmRNA upstream of the resume codon, which is in the A site[30]. Thus, SmpB could be involved in setting the frame. The question remains, are there SmpB mutations that can suppress frameshifting tmRNA mutants and would this point to an interaction between specific SmpB residues and tmRNA bases?

Research Aims

Our general aim was to determine how tmRNA determines the correct frame for *trans*-translation and how its protein binding partner, SmpB, plays a role in that function. To accomplish this, we first wanted to test if there are any SmpB mutations that can suppress mutations in the conserved sequence upstream of the resume codon on tmRNA. This could point to an interaction between specific SmpB residues and tmRNA bases. Finally, we also wanted to look for SmpB mutations through rational mutation which affect frame choice on tmRNA.

Chapter 2: Experimental Procedures

Materials: Restriction enzymes were purchased from New England Biolabs, as was T4 DNA Ligase and Calf Intestinal Phosphatase (CIP). All PCR and plasmid purifications were done using the Qiagen Quick Purification kit. The MegaX DH10B competent cells were from Invitrogen. The mouse anti-His_6 antibody was purchased from Cell Signaling Technology while the rabbit anti-GST antibody was from Sigma. Both secondary antibodies (anti-mouse IRDye 800 and anti-rabbit IRDye 680) were from LI-COR Biosciences.

Constructing SmpB Tail Library – For the SmpB tail library, the *smpB* gene was amplified by PCR from the pKT2-SmpB-promoter-KanR37s 16 plasmid using two primers. The first, 5'libEagI ATT ACC GAT CGG CCG GAT TC, amplifies from the 5' end of the gene and contains an EagI restriction site (underlined) for cloning into the pKT2-SmpB-dummy-Cat plasmid, which contains all of the necessary components of the KanR assay (see below) except SmpB but it does contains cloning sites for SmpB. The second primer binds towards the 3'-end of SmpB. It contains an area that has a 20% mutation rate, from Asp137 to the end of the protein. The primer is 3'80WTSmpB (SpeI): CCT TTC ACT AGT TTA 567 787 776 788 888 658 758 567 876 888 586 656 687 665 886 767

686 888 758 586 875 567 888 586 GTG CTG TTT CTT ACC TTT GG, where 5 represents 80%A, 6 is 80% C, 7 is 80% G and 8 is 80% T. The primer also introduces a SpeI restriction site (underlined) to be used for cloning into pKT2-SmpB-dummy-Cat.

The PCR product was gel purified to eliminate any nonspecific PCR products before it was digested with SpeI and EagI restriction enzymes. The pKT2-SmpB-Dummy-Cat plasmid contains both SpeI and EagI cloning sites for SmpB and was also digested and treated with calf intestinal phosphatase (CIP). The library PCR insert and the pKT2-SmpB-Dummy-Cat backbone were gel purified prior to ligation with T4 ligase. A small ligation using 50 ng of backbone and 10ng insert was set up along with a control reaction which lacked any library PCR insert. After incubation at 16°C for 4 hours, DH10B competent cells were transformed using 1 μL of the ligation or the control reaction and then the cells were plated on ampicillin. If the libraries had a good ratio between the ligation and control reactions, a large ligation was set up using 1-2 μg of backbone and incubated at 16°C overnight. The large ligation was introduced into MegaX DH10B competent cells. After the transformed cells were rescued at 37 °C for 1 hour, 1 μL of the transformed library was plated on a quantitation plate to determine the approximate size of each library.

KanR assay for tmRNA activity – In the KanR assay, functional tmRNA molecules that tag proteins in the correct frame rescue ribosomes stalled on a truncated kanamycin resistance protein (KanR) to synthesize full-length KanR, rendering the cells kanamycin resistant (Figure 8)[29]. To test the normal level of survival of WT and mutant tmRNA on

kanamycin, the pKT2-SmpB-promoter-KanR37s 16 plasmid was used. This plasmid contains wild-type SmpB, tmRNA and a truncated KanR protein lacking the last 15 amino acids, with the sequence Ser-Glu-Pro-Opal added to the C-terminus to induce ribosome stalling. Expression of this protein is driven from an arabinose-inducible promoter. The tmRNA template tag sequence was mutated to encode the last 14 amino acids of the truncated KanR protein, ANKLQFHLMLDEFF, instead of the normal degradation tag, ANDENYALAA. The plasmid was introduced into the selection strain, Δ*ssrA*Δ*smpB*. The rescued transformants were plated on ampicillin. A culture was inoculated from a single colony and grown to saturation.

Saturated cultures were diluted to an OD_{600} of approximately 0.3 in fresh media containing 2% arabinose and appropriate antibiotics then grown for 4 hours to induce expression of the KanR protein. The cells were plated onto selective media. Wild-type tmRNA and several tmRNA mutants were selected at high stringency; 2xYT, ampicillin (and chloramphenicol for the UGU library), 2% arabinose, and 30 μg/mL kanamycin. Growth comparisons (selective vs. non-selective plates) were made after incubation for 24 h at 37 °C. Other tmRNA mutants were assayed at low stringency: 15 μg/mL kanamycin at 25 °C and scored after 48 hours.

Amplification of smpB – After each round of selection, any surviving colonies were scraped and the plasmids purified. The mutant *smpB* gene from each round of library selection was amplified using PCR before each subsequent round of selection to eliminate any contamination. The upstream primer GGT ATC AAC AGG GAC ACC

AGG and the downstream primer, GCT CAG GAG GCC TGG CTC G amplified *smpB* and the PCR product was digested using EagI and SpeI. The SmpB insert was ligated with fresh pKT2-SmpB-Cat-tmRNA plasmid backbone with the corresponding tmRNA mutation and then introduced into the selection strain for additional selection in the KanR assay. This was done to eliminate any plasmids which contained the incorrect tmRNA mutation. The same protocol was used to clone sequenced SmpB mutants into fresh backbone before being tested individually in the KanR assay. Several were also tested against different tmRNA mutations.

Constructing the error-prone library plasmids – The libraries were made using our pKT2-SmpB-promoter-KanR/37s 16 plasmid mentioned previously with *ssrA* mutated with the A86C, U85A, UGU –1 triplet, and Δ89C mutations. The pKT2-SmpB-promoter-KanR/37s 16 plasmid with the A86C mutant tmRNA also contains a *cat* gene for chloramphenicol resistance to eliminate any contamination from the other plasmids which survive at a higher stringency than A86C.

The cloning sites EagI and EcoRV were used to clone in the error-prone SmpB. There was a second EcoRV site present in the plasmid that had to be removed using QuickChange PCR (QCh). Two primers, 566 and 567, were used. 566: ACC TGG CAG ACA GCA ATT TT**A** ATA TCG CCA GCG TCG CAC AG and 567: CTG TGC GAC GCT GGC GAT AT**T** AAA ATT GCT GTC TGC CAG GT. The mutated nucleotide is shown in bold and underlined. For the QCh PCR, two reactions were set up with half of the PCR reaction in each tube and only one primer present in each. Each reaction contained

1X PCR buffer, 2mM $MgSO_4$, 0.25mM dNTP mixture, 50-100 ng DNA template and 1ug of the designated primer. The PCR reactions proceeded for 5 rounds separately before the two reaction mixtures were combined and the PCR reaction went an additional 20 cycles with the two primers both present. The PCR was purified using Qiagen quick purification kit and digested with DpnI to eliminate any template DNA. The digested PCR product was purified once more before DH10B *E. coli* cells were transformed with 5 μL of the QCh DNA. The cells were rescued and all was plated on ampicillin plates. Several colonies were picked and the plasmid purified and digested with EcoRV. The digested DNA was run out on a 0.7% gel. The samples which ran as a single band were then sequenced to be sure that the mutation was present.

The Δ89C tmRNA mutation had not been used previously and needed to be constructed. 89C is flanked by two restriction sites (SphI and PstI), approximately 60 base pairs apart. Two primers were made which contained the area of interest and both restriction sites. Primer 607, CAA GGT *GCA TGC* CGA GGG GCG GTT GGC CTC GTA AAA AGC CGC AAA AAA TAG TGC AAA TAA A*CT GCA G*TT TCA T, and primer 608, ATG AAA *CTG CAG* TTT ATT TGC ACT ATT TTT TGC GGC TTT TTA CGA GGC CAA CCG CCC CTC G*GC ATG C*AC CTT G, were annealed to one another and digested with SphI and PstI (the restriction sites are underlined and italicized). The pKT2-SmpB-promoter-KanR/37s 16 plasmid was also digested with SphI and PstI. The digested plasmid was gel purified and then ligated with the digested primers. DH10B competent cells were transformed with the ligation and a control which lacked the primer insert.

Several colonies were grown up in liquid culture and the plasmid was sequenced to confirm the Δ89C mutation.

The error-prone library was generated using an error-prone PCR reaction which included 1X PCR Buffer, 7 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.1% gelatin, DNA template, an error-prone dNTP mixture, 1 μg upstream and downstream primer, and Taq polymerase. The 10x dNTP mixture was made up of 2 mM dGTP, 2 mM dATP, 10 mM dCTP and 10 mM dTTP. The upstream primer, 392, had the sequence GGT ATC AAC AGG GAC ACC AGG and the downstream primer, 470, CCA GTC ACG TAG CGA AGA TC. There were four library plasmids to make, pKT2-SmpB-promoter-KanR/37s 16 plasmid with A86C, U85A, UGU, and Δ89C tmRNAs. Each error-prone PCR used a different template to cut down on contamination between tmRNA mutants. The PCR products were purified, digested with DpnI to eliminate template DNA and then purified again. The digested PCRs were run on a gel to quantitate before they were digested with EagI and EcoRV.

The corresponding pKT2-SmpB-promoter-KanR/37s 16 tmRNA mutant plasmids were also digested with EagI and EcoRV and all plasmid backbones and PCR inserts were gel purified prior to ligation. The plasmid libraries were introduced into MegaX DH10B competent cells by electroporation.

Immunoblot analysis of frame-shifting – The –1 or +1 misreading plasmid pDH210 express tmRNA with frameshifted tags such that the ANDH_6D tag is only added to stalled nascent peptides if the ribosome reads tmRNA in the correct frame. pDH210 also

expresses the GST protein with the sequence Ser-Glu-Pro-Opal added to the C-terminus, causing stalling during translational termination. Δ*ssrA*Δ*smpB* cells containing one tmRNA/GST plasmid and one SmpB plasmid (pDH113) were grown in ampicillin and tetracycline to an OD_{600} of 0.5. The expression of GST was induced with 1 mM IPTG. After 2.5 h, the cells were pelleted and lysed with SDS. Protein in the crude lysine was quantified via Lowry assay and each sample was resolved by 10% SDS-PAGE. The protein was transferred to PVDF membrane and His_6-tagged GST was bound by a mouse anti-His_6 antibody. Binding of a rabbit anti-GST antibody was used to control for protein expression and loading. Fluorescent secondary antibodies (anti-mouse IRDye 800 and anti-rabbit IRDye 680) were added and the blot was visualized with an Odyssey Infrared Imaging System (LI-COR Biosciences).

Alanine-scanning mutants – Single alanine mutations of the SmpB protein were made using Quickchange PCR. Primers were designed with 20-25 nucleotides on each side of the point of mutation. PCR reactions contained 1X PCR buffer, 2 mM MgSO4, 0.5 mM dNTP mixture, 50-100 ng pDH113 DNA template and 1 μL Platinum *Pfx* polymerase. This reaction is split into two, with 50 μL in each reaction. One primer is added to each of these reactions. After 5-10 rounds, the two reactions are combined so that both primers are present. The reaction continues until there is a total of 25 rounds. The PCR product is purified and digested with DpnI for at least 10 hours at 37 °C to eliminate the high level of background. The PCR product was purified again after digestion and DH10B was transformed with 3-5 μL of each QCh product. Mutations

were verified by sequencing. Several double and triple mutations were made in the same way, using a template DNA that already contained at least one of the desired mutations and primers that aligned with the desired product. The mutated pDH113 SmpB plasmids were introduced into the ΔSmpBΔSsrA strain along with the pDH210 plasmid in the 0, +1 and –1 frames. Western blot analysis was performed as described above.

Chapter 3: Results

Genetic selection of an SmpB tail mutant library

It is still unknown how the ribosome is able to select the correct resume codon when translating the tmRNA peptide tag. The tmRNA sequence found immediately upstream of the resume codon has been implicated in setting the correct frame[42, 52]. Mutations in this upstream region are known to result in frameshifting. Several studies suggest that the upstream region binds to an unknown ligand in order to set the correct translational frame. The most likely candidate for this ligand is the SmpB protein.

Crystal structures of tmRNA in complex with SmpB place the SmpB protein near the decoding center in the A site and in the mRNA channel in the P site[36, 39]. In particular, the SmpB C-terminal tail extends from the bottom of the protein, opposite of where SmpB binds tmRNA. Although the tail is unstructured, it is predicted to play a key role in the interaction between SmpB and the 30S subunit. The SmpB tail contains many conserved amino acid residues (Figure 6) and is known to be essential for *trans*-translation[38, 60]. Mutations in the tail decrease tagging by tmRNA[38]. One proposed function of the SmpB tail is to activate the decoding center of the ribosome to allow

accommodation of tmRNA into the ribosomal A site. We hypothesized that the C-terminal tail domain of SmpB also possessed a second function: that it interacts with the upstream region of tmRNA to set the translational frame for the tmRNA template.

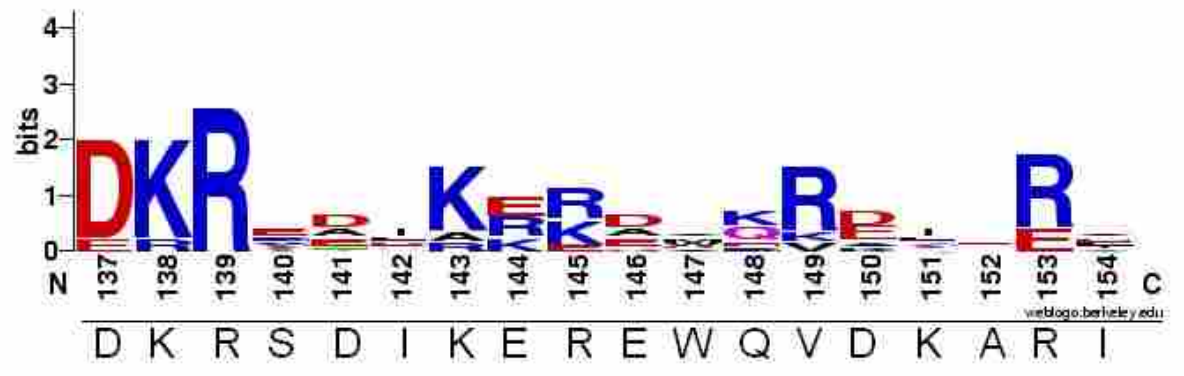


Figure 6: Graphical sequence logo representation of SmpB C-terminal tail amino acid conservation. Generated by Weblogo[66]. The residue numbers correspond to *E. coli* alignment and the C-terminal tail sequence of *E. coli* is printed below the alignment. Alignment only extends to I154 since many SmpB proteins end at this point.

We wanted to identify a genetic or functional interaction between SmpB and the upstream region of tmRNA. To do this, we made tmRNA mutants that have lowered survival in our selection due to defects in frame setting. The tmRNA mutants were paired with libraries of SmpB tail mutants in order to isolate any SmpB mutants that cause increased survival with the tmRNA mutants. SmpB mutants which rescue tmRNA activity will help to identify residues on SmpB that interact with tmRNA in order to set the correct translational frame.

First, we identified tmRNA upstream region mutations that affect the choice of reading frame. After the tmRNA–SmpB complex moves into the P site of the ribosome, the resume codon is positioned in the A site in preparation for pairing with a canonical tRNA. If the resume codon is in the A site, we hypothesized that the triplet immediately upstream, the –1 triplet, must be positioned in the P site near the bottom of SmpB where the tail exits the protein (Figure 7). For this reason, we chose to test the tmRNA –1 triplet mutation UGU. The mutation of the –1 triplet from GUC to UGU has been shown to cause an increase in –1 frameshifting compared to wild-type tmRNA[67]. We also chose A86C because this mutation has been shown to greatly affect tmRNA efficiency and cause frameshifting to the +1 frame[42, 67] and A86 is completely conserved in all known tmRNA sequences (Figure 5).

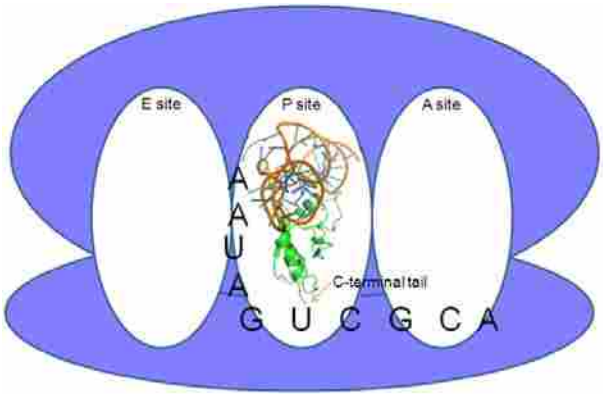


Figure 7: Model of placement of SmpB and the upstream region in the ribosome. The SmpB-tmRNA complex is located in the P site and the resume codon is placed in the A site. If the –1 triplet (GUC) binds in the P site, beneath the complex, the resume codon (GCA) would be naturally positioned in the A site for resumption of translation. In this model, the SmpB tail would interact with the upstream region of tmRNA.

To test the activity of our tmRNA mutants we used the KanR assay, an *in vivo* genetic selection that ties the life of the cell to the function of tmRNA (Figure 8). Functional tmRNA molecules rescue ribosomes stalled on a truncated KanR protein and tag the peptide with the remaining 15 amino acids of the protein, producing full-length KanR and making the cells kanamycin resistant[29, 67]. Using the KanR assay we sought to identify SmpB tail mutants that restore the function to the frameshifting tmRNA mutants UGU and A86C.

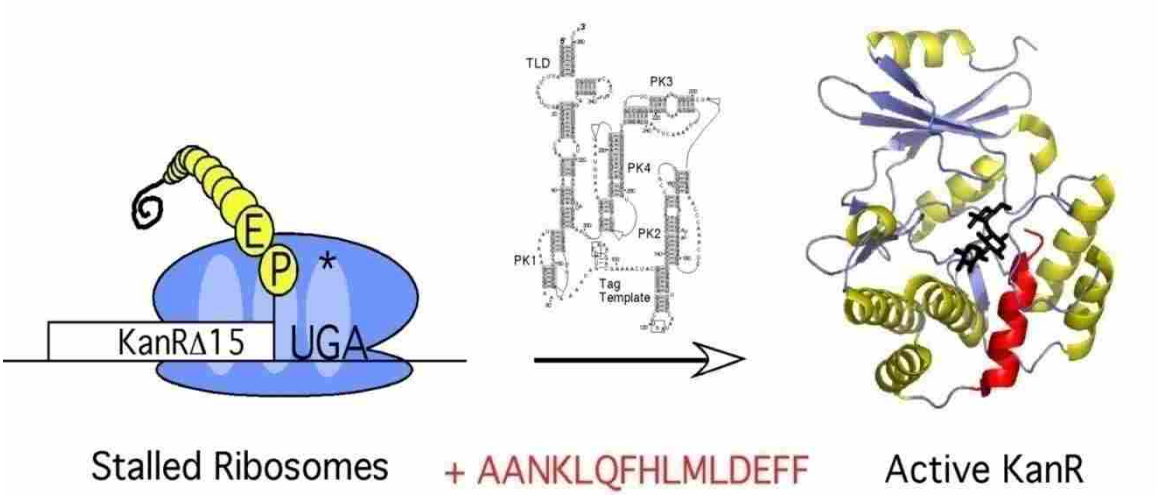


Figure 8: KanR Genetic Selection for tmRNA Activity. Ribosomes stall on a truncated kanR (kanRΔ15) template at a Glu-Pro-(Opal) stalling sequence. Active tmRNA molecules with a mutant template sequence add the final 15 amino acids of KanR (shown in red) to the truncated protein (yellow), resulting in a full-length, functional KanR protein. Only tmRNA that is active and tags in the correct frame will complete the KanR protein and thus tmRNA function is linked to KanR activity and cellular survival on kanamycin (Adapted from Tanner *et al*, 2006)[29].

To lay the groundwork for our selection, we first determined the background survival rates of the UGU and A86C tmRNA mutants in the KanR assay when paired with WT SmpB. The cells with the UGU mutation survived at near wild-type level on the low stringency selective plates (15 μg/mL kanamycin at 25 °C) so they were assayed again at higher stringency (30 μg/mL kanamycin at 37 °C). UGU tmRNA on high stringency kanamycin plates has a background survival of approximately 1 in 10^4 colony forming units (cfu) plated. The A86C mutant tmRNA has very low activity; A86C cells have a background survival of approximately 2 in 10^6 on low stringency kanamycin plates. In contrast, wild-type tmRNA exhibited approximately 100% survival in both high and low stringency testing.

A library of SmpB tail mutants was constructed by mutating the SmpB tail region at a 20% mutation rate per nucleotide from D137 to the end of the protein (R160):

D K R S D I K E R E W Q V D K A R I M K N A H R

Mutant *smpB* genes were cloned onto plasmids expressing either UGU or A86C mutant tmRNAs. Both libraries had over 10^6 mutants. The libraries were induced in culture for several hours with arabinose and dilutions were plated on kanamycin for selection. Control dilutions were plated without kanamycin on glucose to represent the total number of colonies selected on the kanamycin plates. The UGU and A86C libraries were both screened several times but there was no increase in cellular survival compared to wild-type SmpB.

We were unable to find an SmpB mutant which suppressed the UGU or A86C tmRNA mutants in either of our two SmpB tail libraries. It could be that our libraries did not give adequate coverage of mutations in this region to find an SmpB mutant to restore survival in the KanR assay. There are 24 amino acids in our library and full coverage of every possible sequence would be impossible to create with our method of library construction. We mutated approximately 70 nucleotides at 20% mutation per base so the likelihood of finding a single suppressor mutation ($0.8^{70} \sim 10^{-7}$) was possible with the size of our library. Several of our unselected library plasmids were sequenced and all had at least 10 mutated nucleotides in the tail region. Also, we mutated a highly conserved region of SmpB and mutations in this region have previously been shown to be detrimental to SmpB and tmRNA function[38].

A new tmRNA-SmpB crystal structure was published which showed SmpB truncated at K123 (K133 in *E. coli)* in complex with the tRNA-like-domain of tmRNA from *Thermus thermophilus (*Figure 2*)*[30]. We manually fitted the SmpB-tmRNA (TLD) complex structure to the P site tRNA from Ramakrishnan's structure of the *T. thermophilus* 70S ribosome complexed with mRNA, A-, P-, and E-site tRNAs[31]. From this fitting, we see that K-123 descends into the normal mRNA channel in the P site (Figure 9). The SmpB tail extends from this position.

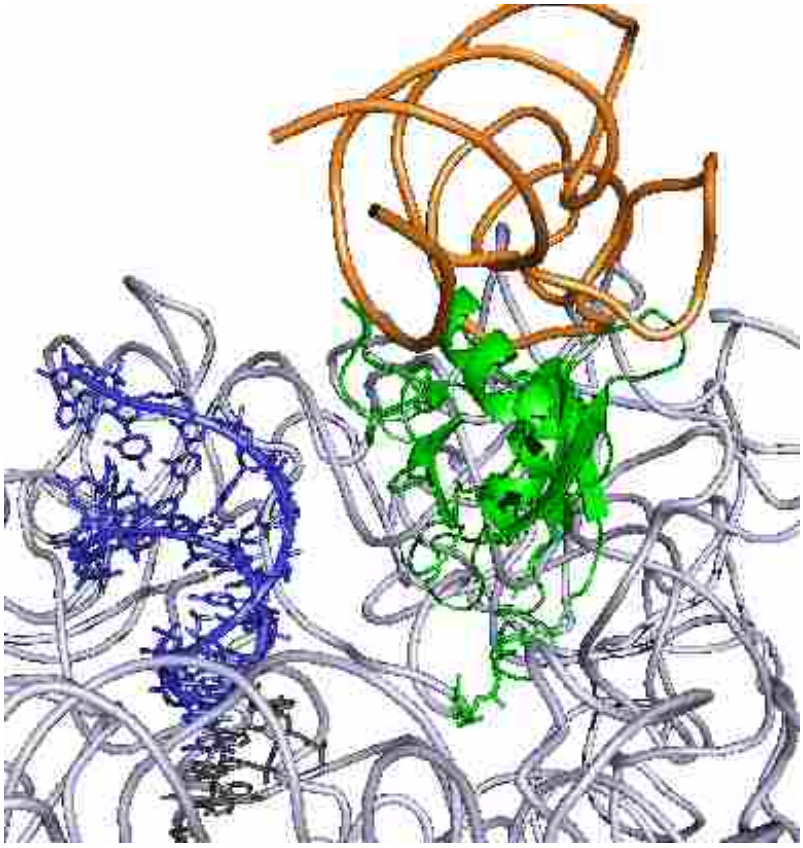


Figure 9: SmpB-tmRNA complex fitted into ribosomal P site. The SmpB-tmRNA (green and orange respectively) structure from Bessho *et al.* is fitted into the structure from Selmer *et al.* of the *T. thermophilus* 16S –site[30, 31]. The 16S is light blue, A site tRNA is dark blue, and A site mRNA is black.

It is unlikely that the SmpB tail extends very far into the A site since the resume codon and a canonical tRNA occupy the A site. The tail needs to fold underneath the SmpB protein, turning back towards the E site of the ribosome. The positioning of the SmpB protein in the P site mRNA channel suggests that there would be little room beneath the SmpB-tmRNA complex for the upstream region to extend. Two other studies suggest that the SmpB tail runs along the mRNA pathway in the P site, which would not allow room for tmRNA to pass below SmpB[39, 64]. The SmpB-tmRNA complex

fits tightly into the P site, leaving no room for the tmRNA upstream region to extend around the complex either. Instead, the tmRNA upstream region is probably located between the A and P sites, extending up to the TLD of tmRNA between the SmpB protein and the A site tRNA.

A paper by Konno *et al* reported that the nucleotide U85 in the tmRNA upstream region is protected from chemical modification in the presence of SmpB, suggesting an interaction between the upstream region of tmRNA and the SmpB protein. SmpB lacking the C-terminal tail (Δ133-160) still exhibits this protection[62]. This implies that the interaction site on SmpB must be located upstream of K133. This is consistent with our failure to find SmpB tail mutants that suppress frame-setting defects in the libraries described above.

Genetic selection of a library of the SmpB gene

A new library of SmpB residues 1-142 (out of 160) was constructed using error-prone PCR on the *smpB* gene. The SmpB mutant library was tested against UGU and A86C tmRNA, as before. We also chose to look at three other tmRNA mutations. The first, U85A, has been shown to cause –1 frameshifting[42]. In Konno's report, they showed the chemical protection at U85 could be shifted to 84 or 86 with the mutations A84U/U85G and A86U respectively. U85 is conserved, though not as much as A86 (Figure 5). We also wanted to test the mutation A86G. In the chemical modification assay, the A86C mutant showed a very different modification pattern than WT or the

other mutants, which may suggest that the A86C mutation affects the conformation of the upstream region of tmRNA. For this reason we decided to test A86G as well as A86C to see if A86G would yield SmpB suppressor mutants that A86C did not. Finally, we constructed a Δ89C tmRNA mutant. In order for Δ89C tmRNA to produce active KanR, –1 frameshifting would have to occur. This mutant would help us identify any SmpB mutants that cause high levels of –1 frameshifting.

Before the libraries were constructed, the background survival rates of cells containing U85A, A86G and Δ89C tmRNA mutants with WT SmpB were determined. U85A mutants were tested on high stringency kanamycin plates and had a background survival of approximately 2 in 10^5. The A86G mutants survived at ~10% on 30 μg/mL kanamycin and 2 in 10^3 on 60 μg/mL kanamycin. This level of background survival is too high for the KanR assay so this tmRNA mutant was discarded. The Δ89C mutant had a background survival of ~1 in 10^6 on low stringency 15 μg/mL kanamycin plates.

SmpB error-prone mutant libraries: The mutant SmpB libraries were constructed by amplifying the *smpB* gene using error-prone PCR. Mutants were sequenced and showed a mutation rate of approximately 2-3 nucleotide mutations per *smpB* gene. The PCR products were cloned into plasmids expressing a mutant tmRNA. All of the libraries constructed were at least 5×10^7 in size. The libraries were grown and induced as described in the previous section. Cultures were plated on both selection and control plates to determine how many colonies were selected. Both the U85A and Δ89C mutant

SmpB libraries were screened in the KanR assay several times but the survival level was never above background levels.

The UGU and A86C libraries each had SmpB suppressor mutants which resulted in higher cellular survival in the KanR assay. The first time that the UGU error-prone library was plated, the cellular survival was up 10-fold. All of the colonies were scraped and grown up in culture. The plasmids were purified and the *smpB* gene was amplified by PCR and cloned into fresh plasmid backbone with the UGU tmRNA mutation after each round of selection to eliminate any contamination. The new plasmids were introduced into the selection strain and selected again. The percentage of surviving colonies in each round increased as the desired clones became enriched over the background survival. After three rounds of selection, there was a 25% survival rate and we picked 28 colonies to sequence and found 6 different mutants.

The first time the A86C library was plated, the cellular survival increased 100-fold over background levels. As with the UGU library, the colonies were scraped, the plasmids recloned, and then selected. After only 2 rounds, the survival rate was 100% for the SmpB mutants. We picked 16 colonies from round 1 and 20 colonies from round 2. We found three different mutants.

Suppressor SmpB mutants from UGU library: After three rounds of selection, several SmpB mutants were found which suppress UGU loss of function. Six of these mutants were chosen for further testing (Figure 10). Survival of these mutants on 30 μg/mL

kanamycin varied from around 20% to nearly 100% compared to UGU mutant background survival of 1 in 10^4.

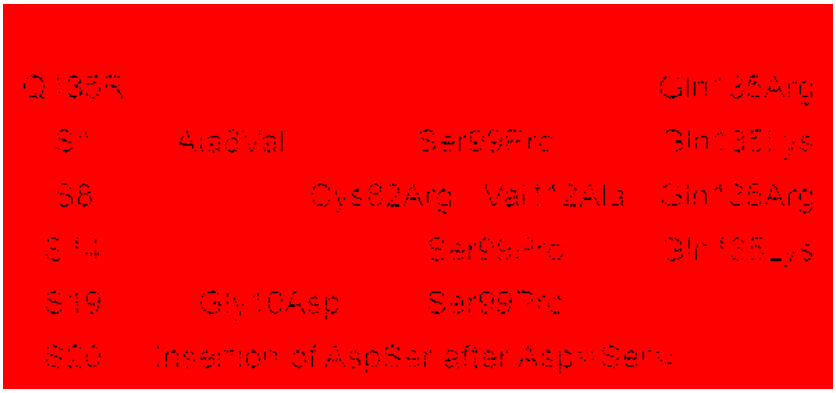


Figure 10: SmpB Suppressor Mutants from UGU library.

The SmpB mutant Q135R was tested with the tmRNA mutations U85A and A86C to determine if the suppressing effect of Q135R is specific to UGU, or if it would suppress additional tmRNA mutations as well. The survival of cells containing U85A tmRNA increased from 2 in 10^5 to 3 in 10^3 when paired with Q135R SmpB– an increase of approximately 150-fold. A86C tmRNA, on the other hand, showed no increase in activity tested alongside Q135R SmpB. Both of these are much lower than the ~6000-fold increase in survival seen with the Q135R and the UGU tmRNA mutant.

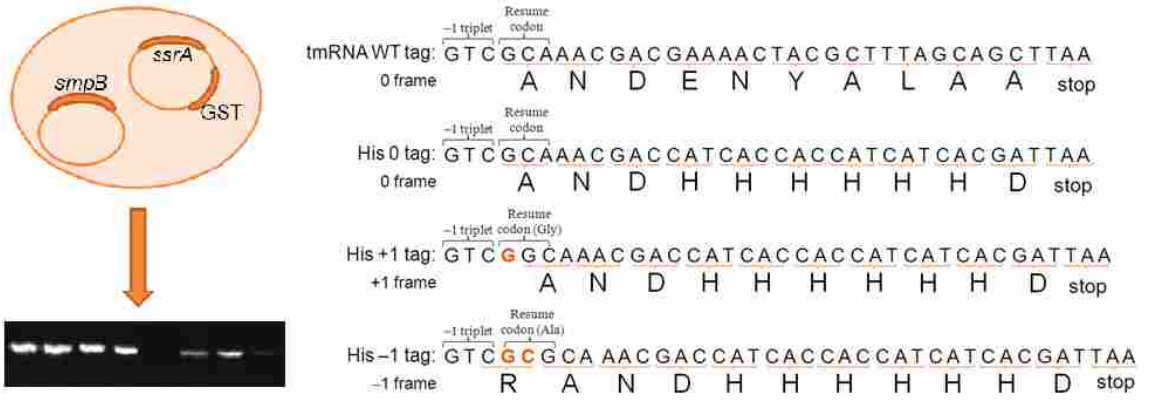


Figure 11: Immunoblot analysis of frame choice. Two plasmids are introduced into Δ*ssrA*Δ*smpB E. coli* cells. One contains *smpB*, the other *ssrA* and *gst* with a EP(Opal) stalling sequence. The tmRNA WT tag (upper right) has been replaced by one of three His6 tags (right). If tagging occurs in the appropriate frame, a 6-Histidine tag is added to the GST protein and can be visualized on an immunoblot using an anti-His antibody and a fluorescent secondary antibody.

Immunoblots of the UGU suppressor mutations were performed looking at tagging in both the 0 and –1 frame. The GST protein that ends with a stall-inducing sequence (Glu-Pro-Opal) at the C-terminus served as a substrate for tagging. The addition of the ANDH$_6$D tag was monitored by immunoblot with anti-His$_6$ antibodies (Figure 11). UGU has previously been shown to –1 frameshift[42, 67]. From the immunoblot results it appears that our SmpB mutants have little to no effect on tagging levels in the 0 frame, either with UGU or WT tmRNA (Figure 12). We do, however, see some effect in the –1 frame. The mutations Q135R and S8 cause an increase in –1 frameshifting with the UGU mutation compared to WT SmpB (Figure 12). The mutation S19 caused increased –1 frameshifting with both tmRNAs (Figure 12). S1, S14 and S20 had no effect (data not shown).

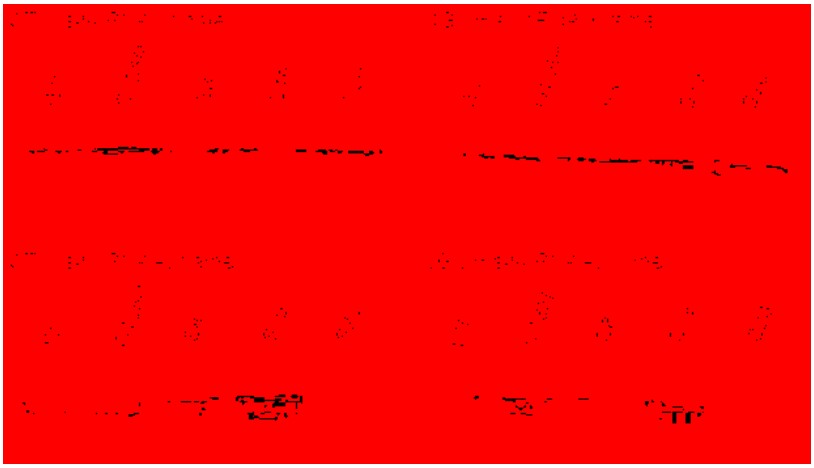


Figure 12: The effects of UGU suppressor mutants on frame selection with WT and UGU tmRNA. See Figure 11 for more details on methods. The intensity of the band represents the amount of tagging that occurs in either the 0 frame (top) or –1 frame (bottom) with WT (left) or UGU (right) tmRNA and WT or mutant SmpB. The tmRNA and frame are listed above each blot. The SmpB mutant is specified over each band.

Suppressor SmpB mutants from A86C library: Three SmpB mutants were identified which suppress the A86C mutation in the KanR assay (Figure 13). All of the mutants were tested for survival on 15 μg/mL kanamycin at room temperature. Survival varied from ~30-80% compared to A86C background cellular survival of ~2 in 10^6. The SmpB mutants were tested with both the UGU and WT tmRNA. The suppressor mutants caused no change in the level of survival of either of these. This suggests that the SmpB mutations are specific to the A86C tmRNA mutation. Since the A86C suppressor mutations retain activity on wild-type, it appears that their specificity for the upstream sequence is broadened, and not altered.

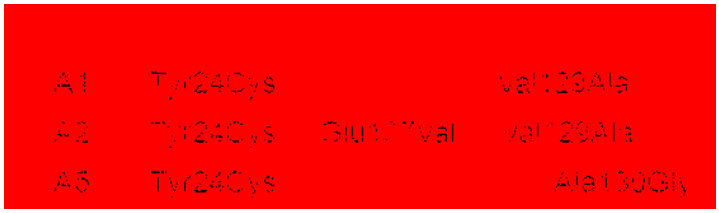


Figure 13: SmpB Suppressor Mutants from A86C library.

The tmRNA mutant A86C has been shown to cause +1 frameshifting. The SmpB suppressor mutants (Figure 13) were tested using immunoblots with both WT and A86C His-tagged tmRNA in the 0 and +1 frames to determine relative tagging in each frame (Figure 14). All three mutants show a significant increase in the 0 frame tagging compared to A86C with WT SmpB, which shows no visible tagging on the immunoblot (Figure 14B). The three SmpB mutations cause a decrease of tagging in the +1 frame compared to A86C tmRNA and WT SmpB (Figure 14D). These data match the genetic evidence and show that SmpB plays a role in setting the frame for the translation of tmRNA.

The A2 mutant shows the greatest increase in 0 frame tagging and the greatest decrease in +1 frame tagging and appears to be the most active of the three suppressor mutants. The only difference between the A1 and the A2 mutant is the additional mutation of Glu107Val in A2. A5 is very similar to A1, with the Ala130Gly mutation replacing the Val129A mutation. Thus it appears that the Glu107Val mutation in A2 results in a gain of function in the context of the other mutations. The suppressor

mutations also showed a slight decrease in 0 frame tagging and no +1 frameshifting with WT tmRNA (Figure 14A and C).

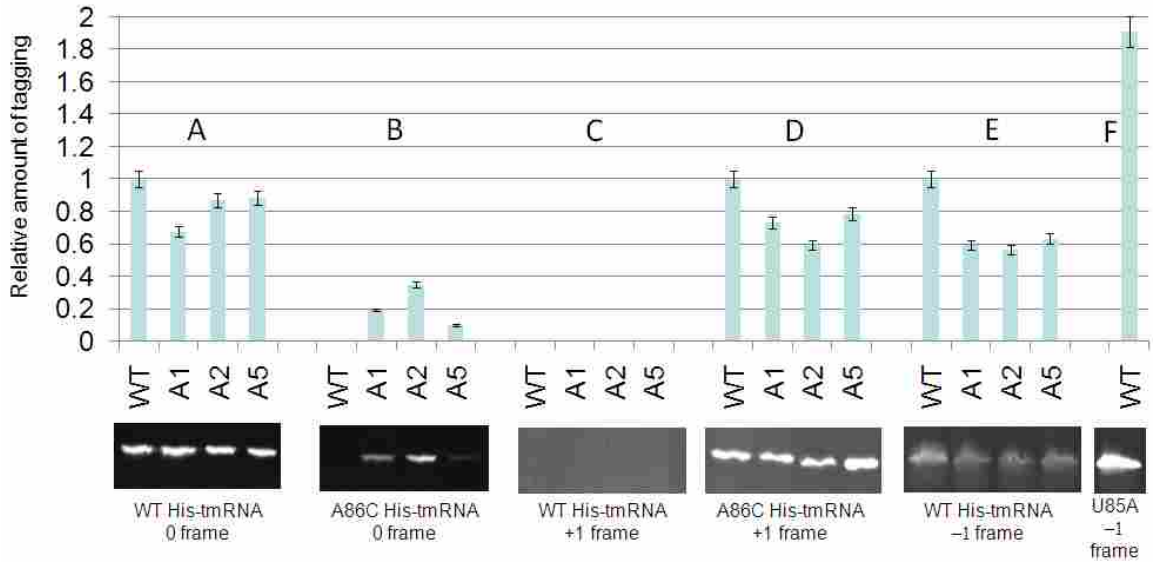


Figure 14: The effects of A86C suppressor mutants on frame selection with WT and A86C tmRNA. Chart of relative amount of His-tagged GST protein compared to the total amount of GST loaded. The values were normalized with WT SmpB tagging equal to 1. The SmpB mutant is listed below each bar and the tmRNA used is listed below each blot. A) WT 0, B) A86C 0, C) WT +1, D) A86C +1, E) WT –1 frame with WT, A1, A2 and A5 SmpB. The last lane F) is U85A –1 tmRNA with WT SmpB and acts as a control for –1 tagging.

We wanted to test whether the SmpB suppressor mutants A1, A2 and A5 cause an increase in 0 frame tagging for A86C tmRNA specifically or if they disrupt an interaction to cause non-specific tagging in all three frames. The A1, A2 and A5 SmpB mutants were tested with the WT –1 tmRNA plasmid using immunoblots to determine the amount of tagging in the –1 frame. Each of the mutations cause a decrease in –1 frameshifting compared to WT SmpB (Figure 14E). We conclude that the suppressor mutations

decrease the efficiency of tagging when paired with WT tmRNA, decreasing the amount of tagging in both the 0 and –1 frames.

The A86C mutant has been studied in several previous reports[42, 62, 75]. The mutation of the adenosine to a cytosine results in significant +1 frameshifting while the U85A mutation causes extensive –1 frameshifting[42, 67]. If A86 is the tmRNA residue which establishes the frame for *trans*-translation, it could be that in the case of U85A, the adenosine now located at position 85 is mistaken for A86, resulting in a –1 frameshift for resuming translation. In the absence of an adenosine, as is the case for the A86C mutation, an interaction could be made with G87 instead, as it is the nearest purine available. This would explain the +1 frameshifting caused by the A86C mutant. If there is no available purine in this region, we hypothesized that we would see an increase in cellular survival in the KanR assay compared to A86C tmRNA since the tagging would not be prejudiced to the +1 frame. To test this possibility, we constructed a double mutant A86CG87C and tested the cellular survival on Kan15 with WT SmpB. The results showed very similar cellular survival levels of the A86CG87C mutation and the A86C mutation in the KanR assay.

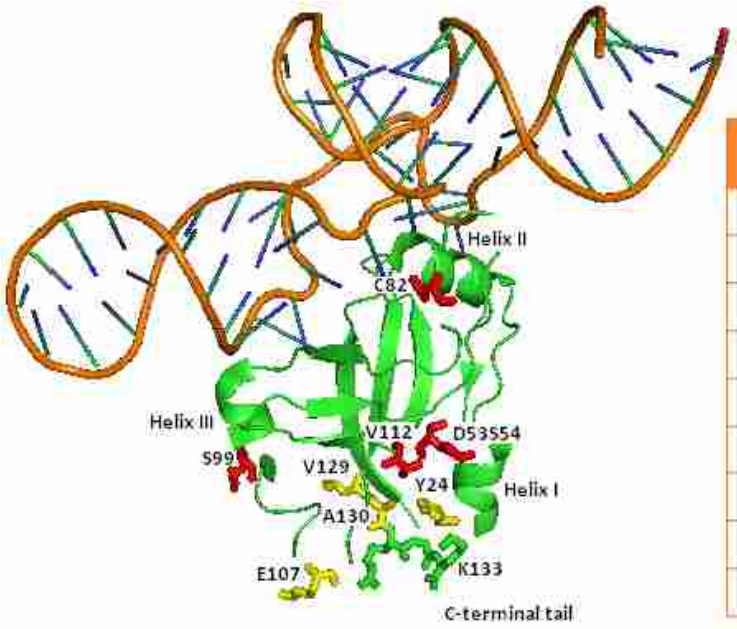
Finally, the mutation Y24C appears in all three of the SmpB suppressor mutants. It is unlikely that Y24 plays a significant role in normal frame-setting since all of our SmpB mutants tag in the 0 frame on WT tmRNA (Figure 14A). They each survive at wild-type levels in the KanR assay against WT (A86) tmRNA, suggesting that the normal frame-setting mechanism is undisturbed when our SmpB mutants are present. It

could be that the Y24C mutation is actually forming an interaction with 86C. In this case, the mutation of Y24 to a cysteine would be important. To test this possibility, we replaced the Y24C mutation in the A2 mutant with Y24A. This SmpB mutant gave similar cellular survival levels to the original A2 mutant in the KanR assay, suggesting the Y24C is a loss-of-function mutation, and the altering of the large tyrosine side chain is sufficient.

The A86C suppressor mutants that we found have an affect on frame choice with the A86C mutant tmRNA, which suggests a link between SmpB and framesetting. What effect do our mutants have on surrounding structures? This led us to look at the structures located near the SmpB mutants to determine if changes in SmpB structural elements would affect frame choice on WT tmRNA.

Rational mutation of SmpB protein to determine effect on frame choice

Using the *T. thermophilus* structure from Bessho *et al.*, the location of each of our mutations was determined using an alignment of the *T. thermophilus* and *E. coli* SmpB sequences (Figure 15)[30]. The clustering of our UGU and A86C suppressor mutants led us to look at helix 1 and helix 3.



E. coli	*Thermus thermophilus*
Y24	Y14
D53 S54	G43 S44
C82	V71
S99	R88
E107	K96
V112	V101
V129	L119
A130	A120
Q135	A125

Figure 15: SmpB-tmRNA complex in P site with suppressor mutants in yellow and red. The SmpB-tmRNA (TLD) structure from Bessho *et al*[30]. This side faces the A site. UGU suppressor mutants are in red and the A86C suppressor mutants are in green. The chart shows the mutations in *E. coli* and the corresponding amino acids in *T. thermophilus.*

Helix 1 contains several positively charged residues and is predicted to be an RNA binding domain. The tmRNA in the SmpB-tmRNA complex makes crystal packing interactions with this region, supporting this prediction[30]. Many of the residues in helix 1 are conserved and are well positioned to bind the ribosome (Figure 16). Y24 (from mutants A1, A2 and A5) is located at the base of helix 1, Ala130 (from A5) points up on the side of SmpB facing the A site, sandwiched between Y24 and T110. These interactions could be important for positioning helix 1. V112 (from S8) is also positioned to interact with Y24.

Several of the other mutations are located near or interact with helix 3 (Figure 16). In an older co-crystal structure of *Aquifex aeolicus* SmpB-tmRNA, tmRNA makes a crystal packing structure with SmpB helix 3 instead of helix 1 as in the new *T. thermophilus* structure[30, 36]. This suggests that helix 3 is an RNA binding site as well. Our V129 mutant from A1 and A2 packs against the highly conserved L100 of helix 3. E107 from A2 is located at the end of helix 3 pointing out toward the ribosome, on the opposite face of Y24. S99 from the S1, S14 and S19 mutants is located in the middle of helix 3 and a mutation of serine to proline would likely disrupt the helical structure.

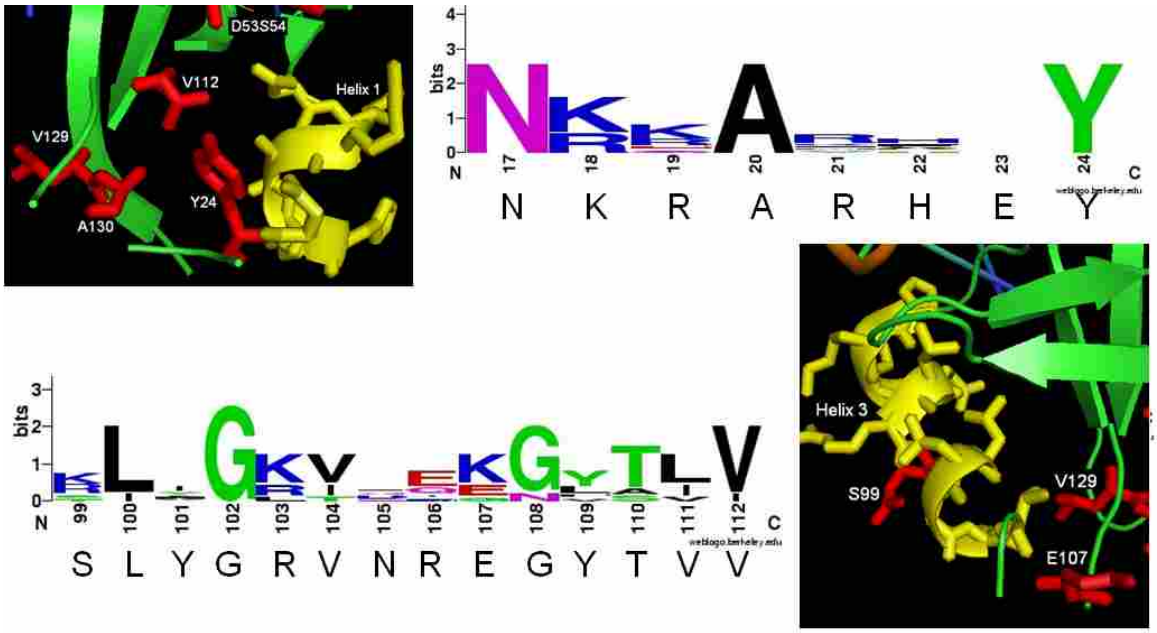


Figure 16: Helix 1 and 3 structures and sequence alignments. Helix 1 and Helix 3 are shown in yellow while the SmpB suppressor mutations are in red. Graphical sequence logos were generated by WebLogo[66].

Helix 1 and helix 3 are well positioned to bind to the ribosome on either side to position SmpB in the P site. Our mutants may function by altering the positioning or structure of helix 1 and helix 3, changing the interaction with the ribosome and altering the arrangement of SmpB within the P site. To characterize the interactions between the ribosome/tmRNA and helix 1, we made N17A, K18A, R19A, A20V, R21A, H22A, E23A and Y24A single mutations for helix 1 and S99P, L100A, G108A, Y109A and T110A single mutations for helix 3. The mutations were characterized by measuring the amount of tagging that took place in all three frames.

Surprisingly, most of these mutants showed no effect on tagging in the 0 frame (Figure 17A). Helix 1 has several positively charged amino acids that make it a good candidate for an RNA binding region. Since mutating each of those residues singly appears to have no effect, we made two double mutants, $K_{18}A/R_{19}A$ (1819) and $K_{18}A/R_{21}A$ (1821), and a triple mutant, $K_{18}A/R_{19}A/R_{21}A$ (KRR). These did not affect tagging in the 0 frame either (Figure 17A). None of the mutations increased –1 tagging though several appeared to decrease tagging in the –1 frame (Figure 17B). E23A, L108A and Y109A showed a significant decrease in –1 tagging. None of the mutants caused +1 frameshifting (data not shown). The immunoblot frame-choice results were surprising since many of the mutated residues are highly conserved yet seem to have little effect on SmpB function or frame choice.

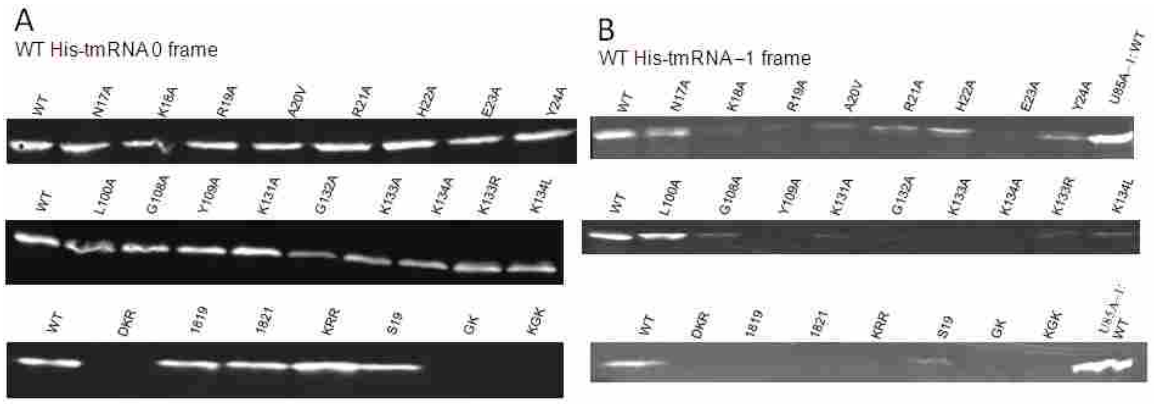


Figure 17: SmpB mutant immunoblots. All of our SmpB mutations were tested along with WT tmRNA-His. The 0 frame blots are on the left (A) and the –1 blots are on the right (B). The first row shows the helix 1 mutations. The second has several helix 3 mutations (L100A, G108A and Y109A) and then shows the tail mutants from K131-K134. G132 is the only single mutation which seems to have an affect on 0 frame tagging efficiency. T110A is not shown but it too has no effect on 0 frame tagging and decreases tagging in the –1 frame. The third row shows several tail mutants that have no discernible tmRNA tagging activity (DKR, GK and KGK). It also shows some double and triple mutations from helix 1 which have no affect on tagging levels in the 0 frame (1819, 1821, KRR). S19 is a UGU suppressor mutant from the error-prone SmpB library.

Another possibility is that our mutants work by affecting the position of the SmpB C-terminal tail. It has already been shown that the tail is important for SmpB function and that it binds in the P site mRNA channel[39, 64]. Many of our mutants, especially those from the A86C suppressor mutants, are clustered just before the beginning of the tail, at the site where it exits the body of the protein. It may be that these amino acids position the tail correctly so that it can place the protein properly in the P site by binding to the mRNA channel of the ribosome. In order to test the role of the C-terminal tail in framesetting, we made the following mutations: K131A, G132A, K133A, K134A, K133R,

K134L, as well as the $G_{132}K_{133}$ double alanine mutant and the $K_{131}G_{132}K_{133}$ and $D_{137}K_{138}R_{139}$ triple alanine mutants (for alignment see Figure 18).

The only single mutant which decreased tagging in the 0 frame was G132A (Figure 17A, middle). The GK, KGK and DKR tail mutants showed no tagging in any of the frames. Many of the mutations showed a significant decrease in tagging in the –1frame (Figure 17). This implies that our frameshifting immunoblot assay may not be giving us reliable results for the –1 frame but our tail mutants are showing an effect on tagging efficiency in the 0 frame.

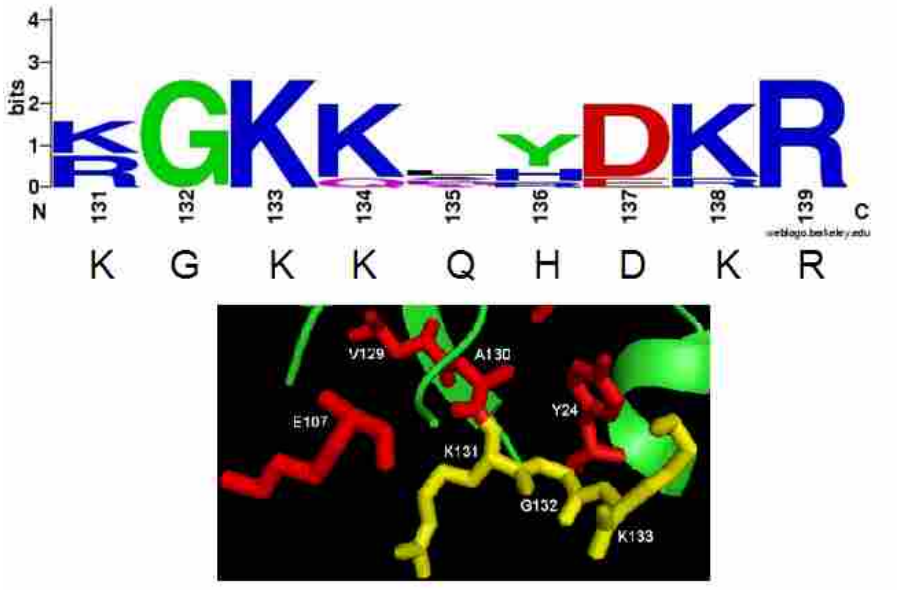


Figure 18: C-terminal tail sequence alignment and structure. The base of the C-terminal tail is shown in yellow while the SmpB suppressor mutations from the A86C library are in red. This shows several of our mutations in close proximity to G132 and the surrounding lysines. Graphical sequence logos were generated by WebLogo[66].

Chapter 4: Discussion

The entry of tmRNA into stalled ribosomes to serve first as a tRNA and then as an mRNA template poses an interesting question. How does the ribosome resume translation on tmRNA at the proper site and in the proper frame without the aid of a start codon or an initiator tRNA? The upstream region of tmRNA and the SmpB protein have both been implicated as important components of determining proper frame for *trans*-translation. To look for possible interactions between the upstream region and SmpB, we used a genetic selection that ties the life of an *E. coli* cell to tmRNA tagging activity. KanR polypeptides which lack the critical C-terminal sequence are stalled on ribosomes. An altered tmRNA that codes for the missing amino acids rescues the stalled ribosomes to produce full-length, functional KanR. The cells can then survive on kanamycin plates. Only cells that contain functional tmRNA—that tag in the correct frame—can survive. We made libraries of SmpB mutants to select for any that would suppress upstream tmRNA mutants that cannot facilitate proper peptide tagging.

We studied two different tmRNA upstream mutants: UGU and A86C. The decreased survival of cells with the tmRNA mutant UGU in the KanR assay is not entirely understood. We showed that UGU causes high levels of –1 frameshifting which

may decrease the amount of protein tagged in the correct frame. It has been shown that cells with the UGU mutant can support phage *λimmP22 c2-dis* plaque formation, which suggests that tmRNA is functional[67]. It has been determined that the critical function of tmRNA in phage survival is ribosome release. Degradation of the protein by protease recognition of the tag is not required for phage survival so the frame of tagging may not matter as it does in the KanR assay. The tmRNA UGU mutant also shows high levels of 0 frame tagging in our immunoblots. It may be that UGU tmRNA behaves differently due to differences in the template sequence in the KanR and His-tag tmRNA. Since it is unknown how UGU affects tmRNA function and peptide tagging, it is unclear why the selected SmpB mutations rescue the decreased tagging of the UGU mutant in the KanR assay. Several of these SmpB mutations showed little effect on tagging in the 0 frame and several appear to increase tagging in the –1 frame. It is interesting that a number of these mutations are clustered near the A86C library SmpB mutants.

The A86C mutant has been shown to be detrimental to tmRNA activity and to cause high levels of +1 frameshifting[42, 67]. The suppressor mutants that we found show an obvious effect on frame in context of the A86C mutation, greatly increasing 0 frame tagging and decreasing the +1 frame tagging. These mutants have little or no effect with wild-type tmRNA, which suggests that if there is an interaction between the upstream region of tmRNA and the SmpB protein, we have not disrupted it. Instead, our SmpB mutations seem to only alter the function of the A86C mutant. All three of the suppressor mutants isolated from the A86C library contained the mutation Y24C. We

determined that it is not the cysteine side chain forming a new interaction which causes the suppression of the A86C mutant since the mutation Y24A works just as well in the context of the other amino acid mutations. This implies that it is not the addition of the cysteine at this position that suppresses the A86C tmRNA mutation, but rather the removal of the tyrosine side chain. Also, it could be that our SmpB mutations actually evolved a new frame-setting interaction with the cytosine now located at A86C. This could be determined by testing another tmRNA mutant, A86U, which has been shown to cause +1 frameshifting as well. Testing A86U with our suppressor mutants would show us if their effect is dependent on a cytosine at position 86 or if they suppress another A86 mutation as well. It would also be beneficial to test each of the SmpB mutations individually and in pairs to determine which SmpB mutations contribute to the restored activity of tmRNA or if some are dispensable.

Many of our suppressor mutants are clustered near the junction of SmpB helix 1 and helix 3 and are near the position where the unstructured C-terminal tail exits the body of the protein (Figure 15). Based on the proximity of our mutants to these structural elements, we chose to look for additional SmpB mutations which caused aberrant frame choice by alanine scanning these three areas: helix 1, helix 3 and the tail region. Our original theory was that helix 1 and helix 3 could be binding to the ribosome to position the SmpB-tmRNA complex in the P site and thus help to set the frame. Mutation of each of the helix 1 residues to alanine did not yield an effect on tmRNA tagging in the 0 frame. Even mutating K18, R19 and R21— a putative RNA

binding region— simultaneously to alanine showed no affect. Another possible experiment would be to mutate these residues once more using negatively charged amino acids to replace the positive charge, rather than neutral alanine. We saw no effect when the helix 3 residues were mutated to alanine. This suggests that in the case of *E. coli* SmpB, helix 1 and 3 are not important for framesetting or that they need to be more heavily mutated before any effect can be seen.

A second way of thinking about the function of our selected mutants is that they act by positioning the C-terminal tail of SmpB. Although the C-terminal tail of SmpB has previously been implicated in playing a crucial role in *trans*-translation[38], its position, structure and behavior in the ribosome remains poorly understood. The suppressor mutants we identified cluster around the point where the tail extends from the body of the protein (Figure 18). In the Bessho *et al.* co-crystal structure of tmRNA and SmpB, there are two different SmpB-tmRNA dimers in the unit cell, each with slightly different configurations[30]. In one, the C-terminal tail comes up to stack against Y24 rather than extending straight down into the mRNA channel, suggesting that our mutants, especially Y24, would be in a position to interact with or stack against the tail, altering the way it extends from the body of the protein.

The SmpB C-terminal tail has been shown to interact with the 16S rRNA in the P site. Perhaps this interaction is what anchors the SmpB-tmRNA complex. Two separate studies report that SmpB interacts with the mRNA channel in the P site. In one, ribosomes containing SmpB were isolated and ribosomal RNA was chemically modified

with kethoxal, dimethyl sulfate, and hydroxyl radicals. Protection was isolated to the P site near the mRNA channel[39]. The second study tethered Fe(II) to various SmpB C-terminal tail residues and identified sites of SmpB binding to the ribosome by directed hydroxyl radical probing[64]. This revealed an SmpB-tail binding site in the P site of the ribosome located almost exclusively around the region of the codon-anticodon interaction. The tail residues involved were 152, 155 and 159. Since the region of the tail which is binding to the P site is towards the end of the tail, we speculate that when the SmpB protein is moved into the P site, the tail remains down in the mRNA channel and has to fold back on underneath itself to extend beneath the body of the protein in the P site. Interactions between the tail and the ribosome could position the SmpB-tmRNA complex in the P site and help to set the translational frame of the tmRNA template tag. Our initial results of our tail mutants look promising. The single alanine mutations did not have much effect on tmRNA function except in the case of G132A, which showed a marked decrease in 0 frame tagging. A double mutant of G132A/K133A showed no tagging, as did the K131/G132/K133 triple alanine mutation. SmpB amino acids G132 and K133 appear to be very important for peptide-tagging by tmRNA. It may be that the positively charged residues act as an RNA binding domain and the glycine is important for flexibility in the tail. Both G132 and K133 are located in close proximity to our suppressor mutations, especially Y24.

Further characterization of the SmpB C-terminal tail would be beneficial to determine the function of the tail in tmRNA-mediated tagging and possibly frame-

setting. It is not clear from the present data whether mutations in the tail would have an effect on frame choice or if the tail only affects the efficiency of tagging. The area surrounding the K_{131}GKK sequence seems the most promising for further study since we saw partial loss of function with the G132A mutant and total loss of function with the G132A/K133A. Preliminary results suggest that the GK mutant is active in binding tmRNA and the ribosome, accommodation into the A site and peptidyl transfer of the initial alanine but prevents activity downstream of the first peptidyl transfer reaction. To further characterize the role of these residues, we want to look at how the length of the tail affects function and frame. This will be done by adding or deleting amino acids upstream of the KGKK sequence. If the positioning of GK is important for function, perhaps moving them by lengthening or shortening the tail will affect frame selection on the tmRNA tag template.

The immunoblot results from all of our mutants suggest that the –1 tagging for wild-type tmRNA and SmpB seems to be too high since many of our mutants appear to improve frame choice and show lower levels of –1 frameshifting. Also, in another frame-shifting study that was been done *in vitro*, no –1 frameshifting with WT tmRNA was reported[42]. The high levels of –1 frameshifting that we saw with WT tmRNA caused us to look at how the –1 tmRNA His-tag was designed. The high level of –1 frameshifting with WT tmRNA that we see could be an artifact of our mutated tag template in the –1 misreading plasmid, pDH210. The tag was altered by inserting two nucleotides into the resume codon of the tag so that when –1 frameshifting occurred, the

His_6-tag would be added. By changing the sequence around the resume codon, we may be inducing –1 frameshifting somehow, though it is still unclear why any of our SmpB mutations would cause a decrease in –1 tagging. A new –1 misreading tmRNA will be designed to determine if WT –1 frameshifting can be decreased in a different context.

The A86C suppressor mutants that we have found and their ability to affect frame-choice in the context of the A86C tmRNA mutation provides strong support for a model where interactions between SmpB and the upstream region of tmRNA are responsible for the selecting the correct translational frame for the tmRNA tag template sequence. Having found SmpB mutants which affect frame on A86C tmRNA, we had hoped to find SmpB mutants which would affect frame-choice on WT (A86) tmRNA as well. So far we have not been successful. While some of our mutations have affected tmRNA tagging efficiency, none have been shown to affect frame other than a decrease in –1 frameshifting. It may be that we will not be able to find such SmpB mutants in the manner that we have been attempting. Our suppressor mutants have little or no affect on frame with WT tmRNA. Their affect is limited to the A86C tmRNA mutant.

Additional work needs to be done to determine how our SmpB mutants cause the suppression of the A86C tmRNA mutation. One possibility is to do a chemical modification assay of A86C with WT SmpB and our mutant SmpB proteins to see if the protection pattern is altered. Konno *et al.* saw that with WT tmRNA and WT SmpB, there was protection of the tmRNA residue U85[62]. It would be interesting to test the selected SmpB mutants with WT and A86C tmRNA. Our suppressor mutants may

restore the protection of U85 seen with WT tmRNA or they may show different interaction sites with A86C tmRNA and the suppressor SmpB mutants. These results could help to determine where interactions are taking place, pointing to a more specific interaction site between SmpB and tmRNA.

The function of SmpB in conjunction tmRNA may have a new dimension. SmpB is known to interact with the TLD to help tmRNA function as a tRNA through aminoacylation, binding to the ribosome and accommodation prior to peptidyl-transfer. Our results suggest a second interaction that contributes to the mRNA function of tmRNA. Previous work has shown a link between the upstream region of tmRNA and framesetting and it appears that this region must interact with a structural element in order to place the resume codon correctly. The best candidate for that structural element is SmpB. Our results indicate a functional interaction between SmpB and the upstream region of tmRNA that affects frame choice. These results give new insight into *trans*-translation. Further exploration of the specific interactions between SmpB and tmRNA will help to clarify how the template is translated in the correct frame.

References

1. Nissen, P.; Hansen, J.; Ban, N.; Moore, P. B.; Steitz, T. A., The structural basis of ribosome activity in peptide bond synthesis. *Science* **2000,** 289, (5481), 920-30.

2. Kozak, M., Initiation of translation in prokaryotes and eukaryotes. *Gene* **1999,** 234, (2), 187-208.

3. Andersen, G. R.; Nissen, P.; Nyborg, J., Elongation factors in protein biosynthesis. *Trends Biochem Sci* **2003,** 28, (8), 434-41.

4. Steitz, T. A., A structural understanding of the dynamic ribosome machine. *Nat Rev Mol Cell Biol* **2008,** 9, (3), 242-53.

5. Cochella, L.; Green, R., An active role for tRNA in decoding beyond codon:anticodon pairing. *Science* **2005,** 308, (5725), 1178-80.

6. Rodnina, M. V.; Wintermeyer, W., Fidelity of aminoacyl-tRNA selection on the ribosome: kinetic and structural mechanisms. *Annu Rev Biochem* **2001,** 70, 415-35.

7. Rodnina, M. V.; Wintermeyer, W., Ribosome fidelity: tRNA discrimination, proofreading and induced fit. *Trends Biochem Sci* **2001,** 26, (2), 124-30.

8. Weinger, J. S.; Parnell, K. M.; Dorner, S.; Green, R.; Strobel, S. A., Substrate-assisted catalysis of peptide bond formation by the ribosome. *Nat Struct Mol Biol* **2004,** 11, (11), 1101-6.

9. Joseph, S., After the ribosome structure: how does translocation work? *Rna* **2003,** 9, (2), 160-4.

10. Petry, S.; Weixlbaumer, A.; Ramakrishnan, V., The termination of translation. *Curr Opin Struct Biol* **2008,** 18, (1), 70-7.

11. Keiler, K. C.; Waller, P. R.; Sauer, R. T., Role of a peptide tagging system in degradation of proteins synthesized from damaged messenger RNA. *Science* **1996,** 271, (5251), 990-3.

12. Roche, E. D.; Sauer, R. T., SsrA-mediated peptide tagging caused by rare codons and tRNA scarcity. *Embo J* **1999,** 18, (16), 4579-89.

13. Roche, E. D.; Sauer, R. T., Identification of endogenous SsrA-tagged proteins reveals tagging at positions corresponding to stop codons. *J Biol Chem* **2001,** 276, (30), 28509-15.

14. Hayes, C. S.; Bose, B.; Sauer, R. T., Proline residues at the C terminus of nascent chains induce SsrA tagging during translation termination. *J Biol Chem* **2002,** 277, (37), 33825-32.

15. Hayes, C. S.; Bose, B.; Sauer, R. T., Stop codons preceded by rare arginine codons are efficient determinants of SsrA tagging in Escherichia coli. *Proc Natl Acad Sci U S A* **2002,** 99, (6), 3440-5.

16. Hayes, C. S.; Sauer, R. T., Cleavage of the A site mRNA codon during ribosome pausing provides a mechanism for translational quality control. *Mol Cell* **2003,** 12, (4), 903-11.

17. Gottesman, S.; Roche, E.; Zhou, Y.; Sauer, R. T., The ClpXP and ClpAP proteases degrade proteins with carboxy-terminal peptide tails added by the SsrA-tagging system. *Genes Dev* **1998,** 12, (9), 1338-47.

18. Sunohara, T.; Jojima, K.; Yamamoto, Y.; Inada, T.; Aiba, H., Nascent-peptide-mediated ribosome stalling at a stop codon induces mRNA cleavage resulting in nonstop mRNA that is recognized by tmRNA. *Rna* **2004,** 10, (3), 378-86.

19. Sunohara, T.; Jojima, K.; Tagami, H.; Inada, T.; Aiba, H., Ribosome stalling during translation elongation induces cleavage of mRNA being translated in Escherichia coli. *J Biol Chem* **2004,** 279, (15), 15368-75.

20. Hallier, M.; Ivanova, N.; Rametti, A.; Pavlov, M.; Ehrenberg, M.; Felden, B., Pre-binding of small protein B to a stalled ribosome triggers trans-translation. *J Biol Chem* **2004,** 279, (25), 25978-85.

21. Lee, S. Y.; Bailey, S. C.; Apirion, D., Small stable RNAs from Escherichia coli: evidence for the existence of new molecules and for a new ribonucleoprotein particle containing 6S RNA. *J Bacteriol* **1978,** 133, (2), 1015-23.

22. Chauhan, A. K.; Apirion, D., The gene for a small stable RNA (10Sa RNA) of Escherichia coli. *Mol Microbiol* **1989,** 3, (11), 1481-5.

23. Komine, Y.; Kitabatake, M.; T., Y.; Nishikawa, K.; Inokuchi, H., A tRNA-like structure is present in 10Sa RNA, a small stable RNA from Escherichia coli. *Proc Natl Acad Sci U S A* **1994,** 91, (20), 9223-7.

24. Tu, G. F.; Reid, G. E.; Zhang, J. G.; Moritz, R. L.; Simpson, R. J., C-terminal extension of truncated recombinant proteins in Escherichia coli with a 10Sa RNA decapeptide. *J Biol Chem* **1995,** 270, (16), 9322-6.

25. Keiler, K. C.; Sauer, R. T., Sequence determinants of C-terminal substrate recognition by the Tsp protease. *J Biol Chem* **1996,** 271, (5), 2589-93.

26. Retallack, D. M.; Johnson, L. L.; Friedman, D. I., Role for 10Sa RNA in the growth of lambda-P22 hybrid phage. *J Bacteriol* **1994,** 176, (7), 2082-9.

27. Felden, B.; Himeno, H.; Muto, A.; McCutcheon, J. P.; Atkins, J. F.; Gesteland, R. F., Probing the structure of the Escherichia coli 10Sa RNA (tmRNA). *Rna* **1997,** 3, (1), 89-103.

28. Williams, K. P.; Bartel, D. P., Phylogenetic analysis of tmRNA secondary structure. *Rna* **1996,** 2, (12), 1306-10.

29. Tanner, D. R.; Dewey, J. D.; Miller, M. R.; Buskirk, A. R., Genetic analysis of the structure and function of transfer messenger RNA pseudoknot 1. *J Biol Chem* **2006,** 281, (15), 10561-6.

30. Bessho, Y.; Shibata, R.; Sekine, S.; Murayama, K.; Higashijima, K.; Hori-Takemoto, C.; Shirouzu, M.; Kuramitsu, S.; Yokoyama, S., Structural basis for functional mimicry of long-variable-arm tRNA by transfer-messenger RNA. *Proc Natl Acad Sci U S A* **2007,** 104, (20), 8293-8.

31. Selmer, M.; Dunham, C. M.; Murphy, F. V. t.; Weixlbaumer, A.; Petry, S.; Kelley, A. C.; Weir, J. R.; Ramakrishnan, V., Structure of the 70S ribosome complexed with mRNA and tRNA. *Science* **2006,** 313, (5795), 1935-42.

32. Rudinger-Thirion, J.; Giege, R.; Felden, B., Aminoacylated tmRNA from Escherichia coli interacts with prokaryotic elongation factor Tu. *Rna* **1999,** 5, (8), 989-92.

33. Shimizu, Y.; Ueda, T., The role of SmpB protein in trans-translation. *FEBS Lett* **2002,** 514, (1), 74-7.

34. Tadaki, T.; Fukushima, M.; Ushida, C.; Himeno, H.; Muto, A., Interaction of 10Sa RNA with ribosomes in Escherichia coli. *FEBS Lett* **1996,** 399, (3), 223-6.

35. Shimizu, Y.; Ueda, T., SmpB triggers GTP hydrolysis of elongation factor Tu on ribosomes by compensating for the lack of codon-anticodon interaction during trans-translation initiation. *J Biol Chem* **2006,** 281, (23), 15987-96.

36. Gutmann, S.; Haebel, P. W.; Metzinger, L.; Sutter, M.; Felden, B.; Ban, N., Crystal structure of the transfer-RNA domain of transfer-messenger RNA in complex with SmpB. *Nature* **2003,** 424, (6949), 699-703.

37. Jacob, Y.; Sharkady, S. M.; Bhardwaj, K.; Sanda, A.; Williams, K. P., Function of the SmpB Tail in tmRNA Translation Revealed by a Nucleus-Encoded Form. *J Biol Chem* **2005,** 280, (7), 5503-5509.

38. Sundermeier, T. R.; Dulebohn, D. P.; Cho, H. J.; Karzai, A. W., A previously uncharacterized role for small protein B (SmpB) in transfer messenger RNA-mediated trans-translation. *Proc Natl Acad Sci U S A* **2005,** 102, (7), 2316-21.

39. Ivanova, N.; Pavlov, M. Y.; Bouakaz, E.; Ehrenberg, M.; Schiavone, L. H., Mapping the interaction of SmpB with ribosomes by footprinting of ribosomal RNA. *Nucleic Acids Res* **2005,** 33, (11), 3529-39.

40. Richards, J.; Mehta, P.; Karzai, A. W., RNase R degrades non-stop mRNAs selectively in an SmpB-tmRNA-dependent manner. *Mol Microbiol* **2006,** 62, (6), 1700-12.

41. Williams, K. P.; Martindale, K. A.; Bartel, D. P., Resuming translation on tmRNA: a unique mode of determining a reading frame. *Embo J* **1999,** 18, (19), 5423-33.

42. Lee, S.; Ishii, M.; Tadaki, T.; Muto, A.; Himeno, H., Determinants on tmRNA for initiating efficient and precise trans-translation: some mutations upstream of the tag-encoding sequence of Escherichia coli tmRNA shift the initiation point of trans-translation in vitro. *Rna* **2001,** 7, (7), 999-1012.

43. Herman, C.; Thevenet, D.; Bouloc, P.; Walker, G. C.; D'Ari, R., Degradation of carboxy-terminal-tagged cytoplasmic proteins by the Escherichia coli protease HflB (FtsH). *Genes Dev* **1998,** 12, (9), 1348-55.

44. Flynn, J. M.; Levchenko, I.; Seidel, M.; Wickner, S. H.; Sauer, R. T.; Baker, T. A., Overlapping recognition determinants within the ssrA degradation tag allow modulation of proteolysis. *Proc Natl Acad Sci U S A* **2001,** 98, (19), 10584-9.

45. Levchenko, I.; Seidel, M.; Sauer, R. T.; Baker, T. A., A specificity-enhancing factor for the ClpXP degradation machine. *Science* **2000,** 289, (5488), 2354-6.

46. Dulebohn, D.; Choy, J.; Sundermeier, T.; Okan, N.; Karzai, A. W., Trans-translation: the tmRNA-mediated surveillance mechanism for ribosome rescue, directed protein degradation, and nonstop mRNA decay. *Biochemistry* **2007,** 46, (16), 4681-93.

47. Dong, G.; Nowakowski, J.; Hoffman, D. W., Structure of small protein B: the protein component of the tmRNA-SmpB system for ribosome rescue. *Embo J* **2002,** 21, (7), 1845-54.

48. Someya, T.; Nameki, N.; Hosoi, H.; Suzuki, S.; Hatanaka, H.; Fujii, M.; Terada, T.; Shirouzu, M.; Inoue, Y.; Shibata, T.; Kuramitsu, S.; Yokoyama, S.; Kawai, G., Solution structure of a tmRNA-binding protein, SmpB, from Thermus thermophilus. *FEBS Lett* **2003,** 535, (1-3), 94-100.

49. Barends, S.; Karzai, A. W.; Sauer, R. T.; Wower, J.; Kraal, B., Simultaneous and functional binding of SmpB and EF-Tu-TP to the alanyl acceptor arm of tmRNA. *J Mol Biol* **2001,** 314, (1), 9-21.

50. Hanawa-Suetsugu, K.; Takagi, M.; Inokuchi, H.; Himeno, H.; Muto, A., SmpB functions in various steps of trans-translation. *Nucleic Acids Res* **2002,** 30, (7), 1620-9.

51. Karzai, A. W.; Susskind, M. M.; Sauer, R. T., SmpB, a unique RNA-binding protein essential for the peptide-tagging activity of SsrA (tmRNA). *Embo J* **1999,** 18, (13), 3793-9.

52. Metzinger, L.; Hallier, M.; Felden, B., Independent binding sites of small protein B onto transfer-messenger RNA during trans-translation. *Nucleic Acids Res* **2005,** 33, (8), 2384-94.

53. Wower, J.; Zwieb, C. W.; Hoffman, D. W.; Wower, I. K., SmpB: A Protein that Binds to Double-Stranded Segments in tmRNA and tRNA. *Biochemistry* **2002,** 41, 8826-8836.

54. Valle, M.; Gillet, R.; Kaur, S.; Henne, A.; Ramakrishnan, V.; Frank, J., Visulaizing tmRNA Entry into a Stalled Ribosome. *Science* **2003,** 300, 127-130.

55. Nameki, N.; Someya, T.; Okano, S.; Suemasa, R.; Kimoto, M.; Hanawa-Suetsugu, K.; Terada, T.; Shirouzu, M.; Hirao, I.; Takaku, H.; Himeno, H.; Muto, A.; Kuramitsu, S.; Yokoyama, S.; Kawai, G., Interaction Analysis between tmRNA and SmpB from *Thermus thermophilus*. *J. Biochem* **2005,** 138, 729-739.

56. Kaur, S.; Gillet, R.; Li, W.; Gursky, R.; Frank, J., Cryo-EM visualization of transfer messenger RNA with two SmpBs in a stalled ribosome. *Proc Natl Acad Sci U S A* **2006,** 103, (44), 16484-9.

57. Valle, M.; Gillet, R.; Kaur, S.; Henne, A.; Ramakrishnan, V.; Frank, J., Visualizing tmRNA entry into a stalled ribosome. *Science* **2003,** 300, (5616), 127-30.

58. Sundermeier, T. R.; Karzai, A. W., Functional SmpB-ribosome interactions require tmRNA. *J Biol Chem* **2007,** 282, (48), 34779-86.

59. Moore, S. D.; Sauer, R. T., The tmRNA system for translational surveillance and ribosome rescue. *Annu Rev Biochem* **2007,** 76, 101-24.

60. Jacob, Y.; Seif, E.; Paquet, P. O.; Lang, B. F., Loss of the mRNA-like region in mitochondrial tmRNAs of jakobids. *Rna* **2004,** 10, (4), 605-14.

61. Lim, V. I.; Garber, M. B., Analysis of recognition of transfer-messenger RNA by the ribosomal decoding center. *J Mol Biol* **2005,** 346, (2), 395-8.

62. Konno, T.; Kurita, D.; Takada, K.; Muto, A.; Himeno, H., A functional interaction of SmpB with tmRNA for determination of the resuming point of trans-translation. *Rna* **2007,** 13, (10), 1723-31.

63. Shpanchenko, O. V.; Zvereva, M. I.; Ivanov, P. V.; Bugaeva, E. Y.; Rozov, A. S.; Bogdanov, A. A.; Kalkum, M.; Isaksson, L. A.; Nierhaus, K. H.; Dontsova, O. A., Stepping Transfer Messenger RNA through the Ribosome. *J Biol Chem* **2005,** 280, (18), 18368-18374.

64. Kurita, D.; Sasaki, R.; Muto, A.; Himeno, H., Interaction of SmpB with ribosome from directed hydroxyl radical probing. *Nucleic Acids Res* **2007**.

65. Nameki, N.; Tadaki, T.; Himeno, H.; Muto, A., Three of four pseudoknots in tmRNA are interchangeable and are substitutable with single-stranded RNAs. *FEBS Lett* **2000,** 470, (3), 345-9.

66. Crooks, G. E.; Hon, G.; Chandonia, J. M.; Brenner, S. E., WebLogo: a sequence logo generator. *Genome Res* **2004,** 14, (6), 1188-90.

67. Miller, M. R.; Healey, D. W.; Robison, S. G.; Dewey, J. D.; Buskirk, A. R., The role of upstream sequences in selecting the reading frame on tmRNA. *BMC Biol* **2008,** 6, (1), 29.

68. O'Connor, L.; Joy, J.; Kane, M.; Smith, T.; Maher, M., Rapid polymerase chain reaction/DNA probe membrane-based assay for the detection of Listeria and Listeria monocytogenes in food. *J Food Prot* **2000,** 63, (3), 337-42.

69. Wower, I. K.; Zwieb, C. W.; Guven, S. A.; Wower, J., Binding and cross-linking of tmRNA to ribosomal protein S1, on and off the Escherichia coli ribosome. *Embo J* **2000,** 19, (23), 6612-21.

70. Bordeau, V.; Felden, B., Ribosomal protein S1 induces a conformational change of tmRNA; more than one protein S1 per molecule of tmRNA. *Biochimie* **2002,** 84, (8), 723-9.

71. Saguy, M.; Gillet, R.; Skorski, P.; Hermann-Le Denmat, S.; Felden, B., Ribosomal protein S1 influences trans-translation in vitro and in vivo. *Nucleic Acids Res* **2007,** 35, (7), 2368-76.

72. McGinness, K. E.; Sauer, R. T., Ribosomal protein S1 binds mRNA and tmRNA similarly but plays distinct roles in translation of these molecules. *Proc Natl Acad Sci U S A* **2004,** 101, (37), 13454-9.

73. Qi, H.; Shimizu, Y.; Ueda, T., Ribosomal protein S1 is not essential for the trans-translation machinery. *J Mol Biol* **2007,** 368, (3), 845-52.

74. Ringquist, S.; Jones, T.; Snyder, E. E.; Gibson, T.; Boni, I.; Gold, L., High-affinity RNA ligands to Escherichia coli ribosomes and ribosomal protein S1: comparison of natural and unnatural binding sites. *Biochemistry* **1995,** 34, (11), 3640-8.

75. Takahashi, T.; Konno, T.; Muto, A.; Himeno, H., Various effects of paromomycin on tmRNA-directed trans-translation. *J Biol Chem* **2003,** 278, (30), 27672-80.