

Distinct tmRNA sequence elements facilitate RNase R engagement on rescued ribosomes for selective nonstop mRNA decay

Krithika Venkataraman, Hina Zafar and A. Wali Karzai*

Department of Biochemistry and Cell Biology, Center for Infectious Diseases, Stony Brook University, Stony Brook, New York, NY 11794, USA

Received May 22, 2014; Revised August 20, 2014; Accepted August 21, 2014

ABSTRACT

***trans*-Translation, orchestrated by SmpB and tmRNA, is the principal eubacterial pathway for resolving stalled translation complexes. RNase R, the leading nonstop mRNA surveillance factor, is recruited to stalled ribosomes in a *trans*-translation dependent process. To elucidate the contributions of SmpB and tmRNA to RNase R recruitment, we evaluated *Escherichia coli*–*Francisella tularensis* chimeric variants of tmRNA and SmpB. This evaluation showed that while the hybrid tmRNA supported nascent polypeptide tagging and ribosome rescue, it suffered defects in facilitating RNase R recruitment to stalled ribosomes. To gain further insights, we used established tmRNA and SmpB variants that impact distinct stages of the *trans*-translation process. Analysis of select tmRNA variants revealed that the sequence composition and positioning of the ultimate and penultimate codons of the tmRNA ORF play a crucial role in recruiting RNase R to rescued ribosomes. Evaluation of defined SmpB C-terminal tail variants highlighted the importance of establishing the tmRNA reading frame, and provided valuable clues into the timing of RNase R recruitment to rescued ribosomes. Taken together, these studies demonstrate that productive RNase R-ribosomes engagement requires active *trans*-translation, and suggest that RNase R captures the emerging nonstop mRNA at an early stage after establishment of the tmRNA ORF as the surrogate mRNA template.**

INTRODUCTION

Ribosomes perform the essential task of protein synthesis in all cells. The translation machinery uses genetic information encoded within messenger RNAs (mRNAs) to synthesize new polypeptides. However, defective mRNAs that lack

some of the required information content arise routinely and cause a myriad of problems for the cell. For instance, generation of nonstop mRNAs, mRNAs that lack in-frame stop codons, trigger ribosome stalling and sequestration of the components of the translation machinery (1–6). Such unproductive sequestration of ribosomes could lead to severe reduction in the protein-synthesis capacity of the cell, diminishing its ability to respond to environmental changes and compete for resources with its neighbors.

Nature has evolved a variety of quality control mechanisms to counteract futile ribosome-stalling events, and ensure the efficiency and accuracy of protein synthesis. Among the three pathways discovered in *bacteria*, *trans*-translation is the major mode of translational quality control. The importance of this system is exemplified by the fact that it is essential for survival and/or virulence of several pathogenic bacterial species (7–11). The two key components of this highly conserved system are transfer-mRNA (tmRNA) encoded by the *ssrA* gene (12) and its protein partner Small protein B (SmpB) (13). The 3' and 5' ends of tmRNA fold to form a tRNA-like domain that is recognized and aminoacylated by alanyl-tRNA synthetase. tmRNA also contains an mRNA-like domain with a short open reading frame (ORF) in lieu of the anticodon loop. SmpB is a small globular protein that binds to the tRNA-like domain of tmRNA and delivers it to stalled ribosomes (13). Previous studies have shown that the C-terminal tail of SmpB plays an important role in proper accommodation and accurate decoding of the tmRNA ORF (14,15). Together with elongation factor Tu (EF-Tu) and guanosine triphosphate (GTP), alanine-charged tmRNA and SmpB form a quaternary complex that recognizes stalled ribosomes with an empty A-site, devoid of coding information. Once tmRNA is accommodated in the A-site, the nascent polypeptide chain is transferred to the alanine residue on the tRNA-like domain of tmRNA. By a mechanism that is facilitated in part by amino acid residues in the C-terminal tail of SmpB, the ribosome switches from the defective mRNA template, and starts decoding the tmRNA ORF as a surrogate template (15). The tmRNA ORF also encodes for

*To whom correspondence should be addressed. Tel: +631 632 1688; Fax: +631 632 8575; Email: wali.karzai@stonybrook.edu

an in-frame stop codon that ensures proper termination of protein synthesis, ribosome rescue and recycling of components of the translation machinery. As a consequence, the nascent polypeptide is co-translationally appended with a short peptide sequence (the *ssrA* tag or degron) encoded by the tmRNA ORF, marking the nascent polypeptide for degradation by various cellular proteases (12,16–19).

Persistence of aberrant transcripts in the cell could lead to futile cycles of translation. Targeted elimination of such defective mRNAs is a distinct outcome of the *trans*-translation process. In order to capture and degrade aberrant mRNAs, RNase R, a 3′-5′ exoribonuclease, is recruited to SmpB–tmRNA rescued ribosomes (20). Recent studies have demonstrated that the unique C-terminal lysine-rich domain of RNase R is essential for the *trans*-translation related activity of RNase R (20–22). Investigations into the importance of the tmRNA ORF in nonstop mRNA decay revealed that mutating the last two codons in the tmRNA ORF increase the half-life of nonstop mRNAs in the cell (21). Although it is known that SmpB and tmRNA are essential for selective degradation of defective mRNAs, their precise role in enabling RNase R to perform this targeted task is not clearly understood. It should be noted that although homologues of SmpB, tmRNA and RNase R are present in all sequenced bacterial species (4,5,18,23–27), a systematic study of the conservation of their individual functions has not been performed. Our data suggest that these surveillance factors are active in *Escherichia coli*, *Yersinia pseudotuberculosis*, *Yersinia pestis* and *Francisella tularensis* (7,8,28,29), implying that their individual functions in translation quality assurance might be similarly conserved. Studies in *Caulobacter crescentus* are consistent with this conclusion (30).

The SmpB–tmRNA ribosome rescue complex recognizes and binds stalled ribosome with an empty A-site decoding center. A recent structural model of the ribosome-bound SmpB–tmRNA complex revealed the details of the initial A-site binding stage of the *trans*-translation process (31). Consistent with biochemical studies, upon binding to the stalled ribosome, the C-terminal tail of SmpB occupies the empty mRNA channel, presumably making critical contacts with components of the channel and the A-site decoding center (14,15,32). These putative contacts must subsequently be disrupted in order for the tmRNA ORF to be established as the surrogate mRNA template. Biochemical analyses of SmpB protein show that mutations in the hinge region, especially the highly conserved Gly132 and residues Ile153 and Met154 in the C-terminal tail, abolish proper establishment of tmRNA ORF and the subsequent peptide tagging activity (14,15). The functions of SmpB and tmRNA in directed-proteolysis of nascent polypeptide have been studied in great detail. However, the mechanistic details of when and how the SmpB–tmRNA system facilitates proper engagement of RNase R has remained unexplored.

In studies described here, we present biochemical evidence that provide insights into the effect of SmpB and tmRNA on the timing and productive engagement of RNase R on rescued ribosomes. In an effort to decipher the contributions of specific SmpB and tmRNA sequence elements to nonstop mRNA decay, we designed hybrid versions of SmpB and tmRNA, from genetically distant bacterial

species, in which the mRNA-like domain of tmRNA or the C-terminal tail of SmpB from *E. coli* was substituted with the equivalent sequences from *F. tularensis*. From the study of hybrid constructs, we observed that while the tmRNA ORF hybrid was functionally active in rescuing stalled ribosomes and tagging the associated nascent polypeptides, it exhibited significant defects in the recruitment of RNase R to stalled ribosomes. Furthermore, these defects were not mitigated when the cognate SmpB C-terminal tail hybrid was co-expressed. Investigation of the tmRNA hybrid suggested that the sequence composition, positioning, and length of the tmRNA ORF play a key role in RNase R recruitment to the translation machinery. To gain further mechanistic insights, we used defined SmpB and tmRNA variants that impact distinct stages of the *trans*-translation process. This analysis revealed that the identity and positioning of sequence elements within the distal part of the tmRNA ORF play decisive roles in the facilitating RNase R enrichment on stalled ribosomes to initiate nonstop mRNA decay. Taken together, our investigations demonstrate that productive engagement of RNase R with rescued ribosome requires the presence of specific tmRNA sequence elements, and occurs at an early stage of the *trans*-translation process after establishment of the tmRNA ORF as the surrogate template.

MATERIALS AND METHODS

Strains and plasmid

Escherichia coli strain W3110 *rnr::kan^r ΔsmpBssrA* was obtained by P1 transduction using Keio deletion strains as donors (33). The coding region of the N-terminal domain of λ-cI protein was used as the reporter gene (pλ-*cI*-nonstop or pλ-*cI*-NS). A corresponding stop reporter plasmid (pλ-*cI*-stop or pλ-*cI*-S) was synthesized by introducing two tandem stop codons (TAA) before the transcriptional terminator sequence by polymerase chain reaction (PCR) based site-directed mutagenesis. Both reporter constructs have a six-histidine (His6) epitope tag at the 5′ end of their coding region. The pACYCDuet-1 plasmid, harboring the *E. coli smpB-ssrA* genes under their native promoter in MCS-1 and *E. coli* or *F. tularensis*-derived *rnr* gene under the P_{BAD} promoter in MCS-2, was used as a template to synthesize single and double hybrids of SmpB and tmRNA (pBA-R). *E. coli*-*F. tularensis* hybrid of SmpB (SmpB^{FT}) was synthesized by swapping the region coding for residues 128–160 of *E. coli* SmpB with the region coding for residues 128–157 of *F. tularensis* SmpB. tmRNA ORF hybrid (tmRNA^{FT}) was synthesized by swapping tmRNA ORF and helix 5 of *E. coli* (bases 90–150) with that of *F. tularensis* (bases 110–200). SmpB I154D/M155E and SmpB G132E variants were obtained by PCR based site-directed mutagenesis. The last two alanine codons of *E. coli* tmRNA ORF in pBA-R plasmid were mutagenized to two aspartic acid codons (pBA^{DD}-R) by PCR based site-directed mutagenesis. To generate pBA^{ED}-R plasmid, encoding the tmRNA^{ED} variant, codons for aspartic acid and glutamic acid at positions three and four of the tmRNA ORF were swapped by PCR based site-directed mutagenesis. The plasmid constructs used in this study are listed in the Table 1.

Table 1. List of plasmids used in this study

Plasmid	MCS1	MCS2
pBA-R	SmpB-tmRNA from <i>Escherichia coli</i>	RNase R from <i>E. coli</i>
pB ^{FT} A-R	SmpB C-terminal tail swapped with that of <i>Francisella tularensis</i> (residues 129–157)	RNase R from <i>E. coli</i>
pBA ^{FT} -R	tmRNA ORF swapped with that of <i>F. tularensis</i>	RNase R from <i>E. coli</i>
pBA-R ^{FT}	SmpB-tmRNA from <i>E. coli</i>	RNase R from <i>F. tularensis</i>
pB ^{FT} A-R ^{FT}	SmpB C-terminal tail swapped with that of <i>F. tularensis</i>	RNase R from <i>F. tularensis</i>
pBA ^{FT} -R ^{FT}	tmRNA ORF swapped with that of <i>F. tularensis</i>	RNase R from <i>F. tularensis</i>
pB ^{DE} A-R	SmpB variant I154D and M155E	RNase R from <i>E. coli</i>
pB ^{G132E} A-R	SmpB variant G132E	RNase R from <i>E. coli</i>
pBA ^{DD} -R	tmRNA ^{DD} (A9D and A10D)	RNase R from <i>E. coli</i>
pBA ^{ED} -R	tmRNA ^{ED} (D3E and E4D)	RNase R from <i>E. coli</i>

Northern blot analysis

Escherichia coli W3110 *rnr::kan^r ΔsmpBssrA* harboring pBA-R, pBA-R^{FT} or pBA-R based SmpB–tmRNA variants was co-transformed with the pλ-*cI*-NS or pλ-*cI*-S reporter mRNA expression plasmids. Overnight cultures of the strains were diluted 1:100× in Luria-Bertani Broth containing 0.01% arabinose, 100 μg/ml of Ampicillin and 30 μg/ml of Chloramphenicol. The cultures were grown at 37°C, with vigorous shaking at 180 rpm. Upon reaching mid-log OD₆₀₀, the reporter gene expression was induced by the addition of 1 mM final concentration of isopropyl β-D-thiogalactoside (IPTG) for one hour. For rifampicin chase assay, 250 μl of rifampicin (34 mg/ml) was used to inhibit transcription, and the assay was performed as described (20). Equal number of cells were harvested and total RNA was isolated using TRI Reagent as per manufacturer's instruction (MRC Inc.) Equal amounts of total RNA from each sample were resolved by electrophoresis on 1.5% formaldehyde-agarose gels. The RNA was transferred to Hybond N+ membrane (GE Healthcare Life Sciences) overnight. Biotin-labeled DNA oligonucleotide probe 5'-TTCATAAATTGCTTTAAGGC GACGTGCGTCTCAAGCTGCTCTTGTGTTA-3' was hybridized to the membrane for 16 h at 42°C. The membrane was developed using BrightStar[®] BioDetect[™] Non-isotopic Detection Kit (Applied Biosystems) and exposed to HyBlot CL[®] autoradiography films. The bands were scanned and quantified using Image-J (<http://rsbweb.nih.gov/ij/>).

Western blot analysis

Harvested cell pellets were resuspended in 1X Laemmli sample buffer, and boiled at 100°C for 30 min. For the purpose of analyzing the levels of RNase R, the samples were resolved by electrophoresis on 10% sodium dodecyl sulphate (SDS)-polyacrylamide gels. Resolved proteins were transferred to polyvinylidene difluoride (PVDF) membrane. Western blot analysis was performed using primary polyclonal anti-RNase R antibody and secondary donkey anti-rabbit antibody conjugated to horseradish peroxidase (HRP). The bands were quantified using Image-J. To analyze lambda reporter gene expression, the samples were resolved by electrophoresis on 15% Tris–Tricine polyacrylamide gels. Resolved proteins were transferred to PVDF membranes. Western blot analysis was performed using primary monoclonal anti-His6 antibody and secondary goat

anti-mouse antibody conjugated to a fluorescent dye. The bands were detected using the Odyssey Infrared Imaging System.

Ribosome enrichment assay

This assay was performed as per previously published protocol (20,34). *E. coli* W3110 *rnr::kan^r ΔsmpB ΔssrA* harboring pBA-R, pBA-R^{FT} or pBA-R based SmpB–tmRNA variants was co-transformed with pλ-*cI*-NS or pλ-*cI*-S. Cells were grown in Luria-Bertani (Miller) broth containing 100 μg/ml of Ampicillin, 30 μg/ml of Chloramphenicol and 0.02% arabinose until OD₆₀₀ ~0.8. Reporter gene expression was induced using 1 mM final concentration of IPTG for one hour. Harvested cells were resuspended in enrichment buffer (50 mM Tris–HCl pH 7.5, 300 mM NH₄Cl, 20 mM MgCl₂, 2 mM β-ME and 10 mM Imidazole) and lysed by French press. Clarified cell lysate was layered onto 32% sucrose solution in enrichment buffer and ribosomes were pelleted by ultracentrifugation at 100 000 × g for 16–18 h. Ribosomes translating the reporter mRNA were separated from the tight-coupled ribosomes by Ni²⁺-Nitrilotriacetic acid (NTA) chromatography. Reporter-enriched ribosomes were eluted with 250 mM imidazole in the lysis buffer. Specific rescue factors, RNase R or SmpB, associated with the ribosomes were detected by western blot analysis.

RESULTS

Nonstop mRNA accumulates in the presence of tmRNA^{FT} hybrid

SmpB and tmRNA perform the intricate task of *trans*-translation. A unique consequence of *trans*-translation is the selective elimination of aberrant mRNAs that cause ribosome stalling. Given the central role played by the C-terminal tail of SmpB and the mRNA-like domain of tmRNA, we sought to ascertain whether specific sequence determinants within these regions contributed to nonstop mRNA decay. To identify key SmpB and tmRNA sequence elements that facilitate nonstop mRNA decay, we engineered an SmpB hybrid composed of the RNA-binding core of the *E. coli* SmpB protein and the C-terminal tail of the *F. tularensis* SmpB, generating SmpB^{FT} and a tmRNA hybrid where the *ssrA*-peptide coding sequence of *E. coli* tmRNA was replaced with the corresponding sequence of *F. tularensis* tmRNA, generating tmRNA^{FT}. Using various combina-

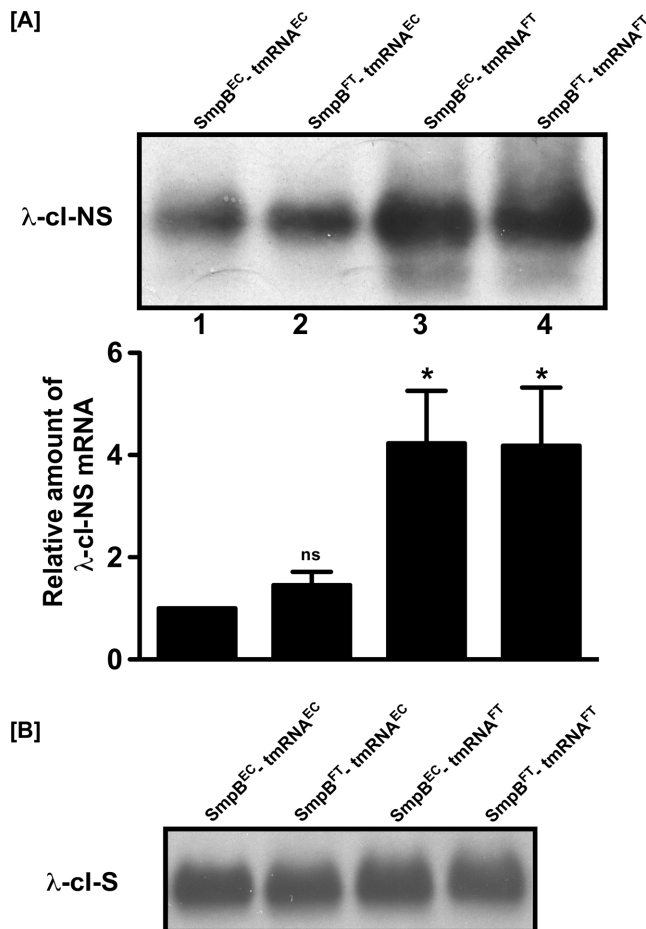


Figure 1. tmRNA ORF alterations lead to accumulation of nonstop mRNA. (A) The top panel depicts a representative northern blot showing the steady state abundance of the λ -cl-NS nonstop mRNA in the presence of the hybrid variants of SmpB and tmRNA. The accumulation of nonstop mRNA in the presence of single and double tmRNA ORF hybrids (lanes 3 and 4) is \sim 4-fold higher than that of *Escherichia coli* derived SmpB-tmRNA. *P*-values were calculated by performing Student's *t*-test analysis with SmpB-tmRNA and SmpB^{FT}-tmRNA (*ns*, not statistically significant), SmpB-tmRNA and SmpB-tmRNA^{FT} ($*P = 0.035$), or SmpB-tmRNA and SmpB^{FT}-tmRNA^{FT} ($*P = 0.049$). The accompanying graph represents the fold change in the steady state level of λ -cl-NS mRNA in the presence of indicated SmpB and tmRNA variants with respect to SmpB-tmRNA. The data are from three independent experiments (mean \pm SEM, standard error of means). (B) A representative northern blot showing similar steady state levels of the control λ -cl-S stop mRNA in the presence of indicated SmpB and tmRNA hybrids.

tions of single and double hybrid constructs in an *smpB*ssrA deficient strain, we determined the steady state abundance of the λ -cl-NS nonstop mRNA—which lacks an in-frame stop codon and promotes ribosome stalling—as a reporter of SmpB-tmRNA mediated ribosome rescue and nonstop mRNA decay (Figure 1A). As a negative control throughout these studies, we used the related stop codon containing λ -cl-S mRNA, which does not elicit ribosome stalling or require SmpB-tmRNA activity. Consistent with previous studies, analysis of these reporter mRNAs showed that the λ -cl-NS mRNA does not accumulate in the presence of wild-type (WT) *E. coli* surveillance factors (SmpB, tmRNA and RNase R). By contrast, there is \sim 4-fold increase

in the accumulation of λ -cl-NS mRNA in the presence of the tmRNA^{FT} hybrid, suggesting that specific tmRNA determinants are required for proper engagement of RNase R on stalled ribosomes. We have recently shown that while the tmRNA^{FT} hybrid could productively engage stalled ribosomes, it is unable to accurately establish the correct tmRNA reading frame (15). However, cognate hybrid SmpB (SmpB^{FT}), carrying the *F. tularensis* C-terminal tail, is required to restore accurate tagging activity. Since the *F. tularensis* tmRNA ORF sequence is highly divergent from that of *E. coli* tmRNA (Supplementary Figure S1), it was possible that *E. coli* SmpB was unable to make functionally relevant contacts with tmRNA^{FT} to promote nonstop mRNA degradation. We reasoned that the observed nonstop mRNA decay defect of tmRNA^{FT} might be due to the absence of cognate SmpB^{FT}. Remarkably, co-expression of the SmpB^{FT} and tmRNA^{FT} hybrids did not mitigate the observed nonstop mRNA degradation defect associated with tmRNA^{FT} (Figure 1A). In contrast, expression of SmpB^{FT} alongside *E. coli* tmRNA (SmpB^{FT}-tmRNA) resulted in clearance of the nonstop mRNA to levels indistinguishable from cells expressing WT *E. coli* SmpB and tmRNA (Figure 1A). This result indicated that SmpB^{FT} makes the required contacts with *E. coli* tmRNA, and likely the translation machinery, to facilitate RNase R enrichment and nonstop mRNA decay. To rule out the possibility that there was a general increase in the reporter mRNA abundance in the presence of the tmRNA^{FT} hybrid, we analyzed the steady state level of the corresponding stop-codon containing control reporter mRNA (λ -cl-S) by northern blot analysis. This evaluation showed that the levels of λ -cl-S mRNA in the presence of single and double hybrids were indistinguishable from cells expressing WT SmpB and tmRNA (Figure 1B), suggesting that the observed mRNA decay defect was specific to the *trans*-translation related deficiencies of the tmRNA^{FT} hybrid. Taken together, these data suggested that swapping the *E. coli* tmRNA ORF with the corresponding *F. tularensis* sequence selectively impacted nonstop mRNA decay.

SmpB^{FT} and tmRNA^{FT} hybrids are active in tagging and ribosome rescue

Previous studies have shown that tmRNA nucleotides 86–122, in the region encompassing the mRNA-like domain, play a crucial role in the peptide tagging activity of tmRNA (15,21). Therefore, it was possible that the tmRNA^{FT} hybrid was compromised in its ability to participate in *trans*-translation. To assess this possibility, we examined the capacity of the SmpB-tmRNA^{FT} single and SmpB^{FT}-tmRNA^{FT} double hybrid to co-translationally tag the λ -cl protein, the product of the nonstop reporter mRNA. As expected, and consistent with previous observations (15), this analysis showed that both hybrid constructs were equally proficient in co-translationally appending the *ssrA* degron to the reporter polypeptide, demonstrating that stalled ribosomes were successfully recognized and rescued by the SmpB^{FT}-tmRNA^{FT} double hybrid (Figure 2A).

SmpB and tmRNA regulate the intracellular stability of RNase R (35). Therefore, we examined whether the absence of either cognate *E. coli* SmpB or *E. coli* tmRNA altered

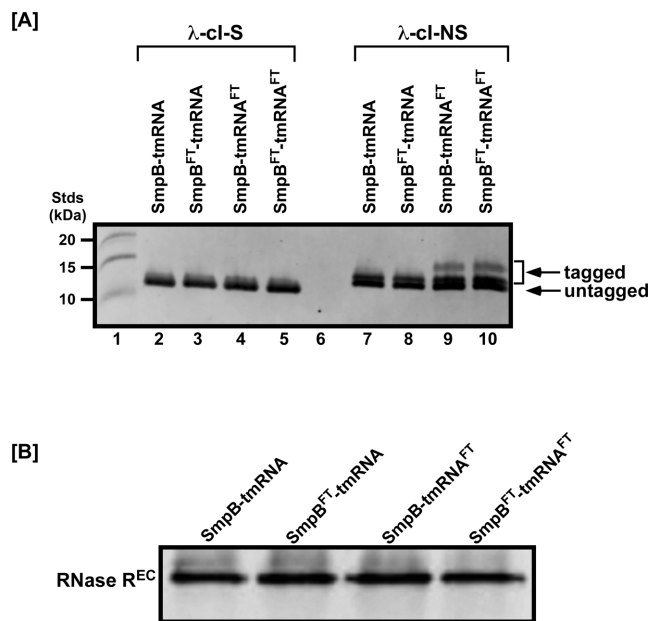


Figure 2. tmRNA ORF alterations do not affect the tagging activity or the steady state levels of RNase R. (A) A representative western blot showing the expression levels of the λ -cl-S stop (lanes 2–5) and nonstop (lanes 7–10) reporter protein in the presence of indicated SmpB and tmRNA hybrids. The λ -cl reporter protein is tagged in the presence of SmpB–tmRNA hybrids whereas the corresponding stop reporter is untagged. (B) A representative western blot showing similar steady state levels of *Escherichia coli* RNase R (RNase R^{EC}) in the presence of indicated SmpB–tmRNA hybrids.

the *in vivo* stability of RNase R, thus resulting in the observed differences in the abundance of nonstop mRNA. To assess this possibility, we compared the steady state levels of RNase R in cells expressing SmpB–tmRNA with cells expressing various combinations of the SmpB^{FT} and tmRNA^{FT} hybrids. This analysis showed that the SmpB^{FT} and tmRNA^{FT} hybrids had no measurable impact on the steady state level of RNase R (Figure 2B).

The tmRNA^{FT} hybrid affects RNase R enrichment on stalled ribosomes

We reasoned that the observed accumulation of the nonstop mRNA in cells expressing tmRNA^{FT} could be due to a defect in facilitating recruitment of RNase R to stalled ribosomes. This possibility was compelling as it suggested that the SmpB–tmRNA mediated peptide tagging and ribosome rescue were insufficient for productive engagement of RNase R on rescued ribosomes. Moreover, it suggested that tmRNA sequence elements must make additional discrete contacts with the translation machinery to promote effective RNase R engagement-contacts that could not be made by the divergent mRNA-like domain of tmRNA^{FT}. Furthermore, these contacts must be unique to the tmRNA ORF and independent of SmpB, since co-expressing SmpB^{FT} did not alleviate the nonstop mRNA defect of tmRNA^{FT}. The inability to establish such crucial contacts could lead to accumulation of nonstop mRNA in the cell. In order to ascertain whether RNase R could productively engage tmRNA^{FT}-rescued ribosomes, we performed ribo-

some enrichment assays (20,34,36). Ribosomes decoding either the λ -cl-NS or the control λ -cl-S reporter mRNAs, both of which carry an N-terminal His6 epitope tag, were captured by Ni²⁺-NTA affinity chromatography from individual pools of tight-coupled ribosomes under high stringency conditions (34). The captured ribosomes were subjected to western blot analysis to assess the recruitment of RNase R to rescued ribosomes in the presence of the various combinations of single and double SmpB–tmRNA hybrids (Figure 3). Using this analysis, we observed a 3-fold enrichment of RNase R on ribosomes in the presence of the SmpB^{FT}–tmRNA hybrid, suggesting that RNase R was able to productively engage tmRNA-rescued ribosome and capture the nonstop mRNA. In contrast, RNase R failed to productively engage on ribosomes rescued by the SmpB–tmRNA^{FT} and SmpB^{FT}–tmRNA^{FT} hybrids (Figure 3A and B). These results suggested that the tmRNA^{FT} hybrid, despite being capable of peptide tagging and ribosome rescue, is unable to establish the necessary contacts required for facilitating RNase R recruitment to rescued ribosomes. We routinely observe a low background level association of RNase R with ribosomes translating the control λ -cl-S reporter mRNA. Therefore, we report enrichment of RNase R on ribosome translating the λ -cl-NS mRNA relative to the control λ -cl-S reporter. Furthermore, to ensure that we were evaluating SmpB–tmRNA rescued ribosomes, we also assessed enrichment of the SmpB–tmRNA rescue complex on ribosomes translating the λ -cl-S and λ -cl-NS reporter mRNAs. Consistent with previous studies (14,34,36), this analysis showed the expected enrichment of SmpB on ribosomes translating the λ -cl-NS reporter mRNA (Figure 3C). Curiously, ribosomes rescued from the λ -cl-NS nonstop mRNA by the tmRNA^{FT} hybrid routinely yielded RNase R enrichment levels that were below the control λ -cl-S reporter. Taken together, these data suggest that the tmRNA ORF contains additional elements that make discrete functional contacts with the translation machinery to facilitate effective engagement of RNase R on rescued ribosomes. We infer from these findings that peptide tagging and the productive engagement of RNase R on stalled ribosomes are two independent attributes of the SmpB–tmRNA mediated *trans*-translation process.

RNase R^{FT} does not mitigate the nonstop mRNA decay defect of tmRNA^{FT}

It was conceivable that the observed nonstop mRNA decay defect of the tmRNA^{FT} hybrid was due to incompatibility of the *E. coli* RNase R (RNase R^{EC}) with sequence elements within the mRNA-like domain of tmRNA^{FT}. Alternatively, it was also possible that changes in the coding region of tmRNA^{FT} had an indirect effect on RNase R recruitment, owing to alterations in interactions of the mRNA-like domain of tmRNA^{FT} with the translation machinery. To distinguish between these possibilities, we expressed *F. tularensis* RNase R (RNase R^{FT}) along with various combinations of SmpB and tmRNA hybrids, and analyzed the steady state level of the λ -cl-NS reporter mRNA. Expression of RNase R^{FT} and *E. coli* SmpB–tmRNA resulted in a basal level accumulation of the λ -cl-NS reporter that was indistinguishable from that of RNase R^{EC} (Figure 4A). Remark-

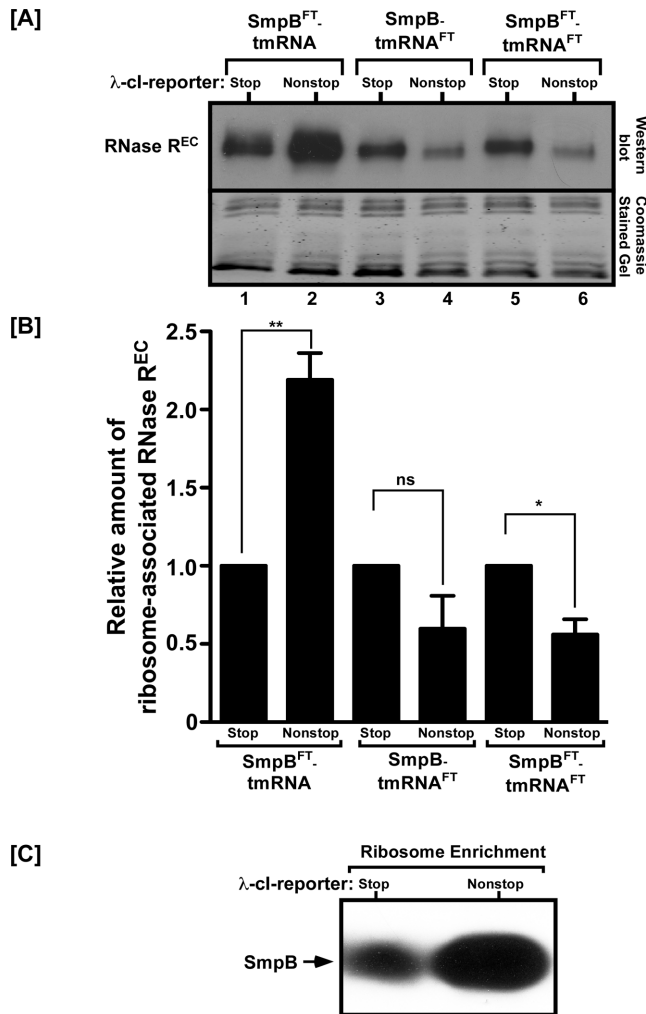


Figure 3. Ribosomes rescued by hybrid tmRNA exhibit defects in RNase R recruitment. (A) Total ribosomes were obtained from cells co-expressing the indicated SmpB and tmRNA hybrid variants with the λ-cl-S or λ-cl-NS reporter mRNAs. Ribosomes translating the reporter mRNAs were isolated from the total ribosomes pool using Ni²⁺-NTA affinity chromatography. Equal numbers of captured ribosomes were resolved by electrophoresis on 10% SDS-polyacrylamide gels. Western blot analysis was performed to detect the presence of *Escherichia coli* RNase R (RNase R^{EC}). The intensity of the band corresponding to RNase R^{EC} was quantified using ImageJ. The top panel shows a representative western blot, using an RNase R^{EC} specific antibody, and the bottom panel shows a section of the corresponding Coomassie stained gel displaying equal protein loading. (B) The graph represents fold RNase R^{EC} enrichment on ribosomes translating the λ-cl-NS reporter compared to ribosomes translating the control λ-cl-S reporter (mean ± SEM). *P*-values were calculated by performing Student's *t*-test analysis on the association level of RNase R^{EC} with ribosomes translating λ-cl-S and λ-cl-NS in the presence of SmpB^{FT}-tmRNA (***P* = 0.0022, *n* = 5), SmpB-tmRNA^{FT} (ns, *n* = 4), or SmpB^{FT}-tmRNA^{FT} (**P* = 0.01, *n* = 5). (C) A representative western blot showing SmpB^{EC} is enriched on ribosomes translating the λ-cl-NS reporter. Ribosomes translating the stop and nonstop reporter mRNAs were isolated from the total ribosomes pool using Ni²⁺-NTA affinity chromatography. Equal numbers of captured ribosomes were resolved by electrophoresis on 10% SDS-polyacrylamide gels. Western blot analysis was performed to detect the presence of *E. coli* SmpB.

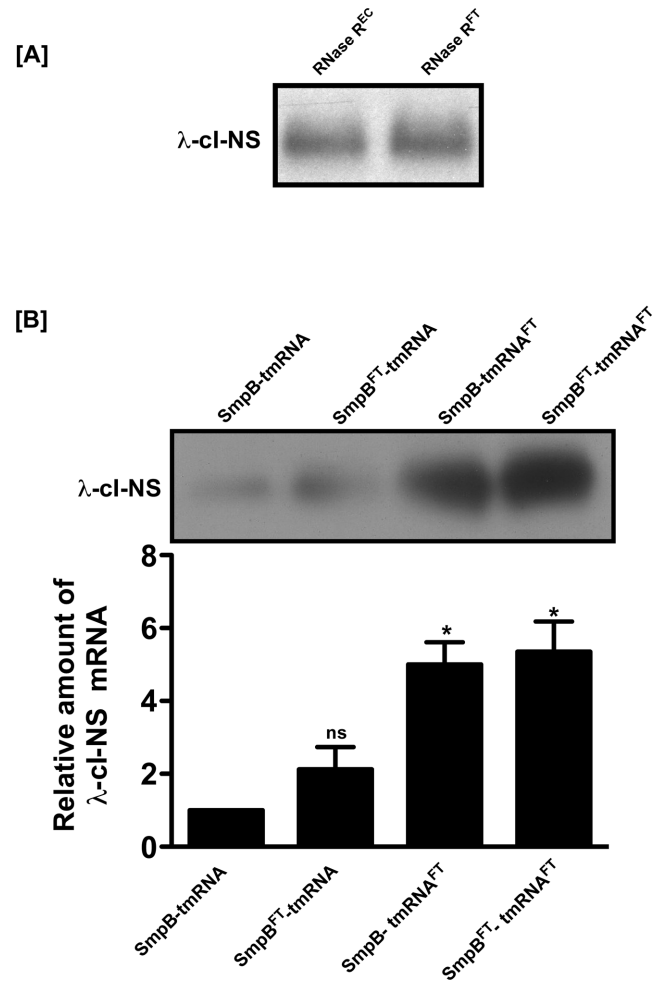


Figure 4. tmRNA ORF alterations lead to nonstop mRNA accumulation in the presence of *Francisella tularensis* RNase R. (A) A representative northern blot showing similar steady state abundance of λ-cl-NS nonstop mRNA in the presence of *Escherichia coli*- and *F. tularensis*-derived RNase R (RNase R^{EC} and RNase R^{FT}), respectively. (B) The top panel depicts a representative northern blot showing the steady state level of λ-cl-NS nonstop mRNA in the presence of the indicated SmpB and tmRNA hybrid variants and RNase R^{FT}. The level of nonstop mRNA accumulation in the presence of single and double tmRNA ORF hybrids (lanes 3 and 4) is ~5-fold higher than that of *E. coli* derived SmpB-tmRNA. *P*-values were calculated by performing Student's *t*-test analysis with SmpB-tmRNA and SmpB^{FT}-tmRNA (*P* = ns), SmpB-tmRNA and SmpB-tmRNA^{FT} (**P* = 0.007), or SmpB-tmRNA and SmpB^{FT}-tmRNA^{FT} (**P* = 0.0135). The accompanying graph shows the fold change in the steady state level of λ-cl-NS mRNA in the presence of indicated SmpB and tmRNA variants with respect to WT *E. coli* SmpB-tmRNA. The data are from four independent experiments (mean ± SEM).

ably, co-expression of RNase R^{FT} with the SmpB^{FT} hybrid (SmpB^{FT}-tmRNA) resulted in low basal level accumulation of the λ-cl-NS reporter, indicating that RNase R^{FT} was functional in the context of the *E. coli* trans-translation and that the difference between the C-terminal tails of *E. coli* and *F. tularensis* SmpB did not play a role in facilitating RNase R enrichment. Surprisingly, co-expression of RNase R^{FT} with tmRNA^{FT} (SmpB-tmRNA^{FT}) resulted in accumulation of ~5-fold higher levels of the nonstop mRNA in these cells (Figure 4B). Furthermore, the nonstop mRNA

accumulation defect elicited by tmRNA^{FT} was not alleviated in the presence of SmpB^{FT}, wherein the functional pair was operating in concert to rescue stalled ribosome (Figure 4B). These data suggested that distinct sequence elements within the mRNA-like domain of tmRNA make specific contacts with the translation apparatus to facilitate RNase R recruitment.

tmRNA ORF elements are essential for RNase R recruitment to stalled ribosomes

We inferred from the aforementioned observations that the key elements involved in facilitating productive engagement of RNase R with stalled ribosomes reside within the tmRNA ORF. We reasoned that these sequence elements might play an important role in facilitating RNase R recruitment to stalled ribosomes. To test this hypothesis, we used a tmRNA variant where the two terminal alanine codons are changed to aspartic acid codons (tmRNA^{A9D/A10D}, henceforth tmRNA^{DD}). The tmRNA^{DD} variant is known to be proficient in peptide tagging and ribosome rescue (8,12,16). To examine the effect of these defined tmRNA sequence changes on selective nonstop mRNA decay, we performed ribosome enrichment and nonstop mRNA stability assays in *smpB* *ssrA* *rnj::kan* cells expressing *E. coli* RNase R, SmpB and tmRNA^{DD}. We evaluated the association of RNase R with ribosomes stalled on the λ -ci-NS reporter. We observed that introduction of these modest tmRNA ORF changes were sufficient to disrupt the productive recruitment of RNase R to tmRNA-rescued ribosomes (Figure 5). Furthermore, the disruptive effect of the tmRNA^{DD} variant was specific to this segment of the tmRNA ORF, as equivalent alterations elsewhere, for instance the third and fourth codons (tmRNA^{D3E/E4D}, henceforth tmRNA^{ED}), did not hamper RNase R association with stalled ribosomes (Figure 5). We also examined stability of the λ -ci-NS reporter mRNA in the presence of the tmRNA^{DD} variant and observed a roughly 2.5-fold increase in the steady-state level (Figure 6) and half-life ($T_{1/2}$) of the reporter mRNA (Supplementary Figure S2). These data indicated that specific tmRNA sequence elements were important for facilitating RNase R ribosome enrichment and the ensuing nonstop mRNA decay.

RNase R captures defective mRNA after establishment of the tmRNA ORF

Previous studies have demonstrated that enrichment of RNase R on stalled ribosomes is dependent on the activity of the SmpB–tmRNA rescue complex (20–22,37,38). Yet, the precise step at which RNase R performs its function has not been determined. In an effort to elucidate the mechanistic time frame during which RNase R initiates defective mRNA degradation, we utilized SmpB mutants that impair specific stages of the *trans*-translation process. We have shown that key residues in the C-terminal tail of SmpB play a critical role in engaging the ribosome and establishing the tmRNA ORF (14,15). For instance, glycine 132 (Gly132) acts as a hinge that confers flexibility on the C-terminal tail of SmpB, thus enabling proper positioning and establishment of the correct reading frame on the tmRNA ORF

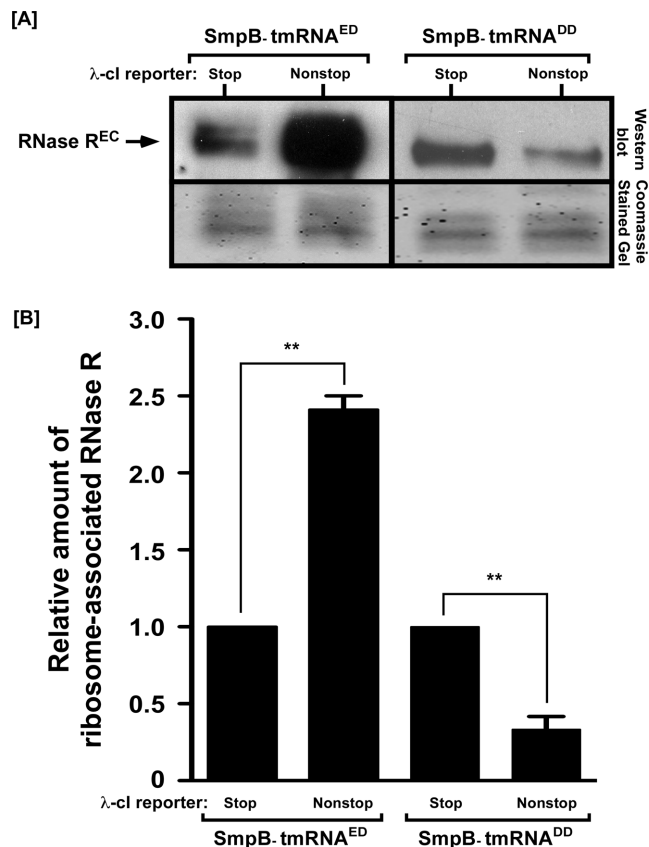


Figure 5. *Escherichia coli* RNase R exhibits defects in enrichment on ribosomes rescued by tmRNA^{DD}. (A) Total ribosomes were obtained from cells expressing the tmRNA^{ED} or the tmRNA^{DD} variants with the λ -ci-S or λ -ci-NS reporter mRNAs. Ribosomes translating the reporter mRNAs were isolated from the total ribosomes pool using Ni²⁺-NTA affinity chromatography. Equal numbers of ribosomes were resolved by electrophoresis on 10% SDS-polyacrylamide gels. Western blot analysis was performed to detect the presence of wild type *E. coli* RNase R. The intensity of the band corresponding to RNase R was quantified using ImageJ. The top panel shows a representative Western blot, using an RNase R specific antibody, and the bottom panel shows a section of the Coomassie stained gel displaying equal protein loading. (B) The graph represents fold RNase R enrichment on ribosomes translating the λ -ci-NS reporter compared to ribosomes translating the control λ -ci-S reporter (mean \pm SEM). *P*-values were calculated by performing Student's *t*-test analysis on the association level of RNase R with ribosomes translating λ -ci-S and λ -ci-NS mRNAs in the presence of tmRNA^{ED} (***P* = 0.004, *n* = 3) or tmRNA^{DD} (***P* = 0.0042, *n* = 4).

(15). Substitution of Gly132 with glutamate (SmpB^{G132E}) prevents accommodation of tmRNA in the ribosomal A-site. Isoleucine 154 and Methionine 155 have been shown to play a crucial role in the tmRNA tagging activity (14). Substitution of these SmpB residues with negatively charged aspartic acid and glutamic acid (SmpB^{DE}) results in substantial defects in its *trans*-translation function, with retention of very low level of tagging activity (14). Interestingly, both SmpB C-terminal tail variants are fully capable of binding tmRNA and delivering it to stalled ribosomes.

We sought to determine the effect of these SmpB variants on selective nonstop mRNA decay by RNase R. To this end, we evaluated the steady state abundance of the λ -ci-NS reporter mRNA in the presence of WT *E. coli* RNase

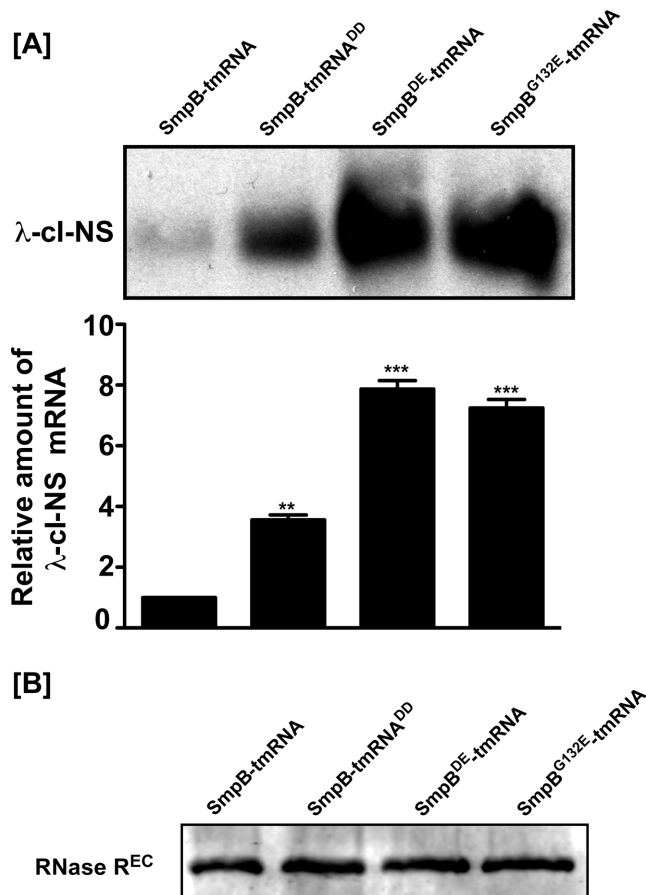


Figure 6. RNase R initiates nonstop mRNA decay after establishment of the tmRNA reading frame. (A) A representative northern blot showing the steady state abundance of λ -cl-NS nonstop mRNA in the presence of SmpB-tmRNA, SmpB-tmRNA^{DD}, SmpB^{DE}-tmRNA and SmpB^{G132E}-tmRNA. The accompanying graph shows the relative steady state abundance of the λ -cl-NS nonstop mRNA in the presence of indicated variants with reference to SmpB-tmRNA. RNase R exhibits severe defect in degrading nonstop mRNA when ribosomes are rescued by SmpB^{DE}-tmRNA or SmpB^{G132E}-tmRNA. RNase R exhibits a moderate defect in degrading nonstop mRNA in the presence of SmpB-tmRNA^{DD}, wherein the reading frame is accurately established. *P*-values were calculated by performing Student's *t*-test analysis on the relative steady state abundance of the λ -cl-NS nonstop mRNA in the presence of SmpB-tmRNA and SmpB-tmRNA^{DD} (***P* = 0.0039), SmpB-tmRNA and SmpB^{DE}-tmRNA (***P* = 0.0017) or SmpB-tmRNA and SmpB^{G132E}-tmRNA (***P* = 0.002). The data are from three independent experiments (mean \pm SEM). (B) A representative western blot showing similar expression levels of RNase R in the presence of indicated SmpB and tmRNA variants.

R and either the SmpB^{DE} or SmpB^{G132E} variant and observed that RNase R-mediated nonstop mRNA degradation was markedly hindered (Figure 6A). The level of nonstop mRNA accumulation in the presence of the SmpB^{DE} and SmpB^{G132E} variants was \sim 2.5-fold higher than that of tmRNA^{DD} variant. This difference in nonstop reporter accumulation cannot be attributed to the variation in the steady state expression level of RNase R in presence of SmpB and tmRNA variants (Figure 6B), and was thus likely due to a defect in the specific capture of the nonstop mRNA. To scrutinize this inference, we performed ribosome enrichment assay in the presence of the SmpB^{DE}

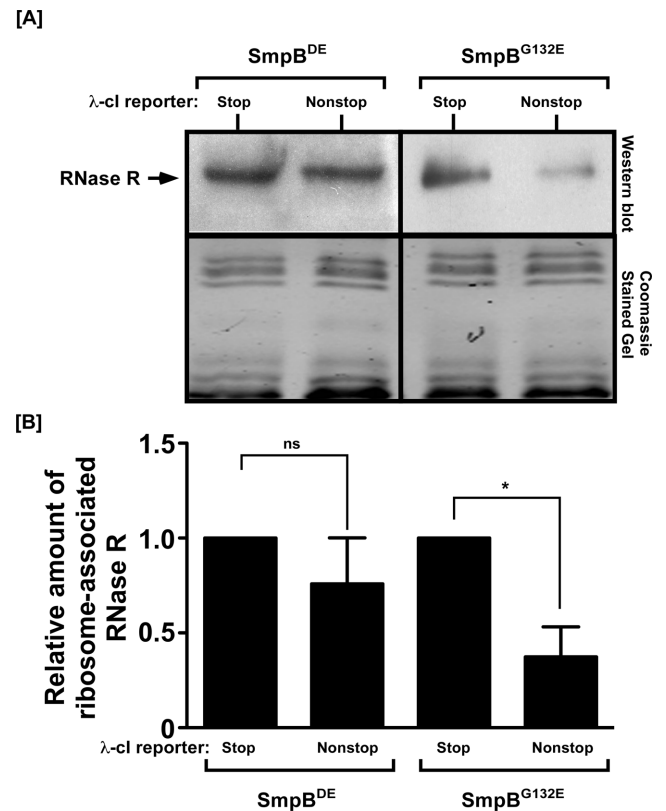


Figure 7. *Escherichia coli* RNase R exhibits defects in enrichment on ribosomes rescued by SmpB C-terminal tail variants. (A) Total ribosomes were obtained from cells co-expressing SmpB^{DE} or SmpB^{G132E} variants with the λ -cl-S or λ -cl-NS reporter mRNAs. Ribosomes translating the reporter mRNAs were isolated from the total ribosomes pool using Ni²⁺-NTA affinity chromatography. Equal numbers of ribosomes were resolved by electrophoresis on 10% SDS-polyacrylamide gels. Western blot analysis was performed to detect the presence of RNase R. The intensity of the band corresponding to RNase R was quantified using ImageJ. The top panel shows a representative western blot, using an RNase R specific antibody, and the bottom panel shows a section of the corresponding Coomassie stained gel displaying equal protein loading. (B) The graph represents fold RNase R enrichment on ribosomes translating the λ -cl-NS reporter compared to ribosomes translating the control λ -cl-S reporter. The data are from three independent experiments (mean \pm SEM). *P*-values were calculated by performing Student's *t*-test analysis on the association level of RNase R with ribosomes translating λ -cl-S and λ -cl-NS in the presence of SmpB^{DE} (*P* = ns) and SmpB^{G132E} (**P* = 0.022).

and SmpB^{G132E} variants and assessed RNase R recruitment to ribosomes translating the λ -cl-NS reporter mRNA. This analysis revealed that both SmpB variants were incapable of facilitating RNase R enrichment on stalled ribosomes (Figure 7). Although there was a substantial defect in RNase R enrichment with the SmpB C-terminal tail and tmRNA ORF variants (Figure 7), we consistently observed a significantly greater defect in nonstop mRNA degradation in the presence of SmpB^{DE} and SmpB^{G132E}, as compared to tmRNA^{DD} (Figure 6A). We reasoned that since the peptide tagging activity and ORF establishment activities of the tmRNA^{DD} variant are not compromised, stalled ribosomes undergo proper termination, and the ribosomal subunits are efficiently recycled. As a result, the aberrant mRNA is exposed to cellular ribonucleases for degradation,

resulting in the observed relatively lower abundance of nonstop mRNA with the tmRNA^{DD} variant as compared to the SmpB^{DE} and SmpB^{G132E} variants.

We infer from the sum of these results that the productive recruitment of RNase R to stalled ribosomes requires accurate establishment of the tmRNA ORF. We postulate that specific sequence elements in the distal portion of the mRNA-like domain of tmRNA makes distinct contacts with the translation apparatus to facilitate optimal engagement of RNase R on stalled ribosomes, thus enabling the specific capture and targeted decay of nonstop mRNAs.

DISCUSSION

The SmpB–tmRNA complex orchestrates the elaborate process of *trans*-translation, marshaling stalled ribosome to resume translation and proceed to normal termination and recycling. A hallmark of *trans*-translation is the RNase R-mediated targeted decay of nonstop mRNAs. Given that recruitment of RNase R to stalled ribosomes is reliant on SmpB and tmRNA, it has been of particular interest to delineate SmpB and tmRNA sequence elements that influence this targeted RNase R function. Our investigations reveal that the mere recognition of stalled ribosomes by the SmpB–tmRNA complex is insufficient for productive recruitment of RNase R to rescued ribosomes. The robust degradation of nonstop mRNA by the *E. coli* and *F. tularensis* RNase R, in the context of SmpB^{FT}–tmRNA^{EC}, demonstrates the requirement for active *trans*-translation in facilitating recruitment of RNase R to stalled ribosomes. This result also suggests that a functional SmpB C-terminal tail, capable of promoting establishment of the tmRNA ORF, is sufficient to engage RNase R in nonstop mRNA decay. Analyses of defined SmpB C-terminal tail variants, with distinct defects in establishment of the tmRNA ORF, demonstrate that RNase R-mediated nonstop mRNA decay requires establishment of the tmRNA ORF. SmpB C-terminal tail variants that are competent in tmRNA binding and ribosome recognition but fail to support establishment of the tmRNA ORF as the surrogate template also fail to facilitate productive engagement of RNase R on rescued ribosomes. We interpret these data to signify that RNase R is recruited to rescued-ribosomes at the same time or soon after establishment of the tmRNA ORF as a surrogate template.

Analysis of tmRNA hybrids and well-known tmRNA ORF variants, that are fully competent in peptide tagging and ribosome rescue, uncovered a novel role for distal elements of the tmRNA ORF that are required for making crucial contacts with the stalled translation machinery and recruitment of RNase R. Studies of the SmpB^{FT} and tmRNA^{FT} hybrids suggest that unique sequence elements of tmRNA interact directly with components of the ribosomal 30S subunit rather than RNase R. The observation that the SmpB^{FT} hybrid could not compensate for tmRNA^{FT} associated nonstop mRNA decay defect supports this conclusion. The fact that the divergent RNase R^{FT} was fully proficient in nonstop mRNA decay in the presence of *E. coli* tmRNA but not in the presence of the tmRNA^{FT} hybrid suggested that direct contacts of RNase R with the cognate tmRNA ORF are not required for productive engage-

ment of RNase R on stalled ribosomes. The mRNA-like region and helix 5 of tmRNA^{FT} differs significantly from *E. coli* tmRNA, both in length and nucleotide composition (Supplementary Figure S1). While the tmRNA^{EC} ORF encodes for a 10 amino acid tag, the tmRNA^{FT} ORF is greater than twice the length, encoding for a 22 amino acid tag. Although the last two codons of both the *E. coli* and *F. tularensis* tmRNA ORF encode for alanine, the sequence of the two synonymous alanine codons is dissimilar—the tmRNA^{FT} ORF uses 5'-GCU-GCC-3' whereas the *E. coli* tmRNA ORF uses 5'-GCA-GCU-3' as alanine codons. We reason that these differences in length and sequence composition result in a less than ideal positioning of the ultimate and penultimate codons and helix 5 with respect to the translation machinery, perhaps components of the mRNA entry channel, thus rendering tmRNA^{FT} deficient in facilitating RNase R recruitment and nonstop mRNA decay. We infer from these observations that the putative contacts made by tmRNA ORF with the translation apparatus are not essential for the peptide tagging and ribosome rescue activities. Instead, these contacts are a prerequisite for facilitating productive engagement of RNase R with rescued ribosomes. We propose that the tmRNA ORF, more specifically the ultimate and penultimate codons, make necessary contacts with the stalled translation apparatus to set the stage for RNase R recruitment and selective nonstop mRNA decay. We postulate that interactions of the tmRNA ORF with 30S ribosomal RNA or protein(s) plays a significant role in coordinating this process. The finding that the divergent RNase R^{FT} can support nonstop mRNA decay in the presence of tmRNA^{EC} but not in the presence of tmRNA^{FT} is consistent with the postulate that critical contacts with the stalled translation machinery are made by the distal part of the tmRNA ORF.

Ribosomes are dynamic molecular machines capable of exploring a range of functional conformational states. The structural dynamics of ribosomal 30S and 50S subunits play pivotal roles at various stages of the decoding process. Recent biochemical and structural studies confirm the capacity of the ribosome to explore a number of previously undetected conformations in response to distinct translation factors or RNA sequence signals (31,39–43). For instance, a hyper-rotated state is observed for ribosomes translating frame-shift inducing structured mRNAs, with the structured sequences poised at the mRNA entry channel (40). The inference drawn from these and related studies could be that interactions of structured mRNAs with components of the mRNA entry channel stimulate a conformational state that influences frame-shifting efficiency. Most relevant to our findings, a recent cryo-EM study sheds new light on the dynamic structural changes that occur in the 30S subunit during accommodation of the mRNA-like domain of tmRNA (31). Of particular interest is the large movement of the 30S latch (wide-open latch state)—resulting in a gap of ~20Å between helix 34 and the G530 region of the 30S subunit—that permits the loading of mRNA-like domain of tmRNA into the ribosomal mRNA entry channel. Intriguingly, the distal part of the tmRNA ORF, where the last two codons are located, appears to interact directly with components of the mRNA entry channel during this stage of *trans*-translation. Specific interactions of the distal segment of the

mRNA-like domain of tmRNA with elements of the 30S subunit provides a plausible explanation for the observed species-specific nature of the impact of the tmRNA ORF on nonstop mRNA decay. We favor this interpretation and propose that crucial interactions of the ultimate and penultimate codons of the tmRNA ORF with the 30S subunit might induce unique conformational states of the 30S subunit that are essential for RNase R to engage rescued ribosomes and gain access to the 3' end of the defective mRNA.

In light of these findings, there are two possible mechanisms for RNase R to engage rescued ribosomes. One pathway could be analogous to eukaryotic nonstop mRNA decay, where Ski7p, the SKI protein complex and the exosome gain access to ribosomal A-site, capture nonstop mRNA, and promote ribosomal subunit disassembly (44,45). The alternative pathway could involve selective recruitment of RNase R to the vicinity of the mRNA exit channel, where the ribonuclease is strategically positioned to capture the emerging nonstop mRNA. Since establishment of the tmRNA reading frame and commencement of the *trans*-translation process are prerequisites for recruitment of RNase R, it is unlikely that the nonstop mRNA would be captured via an exosome-like pathway. We reason that the bacterial nonstop mRNA surveillance system is fundamentally distinct from the known eukaryotic surveillance pathways, as it requires continuation of translation for marking of the aberrant nascent polypeptide for proteolysis and relies on normal translation termination, on a stop codon provided by the tmRNA ORF, for ribosome recycling. While further experiments are necessary to decipher the intricate details of this process, it is tempting to speculate that select contacts of the distal part of the tmRNA ORF with the components of the mRNA entry channel promote a unique conformational state of the 30S subunit that provides a spatio-temporally controlled window of opportunity for RNase R to bind in the vicinity of the mRNA exit channel and capture the emerging nonstop mRNA.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGMENT

We thank all members of the Karzai laboratory for helpful discussions and critical evaluation of the manuscript.

FUNDING

National Institutes of Health [R01 GM065319 to A.W.K.]. Funding for open access charge: National Institute of Health [R01 GM065319 to A.W.K.].

Conflict of interest statement. None declared.

REFERENCES

- Karzai, A.W., Roche, E.D. and Sauer, R.T. (2000) The SsrA-SmpB system for protein tagging, directed degradation and ribosome rescue. *Nat. Struct. Biol.*, **7**, 449–455.
- Withey, J.H. and Friedman, D.I. (2003) A salvage pathway for protein structures: tmRNA and trans-translation. *Annu. Rev. Microbiol.*, **57**, 101–123.
- Dulebohn, D., Choy, J., Sundermeier, T., Okan, N. and Karzai, A.W. (2007) Trans-translation: the tmRNA-mediated surveillance mechanism for ribosome rescue, directed protein degradation, and nonstop mRNA decay. *Biochemistry*, **46**, 4681–4693.
- Barends, S., Kraal, B. and van Wezel, G.P. (2011) The tmRNA-tagging mechanism and the control of gene expression: a review. *Wiley Interdiscip. Rev. RNA*, **2**, 233–246.
- Hayes, C.S. and Keiler, K.C. (2010) Beyond ribosome rescue: tmRNA and co-translational processes. *FEBS Lett.*, **584**, 413–419.
- Moore, S.D. and Sauer, R.T. (2007) The tmRNA system for translational surveillance and ribosome rescue. *Annu. Rev. Biochem.*, **76**, 101–124.
- Svetlanov, A., Puri, N., Mena, P., Koller, A. and Karzai, A.W. (2012) Francisella tularensis tmRNA system mutants are vulnerable to stress, avirulent in mice, and provide effective immune protection. *Mol. Microbiol.*, **85**, 122–141.
- Okan, N.A., Bliska, J.B. and Karzai, A.W. (2006) A Role for the SmpB-SsrA system in Yersinia pseudotuberculosis pathogenesis. *PLoS Pathog.*, **2**, e6.
- Mann, B., van Opijnen, T., Wang, J., Obert, C., Wang, Y.D., Carter, R., McGoldrick, D.J., Ridout, G., Camilli, A., Tuomanen, E.I. *et al.* (2012) Control of virulence by small RNAs in Streptococcus pneumoniae. *PLoS Pathog.*, **8**, e1002788.
- Huang, C., Wolfgang, M.C., Withey, J., Koomey, M. and Friedman, D.I. (2000) Charged tmRNA but not tmRNA-mediated proteolysis is essential for Neisseria gonorrhoeae viability. *EMBO J.*, **19**, 1098–1107.
- Okan, N.A., Mena, P., Benach, J.L., Bliska, J.B. and Karzai, A.W. (2010) The smpB-ssrA mutant of Yersinia pestis functions as a live attenuated vaccine to protect mice against pulmonary plague infection. *Infect. Immun.*, **78**, 1284–1293.
- Keiler, K.C., Waller, P.R. and Sauer, R.T. (1996) Role of a peptide tagging system in degradation of proteins synthesized from damaged messenger RNA. *Science*, **271**, 990–993.
- Karzai, A.W., Susskind, M.M. and Sauer, R.T. (1999) SmpB, a unique RNA-binding protein essential for the peptide-tagging activity of SsrA (tmRNA). *EMBO J.*, **18**, 3793–3799.
- Sundermeier, T.R., Dulebohn, D.P., Cho, H.J. and Karzai, A.W. (2005) A previously uncharacterized role for small protein B (SmpB) in transfer messenger RNA-mediated trans-translation. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 2316–2321.
- Camenares, D., Dulebohn, D.P., Svetlanov, A. and Karzai, A.W. (2013) Active and accurate trans-translation requires distinct determinants in the C-terminal tail of SmpB protein and the mRNA-like domain of transfer messenger RNA (tmRNA). *J. Biol. Chem.*, **288**, 30527–30542.
- Gottesman, S., Roche, E., Zhou, Y. and Sauer, R.T. (1998) The ClpXP and ClpAP proteases degrade proteins with carboxy-terminal peptide tails added by the SsrA-tagging system. *Genes Dev.*, **12**, 1338–1347.
- Choy, J.S., Aung, L.L. and Karzai, A.W. (2007) Lon protease degrades transfer-messenger RNA-tagged proteins. *J. Bacteriol.*, **189**, 6564–6571.
- Ge, Z. and Karzai, A.W. (2009) Co-evolution of multipartite interactions between an extended tmRNA tag and a robust Lon protease in Mycoplasma. *Mol. Microbiol.*, **74**, 1083–1099.
- Herman, C., Thevenet, D., Boulloc, P., Walker, G.C. and D'Ari, R. (1998) Degradation of carboxy-terminal-tagged cytoplasmic proteins by the Escherichia coli protease HflB (FtsH). *Genes Dev.*, **12**, 1348–1355.
- Ge, Z., Mehta, P., Richards, J. and Karzai, A.W. (2010) Non-stop mRNA decay initiates at the ribosome. *Mol. Microbiol.*, **78**, 1159–1170.
- Mehta, P., Richards, J. and Karzai, A.W. (2006) tmRNA determinants required for facilitating nonstop mRNA decay. *RNA*, **12**, 2187–2198.
- Venkataraman, K., Guja, K.E., Garcia-Diaz, M. and Karzai, A.W. (2014) Non-stop mRNA decay: a special attribute of trans-translation mediated ribosome rescue. *Front. Microbiol.*, **5**, 93.
- Janssen, B.D. and Hayes, C.S. (2012) The tmRNA ribosome-rescue system. *Adv. Protein Chem. Struct. Biol.*, **86**, 151–191.
- Karzai, A.W., Roche, E.D. and Sauer, R.T. (2000) The SsrA-SmpB system for protein tagging, directed degradation and ribosome rescue. *Nat. Struct. Biol.*, **7**, 449–455.
- Keiler, K.C. (2008) Biology of trans-translation. *Annu. Rev. Microbiol.*, **62**, 133–151.

26. Keiler, K.C., Shapiro, L. and Williams, K.P. (2000) tmRNAs that encode proteolysis-inducing tags are found in all known bacterial genomes: A two-piece tmRNA functions in *Caulobacter*. *Proc. Natl. Acad. Sci. U.S.A.*, **97**, 7778–7783.
27. Moore, S.D. and Sauer, R.T. (2007) The tmRNA system for translational surveillance and ribosome rescue. *Annu. Rev. Biochem.*, **76**, 101–124.
28. Karzai, A.W., Susskind, M.M. and Sauer, R.T. (1999) SmpB, a unique RNA-binding protein essential for the peptide-tagging activity of SsrA (tmRNA). *EMBO J.*, **18**, 3793–3799.
29. Okan, N.A., Mena, P., Benach, J.L., Bliska, J.B. and Karzai, A.W. (2010) The smpB-ssrA mutant of *Yersinia pestis* functions as a live attenuated vaccine to protect mice against pulmonary plague infection. *Infect. Immun.*, **78**, 1284–1293.
30. Hong, S.J., Tran, Q.A. and Keiler, K.C. (2005) Cell cycle-regulated degradation of tmRNA is controlled by RNase R and SmpB. *Mol. Microbiol.*, **57**, 565–575.
31. Ramrath, D.J.F., Yamamoto, H., Rother, K., Wittek, D., Pech, M., Mielke, T., Loerke, J., Scheerer, P., Ivanov, P., Teraoka, Y. *et al.* (2012) The complex of tmRNA-SmpB and EF-G on translocating ribosomes. *Nature*, **485**, 526–529.
32. Kurita, D., Sasaki, R., Muto, A. and Himeno, H. (2007) Interaction of SmpB with ribosome from directed hydroxyl radical probing. *Nucleic Acids Res.*, **35**, 7248–7255.
33. Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K.A., Tomita, M., Wanner, B.L. and Mori, H. (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.*, **2**, 2006 0008.
34. Mehta, P., Woo, P., Venkataraman, K. and Karzai, A.W. (2012) Ribosome purification approaches for studying interactions of regulatory proteins and RNAs with the ribosome. *Methods Mol. Biol.*, **905**, 273–289.
35. Liang, W. and Deutscher, M.P. (2010) A novel mechanism for ribonuclease regulation: transfer-messenger RNA (tmRNA) and its associated protein SmpB regulate the stability of RNase R. *J. Biol. Chem.*, **285**, 29054–29058.
36. Sundermeier, T., Ge, Z., Richards, J., Dulebohn, D. and Karzai, A.W. (2008) Studying tmRNA-mediated surveillance and nonstop mRNA decay. *Methods Enzymol.*, **447**, 329–358.
37. Richards, J., Mehta, P. and Karzai, A.W. (2006) RNase R degrades non-stop mRNAs selectively in an SmpB-tmRNA-dependent manner. *Mol. Microbiol.*, **62**, 1700–1712.
38. Richards, J., Sundermeier, T., Svetlanov, A. and Karzai, A.W. (2008) Quality control of bacterial mRNA decoding and decay. *Biochim. Biophys. Acta*, **1779**, 574–582.
39. Cornish, P.V., Ermolenko, D.N., Staple, D.W., Hoang, L., Hickerson, R.P., Noller, H.F. and Ha, T. (2009) Following movement of the L1 stalk between three functional states in single ribosomes. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 2571–2576.
40. Qin, P., Yu, D., Zuo, X. and Cornish, P.V. (2014) Structured mRNA induces the ribosome into a hyper-rotated state. *EMBO Rep.*, **15**, 185–190.
41. Zhou, J., Lancaster, L., Donohue, J.P. and Noller, H.F. (2013) Crystal structures of EF-G-ribosome complexes trapped in intermediate states of translocation. *Science*, **340**, 154.
42. Ratje, A.H., Loerke, J., Mikolajka, A., Brunner, M., Hildebrand, P.W., Starosta, A.L., Donhofer, A., Connell, S.R., Fucini, P., Mielke, T. *et al.* (2010) Head swivel on the ribosome facilitates translocation by means of intra-subunit tRNA hybrid sites. *Nature*, **468**, 713–716.
43. Koutmou, K.S., McDonald, M.E., Brunelle, J.L. and Green, R. (2014) RF3:GTP promotes rapid dissociation of the class 1 termination factor. *RNA*, **20**, 609–620.
44. Shoemaker, C.J. and Green, R. (2012) Translation drives mRNA quality control. *Nat. Struct. Mol. Biol.*, **19**, 594–601.
45. Lykke-Andersen, J. and Bennett, E.J. (2014) Protecting the proteome: eukaryotic cotranslational quality control pathways. *J. Cell. Biol.*, **204**, 467–476.