A-Site mRNA Cleavage Is Not Required for tmRNA-Mediated ssrA-Peptide Tagging

Brian D. Janssen¹, Fernando Garza-Sánchez¹, Christopher S. Hayes¹,²*

¹ Department of Molecular, Cellular and Developmental Biology, University of California Santa Barbara, Santa Barbara, California, United States of America, ² Biomolecular Science and Engineering Program, University of California Santa Barbara, Santa Barbara, California, United States of America

Abstract

In Escherichia coli, prolonged translational arrest allows mRNA degradation into the A site of stalled ribosomes. The enzyme that cleaves the A-site codon is not known, but its activity requires RNase II to degrade mRNA downstream of the ribosome. This A-site mRNA cleavage process is thought to function in translation quality control because stalled ribosomes are recycled from A-site truncated transcripts by the tmRNA-SmpB “ribosome rescue” system. During rescue, the tmRNA-encoded ssrA peptide is added to the nascent chain, thereby targeting the tagged protein for degradation after release from the ribosome. Here, we examine the influence of A-site mRNA cleavage upon tmRNA-SmpB activity. Using a model transcript that undergoes stop-codon cleavage in response to inefficient translation termination, we quantify ssrA-peptide tagung of the encoded protein in cells that contain (rnb⁺) or lack (Δrnb) RNase II. A-site mRNA cleavage is reduced approximately three-fold in Δrnb backgrounds, but the efficiency of ssrA-tagging is identical to that of rnb⁺ cells. Additionally, pulse-chase analysis demonstrates that paused ribosomes recycle from the test transcripts at similar rates in rnb⁺ and Δrnb cells. Together, these results indicate that A-site truncated transcripts are not required for tmRNA-SmpB-mediated ribosome rescue and suggest that A-site mRNA cleavage process may play a role in other recycling pathways.

Introduction

Non-stop mRNAs pose a significant molecular quality control problem for all organisms [1]. Non-stop transcripts lack in-frame stop codons and therefore encode incomplete polypeptides that could be deleterious for the cell. Furthermore, ribosomes stall at the 3’-ends of non-stop mRNA because translation termination and subsequent ribosome recycling require an intact A-site stop codon. All bacteria use transfer-messenger RNA (tmRNA) and SmpB to recycle ribosomes from non-stop messages [1,2]. tmRNA is a bi-functional RNA that acts as both a tRNA and mRNA to “rescue” stalled ribosomes and target the associated polypeptides for rapid degradation [3]. The tRNA-like domain of tmRNA is aminoacylated with alanine and allows recognition of stalled ribosomes [3,4]. After the nascent peptide is transferred to tmRNA, the non-stop transcript is released from the ribosome and translation resumes using a short reading frame within tmRNA. In this manner, the tmRNA-encoded ssrA peptide is added to the C-terminus of the nascent chain. The ssrA peptide is recognized by several proteases, which rapidly degrade tagged proteins after release from the ribosome [3,5,6,7]. Because the ssrA coding sequence is terminated with a stop codon, the rescued ribosome is able to undergo normal translation termination and recycling. SmpB is a small tmRNA-binding protein that coordinates the tRNA and mRNA functions of tmRNA [8,9]. The flexible C-terminal tail of SmpB is required for ribosome binding, and recent structural studies indicate that this region mimics the missing A-site codonanticodon helix on stalled ribosomes [10,11,12]. SmpB is also critical for proper presentation of the tmRNA “resume” codon in the A-site after release of the non-stop message [13,14]. Thus, tmRNA-SmpB acts as a translational quality control system that responds to non-processive protein synthesis. Because the tmRNA-SmpB complex provides stalled ribosomes with a stop codon in trans, this process is often termed trans-translation in the literature [15].

tmRNA-SmpB also rescues ribosomes that pause at internal sites within full-length messages. SsrA-peptide tagging activity has been reported for translational pauses that occur at clusters of rare codons [16,17,18], in response to specific nascent peptide sequences [19,20,21,22], and during acute starvation for amino acids [23,24]. In some instances, translational arrest results in cleavage of the A-site codon [24,25,26]. These results suggest that A-site mRNA cleavage is induced to generate non-stop mRNA and promote tmRNA-SmpB recruitment to paused ribosomes. This model is supported by in vitro studies showing that trans-translation occurs more rapidly at ribosomes that have no A-site codon [27]. However, A-site cleaved transcripts are only detected in mutants that lack functional tmRNA-SmpB [25,26]. One explanation for the apparent lack of A-site mRNA cleavage in wild-type cells is that tmRNA-SmpB recycles stalled ribosomes from cleaved transcripts thereby promoting their rapid turnover.
rescue. We find that ssrA-peptide tagging is indistinguishable in [29,31,32]. In this study, we modulate A-site mRNA cleavage to this model, A-site cleavage is suppressed by stable mRNA facilitates the activity of the actual A-site nuclease. In accord with degrades mRNA downstream of the paused ribosome, which then into the ribosome A site and therefore its role in A-site cleavage that another nuclease(s) degrades transcripts to this position in the "toeprint" of the paused ribosome on mRNA, suggesting that are truncated to a position 12 nucleotides downstream of the YbeL-his6-ssrA [25,26,28]. Additionally, not all translational arrests induce A-site mRNA cleavage in E. coli[29]. Translational arrest in E. coli Amb mutants produces transcripts that are truncated to a position 12 nucleotides downstream of the A-site codon [29]. This +12 truncation site probably corresponds to the "toeprint" of the paused ribosome on mRNA, suggesting that another nuclease(s) degrades transcripts to this position in the absence of RNase II. Notably, RNase II cannot degrade mRNAs into the ribosome A site and therefore its role in A-site cleavage must be indirect [29,30]. We have proposed that RNase II degrades mRNA downstream of the paused ribosome, which then facilitates the activity of the actual A-site nuclease. In accord with this model, A-site cleavage is suppressed by stable mRNA structures that are resistant to degradation by RNase II [29,31,32]. In this study, we modulate A-site mRNA cleavage to determine its importance for tmRNA-SmpB mediated ribosome rescue. We find that ssrA-peptide tagging is indistinguishable in \( rnb^+ \) and \( \Delta nrb \) genetic backgrounds. Moreover, the rates of peptidyl-RNA turnover from stalled ribosomes are similar in \( rnb^+ \) and \( \Delta nrb \) cells, indicating the ribosome recycling is largely unaffected by the A-site mRNA cleavage process. Together, these results suggest that mRNA degradation to the 3'-edge of the stalled ribosome is sufficient for efficient tmRNA-SmpB rescue activity.

Materials and Methods

Bacterial strains and plasmids

All bacterial strains were derivatives of E. coli strain X90 and are listed in Table 1. Deletions of \( rnb \), \( pnp \) and \( mrv \) have been described previously [21]. These alleles were introduced into strains CH112, CH113 and CH2385 by phage PI-mediated generalized transduction [33]. The \( rnb \) \( mrv \) double mutant was constructed by removing the kanamycin-resistance cassette [34] from the \( \Delta nrb:\text{kan} \) allele to create CH113 \( \Delta nrb \), followed by phage PI-mediated transduction of the other gene deletions into the \( \Delta nrb \) background. All other gene deletion constructs were transduced from the Keio collection [35] into strains CH113 or CH113 \( \Delta nrb \).

All strains were subjected to whole-cell PCR to confirm chromosomal structure. Plasmid pHis\( \beta \)-YbeL-PP was constructed by amplification of ybeL-PP using oligonucleotides ybeL-\( \text{his}6\)-Nco [5'-TAC CAT GGG CAG CAG CCA TCA TCA TCA TCA TCA TAG TCA TAT GAA GAA GTG TGG TCA] and pET-Eco [5'-CGT TTT CTT CAA GAA TTT TTC TGT TGG ACA GC], followed by digestion with NcoI/EcoRI and ligation to plasmid pET11d.

Protein and RNA analysis

His\( \beta \)-tagged proteins were purified by Ni\( \beta \)-affinity chromatography as described [36]. Cultures were grown to mid-log phase (OD\( \text{600} \) = 0.5) and harvested over ice. Cells were collected by centrifugation and frozen at \(-80^\circ \text{C} \). Frozen cells were broken by freeze-thaw in urea lysis buffer [50% urea – 10 mM Tris-\( \text{HCl} \) (pH 8.0) – 150 mM NaCl] and lysates clarified by centrifugation at 13,000 rpm for 15 min. Lysates were incubated with Ni\( \beta \)-NTA agarose resin on a rotisserie for 1 hr at room temperature. The resin was washed with 20 mL of lysis buffer supplemented with 20 mM imidazole. His\( \beta \)-tagged proteins were eluted with lysis buffer supplemented with 250 mM imidazole. Protein samples were resolved by SDS-PAGE and stained with Coomassie blue or subjected to immunoblot analysis with polyclonal antibodies to the ssrA(DD) peptide. Fluorescent secondary antibodies (anti-rabbit) were obtained from Rockland Immunologicals. Stained gels and immunoblots were visualized and quantified using the Odyssey infrared imager and software package (LiCor). RNA was isolated and analyzed as described previously [21,37]. Transcripts were analyzed by northern blot hybridization using \( ^{32} \text{P} \)-labeled oligonucleotide probe-T7-SD (5' - GTA TAT CTC CCT TCT AAA GTT AAA C) as described [37]. A-site truncation products were quantified using Quantity One software package (BioRad). The reported values for percent A-site truncated mRNA represent the mean ± standard error of the mean (SEM) for three to six independent experiments.

Pulse-chase analysis

E. coli cells were grown to exponential phase in MOPS-buffered defined media [38], pulse labeled with 20 \( \mu \text{Ci/mL} \) of \( ^{35} \text{S} \)-labeled methionine/L-cysteine (MP Biomedicals – 1175 Ci/mmol) and chased with 0.2 mg/mL unlabeled L-methionine/L-cysteine as described [39]. RNA was isolated and run on acid-urea polyacrylamide gels as described [39]. Gels were dried and visualized by phosphorimaging. Radiolabeled peptidyl-RNAs were quantified using Quantity One, and double-exponential decay equations were fitted to the data to estimate rates of peptidyl-RNA turnover. Reported values represent average rates ± SEM for two independent experiments.

Results

A-site mRNA cleavage is not required for tmRNA-mediated peptide tagging

The correlation between A-site mRNA cleavage in ssrA\( ^+ \) cells and ssrA-peptide tagging activity in ssrA\( ^+ \) cells suggests that these processes are linked functionally. To test this model, we asked whether suppression of A-site mRNA cleavage leads to reduced peptide tagging. We chose the previously characterized YbeL-PP protein from E. coli as a model system to study site-specific translational arrest [25,37,40]. YbeL-PP carries a C-terminal Pro-Pro nascent peptide motif that interferes with translation termination [40,41,42]. As a consequence, the ybeL-PP stop codon is cleaved to generate a non-stop message [25], and the nascent chain is tagged with the ssrA peptide [40]. To facilitate the analysis of cleaved ybeL-PP transcripts, we used the flag-(\( \text{myb} \))ybeL-PP mini-gene construct, which encodes a FLAG epitope fused to the C-terminal 49 residues of YbeL-PP (Fig. 1A). The Pro-Pro motif induces ribosome arrest in all genetic contexts tested, and A-site cleavage and ssrA-peptide tagging activities are essentially identical for the full-length and mini-gene ybeL-PP constructs [25,29,37,40]. Full-length flag-(\( \text{myb} \))ybeL-PP transcripts predominate in ssrA\( ^+ \) cells, whereas approximately 30% of the message is truncated at the stop codon in ssrA\( ^- \) cells (Figs. 1B & 1C).

RNase II is required for efficient A-site mRNA cleavage activity, and flag-(\( \text{myb} \))ybeL-PP transcripts are truncated +12 nucleotides downstream of the stop codon when expressed in E. coli ssrA\( ^- \) cells that lack RNase II (encoded by the \( nrb \) gene) (Figs. 1A & 1B) [29]. Quantification of the stop-codon truncated transcripts indicates that these products are approximately three-fold less abundant in ssrA\( ^- \) \( \Delta nrb \) cells (8.1 ± 1.9%) compared with ssrA\( ^+ \) \( nrb \) cells (Fig. 1C). To determine whether diminished A-site cleavage correlates with decreased tmRNA-SmpB activity, we examined ssrA-peptide
**Table 1. Bacterial strains and plasmids.**

| Strain or plasmid | Description | Reference |
|-------------------|-------------|-----------|
| CH12              | X90 (DE3) ssrA::cat, CmR | [40] |
| CH113             | X90 (DE3) ssrA::cat Δme515::kan, CmR KanR | [40] |
| CH1002            | X90 (DE3) ssrA::cat Δmb::kan, CmR KanR | [29] |
| CH1207            | X90 (DE3) ssrA::cat Δmnr::kan, CmR KanR | [29] |
| CH1002            | X90 (DE3) ssrA::cat Δmnb::kan, CmR KanR | [21] |
| CH1208            | X90 (DE3) ssrA::cat Δpnp::kan, CmR KanR | [21] |
| CH1214            | X90 (DE3) ssrA::cat Δrne515::kan, CmR KanR | [21] |
| CH1916            | X90 (DE3) ssrA::cat Δrne515::kan, CmR KanR | [21] |
| CH2385            | X90 (DE3) ssrA(DD) | [24] |
| CH2790            | X90 (DE3) Δmnb::kan, KanR | [21] |
| CH3138            | X90 (DE3) ssrA(DD) Δmnb::kan, KanR | This study |
| CH3139            | X90 (DE3) ssrA(DD) Δmnr::kan, KanR | This study |
| CH3153            | X90 (DE3) ssrA(DD) Δpnp::kan, KanR | This study |
| CH3295            | X90 (DE3) ssrA(DD) Δrne515::kan, CmR KanR | [29] |
| CH3566            | X90 (DE3) ssrA(DD) Δmnb::kan, KanR | This study |
| CH3574            | X90 (DE3) ssrA::cat Δrne515::kan, CmR KanR | [29] |
| CH3575            | X90 (DE3) ssrA::cat Δmnr::kan, CmR KanR | [29] |
| CH3580            | X90 (DE3) ssrA::cat Δmnb::kan, CmR KanR | This study |
| CH4312            | X90 (DE3) ssrA::cat Δrne515::kan, CmR KanR | [29] |
| CH4456            | X90 (DE3) ssrA::cat Δrne515::kan, CmR KanR | This study |
| CH4463            | X90 (DE3) ssrA::cat Δrne515::kan, CmR KanR | [29] |
| CH4465            | X90 (DE3) ssrA::cat Δrne515::kan, CmR KanR | [29] |
| CH4704            | X90 (DE3) ssrA::cat Δrne515::kan, CmR KanR | [29] |
| CH5363            | X90 (DE3) ssrA::cat Δrne515::kan, CmR KanR | This study |
| CH5866            | X90 (DE3) ssrA::cat Δrne515::kan, CmR KanR | This study |
| CH6014            | X90 (DE3) ssrA::cat Δrne515::kan, CmR KanR | This study |
| CH6015            | X90 (DE3) ssrA::cat Δrne515::kan, CmR KanR | This study |
| CH6023            | X90 (DE3) ssrA::cat Δrne515::kan, CmR KanR | This study |
| CH6024            | X90 (DE3) ssrA::cat Δrne515::kan, CmR KanR | This study |
| CH6104            | X90 (DE3) ssrA::cat Δrne515::kan, CmR KanR | This study |
| CH6105            | X90 (DE3) ssrA::cat Δrne515::kan, CmR KanR | This study |
| CH6107            | X90 (DE3) ssrA::cat Δrne515::kan, CmR KanR | This study |
| CH6766            | X90 (DE3) ssrA::cat Δrne515::kan, CmR KanR | This study |
| CH7097            | X90 (DE3) ssrA::cat Δrne515::kan, CmR KanR | This study |
| CH7098            | X90 (DE3) ssrA::cat Δrne515::kan, CmR KanR | This study |
| CH8873            | X90 (DE3) ssrA::cat Δrne515::kan, CmR KanR | This study |
| CH8874            | X90 (DE3) ssrA::cat Δrne515::kan, CmR KanR | This study |
| CH8875            | X90 (DE3) ssrA::cat Δrne515::kan, CmR KanR | This study |
| CH8876            | X90 (DE3) ssrA::cat Δrne515::kan, CmR KanR | This study |
| pHHis6-YbeL-PP    | T7 expression of YbeL(E159P) containing an N-terminal hexahistidine tag, AmpR | This study |
| pFLAG-(m)YbeL-PP  | Expresses FLAG epitope fused to the C-terminal 49 residues of YbeL-PP, AmpR | [29] |
| pKW1              | pACYC184 derived vector, TetR | [16] |
| pKW23             | Plasmid pKW1 derivative that expresses tmRNA(DD), TetR | [16] |

*Abbreviations used: AmpR, ampicillin resistant; CmR, chloramphenicol resistant; KanR, kanamycin resistant, TetR, tetracycline resistant.

doi:10.1371/journal.pone.0081319.t001
tagging of full-length YbeL-PP proteins in *E. coli* ssrA(DD) cells. The *ssrA(DD)* allele specifies a tmRNA variant that encodes the ssrA(DD) peptide tag. This modified tag is resistant to proteolysis and ssrA(DD)-tagged proteins accumulate in the cell [5,6]. Analysis of total His<sub>6</sub>-YbeL-PP protein produced in *ssrA(DD)* cells compared to *ssrA* cells shows that 20±0.7% of the chains are ssrA(DD)-tagged (Figs. 2B & 2C). Somewhat surprisingly, His<sub>6</sub>-YbeL-PP produced in *ssrA(DD)* Δmb cells is tagged at essentially the same level (21±1.2%) as protein from *ssrA(DD) rnb<sup>D</sup>* cells (Figs. 2B & 2C), indicating that the suppression of A-site cleavage activity does not significantly impede tmRNA-SmpB recruitment to stalled ribosomes.

*E. coli* contains two additional 3′-to-5′ exoribonucleases, polynucleotide phosphorylase (PNPase) and RNase R, that play significant roles in mRNA turnover [43,44]. Therefore, we tested cells deleted for *pnp* and *rnb*, which encode PNPase and RNase R, respectively. Transcript processing is slightly reduced in *ssrA* Δ*pnp* cells (23±3.6%), but unaltered in *ssrA* Δ*rnb* cells (30±3.2%) (Figs. 2A & 2C). Similarly, ssrA(DD)-peptide tagging activity is not significantly altered in Δ*pnp* or Δ*rnb* cells (Figs. 2B & 2C). Cells deleted for both *rnb* and *pnp* exhibit the same mRNA processing as Δmb single mutants, but tagging activity may be subtly reduced (18±0.9%) compared to Δmb Δ*rnb* cells (Fig. 2A, 2B & 2C). *E. coli* Δ*pnp* Δ*rnb* double mutants are not viable [45,46], therefore we were unable to test other mutation combinations. Taken together, these results show that RNase II plays a role in *flag-(m)ybeL-PP* transcript processing, but has little effect on tmRNA-SmpB-mediated peptide tagging.

**Role of other RNases and RNA helicases in mRNA processing and tmRNA-mediated peptide tagging**

The presence of +12 truncated transcripts in Δmb cells suggests that another unidentified RNase degrades mRNA to this position in the absence of RNase II. To identify the enzyme responsible for this activity, we examined mRNA processing in strains that are deleted for known RNase genes. RNase gene deletions were transferred into *E. coli* ssrA<sup>D</sup> Δmb cells by transduction and the effects on *flag-(m)ybeL-PP* transcript processing were assessed by northern blot analysis. Deletion of genes encoding RNase I (*rnb*), RNase D (*rnd*), RNase T (*rng*), RNase PH (*rph*), RNase Z (*elaC*), RNase LS (*mla*) and RNase G (*rng*) has only modest effects on A-site mRNA cleavage in the *ssrA* background and +12 cleavage in the *ssrA* Δmb background (Fig. 3A). Additionally, deletion of the C-terminus of RNase E (*rne515*), which organizes the multienzyme RNA “degradosome” [47], does not change the pattern of transcript processing (Fig. 3A). However, deletion of *rnC* (encoding RNase III) in the *ssrA* Δmb background restores A-site mRNA cleavage (Fig. 3A, lowest panel). This latter effect is likely due to ~10-fold up-regulation of PNPase expression in ΔrnC mutants [48]. We have previously shown that PNPase overexpression is sufficient to restore A-site mRNA cleavage in ΔrnC mutants [29], suggesting that the ΔrnC mutation may have the same effect. Quantification of ssrA(DD)-tagged His<sub>6</sub>-YbeL-PP proteins from the RNase deletion strains showed that tagging activity is not significantly altered in most instances (Fig. 3B). However, ssrA(DD)-tagging efficiency was slightly, but reproducibly, reduced in Δmb ΔrnC mutants (Fig. 3B).

RNA helicases are important for the regulation of mRNA translation in eukaryotic cells and could play similar roles in bacteria [49]. In fact, the DEAD-box helicase, RhlB, is found within the RNA degradosome, where it facilitates mRNA turnover by unwinding secondary structures [50]. Based on these observations, we screened four DEAD-box helicases, RhlB, RhlE, HrpA and DecD, to determine whether these enzymes influence the A-
We transduced deletion alleles for each helicase into *E. coli* ssrA− cells and examined flag-(m)-ybeL-PP transcript processing. Each helicase deletion strain showed the same pattern of mRNA cleavage as ssrA− cells with the full complement of helicases (Fig. 4A). Mutants lacking RNase II (Δrnb)

**Figure 2.** A-site mRNA cleavage is not correlated with ssrA- peptide tagging activity. A) Northern blot analysis of flag-(m)ybeL-PP transcripts in cells lacking 3′-to-5′ exoribonucleases. Total RNA was isolated from *E. coli* ssrA− cells that lack the indicated RNase genes and probed with a radiolabeled oligonucleotide that hybridizes the 5′-UTR of flag-(m)ybeL-PP message. The lane labeled in vitro contains flag-(m)ybeL-PP mRNA that is truncated at the stop codon. The migration positions of full-length and truncated transcripts are indicated. B) SsrA(DD)-peptide tagging of His6-YbeL-PP. His6-YbeL-PP chains were purified from cells of the indicated genetic backgrounds and resolved by SDS-PAGE and stained with Coomassie blue. C) Quantification of A-site mRNA cleavage and ssrA(DD) tagging efficiency. The percentage of A-site truncated mRNA was determined by quantifying northern blot hybridization signals as described in Methods. The effect of each RNase gene deletion was examined in an ssrA− background, and the data (in white bars) represent the mean ± SEM for at least three independently prepared RNA samples. Full-length and ssrA(DD)-tagged His6-YbeL-PP chains were isolated from ssrA(DD) cells and quantified by densitometry. Tagging efficiency (in gray bars) is reported as the percentage of total chains that carry ssrA(DD) peptides. Reported values represent the mean ± SEM from two independent experiments. doi:10.1371/journal.pone.0081319.g002

**Figure 3.** Effect of RNase deletions on mRNA processing and ssrA(DD)-peptide tagging. A) Northern blot analysis of flag-(m)ybeL-PP transcripts. Total RNA was isolated from *E. coli* ssrA− cells with the indicated genotypes and probed with an oligonucleotide that hybridizes to the 5′-UTR of flag-(m)ybeL-PP message. The lanes labeled in vitro contain flag-(m)ybeL-PP mRNA that is truncated at the stop codon. The migration positions of full-length and truncated transcripts are indicated. B) Quantification of ssrA(DD) tagging efficiency. Full-length and ssrA(DD)-tagged His6-YbeL-PP chains were quantified by densitometry and tagging efficiency reported as the percentage of total chains that carry ssrA(DD) peptides. Reported values represent the mean ± SEM from four independent experiments. doi:10.1371/journal.pone.0081319.g003

site cleavage process. We transduced deletion alleles for each helicase into *E. coli* ssrA− cells and examined flag-(m)-ybeL-PP transcript processing. Each helicase deletion strain showed the same pattern of mRNA cleavage as ssrA− cells with the full complement of helicases (Fig. 4A). Mutants lacking RNase II (Δrnb)
in combination with the individual helicase knockouts did not change the cleavage patterns (Fig. 4A). We also moved the helicase deletions into ssrA(DD) mb+ and ssrA(DD) Δmb backgrounds, but found that these enzymes have little to no effect on ssrA-peptide tagging efficiency (Fig. 4B). Together, these results indicate that RNase II is required for A-site cleavage and that other known RNases and RNA helicases appear to play no role in this process.

YafO toxin does not catalyze +12 cleavage during ribosome arrest

The results presented above also indicate that none of the tested exoribonucleases are individually required for +12 processing in ssrA−Δmb cells. Inouye and colleagues recently characterized a type II toxin/antitoxin (TA) module from E. coli that encodes an RNase with an activity that is similar to the +12 cleavage activity described here. YafO acts on translation initiation complexes to cleave mRNA near the +15 position with respect to the P-site AUG initiation codon [51]. This site corresponds to +12 processing in our system, suggesting that YafO may be activated in response to ribosome pausing. We deleted yafO in ssrA− and ssrA−Δmb backgrounds and examined the effect on flag-(m)ybeL-PP transcript processing, but found no changes in transcript profiles between yafO+ and ΔyafO strains (Fig. 5). Thus, the YafO toxin is not required for +12 cleavage during ribosome arrest.

RNase II activity does not accelerate ribosome recycling

Because A-site cleavage has no discernable effect on ssrA-peptide tagging, this mRNA processing is probably not required for tmRNA-SmpB-mediated ribosome rescue. However, there are at least two other ribosome rescue systems in E. coli [52,53,54], raising the possibility that A-site cleavage facilitates ribosome recycling through an alternative pathway. Therefore, we measured the rates of paused ribosome recycling in mb+ and Δmb backgrounds. Because ribosomes pause during the termination of ybel-PP translation, they carry nascent chains that are covalently linked to P-site tRNA2Pro (Fig. 1A). We have previously shown that peptidyl-tRNA2Pro accumulates in response to translational arrest and can be exploited as a biochemical marker of paused ribosomes [37,39]. We pulse labeled nascent chains with [35S]-labeled methionine/cysteine and monitored their turnover during a chase with excess unlabeled amino acids. As reported previously, peptidyl-prolyl-tRNA2Pro turnover more rapidly in ssrA+ cells compared to ssrA− cells (Table 2) [37], consistent with the role of tmRNA-SmpB in ribosome rescue. Somewhat unexpectedly, peptidyl-prolyl-tRNA2Pro turnover is slower in wild-type ssrA+ mb+ cells compared to the ssrA−Δmb cells (Figs. 6A & 6B, Table 2). However, in the ssrA− background, deletion of mb results in a slightly longer half-life (although almost within error) for peptidyl prolyl-tRNA2Pro (Table 2). Together, these data indicate that the

Figure 4. Effect of RNA helicase deletions on mRNA processing and ssrA(DD)-peptide tagging. A) Northern blot analysis of flag-(m)ybeL-PP transcripts. Total RNA was isolated from E. coli ssrA− cells with the indicated genotypes and probed with an oligonucleotide that hybridizes to the 5′-UTR of flag-(m)ybeL-PP message. The lanes labeled in vitro contain flag-(m)ybeL-PP mRNA that is truncated at the stop codon. The migration positions of full-length and truncated transcripts are indicated. B) Quantification of ssrA(DD) tagging efficiency. Full-length and ssrA(DD)-tagged His6-YbeL-PP chains were quantified by densitometry and tagging efficiency reported as the percentage of total chains that carry ssrA(DD) peptides. Reported values represent the mean ± SEM from two independent experiments. doi:10.1371/journal.pone.0081319.g004

Figure 5. YafO does not mediate +12 processing during translational arrest. Northern blot analysis of flag-(m)ybeL-PP transcripts. Total RNA was isolated from E. coli ssrA− cells with the indicated genotypes and probed with an oligonucleotide that hybridizes to the 5′-UTR of flag-(m)ybeL-PP message. The lane labeled in vitro contains flag-(m)ybeL-PP mRNA that is truncated at the stop codon. The migration positions of full-length and truncated transcripts are indicated. doi:10.1371/journal.pone.0081319.g005
A-site mRNA cleavage process has a modest effect on the rate of paused ribosome recycling.

Discussion

The original model of trans-translation postulated that ribosome arrest at the 3'-end of nonstop mRNA is the signal for tmRNA-SmpB recruitment [3]. This conclusion is supported by subsequent in vitro studies by Ehrenberg and colleagues. The latter work shows that nascent chain transfer to tmRNA occurs most rapidly when 0–6 nucleotides are present downstream of the P-site codon, and that transfer rates diminish with longer transcripts [27]. Although the initial in vitro rate of trans-transfer is close to zero when there are 15 nucleotides downstream of the P-site codon (equivalent to +12 processing in our system), these same reactions approach 50% completion after 1 s of incubation [27]. Our results suggest that degradation of mRNA to the 3'-edge of stalled ribosomes is sufficient for tmRNA-SmpB-mediated rescue in vivo. Perhaps tmRNA-SmpB induces a slow conformational change in the ribosome that allows trans-translation to occur upon prolonged arrest. Structural studies indicate that the C-terminal tail of SmpB interacts with 30S A site, where it is thought to mimic the missing codon:anticodon mini-helix [9,12,55]. Therefore, A-site mRNA must presumably be displaced to accommodate tmRNA-SmpB binding. Although there appears to be a discrepancy between the in vitro and in vivo requirements for trans-translation, it is possible that an unknown cellular factor enhances trans-translation when transcripts extend beyond the stalled ribosome A site. We note that there are several examples of ribosome arrest that do not induce A-site mRNA cleavage [18,19,20,23], suggesting that longer truncated transcripts represent a major pathway for ribosome rescue. A rigorous test of this model awaits identification of the nuclease(s) responsible for +12 mRNA processing.

Most E. coli messages are thought to be first recognized and cleaved by RNase E to produce fragments that are subsequently degraded by 3’-to-5’ exoribonucleases [43]. RNase E preferentially binds monophosphate groups at the 5’-ends of transcripts, and therefore the bulk flow of mRNA degradation proceeds with 5’-to-3’ polarity even though E. coli lacks known 5’-to-3’ exoribonucleases. This strategy minimizes the production of translatable non-stop messages during mRNA turnover. Moreover, mRNA turnover is typically processive without the accumulation of decay intermediates. However, the results presented here and elsewhere show that paused ribosomes stabilize partially degraded transcripts that lack 3’-ends [18,19,20,21,23,24,25,26,28,56]. It is unclear whether these fragments represent normal decay intermediates that are stabilized by stalled ribosomes, or whether they accumulate because the 5’-to-3’ degradation pathway is disrupted by queued ribosomes. In either case, paused ribosomes interfere with processive mRNA decay. When we first discovered A-site mRNA cleavage, we proposed that this activity was critical for mRNA turnover because...
it would accelerate ribosome recycling and expose truncated transcripts to exoribonucleases [25]. Although that original conclusion is not supported by the present study, tmRNA-SmpB activity does indeed hasten mRNA decay. Karzai and colleagues have shown that ribosome rescue leads to rapid degradation of truncated SecM at its 3′ end by RNase R [57,64]. The results presented here now suggest that +12 cleavage is sufficient for these tmRNA-SmpB-dependent effects on mRNA turnover.

If +12 truncated transcripts are sufficient for the tmRNA-SmpB activity, then what is the functional significance of A-site mRNA cleavage? Because A-site cleavage is only detected in secM (or smpB) mutants, it may be a response to prolonged translational arrest in the absence of ribosome rescue. This model suggests that A-site cleavage could play a role in alternative ribosome rescue mediated by ArfA [53]. ArfA functions as a back-up ribosome rescue system that is only deployed when tmRNA-SmpB is overwhelmed or incapacitated [59,60]. ArfA binds to stalled ribosomes and induces nascent chain release by recruiting release factor-2 [RF-2] [61,62]. Because RF-2 activity requires an intact stop codon, it is possible that ArfA-mediated rescue activity requires an incomplete A-site codon [64,66]. Clearly, further study will be required to ascertain the relative importance and functional interactions between these recycling pathways.

Author Contributions
Conceived and designed the experiments: BDJ FGS CSH. Performed the experiments: FGS CSH. Analyzed the data: BDJ FGS. Contributed reagents/materials/analysis tools: BDJ FGS. Wrote the paper: BDJ CSH.

References
1. Janssen BD, Hayes CS (2013) The tmRNA ribosome-rescue system. Adv Protein Chem Struct Biol 86: 151–191.
2. Hayes CS, Keller KC (2010) Beyond ribosome rescue: tmRNA and co-translational processes. FEBS Lett 594: 413–419.
3. Keller KC, Waller PR, Sauer RT (1996) Role of a peptide tagging system in degradation of proteins synthesized from damaged messenger RNA. Science 271: 990–993.
4. Komine Y, Kitabatake M, Yokogawa T, Nishikawa K, Inokuchi H (1994) A tmRNA-like structure is present in 10SA RNA, a small stable RNA from Escherichia coli. Proc Natl Acad Sci U S A 91: 9223–9227.
5. Gottsman S, Roche E, Zhou Y, Sauer RT (1998) The ClpXP and ClpAP proteases degrade proteins with carboxy-terminal peptide tails added by the SsrA-tagging system. Genes Dev 12: 1330–1347.
6. Herman C, Thevenet D, Bouloc P, Walker GC, D’Ari R (1998) Degradation of carboxy-terminal-tagged cytoplasmic proteins by the Escherichia coli protease HflB (FshB). Genes Dev 12: 1348–1355.
7. Choy JS, Aung LL, Karzai AW (2007) Protein degradation during ribosome rescue is mediated by the ClpXP protease complex. J Bacteriol 189: 6564–6571.
8. Carzaniga H, Paschel MY, Felden B, Ehrenberg M (2004) Ribosome rescue by tmRNA requires truncated mRNAs. J Mol Biol 340: 33–41.
9. Zuo Y, Vincenz HA, Zhang J, Wang Y, Deutscher MP, et al. (2006) Structural basis for processivity and single-strand specificity of RnaII. Mol Cell 24: 149–156.
10. Frazão C, McVey CE, Asmar M, Barbosa A, Vournicn C, et al. (2006) Uraavelling the dynamics of RNA degradation by ribonuclease II and its RNA-binding complex. Nature 443: 110–114.
11. Spickler C, Mackie GA (2000) Action of the A-site mRNA during ribosome pausing. Mol Microbiol 73: 892–897.
12. Moore SD (2011) Assembling new tmRNA strains by transduction using plasmid P1, Methods Mol Biol 765: 155–169.
13. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A 97: 6640–6645.
14. Baumann T, Ara T, Hasegawa M, Takai Y, Okunura Y, et al. (2006) Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol 2: 600001.
15. Janssen BD (2009) Kinetics of truncated mRNAs. J Mol Biol 394: 251–267.
16. Neilhardt FC, Bishl PL, Smith DP (1974) Culture medium for enterobacteria. J Bacteriol 119: 736–747.
17. Janssen BD, Diner EJ, Hayes CS (2012) Analysis of aminoacyl- and peptidyl-tRNAs by gel electrophoresis. Methods Mol Biol 905: 291–309.
40. Hayes CS, Bose B, Sauer RT (2002) Proline residues at the C terminus of nascent chains induce SsrA tagging during translation termination. J Biol Chem 277: 33825–33832.

41. Mottagui-Tabar S, Bjornsson A, Isaksson LA (1994) The second to last amino acid in the nascent peptide as a codon context determinant. EMBO J 13: 249–257.

42. Bjornsson A, Mottagui-Tabar S, Isaksson LA (1996) Structure of the C-terminal end of the nascent peptide influences translation termination. EMBO J 15: 1696–1704.

43. Andrade JM, Pobre V, Silva IJ, Domingues S, Araújo CM (2009) The role of 3′-5′ exoribonucleases in RNA degradation. Pog Mol Biol Transl Sci 85: 187–229.

44. Cheng ZF, Deutscher MP (2005) An important role for RNase R in mRNA decay. Mol Cell 17: 313–318.

45. Cheng ZF, Zuo Y, Li Z, Rudd KE, Deutscher MP (1998) The vacB gene required for virulence in Shigella flexneri and Escherichia coli encodes the exoribonuclease RNase R. J Biol Chem 273: 14077–14080.

46. Donovan WP, Kushner SR (1986) Polynucleotide phosphorylase and ribonuclease II are required for cell viability and mRNA turnover in Escherichia coli K-12. Proc Natl Acad Sci U S A 83: 120–124.

47. Vanzo NF, Li YS, Py B, Blum E, Higgins CF, et al. (1998) Ribonuclease E organizes the protein interactions in the Escherichia coli RNA degradosome. Genes Dev 12: 2770–2781.

48. Jarrige AC, Mathy N, Portier C (2001) PNase autocontrols its expression by degrading a double-stranded structure in the pop mRNA leader. EMBO J 20: 6845–6853.

49. Lindner P, Jankowsky E, Deutscher MP (2011) From unwinding to clamping - the DEAD box RNA helicase family. Nat Rev Mol Cell Biol 12: 505–516.

50. Py B, Higgins CF, Krisch HM, Carposis AJ (1996) A DEAD-box RNA helicase in the Escherichia coli RNA degradosome. Nature 381: 169–172.

51. Zhang Y, Yamaguchi Y, Inouye M (2009) Characterization of YafO, an Escherichia coli toxin. J Biol Chem 284: 25522–25531.

52. Chadani Y, Ono K, Kutsukake K, Abo T (2011) Escherichia coli YaeJ protein mediates a novel ribosome-rescue pathway distinct from SsrA- and ArfA-mediated pathways. Mol Microbiol 80: 772–783.

53. Chadani Y, Ono K, Ozawa S, Takahashi Y, Takai K, et al. (2010) Ribosome rescue by Escherichia coli ArfA (YhdL) in the absence of trans-translation system. Mol Microbiol 78: 796–808.

54. Hanf Y, Inaho N, Namiki N (2010) YaeJ is a novel ribosome-associated protein in Escherichia coli that can hydrolyze peptidyl-tRNA on stalled ribosomes. Nucleic Acids Res.

55. Neubauer C, Gillet R, Kelley AG, Ramakrishnan V (2012) Decoding in the absence of a codon by tmRNA and SmpB in the ribosome. Science 335: 1366–1369.

56. Kuroha K, Heiguchi N, Aiba H, Inada T (2009) Analysis of nonstop mRNA translation in the absence of tmRNA in Escherichia coli. Genes Cells 14: 739–749.

57. Ge Z, Mehta P, Richards J, Karzai AW (2010) Non-stop mRNA decay initiates at the ribosome. Mol Microbiol 78: 1159–1170.

58. Richards J, Mehta P, Karzai AW (2006) RNase R degrades non-stop mRNAs selectively in an SmpB-tmRNA-dependent manner. Mol Microbiol 62: 1700–1712.

59. Garza-Sanchez F, Schaub RE, Janssen BD, Hayes CS (2011) tmRNA regulates synthesis of the ArfA ribosome rescue factor. Mol Microbiol 80: 1204–1219.

60. Chadani Y, Matsumoto E, Aso H, Wada T, Kutsukake K, et al. (2011) trans-translation-mediated tight regulation of the expression of the alternative ribosome-rescue factor ArfA in Escherichia coli. Genes Genet Syst 86: 151–163.

61. Chadani Y, Ito K, Kutsukake K, Abo T (2012) ArfA recruits release factor 2 to rescue stalled ribosomes by peptidyl-tRNA hydrolysis in Escherichia coli. Mol Microbiol 86: 57–50.

62. Shimizu Y (2012) ArfA recruits RF2 into stalled ribosomes. J Mol Biol 423: 624–631.

63. Ramadoss NS, Zhou X, Keiler KC (2013) tmRNA is essential in Shigella flexneri. PLoS One 8: e57537.

64. Doerfel LK, Wohlgemuth I, Rothe C, Peske F, Urlaub H, et al. (2013) EF-P is essential for rapid synthesis of proteins containing consecutive proline residues. Science 339: 83–86.

65. Hersch SJ, Wang M, Zou SB, Moon KM, Foster LJ, et al. (2013) Divergent protein motifs direct elongation factor P-mediated translational regulation in Salmonella enterica and Escherichia coli. MBio 4: e00180-00113.

66. Ude S, Lassak J, Starosta AL, Kraenberger T, Wilson DN, et al. (2013) Translation elongation factor EF-P alleviates ribosome stalling at polyproline stretches. Science 339: 82–85.