Optimization of Ribosome Structure and Function by rRNA Base Modification

Jennifer L. Baxter-Roshek, Alexey N. Petrov, Jonathan D. Dinman*

Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, Maryland, United States of America

Background. Translating mRNA sequences into functional proteins is a fundamental process necessary for the viability of organisms throughout all kingdoms of life. The ribosome carries out this process with a delicate balance between speed and accuracy. This work investigates how ribosome structure and function are affected by rRNA base modification. The prevailing view is that rRNA base modifications serve to fine tune ribosome structure and function. Methodology/Principal Findings. To test this hypothesis, yeast strains deficient in rRNA modifications in the ribosomal peptidyltransferase center were monitored for changes in size and translational fidelity. These studies revealed allele-specific sensitivity to translational inhibitors, changes in reading frame maintenance, nonsense suppression and aa-tRNA selection. Ribosomes isolated from two mutants with the most pronounced phenotypic changes had increased affinities for aa-tRNA, and surprisingly, increased rates of peptidyltransfer as monitored by the puromycin assay. rRNA chemical analyses of one of these mutants identified structural changes in five specific bases associated with the ribosomal A-site. Conclusions/Significance. Together, the data suggest that modification of these bases fine tune the structure of the A-site region of the large subunit so as to assure correct positioning of critical rRNA bases involved in aa-tRNA accommodation into the PTC, of the eEF-1A-tRNA-GTP ternary complex with the GTPase associated center, and of the aa-tRNA in the A-site. These findings represent a direct demonstration in support of the prevailing hypothesis that rRNA modifications serve to optimize rRNA structure for production of accurate and efficient ribosomes.

INTRODUCTION

Modification of ribonucleotides represents a way to expand the topological potentials of RNA molecules beyond those afforded by each of the four bases alone. Extensive research into tRNA modification has resulted in mapping of the majority of pseudouridine (Ψ) and 2'-O-methylation (Nm) residues in eukaryotic and archaeal ribosomes, and in identification of the snoRNA molecules that guide their modification. Despite this, little is understood about the functional roles of nucleotide modification. It is known that nucleotide modifications within the ribosome are not located randomly. This is most clearly seen in the ribosomal large subunit, where modifications cluster in highly conserved areas of the ribosome devoted to peptidyl transfer, sites of A- and P-tRNA binding, the peptide exit tunnel and intersubunit bridges [1–4]. This clustering is conserved in organisms ranging from E. coli to humans with the number of modifications increasing with evolutionary complexity [5]. In vitro reconstituted E. coli ribosomes lacking rRNA modifications were severely defective in catalytic activity [6], and global disruption of Ψ or Nm formation in vivo resulted in strong growth defects in yeast [7,8]. These essential modifications tend to be performed by snoRNPs that also harbor components essential for tRNA processing. However, most snoRNAs responsible for guiding tRNA modification can be individually deleted with minimal detriment to the organism [3,9]. In fact, disruption/deletion of pseudouridine synthase proteins responsible for modification of only two or three residues in E. coli did not produce discernable differences in exponential growth rates between wild-type and mutant strains in vivo. However, rRNA modification mutants were strongly out competed by isogenic wild-type strains in competition experiments, suggesting a growth advantage conferred by the modifications [10,11]. The prevailing hypothesis is that, although rRNA modifications are individually dispensable for survival, together they may serve to optimize rRNA structure for production of accurate and efficient ribosomes.

Based on the chemical properties of Ψ and Nm residues, their possible functional roles can be inferred but not established. It has been suggested that Ψ residues may contribute to RNA stability by altering potentials for base stacking, and by offering an extra hydrogen bond donor as compared to uridine [12,13]. Nm residues offer protection against hydrolysis by bases and nucleases and can promote RNA structural changes by changing the hydration sphere around the 2' oxygen, blocking sugar edge interactions and favoring the 3'endo ribose configuration [13,14]. Thermodynamic and NMR based studies revealed that a Ψ residue can stabilize an RNA hairpin structure when located at a stem loop junction, and is slightly destabilizing when located in single-stranded loop regions [15]. Recent NMR studies of the highly conserved and highly modified large subunit rRNA (LSU) helix 69 of the human ribosome observed discernable but subtle secondary structure differences between rRNA with and without the modifications [16]. Functional and structural studies have shown that rRNA modification defects can impact on translation rates and ribosome integrity. In E. coli, mutants lacking...

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To whom correspondence should be addressed. E-mail: dinman@umd.edu
methylation of the m'G745 residue located in the LSU exhibited decreased growth rates, decreased rates of polypeptide chain elongation, defects in ribosome profiles, and resistance to viomycin [17]. In yeast, knockout strains were made lacking each of six snoRNA genes that guide pseudouridylation of residues in the PTC of the ribosome, as well as one strain that lacked all six genes [10]. Only one individual mutant, the snR10 deletion strain, had phenotypic defects, but deletion of all six snoRNA genes promoted moderate defects in growth and translation rates, paromomycin hypersensitivity, and changes in ribosome profiles. In vitro DMS studies also revealed altered LSU rRNA structure for the multiple snoRNA deletion strain. Other functional studies have centered around two methylated nucleotides, mU2920 and mG2921, in the A loop of the yeast ribosome. There are two components thought to be involved in the methylation of these rRNA residues: the guide snoRNA snR32, and the site-specific methyltransferase Sbp1p, an essential yeast nucleolar protein. Primer extension analysis revealed a functionally redundant pathway whereby snR52 or Sbp1p could methylate residue Um2920 [19]. Later thin layer chromatography experiments revealed a different mechanism whereby Sbp1p and snR32 were responsible for methylation of Gm2921 and Um2920 respectively, and showing that Sbp1p could methylate residue Um2920 in the absence of snR32 [20]. Despite this discrepancy, it is clear that deleting both snR52 and Sbp1p resulted in strong defects in growth rates, altered polysome profiles, and paromomycin hypersensitivity [19], making Sbp1p an important exception to the snoRNA guided modification rule in eukaryotes. The E. coli homolog of Sbp1p, FtsJ/RrmJ, methylates 25S rRNA residue Um2920, the equivalent of yeast Um2920[21], and deletion of this protein in E. coli resulted in severe growth defects, temperature sensitivity, and altered ribosome profiles [22].

Despite their high level of conservation and distribution in functionally important areas of the ribosome, the functions of individual rRNA modifications belie their importance with a lack of defects in their absence. However, the changes in ribosome profiles and rRNA structures in multiple mutants suggest the intriguing possibility that they may each contribute to refining the structure and function of the translational apparatus. To more fully understand this, several strains harboring single deletion mutations, and one containing two gene deletions of previously characterized snoRNAs known to modify the PTC of the yeast ribosome were first characterized using a wide variety of genetic assays designed to assess translational fidelity. The results show that defects in rRNA modification produce allele specific mutant phenotypes including increased sensitivity to translational inhibitors; defects in virus propagation; changes in translational fidelity as monitored by +1 and −1 PRF, discrimination between cognate- and near-cognate aa-tRNAs, and recognition of termination codons. These analyses led to more detailed biochemical characterization of two mutants, demonstrating their increased affinities for aa-tRNAs and decreased rates of peptidytransfer.

rRNA chemical deprotection studies using a mutant defective in its ability to Nm Gm2921 and Um2920 identified structural changes in five positions. Specifically, at U2923 in the 25S rRNA A-loop in the peptidytransferase center, at A2932 and A2933, which help to coordinate correct folding of the helix 90 – 92 structure, and at C2848 where the tip of helix 89 interacts with the GTPase-associated center, and at C2851, where helix 89 interacts with the T-stem of aa-tRNA. Together, the data suggest that modification of these bases fine tune the structure of the A-site region of the large subunit so as to assure correct positioning of critical rRNA bases involved in aa-tRNA accommodation into the PTC, of the eEF1Aaa-tRNA-GTP ternary complex with the GTPase associated center, and of the aa-tRNA in the A-site. These findings represent the first direct demonstration in support of the prevailing hypothesis that rRNA modifications serve to optimize rRNA structure for production of accurate and efficient ribosomes.

RESULTS
In order to more precisely determine the role of rRNA modifications in the translational fidelity of the ribosome, yeast strains lacking several previously characterized snoRNAs and one protein that modify residues around the peptidytransferase center of the ribosome were chosen for characterization. The strains contain single knockouts of the snoRNAs snR10, snR34, snR37, snR42, and snR46 which together pseudouridylate six rRNA residues in the PTC of the yeast ribosome, with snR34 modifying two of those residues. Single and double knockout strain snR52 and a methylase deficient mutant of the essential yeast protein Spb1, which are responsible for methylation of Gm2921 and Um2920, were also used in this study. Since Sbp1p is an essential yeast protein, a methylase deficient mutant with a D to A substitution afecting the AdoMet-binding site was used [19,20]. Mutant strains snr10A, srb1Dk, and srb1DA/snr52A have slow growth phenotypes. The locations of the modified bases modified are shown in figure 1.

rRNA modification mutants show sensitivity to translation inhibitors
Protein translation inhibitors that specifically interact with the ribosome provide sensitive and convenient probes for changes in ribosome function. Anisomycin, which binds the A-site crevice that normally accepts the amino acid side-chains of A-site bound aminocycl-tRNAs [23] interfering with the binding of 3’ end of the aa-tRNA [24–26], was used to probe the A-site of the peptidytransferase center (PTC). Spirosycin, which binds on top of the CCA end of a P-site bound substrate and interacts with it interfering [27] with the binding of the 3’ end of the peptidy-tRNA[28–30] was also used as a probe for functional changes in the PTC. Previous studies reported that srb1DA mutants were sensitive to paromomycin, and that sparsomycin had no effect [19]. Further, the snr10A mutant was also shown to be sensitive to paromomycin [18]. To obtain drug sensitivity profiles for all of the mutants, standard 10-fold dilution spot assays were performed in the presence of anisomycin or sparsomycin (20 μg/ml each). As shown in Figure 2 and summarized in Table 1, srb1DA/snr52A cells were anisomycin hypersensitive, and snr34Δ and snr46Δ strains were hypersensitive to sparsomycin.

Virus propagation in rRNA modification mutants
The yeast killer virus system composed of the dsRNA L-A helper virus and M1 “killer” satellite viruses, provides a highly sensitive assay for small defects in ribosome function. The L-A viral genome contains two overlapping ORFs, gag and pol, which encode the structural protein and the RNA dependent RNA polymerase respectively. The two ORFs are joined by a programmed −1 ribosomal frameshift (−1 PRF) signal, and a −1 PRF event is required for synthesis a Gag-pol fusion protein. The M1 satellite virus dsRNA genome encodes a secreted toxin. The pre-toxin provides the infected cell with immunity to the toxin, while secretion of the mature toxin results in death of uninfected yeast cells. Alterations in −1 PRF frequencies alter the ratio of structural to enzymatic viral proteins produced for particle assembly thereby interfering with the ability of yeast to maintain the L-A helper and M1 satellite viruses [31]. In addition, M1 propagation is highly sensitive to changes in levels of free large subunits in yeast, and mutants with altered amounts of free ribosomal LSU fail to maintain the M1 virus [32]. To assess the effects of the mutants on
virus propagation, the L-A and M1 viruses were introduced into isogenic wild-type and mutant cells by cytoplasmic mixing, and cells were assayed for their abilities to maintain the killer phenotype (Fig. 3A). The wild-type strains and several of the mutants (snr42D, snr34D, and snr52D) were able to stably maintain the killer virus (K+). However, several of the mutants displayed defective killer phenotypes. The snr37D and snr46D mutants stably maintained the virus, but showed reductions in zones of killer activity phenotypes (Kw). Although the killer phenotype could be initially established in snr10D cells, it was rapidly lost, resulting in K2 phenotype. The killer maintenance defect was most severe in the spb1DA and spb1DA/snr52D mutants where infection could not be established. Previously published data indicates altered ribosome profiles for mutants snr10Δ [18] and spb1DA/snr52Δ [19], which could be a contributing factor to the observed virus propagation defects. To rule out the possibility that defects in the processing or secretion of the killer toxin [32] were responsible for the observed killer phenotypes, double-stranded viral RNA was extracted from wild-type and mutant cells and visualized (Fig. 3B). The analysis revealed that M1 dsRNA abundance correlated with the observed killer phenotypes; i.e. M1 dsRNA was observed in the strains which showed the K+ phenotype and was absent or faint in strains that showed K2 or Kw phenotypes respectively.

rRNA modification mutants cause allele-specific defects in translational fidelity

Assays designed to monitor various aspects of translational fidelity were employed to more precisely determine the role of rRNA modifications in translational fidelity. An in vivo bicistronic dual-luciferase reporter system was used to quantitatively monitor changes in 21 and +1 PRF, suppression of nonsense codons, and fidelity of aa-tRNA selection [33]. The control reporter is a yeast expression vector containing Renilla and firefly luciferase genes, which yields active Renilla and firefly luciferase proteins. Programmed 21 and +1 frameshifting test reporters were constructed by inserting a frameshift signal, L-A or Ty1 respectively, between the Renilla and firefly genes such that firefly luciferase can only be produced in the event of a frameshift. Renilla luciferase serves as an internal control, eliminating effects due to differences in mRNA abundance, mRNA stability or translation rates between the test and control reporters. Nonsense suppression test reporters contained a stop codon (UAA, UAG, or UGA) six codons into the firefly luciferase gene, so that firefly luciferase can only be produced consequent to nonsense suppression. The misincorporation test reporters were created by mutating the firefly luciferase catalytic residue R218 from the wild-type AGA codon to either the near-cognate AGC leucine codon, or the non-cognate TCT leucine codon, so that active firefly luciferase can only synthesized when the incorrect tRNAArg is selected. Recoding
### Table 1. Summary of rRNA modification mutant phenotypes.

| Killer<sup>a</sup> | snr42<sup>−</sup> | snr37<sup>−</sup> | snr10<sup>−</sup> | snr34<sup>−</sup> | snr46<sup>−</sup> | SPB1 | SNR52 | spb1DA | snr52<sup>−</sup> | snr52<sup>−</sup> |
|-------------------|------------------|------------------|------------------|------------------|------------------|------|-------|--------|--------|--------|
| NE                | NE               | NE               | NE               | NE               | NE               | NE   | NE    | NE     | NE     | NE     |
| Drug sens.<sup>b</sup> | Aniso            | NE               | NE               | NE               | NE               | NE   | NE    | NE     | NE     | NE     |
| Sparso            | NE               | NE               | NE               | S                | S                | NE   | NE    | NE     | NE     | NE     |
| Programmed Frameshifting<sup>c</sup> | Recoding (%) | 10.3±0.1 | 11.3±0.1 | 10.6±0.1 | 11.8±0.1 | 12.6±0.1 | 12.1±0.1 | 8.6±0.1 | 9.1±0.1 | 8.9±0.1 | 10.2±0.1 |
| Fold WT | 1.0             | 1.1             | 1.0             | 1.1             | 1.2             | 1.0             | 1.0             | 1.1             | 1.0             | 1.2             |
| ρ-values | 7.0E−02 | 3.0E−01 | 1.5E−03 | 2.7E−03 | 4.4E−04 | 1.2E−01 | 2.9E−01 | 8.0E−08 |
| +1PRF Recoding (%) | 0.02±0.01 | 0.02±0.01 | 0.03±0.01 | 0.03±0.01 | 0.03±0.01 | 0.02±0.01 | 0.02±0.01 | 0.02±0.01 | 0.03±0.01 |
| Fold WT | 1.0             | 0.9             | 1.0             | 1.3             | 1.3             | 1.0             | 0.8             | 0.9             | 1.2             |
| Near Cog. Recoding (%) | 0.1±0.01 | 0.09±0.01 | 0.10±0.01 | 0.13±0.01 | 0.11±0.01 | 0.10±0.01 | 0.05±0.01 | 0.06±0.01 | 0.08±0.01 | 0.1±0.01 |
| Fold WT | 1.0             | 0.9             | 1.0             | 1.3             | 1.1             | 1.0             | 1.1             | 1.4             | 19             |
| ρ-values | 3.5E−37 | 5.0E−17 | 7.9E−32 | 8.0E−21 | 3.9E−29 | 9.7E−22 | 7.4E−18 | 3.1E−25 |
| Nonsense Suppression<sup>e</sup> | UAA Recoding (%) | 0.22±0.01 | 0.05±0.01 | 0.06±0.01 | 0.12±0.01 | 0.25±0.01 | 0.31±0.01 | 4.0±0.1 | 3.6±0.1 | 4.8±0.1 | 1.8±0.1 |
| Fold WT | 1.0             | 0.2             | 0.7             | 0.5             | 1.1             | 1.4             | 1.0             | 0.9             | 1.2             | 0.5             |
| ρ-values | 1.2E−27 | 6.4E−16 | 1.1E−08 | 8.2E−09 | 2.1E−14 | 1.2E−02 | 5.0E−07 | 3.5E−16 |
| UAG Recoding (%) | 0.19±0.01 | 0.23±0.01 | 0.17±0.01 | 0.08±0.01 | 0.27±0.01 | 0.38±0.01 | 4.8±0.1 | 4.2±0.1 | 5.3±0.1 | 18±0.1 |
| Fold WT | 1.0             | 1.2             | 0.4             | 0.4             | 1.4             | 2.0             | 1.0             | 0.9             | 1.1             | 0.4             |
| ρ-values | 4.2E−04 | 4.7E−17 | 2.2E−15 | 6.7E−03 | 8.1E−10 | 8.1E−04 | 1.7E−03 | 3.6E−11 |
| UGA Recoding (%) | 0.55±0.01 | 0.20±0.01 | 0.14±0.01 | 0.27±0.01 | 0.57±0.01 | 0.74±0.01 | 8.8±0.1 | 10.2±0.1 | 9.4±0.1 | 6.8±0.1 |
| Fold WT | 1.0             | 0.4             | 0.3             | 0.5             | 1.0             | 1.3             | 1.0             | 1.2             | 1.1             | 0.8             |
| ρ-values | 2.7E−16 | 3.7E−16 | 7.1E−13 | 1.9E−08 | 4.7E−22 | 2.4E−08 | 2.4E−03 | 2.9E−10 |
| Ribosome Binding<sup>b</sup> | A-site Binding | K<sub>d</sub> (μM) | 1.8±0.4 | 0.3±0.05 | 0.6±0.2 | 0.3±0.1 |
| Fold WT | 1.0             | 0.2             | 1.0             | 0.6             | 0.06             | 0.03             | 0.02             | 0.004             | 0.007             |
| P-site Binding<sup>b</sup> | K<sub>d</sub> (μM) | 0.35±0.05 | 0.33±0.02 | 2.4±0.3 | 2.8±0.4 |
| Fold WT | 1.0             | 1.0             | 1.0             | 1.0             | 1.0             |
| Peptidyl Transfer | K<sub>off</sub> (min<sup>−1</sup>) | 0.04±0.003 | 0.06±0.003 | 0.02±0.004 | 0.04±0.007 |
| Fold WT | 1.0             | 1.5             | 1.0             | 1.6             |

Note that there are two sets of isogenic strains denoted by the heavy bars. "K": stable killer phenotype; "K": weak killer phenotype; "K": unable to maintain killer. "S": hypersensitive. All values are reported percent recoding, fold of the isogenic wild-type, and ρ-values. "S": sensitivity to anisomycin or sparsomycin. NE: no effect; S: sensitive; "K": stable killer phenotype. ρ-values were determined as described in Materials and methods.

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efficiencies were measured for each mutant as described in the Materials and Methods. These translational fidelity data are summarized in Table 1.

The spb1DA/snr52Δ double mutant showed a significant increase in –1 PRF efficiency (1.5 fold of wild-type, p = 2.7 × 10^{-15}), consistent with the inability of this strain to propagate the M1 killer virus. None of the other mutants significantly affected –1 PRF (1.2 fold or less than wild type). Similarly, none of the mutants had a significant effect on +1 PRF (1.2 fold or less). The most dramatic effects of the mutants were observed in their abilities to recognize termination codons. With the exception of snr42A, which was hyperaccurate at UAA and UGA, but not at UAG codons, similar trends for changes in nonsense suppression were observed for all three stop codons in the other mutant strains. For example, the snr37Δ, snr10Δ, and the double mutant spb1DA/snr52Δ strains were hyperaccurate with respect to their ability to recognize all three termination codons, while a significant increase in general nonsense suppression was displayed in the snr6Δ mutant strain. The mutants were also assayed with regard to their ability to discriminate between sense and missense codons by using reporters described in the Supporting Information Materials and Methods S1. The snr10Δ mutant strain slightly enhanced misreading of both near and non-cognate codons (1.3 fold > wild-type), and snr6Δ showed a small increase in selection of non-cognate aa-tRNA (1.3 fold of wild type). The double mutant spb1DA/snr52Δ had a very significant effect on near cognate aa-tRNA misreading (1.9 fold wild-type), but did not significantly affect non-cognate aa-tRNA selection. The spb1DA single mutant actually exhibited a slight (0.8 fold) decrease in non-cognate aa-tRNA selection when compared to its wild-type strain with no effect on near-cognate selection events. The snr32A single mutant showed no change in non-cognate aa-tRNA selection events, but did display a 1.4 fold increase in near-cognate values.

Changes in aminoacyl-tRNA binding and peptidyltransfer rates

Defects in translational fidelity could possibly be due to changes in tRNA binding to the ribosome or rates of peptidyltransfer. The two mutants with the most dramatic phenotypic effects, snr46Δ and spb1DA/snr52Δ, were selected for more detailed biochemical characterization. Ribosomes were isolated from the isogenic wild-type and mutant strains and their affinities for aa- and peptidyl-tRNA as well as peptidyltransfer rates were determined as described in the Supporting Information Materials and Methods S1. Ribosomes from both mutants had increased affinities for [14C]Phe-tRNA (Fig. 4A). Specifically, the Kdapp for snr46Δ ribosomes was 0.3 μM ± 0.05, as compared to 1.8 μM ± 0.4 by ribosomes isolated from the corresponding isogenic wild-type strain. Similarly, ribosomes isolated from the spb1DA/snr52Δ mutant had a Kdapp of 0.3 μM ± 0.1 as compared to 0.6 μM ± 0.2 for the corresponding isogenic wild-type Sb1/ SNR52 strain. In contrast, neither mutant affected binding of Ac-[^14C]Phe-tRNA to the ribosomal P-site (Fig 4B). Peptidyltransfer rates were measured using the puromycin reaction as described in the methods. Surprisingly, both mutant strains promoted increased rates of peptidyltransfer as compared to isogenic wild-type values (Fig. 4C). Specifically, the Kapp of snr46Δ ribosomes was 0.06 min^{-1} ± 0.003 while its isogenic wild-type showed a Kapp value of 0.04 min^{-1} ± 0.003. The wild-type Sb1/SNR52 was shown to have a Kapp of 0.02 min^{-1} ± 0.004 while the mutant strain spb1DA/snr52Δ showed a Kapp of 0.04 min^{-1} ± 0.007.

The spb1DA/snr52Δ mutant promotes changes in key rRNA structural elements that interact with aa-tRNA.

It has been speculated that the post-transcriptional rRNA modification may serve to increase the stability of the local RNA structure or decrease risk of degradation [4,13]. With this in mind, in vitro rRNA structure probing was performed on the wild-type and mutant ribosomes biochemically characterized in the previous section. Mutants snr6Δ and spb1DA/snr52Δ and isogenic wild-type puromycin treated ribosomes were incubated with the chemically modifying agents CMCT, kethoxal and DMS in vitro. rRNAs were extracted and primer extension analyses performed using primers sufficient to transverse the entire PTC i.e. helices 89-93. Figure 5A shows a representative autoradiogram for the wild-type and mutant spb1DA/snr52Δ strains. Differences between wild-type and mutant protection patterns and their nucleotide locations are indicated. Residues C2843 and C2851 in helix 89 were deprotected from DMS, and residue U2923 in the A-loop showed increased protection from CMCT. Weaker, but consistent deprotection patterns of A2932 and A2933 were observed in the corresponding isogenic wild-type and mutant ribosomes (data not shown).

DISCUSSION

It is presently thought that rRNA base modification serves to fine tune the ribosome structure so as to optimize ribosome biogenesis and the various functions carried out by mature ribosomes. The current study has tested this hypothesis by focusing on modified bases in the A-site region of the large subunit. The data presented here provide the most detailed structure/function analysis to date, showing how minor changes in rRNA structure assure correct positioning of critical rRNA bases involved in guiding and placement of aa-tRNAs into the ribosomal A-site.

Figure 3. Many of the rRNA base modification mutants have M1 virus propagation defects. Yeast rRNA modification mutants were tested for their ability to maintain the L-A and M1 viruses. (A) Mutant and isogenic wild-type yeast strains were spotted onto YPAD plates, and allowed to grow at 30 °C, and then replica plated to a seeded lawn of 5X47 indicator cells. Plates were incubated at room temperature for 3–5 days until a zone of inhibition was clearly visible for wild-type cells. (B) Total RNAs were extracted from mutant and isogenic wild-type yeast strains and digested with RNase A under high salt conditions. The resulting double-stranded RNA was separated on a 1% agarose gel and visualized with ethidium bromide. L-A and M1 dsRNAs are indicated. The image was inverted for clarity.

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Changes in aminoacyl-tRNA binding and peptidyltransfer rates

Defects in translational fidelity could possibly be due to changes in tRNA binding to the ribosome or rates of peptidyltransfer. The
The observation of increased rates of peptidyltransfer in the spb1DA/snr52D and snr46D ribosomes may provide the key to understanding the function of these modified bases. Such an observation is unusual because this reaction is normally nearly instantaneous (>$50/$sec) [35]. To understand this, it is necessary to examine the reaction in the context of the puromycin assay, which is approximately 3 orders of magnitude slower than peptidyltransferase assays performed with whole aa-tRNAs. One of the rate limiting aspects of the puromycin reaction is the requirement for this small molecule to diffuse into the A-site of ribosomes that are pre-loaded with peptidyl-tRNA. Thus, a simple way to increase the rate of this reaction would be to increase rates of puromycin diffusion into the active site. This could be accomplished by widening the path through which puromycin must travel in order to access the A-site. The structure probing data from spb1DA/snr52D ribosomes reveals that C2843 and C2851, two critical bases lying along the path taken by the 3' end of the aa-tRNA during accommodation [36], are deprotected (Fig. 5A–C). This is consistent with the model of this channel being more open in this mutant. Accommodating aa-tRNA slides along the side of the helix 90 – 92 structure, and A2032 and A2933 play important roles in coordinating proper folding of this structure. Thus, the observed mild deprotection of these two bases is also consistent with the 'open accommodation channel' model. In addition, hyperprotection of U2923 may be due to this base collapsing into the space normally occupied by the methyl groups attached to the nearby Um2920 and Gm2921 (see Fig. 5D). This movement would drag C2922 along with it. C2922 is one of two bases that form the first "gate" through which accommodating aa-tRNA must pass [36] (shown as orange arrows in Figs. 5C and 5D). Thus, repositioning it toward the 5' side of the A-loop would serve to open this gate, further lessening steric hindrance to puromycin.

This structural model can account for all of the other biochemical and genetic phenotypes associated with the double mutant. Opening of the aa-tRNA gates would enhance rates of diffusion of small molecules, e.g. anisomycin and sparsomycin, into the peptidyltransferase center, resulting in the hypersensitivity to these drugs observed with the spb1DA/snr52D and snr46D mutants respectively. This could also enhance rates of aa-tRNA accommodation, resulting in increased affinity for aa-tRNA, and increased rates of aa-tRNA misincorporation at near-cognate and termination codons. Increased 21 PRF, which involves aa-tRNA slippage could be due to loss of the interaction between C2851 and the T-stem of aa-tRNA, i.e. the aa-tRNA may not be as well fitted into the LSU, perhaps making it more prone to slip. In turn, the inability to propagate the killer virus is consistent with increased −1 PRF [37], although it should be noted that propagation of the M1 satellite virus is extremely sensitive to defects in the translational apparatus independent of changes in −1 PRF [38]. Importantly, +1 PRF, which only involves peptidyl-tRNA slippage [39], was not affected by these mutants, consistent with observation that none of the mutants affected binding of Ac-[14C]Phe to the P-site.
We speculate that the $spb1DA/snr52\Delta$ double mutant displayed the most severe phenotypic defects because loss of methyl groups on 2 adjacent bases had the greatest effects on local hydration spheres, thus producing the most dramatic changes in rRNA structure. This suggests that the other mutants may also alter rRNA structure, but that these were too slight to be detected by the methods employed. However, we suggest that the effects of small structural changes are amplified through chains of biological events, thus producing the observed phenotypes. In particular, the ability of ribosomes to recognize termination codons, and the ability of cells to maintain the killer virus appear to be exquisitely sensitive indicators of subtle changes in the translational apparatus. Our conclusion is that rRNA base modification serves to fine-tune ribosome structure so as to best coordinate the structure of the molecule with its functional requirements.

There are other functionally important regions of the ribosome that contain high densities of rRNA modifications. For example, the loop of helix 69 has at least three modified residues that are too slight to be detected by the methods employed. However, we suggest that the effects of small structural changes are amplified through chains of biological events, thus producing the observed phenotypes. In particular, the ability of ribosomes to recognize termination codons, and the ability of cells to maintain the killer virus appear to be exquisitely sensitive indicators of subtle changes in the translational apparatus. Our conclusion is that rRNA base modification serves to fine-tune ribosome structure so as to best coordinate the structure of the molecule with its functional requirements.

Figure 5. 25S rRNA structure probing analysis in $spb1DA/snr52\Delta$ mutant ribosomes. (A) Puromycin treated ribosomes isolated from isogenic wild-type and $spb1DA/snr52\Delta$ mutant strains were used for in vitro chemical probing of the structure around the peptidyltransferase center of the ribosome, specifically helices 89-93. Reactions were performed in triplicate, representative autoradiographs are shown. U - untreated; C - CMCT; D - DMS; K - Kethoxal. Residues with changes in banding pattern labeled. Strongly modified bases at positions U2923, C2843, and C2851 are indicated, as are the more weakly deprotected A2932 and A2933. The increased intensity corresponding to U2845 (marked by *) is not DMS-specific. (B-D). Locations of residues demonstrating strong changes in protection patterns mapped to LSU rRNA structures. Um2920 and Gm2921 are indicated and color coded. Bases with altered protection patterns are circled in orange. aa-tRNA accommodation ‘gate’ bases [36] are indicated with purple (gate 1) and green (gate 2). The “catalytic” A base (equivalent to $E. coli$ 23S rRNA A2451) is incicated with red. Individual LSU helices are numbered and color coded. (B) Secondary structure of yeast 25S rRNA around the PTC. (C) Three dimensional representation of rRNA bases of interest mapped onto the $E. coli$ PTC [34]. Arrow represents the path the 3’ end of the aa-tRNA travels when entering the A-site. (D) Rotation and zoom in of Panel C. Shows the path into the A-site from the aa-tRNA perspective. Residues demonstrating changes in protection patterns are labeled in orange. Locations of modified bases and A-site ‘gate’ residues are labeled.

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associated with severe telomere dysfunction and defects in pre-rRNA processing [41]. Interestingly, cells with mutant dyskerin activity also demonstrate a defect in translation of messenger RNAs containing IRES elements [42]. It will be interesting to explore the possible relationship between rRNA modification and regulation of IRES dependent translation.

MATERIALS AND METHODS

Detailed descriptions of materials and methods are available in the Supporting Information Materials and Methods S1.

Strains, media, and genetic methods

The S. cerevisiae strains used in this study are presented in Table S1. Escherichia coli strain DH5α was used to amplify plasmids (listed in Table S2), and E. coli transformations were performed using the high-efficiency transformation method [43]. Yeast cells were transformed using the alkali cation method [44]. YPAD and synthetic complete medium (H-), as well as YPG, SD, and 4.7 MB plates used for testing the killer phenotype were prepared and used as described previously [37]. Oligonucleotide primers were purchased from IDT (Coralville, IA) and are listed in Table S3. Yeast deletion strains snr10Δ, 37Δ, 42Δ, 46Δ and isogenic wild-type were provided by M.J. Fournier. Yeast strains snr2ΔA, snb1DA, the double mutant and an isogenic wild-type were provided by G. Lutfalla. Cytoduction of the L-A and M1 killer virus into snRNA knockout strains and subsequent killer virus assays were carried out as previously described [31]. Total RNAs extracted from cytoduced wild-type and snRNA knockout strains were analyzed for the presence of the L-A and M1 16S RNAs, and dual luciferase assays to quantitatively monitor translational recoding in yeast were performed as previously described [33]. The latter involved use of a 0-frame control reporter in combination with −1, or +1 ribosomal frameshift, nonsense suppression, or misincorporation test reporter constructs. Recoding efficiencies and statistical analyses were performed as previously described [45]. Ten-fold dilution spot assays to monitor sensitivity to anisomycin or sparsomycin (20 μg/mL) were performed as previously described [46].

Ribosome biochemistry

S. cerevisiae ribosomes were isolated, yeast aminoacyl-tRNA synthetases were purified, yeast phenylalanyl-tRNAs were ami-

noacylated with [14C]Phe, and Ac-[14C]tRNA was synthesized as previously described [47]. [14C]Phe-tRNA and Ac-[14C]tRNA were purified by HPLC. Peptidyltransfer assays using Ac-[14C]Phe-tRNA and puromycin, and equilibrium binding studies of [14C]Phe-tRNA binding to the ribosomal A-site, and of Ac-[14C]Phe-tRNA to the ribosomal P-site were carried out as previously described [47]. The data were fitted to a one site binding model using Prism Graph Pad software. Chemical protections studies of 25S rRNA employed puromycin treated ribosomes incubated with DMS (dimethyl sulfate), kethoxal or CMCT (1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-
toluenes), and the rRNA modifications were visualized by primer extension reactions using AMV reverse transcriptase (Roche, Mannheim, Germany) and the 32P-end-labeled primers shown in Table S3 were performed as previously described [48,49].

SUPPORTING INFORMATION

Materials and Methods S1

Found at: doi:10.1371/journal.pone.0000174.s001 (0.09 MB DOC)

Table S1 Yeast strains used in this study.

Found at: doi:10.1371/journal.pone.0000174.s002 (0.04 MB DOC)

Table S2 Plasmid list.

Found at: doi:10.1371/journal.pone.0000174.s003 (0.05 MB DOC)

Table S3 Oligonucleotides

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Author Contributions

Conceived and designed the experiments: JD JB. Performed the experiments: JB AP. Analyzed the data: JD JB. Contributed reagents/materials/analysis tools: JB AP. Wrote the paper: JD JB.
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