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The central core region of yeast ribosomal protein L11 is important for subunit joining and translational fidelity

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Abstract

Yeast ribosomal protein L11 is positioned at the intersubunit cleft of the large subunit central protuberance, forming an intersubunit bridge with the small subunit protein S18. Mutants were engineered in the central core region of L11 which interacts with Helix 84 of the 25S rRNA. Numerous mutants in this region conferred 60S subunit biogenesis defects. Specifically, many mutations of F96 and the A66D mutant promoted formation of halfmers as assayed by sucrose density ultracentrifugation. Halfmer formation was not due to deficiency in 60S subunit production, suggesting that the mutants affected subunit-joining. Chemical modification analyses indicated that the A66D mutant, but not the F96 mutants, promoted changes in 25S rRNA structure, suggesting at least two modalities for subunit joining defects. 25S rRNA structural changes were located both adjacent to A66D (in H84), and more distant (in H96-7). While none of the mutants significantly affected ribosome/tRNA binding constants, they did have strong effects on cellular growth at both high and low temperatures, in the presence of translational inhibitors, and promoted changes in translational fidelity. Two distinct mechanisms are proposed by which L11 mutants may affect subunit joining, and identification of the amino acids associated with each of these processes are presented. These findings may have implications for our understanding of multifaceted diseases such as Diamond–Blackfan anemia which have been linked in part with mutations in L11.

Keywords

L11; Virus; Ribosome; Yeast; Initiation; Subunit

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Introduction

The eukaryotic ribosome is a complex macromolecule responsible for translation of mRNA into proteins. Comprised of four rRNA molecules and 79 proteins organized into the large (60S) and small (40S) subunits in yeast, accurate assembly of the ribosome is critical for its proper function. During canonical translation initiation, 40S subunits are complexed with Met-tRNA_i^{Met} in their P-sites with the help of eIF1, eIF1A, eIF3, eIF5, and eIF2:GTP, forming the 43S pre-initiation complex (reviewed in Jackson et al. 2010). This 43S particle is loaded onto the 5' end of the mRNA, assisted by the eIF4F complex and PABP, resulting in the 48S particle (Grifo et al. 1983). ATP-dependent eIF1 and eIF1A coordinate 5'–3' scanning of the 48S particle along the mRNA to the AUG start codon (Kozak 1980; Pestova et al. 1998). Upon proper alignment of the AUG start codon with the anticodon of the initiator tRNA, eIF2 and eIF5 induce GTP hydrolysis, followed by the dissociation of all eIFs, except for eIF1A. Recruitment of the 60S to the SSU is mediated by eIF5B:GTP, which binds to the intersubunit surface of the 40S subunit, i.e., subunit joining (Pestova et al. 2000; Pisarev et al. 2006; Unbehaun et al. 2004). Subsequently, hydrolysis of GTP promotes dissociation of both eIF5B and eIF1A, producing the complete 80S ribosome positioned at the start codon with the Met-tRNA_i^{Met} in the P-site (Acker et al. 2006). Translation elongation proceeds from this stage, with the ribosome moving along the mRNA in the 3' direction, coordinating tRNA binding, peptidyl transfer, and eventual termination at a stop codon.

The availability of near-atomic resolution images of the ribosome has enabled localization of its constituent parts, providing the structural foundation for detailed studies addressing questions pertaining to how interactions between key structural elements affect ribosome function. Our laboratory has used this information to investigate the roles of specific ribosomal proteins and rRNA bases to understand their roles in mediating information flow through the ribosome (Kiparisov et al. 2005; Rakauskaitė and Dinman 2006, 2008; Meskauskas and Dinman 2007, 2008; Meskauskas et al. 2008; Petrov et al. 2008; Rhodin and Dinman 2010). During yeast 60S subunit assembly, the essential ribosomal protein L11 (L5 in *Bacteria* and *Archaea*) forms a complex with 5S rRNA and ribosomal protein L5 (L18 in *Bacteria* and *Archaea*) in association with the assembly factor Rrs1p (Nariai et al. 2005). This complex is transported into the nucleolus where it associates with the 66S pre-ribosomal particle prior to cytoplasmic export and final maturation into the 60S LSU. Within the LSU, L11 contacts 5S rRNA and H84 of the 25S rRNA, and is located at the intersubunit face of the CP (Fig. 1). L11 also interacts with the P-site tRNA through the “L11 P-site loop” and forms the only universal protein–protein intersubunit bridge with ribosomal protein S18 (S13 in *Bacteria* and *Archaea*) (Rhodin and Dinman 2010; Taylor et al. 2009; Yusupov et al. 2001; Spahn et al. 2001).

The yeast *Saccharomyces cerevisiae* underwent a genome duplication event approximately 100 million years ago (Wolfe and Shields 1997; Kellis et al. 2004). While the majority of the duplicated genes were lost, two copies were retained for most ribosomal protein genes. L11 is encoded by the paralogous genes *RPL11A* and *RPL11B*, located on chromosomes 16 and 7, respectively (Leer et al. 1984). The 19-kDa, 174 amino acids long isoforms are

identical except for an alanine (L11A) to threonine (L11B) substitution at the third amino acid position. While early studies of L11 (a.k.a. L16 prior to standardization of nomenclature) in the 1980s and 1990s found the two isoforms to be functionally interchangeable, their expression levels were found to be unequal, with L16A comprising one-third of the total L16 (L11) in the cell, and L16B providing the remaining two-thirds. While expression of either isoform alone is adequate for cell viability, 60S assembly is depressed in both instances, and expression of only L16A resulted in large halfmer peaks in polysome sucrose fractionation studies. Additionally, cells expressing only L16B maintained wild-type growth rates, while those with only L16A displayed slow-growth phenotypes (Rotenberg et al. 1988). When the name change was agreed upon in the late 1990s L16A became known as L11B, and L16B became L11A. However, in our experience, strains expressing *RPL11B* as the sole form of L11 grow at wild-type rates; they do not promote any strong decreases in 60S subunit levels; they have normal polysome profiles lacking halfmer peaks; they maintain the yeast killer virus, which is exquisitely sensitive to 60S biogenesis defects (Ohtake and Wickner 1995); and their translational fidelity phenotypes are comparable to other wild-type strains that we have worked with. In sum, cells expressing *RPL11B* as the sole isoform of L11 are phenotypically indistinguishable from cells expressing both isoforms. This leads us to postulate there may have been a clerical error in the renaming of the L16 isoforms to the modern L11 nomenclature. All mutations and experiments reported in the current study were performed in an *rpl11A rpl11B* double-deletion strain background in which allelic variants of *RPL11B* were expressed under the control of its endogenous promoter from low-copy episomal plasmids.

The current study focuses on critical amino acids located in the central core region of L11, identifying mutants that affect cell growth, translational fidelity, 25S rRNA structure, and subunit joining. The subunit joining defects appear to arise from the disruption of two distinct facets of subunit association. We propose one model where misfolding of L11 disrupts subunit joining at the L11–S18 intersubunit bridge and a second in which the transition from initiation to elongation may be depressed by the perturbation of 60S:eIF5B:GTP binding and/or function.

Materials and methods

The materials and methods including strain generation, tenfold dilution spot assays, assays for translational fidelity, ribosome/tRNA binding, and rRNA structural analyses were previously described in detail in a recent publication (Rhodin and Dinman 2010). Briefly, *Saccharomyces cerevisiae* strains were generated using yeast strain JD1313 (*MAT α rpl11a::HIS3 rpl11b::HIS3 ura3-52 leu2 1 trp1 63 his3 200 Killer⁺ + YCpL11B-URA3*) in which the YCpL11B-URA3 plasmid was replaced with wild type or mutant *RPL11B* flanked by 5' and 3' wild-type UTRs episomally supplied to the cell on a *TRP1-CEN6* plasmid, i.e., YCpL11B-*TRP1*. Dilution spot assays were performed by growing yeast to mid-log growth phase, and spotting them in tenfold serial dilutions from 10⁵ to 10¹ colony-forming units per spot on SD-Trp media and followed by incubation at the indicated temperatures or in the presence of indicated drugs. Dual luciferase reporter plasmids pYDL-control, pYDL-LA-1 programmed ribosomal frameshifting (−1 PRF), pYDL-TyI (+1 PRF), pYDL-UAA (nonsense) (Harger and Dinman 2003), and pYDL-AGC₂₁₈ (missense) (Plant

et al. 2007) were employed in this work. Cells transformed with either control or reporter plasmids were grown overnight in liquid standard defined media to mid log phase ($A_{595} = 0.8\text{--}1.5$) and lysates were collected. Samples were measured using the Stop and Glo dual luciferase reagents with a TD20/20 luminometer (Promega). Control and test lysates were analyzed 6–12 times each per strain depending on the uniformity of the data. Translational recoding rates were calculated by taking the ratio of firefly to *Renilla* for control and test reporters and then dividing the average test ratio by the average control ratio. *t* tests were utilized to determine statistical significance compared with wild-type rates as previously described (Jacobs and Dinman 2004).

Steady-state dissociation rates (K_D) of aa-tRNA to the ribosomal A-site were determined by pre-incubating puromycin-treated salt-washed ribosomes with soluble elongation factors (Dresios et al. 2000), polyuridine, and yeast tRNA^{Phe} to block the P-site, followed by a 30°C incubation with twofold dilutions of yeast [¹⁴C]Phe-tRNA^{Phe}. Free tRNAs were removed by filtration through nitrocellulose filters and tRNA binding was quantified by scintillation counting. K_D values were calculated using one site binding with ligand depletion formula using Graphpad Prism. P-site tRNA K_D s were similarly evaluated with polyU-programmed puromycin-treated salt-washed ribosomes incubated with varying concentrations of [¹⁴C]Ac-Phe-tRNA^{Phe}.

Oligonucleotide primers used for generation of L11 bridge mutants are listed in online resource 1. Oligonucleotide primers used for structural analysis by SHAPE (Wilkinson et al. 2006), dimethyl sulfate (DMS), 1-cyclo-hexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene (CMCT), and kethoxal are listed in online resource 2. Methods for chemical modification and analysis by reverse transcription primer extension using SHAPE, DMS, CMCT, and kethoxal were performed as previously described (Stern et al. 1988; Rhodin and Dinman 2010). The published structures for the 70S ribosome from *E. coli* [PDB accession numbers 2AVY, 2AW4; (Schuwirth et al. 2005)], as well as 80S structures from yeast [3JYV, 3JYW, 3JYX; (Spahn et al. 2004; Taylor et al. 2009)] were employed in the analysis of this work and for figure preparation. Published *Thermus thermophilus* 70S ribosomes containing A-site, P-site, and E-site Phe-tRNAs were also utilized [1G1X (Yusupov et al. 2001)]. All structures were visualized and manipulated using MacPy-Mol software (DeLano 2002). tRNAs molecules were inserted into the yeast ribosome using ribosomal protein S18 as an “anchor” in a tRNAs + S18 object and aligning the yeast and *T. thermophilus* S18 molecules using PyMol. *Thermus thermophilus* S18 was then hidden for purposes of image generation.

Sucrose density gradient fractionation experiments were performed by growing yeast cells in YPAD to mid-log growth phase (OD_{595} of 0.6–1.0). To generate polysome profiles, cycloheximide was added to cultures to a final concentration of 0.1 mg/ml and cultures were placed on ice for 10 min. Cells were pelleted by centrifugation and washed with polysome lysis buffer (20 mM Tris-HCl pH 7.0 at room temperature, 50 mM KCl, 12 mM MgCl₂, 1 mM DTT, 200 µg/ml cycloheximide). Cells were resuspended in polysome lysis buffer and disrupted using Zirconian beads and two 2-min pulses using a Minibead beater. Lysates were clarified by centrifugation, and aliquots of 6.5 absorption units at OD_{260} were frozen at –80°C. Aliquots were layered on top of 10.5 ml 7–47% sucrose gradients (polysome lysis

buffer with 7–47% weight/volume sucrose). Gradients were centrifuged at 40,000 rpm for 100 min at 4°C using a Beckman Coulter SW41Ti rotor. After centrifugation, samples were continuously monitored at 254 nm using an ISCO gradient fractionator Model UA-5. To determine relative ratios of 40S and 60S subunits, cells grown to mid-log growth phase were pelleted by centrifugation and washed with 4°C subunit lysis buffer (50 mM Tris–HCl pH 7.0, 50 mM KCl, 1 mM DTT). Cells were resuspended in subunit lysis buffer, disrupted, and aliquoted as described above. Aliquots were layered on top of 10.5 ml of 7–47% sucrose gradients (20 mM Tris–HCl pH 7.0, 500 mM KCl, 12 mM MgCl₂, 1 mM DTT, 7–47% weight/volume sucrose). Gradients were centrifuged at 40,000 rpm for 180 min at 10°C using a Beckman Coulter SW41Ti rotor. Samples were continuously monitored at 254 nm using an ISCO gradient fractionator Model UA-5.

Results

Generation of *rpL11* alleles and genetic analyses

Recent studies from our laboratory demonstrated that critical amino acids in two distinct regions of L11, i.e., the “L11 P-site loop” and in the B1b/c intersubunit bridge-forming region, participate in communicating information from the decoding center (DC) in the small subunit (SSU) to the peptidyltransferase center (PTC) in the large subunit (LSU) (Rhodin and Dinman 2010, 2011). In both cases, structural changes were observed in H84 of the 25S rRNA. These observations propelled examination of a stretch of amino acids of L11 located in the core of L11 that appear to connect the intersubunit B1b/c bridge with H84. These extend from R92 to F96 (Fig. 1b). Initially, stretches of 3–5 amino acids spanning R₉₂DRNF₉₆ were mutated to polyalanine, poly-arginine, or deleted from L11. This analysis revealed two lethal mutants: deletion of this entire stretch of amino acids (R₉₂DRNF₉₆ →), and substitution to poly-arginine (D₉₃RNF₉₆ → R₉₃RRR₉₆). These experiments were followed by a second round of mutagenesis focusing on R92 and F96 due to their locations within L11 relative to the B1b/c intersubunit bridge region and H84 (R92), or their universal conservation (F96) (online resource 3). A prior random mutagenesis screen also identified A66D as resistant to anisomycin. This mutation is located on the opposite side of, yet still adjacent to the H84 and was also analyzed in this study (Fig. 1b).

The poly-alanine mutants R₉₂DRNF₉₆–A₉₂AAAA₉₆ (92-6A, nomenclature used throughout) and 93-5A were both viable, as were all other single amino acid mutants. Cellular growth phenotypes were monitored by standard tenfold dilution spot analysis on SD-trp media. As baseline growth phenotypes under ideal conditions were determined at 30°C, phenotypic changes at alternative temperatures and conditions were compared with this standard. At 30°C, 93-5A caused a small reduction in growth, as did R92K, R92I, R92Q, F96 , F96S, F96Q, and F96 W. More severe growth impairment was observed in the F96R and F96E mutants (Fig. 2a). Changes in ribosome function are often exacerbated by warmer or colder temperatures due to changes in the thermodynamic and/or kinetic conditions in which inter- and intra-molecular interactions occur. At 20°C kinetics are slowed, and most strains maintained their growth rates relative to wild-type as observed at 30°C, with the exception of 92-6A and F96E, both of which grew slightly better at the lower temperature as compared with 30°C (Fig. 2a). At 37°C wild-type growth rates were

restored in the 93-5A, R92K, and F96 W mutants. In contrast, decreased growth was observed in the 92-6A, F96 , F96R, F96E, F96S, F96Q, F96N, and F96H mutants (Fig. 2a).

Changes affecting mRNA decoding and tRNA binding to the ribosomal A- and P-sites can be monitored by growing yeast in the presence of the small-molecule translational inhibitors paromomycin, anisomycin, and sparsomycin, respectively. Paromomycin binds to the DC, stabilizing near-cognate codon:anticodon interactions, resulting in elevated levels of translational miscoding (Carter et al. 2000). Tenfold serially diluted yeast grown in the presence of 2 mg/ml paromomycin revealed that the 93-5A mutant promoted a small degree of resistance to paromomycin, while the 92-6A and most of the F96 mutants were slightly paromomycin hypersensitive, and the R94A and R92 mutants were not affected by this drug (Fig. 2b). Anisomycin binds to the A-site pocket of the PTC, competing with the 3' end of aa-tRNA as well as affecting peptidyltransfer, and changes in cell growth in the presence of this drug are suggestive of changes in ribosome affinity for aa-tRNA and/or for anisomycin (Grollman 1967; Hansen et al. 2003). The A66D, R92 , R92H, F96 , and F96H mutants were slightly resistant to 40 µg/ml anisomycin, and strong resistance was observed for F96R, F96E, F96S, F96Q, and F96 W (Fig. 2b). Sparsomycin binds to the P-site pocket in conjunction peptidyl-tRNA binding and also interferes with peptidyl transfer (Hansen et al. 2003; Schlunzen et al. 2001). At 30 µg/ml sparsomycin, no changes were observed in either the 92-6A or 93-5A mutants, but R94A was slightly hypersensitive, as were all of the R92 mutants (R92S conferring the strongest effect). All of the F96 mutants were similarly sparsomycin hypersensitive (Fig. 2b). Surprisingly, the F96 single amino acid mutants promoted more pronounced sparsomycin hypersensitivity defects than the 92-6A mutant. The strong sparsomycin hypersensitivity promoted by the F96 mutants as compared with the R94 or R92 mutants support the notion that the universally conserved F96 is important for ribosome stability and function.

The ability of yeast strains expressing L11 mutants to maintain the yeast “Killer” virus provides a rapid, but non-specific assay for changes in translational fidelity (Wickner 1996). The Killer virus is composed of the dsRNA L-A helper virus and the dsRNA M₁ satellite virus. The M₁ satellite virus encodes a secreted toxin that targets the GPI-anchored Kre1p cell wall assembly protein, killing yeast lacking L-A and M₁ as indicated by a zone of growth inhibition produced when Killer⁺ cells are grown adjacent to Killer⁻ indicator strains (Breinig et al. 2002). Defects in either translational fidelity or in LSU biogenesis affect the ability of cells to maintain M₁, resulting in reduction or loss of the Killer phenotype (Killer^w, or Killer⁻) (Dinman et al. 1991; Dinman and Wickner 1992). The L11 92-6A, R92E, R92S, R92H, F96R, F96E, F96S, F96Q, F96H, and A66D mutants all promoted the Killer⁻ phenotype (Fig. 2c). The R92 mutant promoted a Killer^w phenotype.

L11 mutations affect translational fidelity

Killer loss can be caused by changes in translational fidelity (Dinman and Wickner 1992). This parameter was monitored by assays of -1 programmed ribosomal frameshifting (PRF), +1 PRF, readthrough of a UAA termination codon, and suppression of an AGC serine by a near-cognate tRNA that normally decodes an AGA arginine codon. Each of these tests utilized bicistronic *Renilla*-firefly dual luciferase reporter plasmids. The control

(readthrough) reporter encodes a *Renilla*-firefly luciferase fusion protein. In the -1 PRF reporter plasmid, the firefly coding sequence (CDS) is in the -1 reading frame relative to the *Renilla* CDS, and the two are separated by a -1 PRF signal derived from yeast L-A virus so that firefly luciferase is only synthesized subsequent to a -1 PRF event. In the $+1$ PRF reporter, the two luciferase CDSs were similarly separated by the *Ty1* retrotransposon-derived $+1$ PRF signal, and the firefly CDS was in the $+1$ frame. The termination readthrough reporter plasmid encodes for both genes in frame with a UAA codon in between the two CDSs. In the missense suppression reporter the codon for the catalytic arginine_{218-AGA} of the firefly luciferase gene was replaced with an AGC serine codon, causing the firefly luciferase product to be inactive unless an arginyl-tRNA is mistakenly employed to decode this position. Cells were grown to mid-log phase and lysates were monitored by using a luminometer. The ratio of ratios was calculated to determine the rates for each event (Jacobs and Dinman 2004).

-1 PRF can proceed through at least three distinct kinetic pathways, two occurring during translocation events, and one during accommodation of aa-tRNA into the A-site (Harger et al. 2002; Liao et al. 2011). However, in each case the ribosome's A- and P-sites are occupied by tRNA molecules, and specific mRNA signals such as a slippery sequence, spacer region, and mRNA pseudoknot each contribute to determine rates of frameshifting, as can the kinetics of translation elongation, which can affect partitioning of the ribosome with regard to reading frame at the -1 PRF signal. Wild-type rates of -1 PRF were $6.07 \pm 0.16\%$, values that agree with other "wild-type" strains measured in our laboratory [4–8%; see (Harger and Dinman 2003; Jacobs and Dinman 2004)]. Most of the L11 mutants conferred significant changes in -1 PRF. The majority of the mutants promoted decreased rates of -1 PRF, though the F96Q, F96E, and A66D mutants slightly stimulated this phenomenon (Fig. 3). $+1$ PRF requires a different substrate than -1 PRF [reviewed in (Meskauskas et al. 2003)], thus providing an assay for changes in ribosome function in a different biophysical state (Dinman 2006). $+1$ PRF was $10.98 \pm 0.30\%$ in wild-type cells, and again most mutants conferred statistically different changes in this parameter, although the extent of these changes tended to be smaller than for -1 PRF due to intrinsically higher rates of $+1$ PRF (Fig. 3). However, the F96N mutant promoted an ~ 1.7 -fold increase in $+1$ PRF ($18.62 \pm 0.6\%$). Nonsense and missense suppression levels are useful measurements for examining the ability of the ribosome to accurately decode and proofread mRNAs. While decoding occurs in the SSU, far from L11, recent studies have revealed that the two are connected through an informational pathway traversing the B1b/c intersubunit bridge (Rhodin and Dinman 2011). Wild-type levels of termination codon readthrough were $0.137 \pm 0.003\%$, and missense levels were 0.074 ± 0.002 . Assays of mutant cells revealed significant changes in these two aspects of translational fidelity, with ranges from 0.5- to 1.3-fold wild-type levels. Profound increases were promoted by the F96Q and A66D mutants, which promoted rates of nonsense suppression 4.6- and 15-fold higher than wild-type, respectively (Fig. 3). While the A66D and F96Q results aligned well with the observed paromomycin sensitivity data, missense suppression rates were unaffected for F96Q, and other paromomycin sensitive mutants did not promote elevated levels of termination codon readthrough.

A66D and allele-specific F96 mutants confer subunit joining/60S ribosome biogenesis defects

Polysome profiling was performed to examine whether the effects on Killer virus maintenance were related to defects in ribosome biogenesis or subunit joining. Yeast cells were grown to mid-log phase and translation was arrested with cycloheximide. Lysates were clarified, sedimented through sucrose gradients (7–47%), and monitored by continuous spectrophotometry (Fig. 4). The two peaks to the left correspond to the 40S and 60S subunits, respectively. Due to the fact that the parental yeast strain lacked the gene encoding the paralogous ribosomal protein L11A, the 60S subunit peaks were smaller than true wild-type levels, although previous work demonstrated that this level of reduction does not in itself negatively impact cell viability or growth rates (Rotenberg et al. 1988). Further down the gradient (to the right of the 60S peak) is the 80S ribosome peak, followed by peaks corresponding to the polysome fractions. The 92-6A mutant and all R92 mutants mimicked wild-type polysome profiles, but many of the F96 mutants and A66D promoted strong halfmer formation evidenced as shoulders or small second peaks to the right side of the 80S and/or polysome peaks. Halfmers are caused by small subunits stalled on mRNA due to limiting abundance of the LSU and are indicative of 60S subunit ribosome biogenesis defects (Zanchin et al. 1997). This can arise from a decrease in the total number of 60S subunits, thus slowing down the rate of translation initiation as SSUs stall on the mRNA at AUG start codons awaiting a LSU. Alternatively, LSU abundance may not be affected, but the changes in L11's interactions with the SSU protein S18 through the B1b/c bridge may negatively affect LSU joining during initiation. To differentiate between the two models, sucrose density centrifugation was employed to determine the ratios of free 40S and 60S subunits from wild-type and selected mutants. Cells were prepared in the same manner as for polysomes, but cycloheximide was omitted, allowing the majority of translating ribosomes to run off the mRNAs. In addition, lysates were sedimented in a buffer containing a higher salt concentration to promote subunit dissociation, and samples were centrifuged longer to improve peak separation. This prolonged spin especially improved visualization of the 40S peak, separating it from cellular debris at the top of the gradient as observed at the far left of the polysome profiles (Fig. 4), which often confound interpretation of the true heights of the 40S peaks. Figure 5 shows these subunit profiles, with clearly visible 40S, 60S, plus a fraction of remaining 80S peaks. As the 80S fraction contains both subunits in a one-to-one ratio, it can be ignored. Wild-type profiles show that the 60S peak is larger than the 40S peak. Comparative analyses showed that subunit profiles of the mutants generally matched the wild type, with the exception of the F96N mutant. However, even in this mutant, the 60S peak was larger than the 40S peak. These data indicate that the ratios of 40/60S subunits were not substantially altered in the halfmer-promoting mutants, supporting the hypothesis that the L11 mutants affected subunit joining as opposed to 60S subunit steady-state levels.

Biochemical and structural analyses

Changes in rates of PRF and cell growth response to translational inhibitors can result from changes in ribosomal affinities for tRNAs. The dissociation constant between aa-tRNAs and the ribosomal A-site was determined in vitro by binding twofold serial dilutions of [¹⁴C]Phe-tRNA^{Phe} to puromycin-treated salt-washed ribosomes pre-loaded with uncharged

tRNA^{Phe} in their P-sites, polyU, and supplied with soluble binding factors (Dresios et al. 2000). Wild-type K_D values were 103 ± 11 nM. All mutant strains assayed (A66D, 92-6A, F96A, and F96N) had similar K_D values (Fig. 6a, b). P-site tRNA K_D s were measured by binding serial twofold dilutions of Ac-[¹⁴C]Phe-tRNA^{Phe} to empty puromycin-treated salt-washed ribosomes loaded with polyU. Wild-type ribosomes bound this P-site substrate with a K_D of 72.3 ± 7.9 nM (Fig. 6c, d). The 92-6A and F96A mutants fell within wild-type range. A66D ribosomes had a small but statistically significant increase in K_D (89.5 ± 3.8 nM), and F96N promoted a small but significant decrease (48.0 ± 8.3 nM).

Ribosomal RNA chemical protection studies, analyzed by reverse transcriptase primer extension visualized by urea-PAGE, were performed to determine the effects of selected mutants on rRNA structure. 1M7 SHAPE modification was utilized to examine the 92-6A, F96A, and F96N mutants, while CMCT, kethoxal, and DMS were employed for the A66D mutant ribosomes. Approximately, one-third of all bases in the 25S, 5S, and 18S rRNAs were interrogated using SHAPE (online resource 4). Despite this large area of coverage, no changes were observed in 92-6A, F96A, or F96N ribosomes as compared with wild type (data not shown). However, chemical protection studies of the A66D ribosomes revealed that several bases were deprotected from chemical modification relative to wild type (G2677, G3069, and G3116 in H84, H96, and H97, respectively). Additionally, two bases portrayed a pattern of increased protection (A2680 and G3128 in H84 and H97, respectively) (Fig. 7a). The positions of these bases were mapped to the two- and three-dimensional structures of the LSU of the ribosome and are shown in Fig. 7b, c. A table summarizing the phenotypic effects on each mutant is provided in online resource 5.

Discussion

Previous mutagenesis studies demonstrated the involvement of L11 in communicating information between the DC, located in the SSU, and the PTC located in the LSU via the B1b/c bridge (Rhodin and Dinman 2011). The “P-site loop” structural element of L11 is also involved in monitoring and mediating peptidyl tRNA binding to the ribosomal P-site (Rhodin and Dinman 2010). Furthermore, cryo-EM analysis demonstrated that L11 (L5 in bacteria) dynamically interacts with the E-site tRNA (Fischer et al. 2010). The current study focused on amino acids located in the core of L11, specifically those located in close proximity to H84 of the 25S rRNA. A series of mutants were identified that affected cell viability, translational fidelity, ribosome biogenesis, tRNA binding, and rRNA structure. Many of these defects were similar to those conferred by previously described L11 mutants located either in the P-site loop or in the B1b/c bridge region. While these observations suggest that similar mechanistic properties are affected by the L11 core mutants, the unique aspect of this class of mutants is their effects on 60S subunit joining. Thus, these mutants offer additional insight into the role of L11 in the process of translation initiation as well as during the elongation phase of protein synthesis.

Both the R92 and F96 amino acids of L11 were mutated to a variety of amino acids spanning a range of biochemical properties. While mutants at both positions affected cellular growth rates and translational fidelity, the strongest genetic phenotypes were observed in the F96 mutants. Intriguingly, the 92-6A and 93-5A poly-alanine substitutions only promoted

moderate effects on cell growth and sensitivity to translational inhibitors (Fig. 2). Translational fidelity was globally enhanced by the 92-6A mutant (i.e., decreased rates of -1 and $+1$ PRF, UAA termination readthrough and missense suppression, see Fig. 3), correlating with the inability to maintain the Killer virus (Fig. 2c). Notably, this mutant did not affect ribosome biogenesis, ribosome association with tRNAs, or rRNA structure.

Three possible models can be proposed to account for the observed subunit joining defects. The first posits that mutations that alter the intramolecular structure of L11 makes the kinetics of subunit joining less favorable. By this model, all elements required for subunit joining are present at the correct stoichiometric levels, but subtle structural defects in L11 decrease the efficiency of subunit joining or increase the rate of subunit dissociation. Thus, this model proposes that the subunit joining defect is the consequence of inefficient interactions between 60S and 40S subunits. A second possibility is that the structural changes introduced by the A66 or F96 mutants may affect eIF5B association with the 60S subunit. eIF5B promotes recruitment of 60S particles to mRNA-bound 40S particles (Pestova et al. 2000), and eIF5B mutants negatively impact binding rates of 60S subunits to the 40S subunit (Acker et al. 2009). While the precise ribosomal binding site for eIF5B is unknown, it is a large GTPase protein believed to interact with the GTPase activating center, H80, and the P-site pocket at the intersubunit surface of the 60S subunit (Roll-Mecak et al. 2000; Gryk et al. 2002). By this model, altered association of eIF5B with the LSU would reduce rates of 80S formation resulting in the accumulation of halfmers. A third possibility envisions that, while eIF5B may still properly bind free 60S subunits and promote formation of 80S ribosomes, mutations in the core of L11 may depress the rate of GTP hydrolysis by eIF5B subsequent to subunit joining. This could result in stalled complexes containing eIF5B:GTP and/or perhaps promote dissociation of eIF1A from the ribosome, thus blocking progression to elongation. By this scenario, additional 40S subunits could still be loaded onto the mRNA upstream of the start codon, which would appear as halfmers in polysome profiles.

All of the F96 halfmer mutants (F96R, F96S, F96Q, and F96N) exhibited improved growth at 20°C as compared with the optimal growth condition of 30°C and were hypersensitive at 37°C. This is suggestive of intramolecular L11 folding defects rather than on the ability to assemble into intermolecular complexes because higher temperatures increase entropy and intramolecular unfolding rates while low temperatures slow the kinetics of a system, reducing entropy and intermolecular collision rates. In contrast, the halfmer-producing mutant A66D maintained wild-type growth rates at all three temperatures. While the A66D mutant conferred both local (H84) and distant (H96 and H97) changes in rRNA structure, F96N did not. These data suggest the mutations at these two sites affected different aspects of subunit joining. The universally conserved F96 is physically closer to the amino acids directly involved in the B1b/c intersubunit bridge than A66. Thus, we suggest that the F96 mutations affect the conformation of L11 so as to negatively impact formation of the B1b/c bridge without altering 25S rRNA structure. This is consistent with the first model described above. In contrast, the 25S rRNA structural changes associated with the A66D mutant (Fig. 7) are more consistent with the 60S:eIF5B:GTP interaction inhibition, likely acting through models 2 or 3. It should also be noted the level of halfmers in A66D lysates were significantly elevated from those observed in F96 mutants (Fig. 4), supporting the

hypothesis that these two classes of mutants affect subunit joining through different mechanisms. Intriguingly, previous work identified several temperature-sensitive L11 mutants (S97F + A98 V, S119C + G135D, and G135D) all of which also promoted formation of halfmers (Moritz et al. 1991). These mutations are located either at the B1b/c intersubunit bridge region of L11 (S119C), or within a 5-Å radius of each other and position F96 within the three-dimensional structure of L11. While these mutations bolster the notion that the B1b/c bridge region and the core of L11 are critical for proper subunit joining, a deeper characterization of these mutants and their effects on ribosomal structure has not to our knowledge been performed.

Changes in the orientation of H84 have been linked to altered P-site tRNA binding affinities (Rhodin and Dinman 2010, 2011). The moderate increase in P-site tRNA K_D by A66D ribosomes is consistent with this type of defect. This biochemical defect may be due to a conformational change in the L11 P-site loop, which is adjacent to A66, and which interacts with the T-loop of the peptidyl tRNA. It is also possible that F96N may affect positioning of the L11 P-site loop, e.g., drawing it away from the P-site pocket and into closer proximity to H84, thereby increasing the ribosome's affinity for the P-site substrate, as reflected in its lower K_D value for this substrate (Fig. 6). However, direct evidence for this is lacking in the rRNA chemical protection studies.

Collectively, the investigations described in this study have identified critical amino acids in the core of L11 which when altered confer significant defects on translational fidelity, both in maintaining proper reading frame, as well as in the ability of the ribosome to differentiate between cognate, near-cognate, and termination codons. These effects appear to operate independently of the biogenesis/subunit joining defects. These findings add to our growing understanding of the role played by L11 in the eukaryotic ribosome and may offer new insights into diseases previously linked to mutations in L11, e.g., Daimond–Blackfan anemia (linked to L11 among other ribosomal proteins) (Boria et al. 2010; Gazda et al. 2008). Further examination of the structure of L11, particularly regarding mutations of F96 and A66, and its relationship with eIF5B are needed to fully unravel the causes underlying the observed initiation defects.

The authors declare that all experiments and procedures comply with the current laws of the United States of America wherein these studies were performed.

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Fig. 1.

Location of L11 within the ribosome. **a** Crown view of the yeast ribosome LSU. 25S rRNA in gray, unlabeled ribosomal proteins are in blue. L11 (cyan) is positioned at the intersubunit cleft, located in the central protuberance of the LSU where it interacts with 5S rRNA, Helix 84 of 25S rRNA, and the T-loop of the peptidyl-tRNA. PTC denotes the peptidyltransferase center. **b** Close-up view of L11 and neighboring structures. Amino acids of the core region of L11 targeted for mutation are shown as blue spheres. Ribosome structures were generated with PyMol using yeast cryo-EM structures (Taylor et al. 2009) docked with tRNAs from *T. thermophilus* (Yusupov et al. 2001)

Fig. 2.

Phenotypic analyses of the viable L11 mutants. **a** Tenfold dilutions of yeast strains were spotted onto SD-trp media and incubated at temperatures indicated, or **b** on SD-trp media containing paromomycin, anisomycin, or sparsomycin at the indicated concentration and grown at 30°C. **c** Killer virus assays. Wild-type (WT) Killer⁺ cells are identified by a zone of growth inhibition. Killer⁻ cells lack the growth inhibition zone, and decreased radius of the growth zone indicates the Killer^w phenotype

Fig. 3.

L11 mutants alter translational fidelity. Isogenic yeast cells expressing either wild-type or mutant forms of L11B were transformed with dual luciferase reporters and control plasmids and rates of translational recoding were determined. All results are graphed as fold wild-type. -1 PRF was measured using the yeast L-A virus frameshift signal. +1 PRF was directed by the frameshift signal derived from the *Ty1* retrotransposable element. Readthrough measures the percentage of ribosomes suppressing an in-frame UAA termination codon positioned between the *Renilla* and firefly luciferase reporter genes. Missense suppression rates evaluated incorporation of an arginine (AGA) near-cognate amino acid instead of a cognate serine (AGC) at the catalytic codon 218 within the firefly luciferase gene. Error bars denote standard error with WT error bars factored in. Asterisks above samples indicate statistically significant changes as determined by *t* test

Fig. 4.

Polysome profiling. Cycloheximide-arrested cell lysates were sedimented through a 7–47% sucrose gradient and visualized by continuous OD₂₆₀. **Bold-underlined** mutants display halfmers, visualized as shoulders or small peaks to right of primary peaks

Fig. 5.

Subunit profiling. The relative size of 40S versus 60S peaks are unchanged between the natural variation seen in wild-type cells and those of the mutants. 80S peaks can be ignored as they possess one of each subunit. Negative control samples had normal polysome profiles with no halfmers

Fig. 6.

A- and P-site tRNA binding K_D s. **a** Binding of tRNA to the A-site. Ribosomal P-sites were blocked with tRNA^{Phe} at 30°C, then incubated for 35 min with [¹⁴C]Phe-tRNA plus elongation factors and poly(U). 80S-tRNA-poly(U) complexes were bound to nitrocellulose filters and washed with binding buffer. Samples were read by radioactive scintillation counting. Curves were generated using GraphPad Prism 4. **b** A-site tRNA binding K_D values were determined using one site binding with ligand depletion equation. *Error bars* show standard error of variables calculated from three binding reactions per strain. **c** Binding of tRNA to the P-site. 12.5 pmol of salt-washed ribosomes were incubated for 40 min at 30°C with dilutions of N-acetylated-[¹⁴C]Phe-tRNA and poly(U) and processed as described for A-site binding. **d** K_D values for P-site tRNA binding. *Error bars* show standard error of variables calculated from six binding reactions for wild type and three per mutant strain

Fig. 7. Chemical protection analysis of L11 A66D 25S rRNA. **a** Base modifications using *UN* untreated, *DMS* dimethyl sulfate, *KET* Kethoxal, and *CMCT* 1 cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene; showing decreased protection, *filled triangles*; and increased protection, *empty triangles*. **b** Altered bases mapped to two-dimensional structure of 25S rRNA. **c** Changes mapped to three-dimensional structure. *Green bases* have increased protection, *red* have decreased protection