Determining the Region of Rpl10 crucial for Nmd3 release in the 60S Subunit Pathway of Ribosomal Biogenesis

Presented By Jaimin R. Patel

In partial fulfillment of the requirements for graduation with the Dean's Scholars Honors Degree in Biochemistry.

_____	_____
Dr. Arlen Johnson, Ph.D.	Date
Department of Molecular Genetics and Microbiology	
Supervising Professor	
_____	_____
Dr. Karen Browning, Ph.D.	Date
Department of Biochemistry	
Second Reader	
_____	_____
Dr. John Stanton, Ph.D.	Date
Department of Chemistry	
Honors Advisor in Chemistry and Biochemistry	

Abstract

Ribosomes are large structures that synthesize proteins in cells. They are composed of two subunits, the large ribosomal subunit and the small ribosomal subunit, both of which are assembled in the nucleus from ribosomal RNAs and proteins. The subunits are then exported to the cytoplasm where they mature and combine to form a fully functional ribosome. The only way to travel from the nucleus to the cytoplasm is through nuclear pores. Particles that are less than 40kDa in size can diffuse through these pores; however, ribosomes are substantially larger than this. Large particles such as the ribosome require export receptors to interact with the nuclear pore complex and facilitate export from the nucleus. The large ribosomal subunit of the ribosome is exported out of the nucleus with the help of a protein known as Nmd3. Nmd3 interacts with the large ribosomal subunit and provides a nuclear export signal, which recruits export receptors and thereby facilitates subunit export from the nucleus. However, in order for more large ribosomal subunits to be able to be exported from the nucleus, Nmd3 must be released from the ribosome in the cytoplasm and recycled back to the nucleus.

Rpl10 is a protein of the large ribosomal subunit that is required for the release of Nmd3 from the large ribosomal subunit and allows it to be recycled back to the nucleus, though the exact mechanism of Nmd3 release is unknown. A mutant *NMD3 I112T I362T* allele has been shown to suppress a mutant *rpl10 G161D* allele, thus suggesting some type of interaction between the Nmd3 and Rpl10 proteins. Further studies have shown that Nmd3 and Rpl10 do not physically interact with each other; however, Rpl10 has been shown to interact with tRNA and rRNA, thus possibly providing a means by which Rpl10 may indirectly affect Nmd3 release.

A genetic screen was devised to identify mutations in *RPL10* that are dependent on *NMD3 I112T I362T* for survival. These mutations in *RPL10* were mapped onto the atomic structure of Rpl10 in the ribosome. While the mutations did not map to any one location on Rpl10, two clusters of three mutations each were identified, one near the N-terminus of the protein and another near the C-terminus of the protein, both containing residues that could interact with rRNA nearby, as well as residues that could interact with other residues internal to Rpl10 and thus affect the overall structure of the protein. Thus, it may be possible that Rpl10 affects Nmd3 release indirectly through accommodation of Rpl10 onto the ribosome via protein-rRNA interactions that affect Nmd3-rRNA interactions.

Table of Contents

Background

Ribosomes synthesize proteins in cells. They are composed of both a large subunit and a small subunit, with each subunit composed of ribosomal ribonucleic acid (rRNA) and protein. Despite proteins being commonly known as catalysts, the rRNA in ribosomes has been shown to perform all catalytic functions in the ribosome. rRNA in the small ribosomal subunit is used to decode mRNA, while rRNA in the large ribosomal subunit is involved in peptide bond formation.[12,14] The two ribosomal subunits, in addition to being characterized by their size, are also characterized by their sedimentation rates. As such, in eukaryotes the large ribosomal subunit is often referred to as the 60S subunit and the small ribosomal subunit is referred to as the 40S subunit. The 40S subunit contains 18S rRNA and 33 proteins while the 60S subunit contains 25S, 5.8S, and 5S rRNA and 46 proteins.[3,12]

Prokaryotic and eukaryotic ribosomes, while similar in function, have been shown to differ in structure; namely, eukaryotic ribosomes are much larger than their prokaryotic counterparts, and they undergo the processes of initiation, elongation, and termination differently in some aspects when compared to prokaryotes. Eukaryotic ribosomes are at least 40% larger than their prokaryotic counterparts due to rRNA elements known as expansion segments, as well as additional proteins that are not found in prokaryotes.[1] Most of these additional proteins are found on the surface of the ribosome, and it has been shown that 32 of the proteins found in eukaryotes have no homologs in crystal structures of the ribosomes of archaea and bacteria.[1]

Ribosome biogenesis as a whole is a very complex and energy-consuming process. Over 200 *trans*-acting factors, proteins that are not an integral part of the ribosome, are used in the

ribosomal maturation process.[2] The pathway begins when a pre-90S ribosome splits into pre-60S and pre-40S subunits. About 50 non-ribosomal proteins are associated with the earliest nucleolar 60S subunits while none are associated with fully mature 60S subunits, thus indicating the importance of the *trans*-acting factors to the maturation process, as well as the considerable changes that occur in ribosomal structure over biogenesis.[13]

Eukaryotic cells have one problem that must be dealt with that prokaryotic cells do not face: eukaryotic cells contain a membrane-bound nucleus. Because ribosomes are initially assembled in the nucleolus, a subcompartment of the nucleus, they must be transported out of the nucleus, which is bound by the nuclear membrane. The nuclear membrane contains nuclear pores through which particles can pass from the nucleus to the cytoplasm and vice-versa. While molecules less than 40kDa in size can cross the nuclear pore complex by simple diffusion, the ribosome is much larger in size than 40kDa.[5] When exported, the ribosome is not transported across the nuclear membrane as a whole; the large and small ribosomal subunits are assembled and exported from the nucleus independently.[6] Even independently, both subunits are far too big to diffuse through nuclear pores and thus both subunits require the help of carrier proteins for export.

Nucleoporins (Nups) are proteins that line nuclear pores and control macromolecules going into and out of the nucleus. Nups interact hydrophobically with molecules; thus the negatively-charged ribosomal subunits must interact with other proteins that shield this negative charge that is present due to the negatively-charged phosphate backbone of the rRNA in order to pass through these pores. Export of the large ribosomal subunit requires a shuttling adaptor protein

that associates with the pre-60S subunit, Nmd3. The C-terminus of Nmd3 contains a leucine-rich nuclear export signal that is recognized by Crm1, a nuclear export receptor.[6] Crm1 shields the negative charge of the 60S subunit and thus allows it to be exported through the hydrophobic nuclear pores.[10] Due to the size of the large ribosomal subunit, however, multiple export receptors are used. In addition to Crm1, the proteins Arx1 and a Mtr2-Mex67 heterodimer are used as export receptors as well.[5] While Nmd3 helps with nuclear export of the ribosome, it must be recycled back to the nucleus after nuclear export for other ribosomes to be exported.

Several proteins have been shown to be involved in Nmd3 release. Tif6, a protein at the interface of the subunits, must be released from the 60S subunit for Nmd3 to be released.[7] The exact mechanism by which Nmd3 is released is not known exactly, but has been shown to be affected by the proteins Rpl10, Lsg1, and Sqt1.[15] It is currently proposed that Sqt1 helps facilitate Rpl10 loading onto the ribosomal subunit in the cytoplasm, which in turn affects Nmd3 release from the 60S subunit; the GTPase Lsg1 is also thought to play a role in this process, facilitating Nmd3 release.[8]

Nmd3 is bound to the ribosome via contacts with rRNA and it has previously been shown that Rpl10 and Nmd3 do not interact directly.[8] Additionally, it has been proposed that Rpl10 interacts with rRNA, which therein helps promote Nmd3 release from the ribosome. Structurally, the N-terminal domain of Nmd3 is its ribosomal binding domain. Helix 95 of the 25S rRNA of the 60S subunit, to which Nmd3 binds and which is located in the vicinity of Rpl10, could possibly be a means for indirect interaction between Nmd3 and Rpl10.[10] In

bacteria, the possibility has been brought up of Rpl10 interacting with A-site tRNA within the ribosome.[14] It has also been previously shown that the arginine-51 and arginine-56 residues of Rpl10 in bacteria interact with A-site tRNA, thus perhaps introducing the possibility of indirect Rpl10-Nmd3 interaction occurring via Rpl10-A-site tRNA interaction.[3,14] Furthermore, it has been shown that Rpl10 plays an important role in coordinating tRNA movement through the large ribosomal subunit.[9]

Previous studies have been done in an attempt to determine the regions of Rpl10 that are significant for Rpl10 interactions with other proteins.[2,4] Anne Hofer of the Johnson Laboratory did a mutational analysis of *RPL10* in an attempt to determine what proteins Rpl10 interacts with directly or indirectly. Hofer created random mutations in *RPL10* and tested these mutants for interaction with various ribosomal proteins and *trans*-acting factors, including Nmd3. She identified mutations in a central loop of Rpl10 (amino acids 102-112), that affected Nmd3 release from the pre-60S subunit. Further work by another member of the Johnson Laboratory, Cyril Bussiere, determined that this P-site loop affects Tif6 release from the pre-60S subunit. Suppression of these mutants by *TIF6 V192F* allowed Nmd3 release, thus showing that the P-site loop directly affects Tif6 release, which indirectly affects Nmd3 release as Tif6 release is a necessary prerequisite for Nmd3 release.[2,4] Bussiere's work also showed that while the P-site loop of Rpl10 is required for Tif6 release, other distinct features of Rpl10 are necessary for Nmd3 release.[4] What exact features of Rpl10 these are have yet to be determined.

The aim of this study is to perform another mutational analysis of *RPL10*, and to test *rpl10* mutants for suppression by an *NMD3 I112T I362T* vector, which is already known to suppress a

rpl10 G161D mutant.[3] Mutations in *RPL10* that affect Nmd3 release will not be tolerated by wild-type *NMD3*, but suppressed mutations will be tolerated by *NMD3 I112T I362T*. By identifying suppressed *rpl10* mutants, it may be possible to map suppressed mutations to a region of Rpl10 important for Nmd3 release.

Materials and Methods

Strains and Plasmids

All strains were grown at 30°C in rich medium (yeast extract peptone) or dropout medium containing either 2% glucose or 1% galactose as the carbon source. The only yeast strain used in this study was AJY 2104 (matα KanMX::GAL1::RPL10 ade2 ade3 ura3 leu2) (Hofer *et al*, 2007). Plasmids used in this study were: pAJ 24 (LEU2-CEN) (Sikorski *et al*, 1989), pAJ 100 (URA3-CEN) (Sikorski *et al*, 1989), pAJ 755 (URA3-CEN NMD3-GFP) (This Study), pAJ 1197 (LEU2-CEN RPL10-myc) (Hofer *et al*, 2007), pAJ 2240 (URA3-CEN TIF6[V192F]) (Bussiere *et al*, 2011), pAJ 2522 (LEU2-CEN RPL10) (Bussiere *et al*, 2011), pAJ 2541 (URA3-CEN NMD3[I112T,I362T]-GFP) (This Study), and pAJ 2665 (URA3-CEN TIF6) (Bussiere *et al*, 2011).

PCR Mutagenesis

In order to create *rpl10* mutants, PCR mutagenesis was performed on pAJ 1197. Primer AJO 525 (5'-AGCGGATAACAATTTCACACAGGA), a forward primer, and AJO 722 (5'-TCAAGTCTTCCTCGGAGATTAGCTTTTGTTCACCGTTAATTAACC), a reverse primer, were used. Both flanked the *RPL10* gene, thus allowing it to be amplified via PCR. 10 reactions of 25 cycles each were performed using the enzyme Taq Polymerase. Each cycle consisted of 30 seconds at 95°C, 30 seconds at 50°C, and 1 minute and 20 seconds at 72°C.

Restriction Digest

In order to create gapped pAJ 1197 to allow mutant *rpl10* DNA created from PCR mutagenesis to recombine *in vivo* in cells after co-transformation with gapped vector, a restriction digest was

performed on pAJ 1197 with the enzymes BsaBI and SalIHF. Both of these enzymes cut internally in the *RPL10* gene, removing most of the open reading frame. Samples were incubated with 3 units of SalIHF per 1ug of DNA for one hour at 37°C followed by incubation with 3 units of BsaBI per 1ug of DNA for one hour at 60°C. The digest products were run on a 1% agarose gel and the gapped vector was purified from the gel and used in the yeast transformation reaction.

Yeast Transformation

For yeast transformations, AJY 2104 was grown on appropriate plates overnight at 30°C. Using a 1ml glass pipette and 1ml ddH_2O, cells were scrapped off the plate and pipetted into a 1.5ml Eppendorf Tube. These cells were centrifuged at 14000rpm for 15 seconds and the supernatant was discarded. Next, the cells were washed in a 1x Li/TE solution and centrifuged again at 14000rpm for 15 seconds and the supernatant was again discarded. Then, cells were incubated with carrier DNA, 400ng sample DNA, and 5 drops of PEG/Li/TE from a 5ml pipette for 30 minutes at 30°C and 15 minutes at 42°C. 1ml of ddH_2O was then added to the transformation reaction and the reaction was centrifuged at 14000rpm for 15 seconds. The supernatant was discarded and 400ul of ddH_2O was added to the reaction. The reaction was then plated on selective plates that were incubated overnight at 30°C.

Isolating rpl10 mutants that depend on suppression by NMD3 I112T, I362T

In order to identify *rpl10* mutants that were dependent on *NMD3 I112T I362T*, mutagenized *rpl10* was co-transformed, along with BsaBI/SalIHF digested pAJ 1197, into AJY 2104 containing pAJ 2541. This transformation reaction was plated on ura^-leu^-glucose plates to select

for cells that contained both the *rpl10* mutant gene that had been recombined into pAJ 1197 as well as pAJ 2541. In order to conclude that the *rpl10* mutant was dependent on *NMD3 I112T I362T*, it had to be shown that the *rpl10* mutant grew poorly or not at all in the absence of *NMD3 I112T I362T* and the presence of only wild-type *NMD3*. This was done by replica plating colonies from the $ura^{-}leu^{-}$glucose plates to $leu^{-}ura^{+}$glucose plates. This would give colonies the opportunity to lose the pAJ 2541 plasmid, as the URA3 gene was no longer necessary. Then, the leu^{-}glucose plate was replica plated onto leu^{-}5FOA glucose plates, which would ensure that no colonies that contained pAJ 2541 survived. Colonies that were 5FOA-sensitive were the colonies of interest.

Isolating rpl10 Mutant Plasmids

In order to isolate *rpl10* mutant plasmids of interest from cells, 5FOA-sensitive colonies were taken from the leu^{-}glucose plates and streaked for single colonies on leu^{-}FOA galactose plates to select for the mutant *rpl10* plasmid and to ensure elimination of pAJ 2541. Single colonies were used to inoculate 2ml of leu^{-}galactose medium. Cultures were grown to saturation and plasmid DNA was extracted using a Zymo Yeast Plasmid Purification Kit. Because yield from yeast plasmid extraction is low, 5ul of the extracted DNA was transformed into 20ul DH5α bacterial cells to propagate the mutant *rpl10* plasmids. These DH5α cells were grown on LB/Amp plates overnight at 37°C and then a single colony from a plate was used to inoculate 2ml of LB/Amp medium that was grown overnight in a 37°C shaker incubator. The plasmid of interest was isolated from these cells with a GenElute Plasmid Miniprep Kit. Plasmids were re-transformed into AJY 2104 containing pAJ 2541 to confirm their dependence on *NMD3 I112T, I362T*. *rpl10* mutants that showed dependence on *NMD3 I112T I362T* were sequenced using primer AJO 722.

Sucrose Cushions

For sucrose cushions, *rpl10* mutant strains containing pAJ 2541 were grown overnight in 2ml ura⁻leu⁻glucose media in a 30°C shaker incubator. 200ul of this culture was added to 100ml ura⁻ leu⁻glucose media which was grown overnight in a 30°C shaker incubator. When the OD_{600} of this solution was between .4 and .6, the cells were harvested by centrifugation at 5000rpm for 5 minutes at 4C in a Beckham JLA 10.5 rotor. The supernatant was discarded and the pellet was resuspended in the little supernatant remaining, and the cells were then transferred to a 1.5ml Eppendorf tube and spun in a microcentrifuge at 5000rpm for 5 minutes at 4°C. Then, the supernatant was discarded and the cells were stored at -80°C. Next, the cells were washed with 500ul X-buffer (20mM Tris pH 7.4, 100mM NaCl, 6mM $MgCl_2$, 10% glycerol, 0.1% NP40, 1mM PMSF, 1uM pepstatin A, 1uM leupeptin) and spun again at 5000rpm for 5 minutes at 4°C. The cells were then resuspended in 500ul X-buffer and transferred to glass tubes, along with approximately 1ml glass beads. Cells then underwent 5 cycles of vortexing for 30 seconds followed by at least 30 seconds on ice. Cell extracts were transferred to an Eppendorf tube. The glass beads were then washed with 300ul X-buffer and all remaining extracts were transferred to the same Eppendorf tube. Upon checking for greater than 50% cell lysis under a microscope, the extract was then centrifuged at 14000rpm for 10 minutes at 4°C, and the supernatant was transferred to a new prechilled Eppendorf tube. This supernatant was once again centrifuged at 14000rpm for 10 minutes at 4°C, and the supernatant was once again transferred to a new prechilled Eppendorf tube. The protein concentrations of various samples were then normalized by their OD_{280} values, using X-buffer to dilute more concentrated samples. 200ul of sample was then loaded on top of 500ul of 0.5M sucrose prepared in X-buffer. These samples were then centrifuged at 75000rpm for 1 hour at 4°C in a Beckman TLA 100.3 rotor. After the

centrifugation, 40ul of the supernatant was then collected and 10ul of 5X Laemmli Buffer was added to this supernatant sample before the remainder of the supernatant was removed from the tube. The ribosome pellet was then resuspended in 200ul X-buffer and 50ul of 5X Laemmli Buffer was added to this pellet sample.

Western Analysis

Samples produced from sucrose cushions were heated at 100°C for 3 minutes and run on SDS-PAGE gels consisting of a 4% stacking gel and a 10% separating gel. Gels were run at a constant current of 30mA. A membrane transfer was then performed at a constant voltage of 15V for 45 minutes. The nitrocellulose membrane was then incubated in 10ml 5% Milk/TBS for 2 hours at room temperature. Next, the membrane was rinsed with 10ml TBS for 5 minutes, followed by incubation with 2ul primary antibody in 10ml TBST for 2-3 hours. The membrane was then washed three times with 10ml TBST for 5 minutes each, followed by incubation with 0.3ul secondary antibody in 10ml TBST for 2-3 hours. Next, the membrane was again washed three times with 10ml TBST for 5 minutes each. 500ul of A/B solution was then added to the membrane, which was then taped into a cassette and exposed to film.

Results

RPL10 was randomly mutagenized using the low fidelity Taq DNA polymerase as described in the Materials and Methods. Over 4000 transformants were screened and about 200 mutants were picked as potential 5FOA-sensitive mutants. From this initial screen, 21 continued to show dependence on *NMD3 I112T I362T* after streaking for single colonies on leu⁻5FOA glucose plates and comparing growth to AJY 2104 with pAJ 1197. Plasmid DNA was isolated from these 21 mutant strains and propagated in DH5α cells, as stated in the Methods. After the *rpl10* mutant plasmids were isolated from DH5α cells, they were retransformed into AJY 2104 containing pAJ 2541, and 11 *rpl10* mutants continued to show dependence on *NMD3 I112T I362T*. These 11 *rpl10* mutant plasmids were sequenced, and all of these plasmids were found to contain mutations in the open-reading frame of *RPL10*, with the exception of one. To test the growth rates of the *rpl10* mutants compared to wild-type *RPL10* in the presence of wild-type *NMD3*, AJY 2104 was transformed with pAJ 1197, as well as all of the sequenced *rpl10* mutants, and a serial dilution test was performed on all of these transformants. This test identified 4 strains that grew as fast as wild-type *RPL10*, and hence were false positives that should have not been sequenced. The six *rpl10* mutants that were identified to depend on *NMD3 I112T I362T* are listed in Table 1.

Table 1: *NMD3 I112T I362T*-dependent *rpl10* Mutants

Sample	Nucleotide Mutation	Protein Mutation	Growth in rpl10Δ
PCR1	A67G	N23D	+
PCR2	T123C, A135G, A472G	K158E	+
PCR3	T77C, T308C, A550G, C602T	V26A, L103S, K184E, S201F	-
PCR8	A43G, C378G, T443C	K15E, V148A	++
PCR15	A268G	R90G	++
PCR18	T637C	F213L	+

In Table 1, the wild-type *RPL10* growth rate would be rated as +++, with each + signifying a fairly dense spot on the serial dilution test in Figure 1 in the presence of empty vector. To determine the relative strength of dependence of these six *rpl10* mutants on *NMD3 I112T I362T*, AJY 2104 containing one mutant *rpl10* plasmid was transformed with 3 plasmids: pAJ 100 (empty vector), pAJ 755 (wild-type *NMD3*), and pAJ 2541 (*NMD3 I112T I362T*), and a serial dilution test was performed for each of the transformed strains, so as to compare the relative growth rates of the *rpl10* mutants under varying *NMD3* conditions. The results of these serial dilution tests are shown in Figure 1.

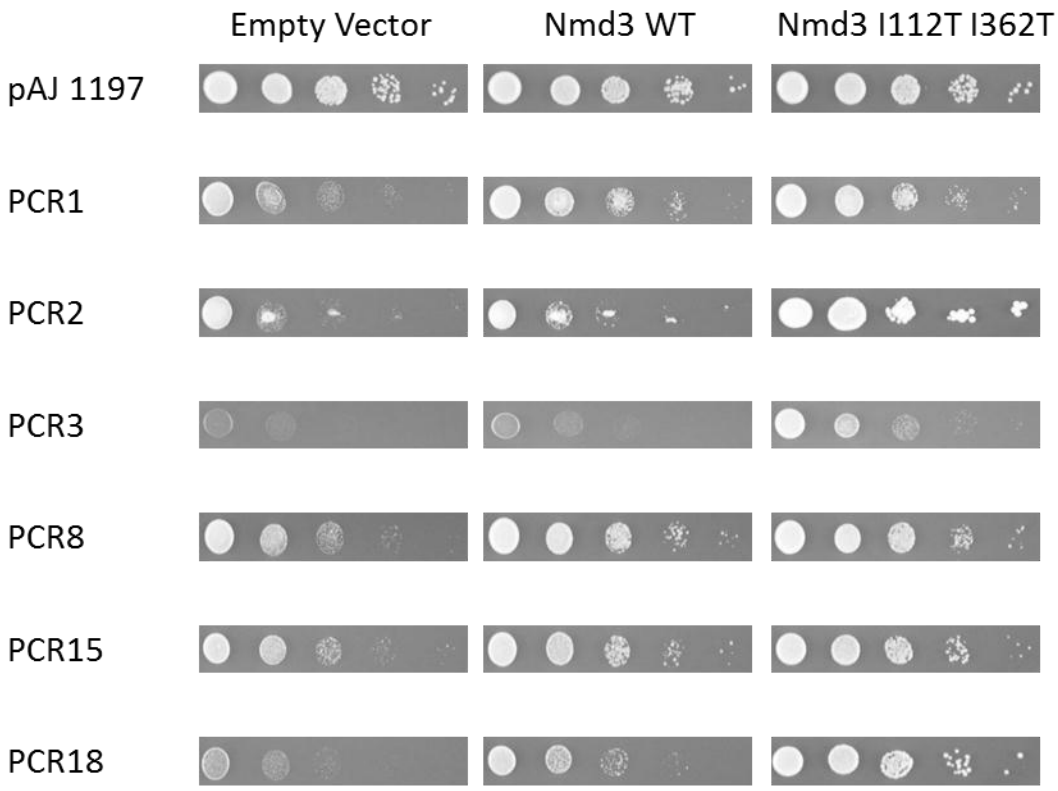

Figure 1: AJY 2104 containing wild-type or mutant *RPL10* was transformed with pAJ 100 (empty vector), pAJ 755 (*NMD3* WT), and pAJ 2541 (*NMD3 I112T I362T*). Transformants were grown in 2ml ura$^-$leu$^-$galactose media overnight in a 30°C shaker incubator. The OD_{600} of samples was taken the next day to standardize concentrations and successive 10-fold dilutions were done for each spot indicated, from left to right. 4ul of cells were spotted on ura$^-$leu$^-$glucose plates, which were incubated at 30°C for 2 days.

As can be seen, the mutant strains did not grow well in the presence of empty vector. Strains grew slightly better in the presence of a wild-type *NMD3* plasmid, but all strains grew best in the presence of *NMD3 I112T I362T*. PCR 3 was the mutant that relied on the *NMD3 I112T I362T* the most, followed PCR 1, 2, and 18, while PCR 8 and PCR 15 only grew slightly better in the presence of the *NMD3 I112T I362T* compared to their growth in the presence of a wild-type *NMD3* plasmid.

Mapping Suppressed Rpl10 Mutations

In order to determine if the suppressed *RPL10* mutations mapped to a common region in the tertiary structure of Rpl10, a crystal structure of the 60S ribosomal subunit was used (PDB codes 3U5D, 3U5E).[16] The suppressed mutations are shown in the following figures. While all mutations will be discussed, firm conclusions cannot be made about mutants containing multiple mutations without subcloning them as individual mutations.

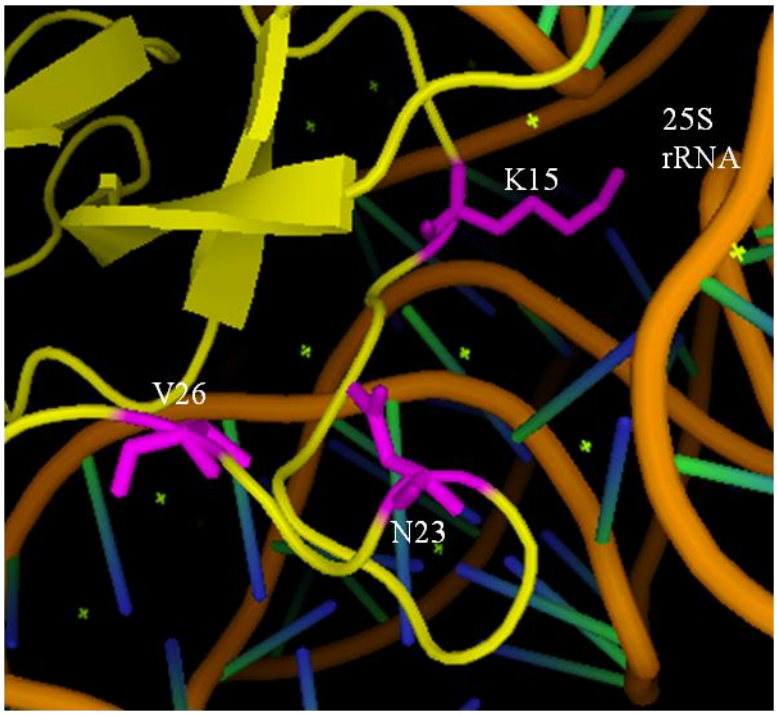

Figure 2: Amino acids K15, N23, and V26 are close to each other in the tertiary structure of Rpl10. The yellow structure is the Rpl10 protein, while *NMD3 I112T I362T*-suppressed residues of Rpl10 are shown in pink. The orange structure is the phosphate backbone of rRNA, while the nitrogen bases of the rRNA are shown in green and blue.

As can be seen in Figure 2, 3 of the suppressed mutations that map in a similar region of the tertiary structure of Rpl10 are K15E, N23D, and V26A. K15E could have a significant effect on protein-rRNA interaction as this would involve substituting a positively-charged amino acid that can interact with the negatively-charged phosphate backbone of rRNA with a negatively-charged amino acid. As shown in Figure 2, the R group of lysine is positioned close to rRNA, thus suggesting an important Rpl10-rRNA electrostatic interaction. This mutation was one of two in PCR8, which was one of the weaker *rpl10* mutants in terms of dependence on *NMD3 I112T I362T*. However, based on its location and possibility to interact with rRNA, it seems that it is an important residue. The mutation N23D substitutes a polar R-group for a negatively-charged R-group. As shown in Figure 2, the R-group of asparagine faces the interior of the protein and

may play an important role in affecting the tertiary structure of Rpl10. N23D is the only mutation in PCR1, which did show fairly strong dependence on *NMD3 I112T I362T*, so this seems to be an important residue. The mutation V26A substitutes a nonpolar amino acid for another nonpolar amino acid and as such, this mutation would not be expected to have a significant effect on Rpl10 structure. Because this mutation is just one of four total in PCR3, it may be possible that *NMD3 I112T I362T* has no effect on this particular mutation.

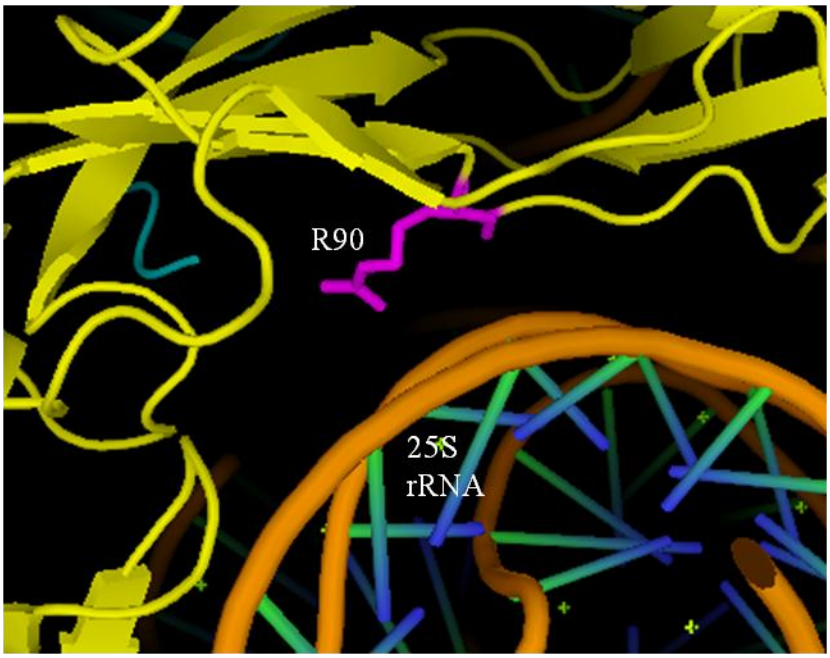

Figure 3: Amino acid R90 had no other suppressed mutations near it in the tertiary structure of Rpl10. The yellow structure is the Rpl10 protein, while the *NMD3 I112T I362T*-suppressed residue of Rpl10 is shown in pink. The orange structure is the phosphate backbone of rRNA, while the nitrogen bases of the rRNA are shown in green and blue.

As can be seen in Figure 3, the *rpl10* mutation R90G mapped to a region of Rpl10 that no other suppressed mutations did. R90G substitutes a positively-charged amino acid for a polar one. Based on Figure 3, it can be hypothesized that the positively-charged arginine interacts electrostatically with the negatively-charged phosphate backbone of rRNA. R90G was the only suppressed mutation in PCR 15, which was one of the strains that only showed slightly better

growth in the presence of *NMD3 I112T I362T* compared to growth in the presence of wild-type *NMD3*. However, while R90 may not be a crucial residue to Rpl10 function in terms of Nmd3 release, the possibility of its interaction with rRNA is evident.

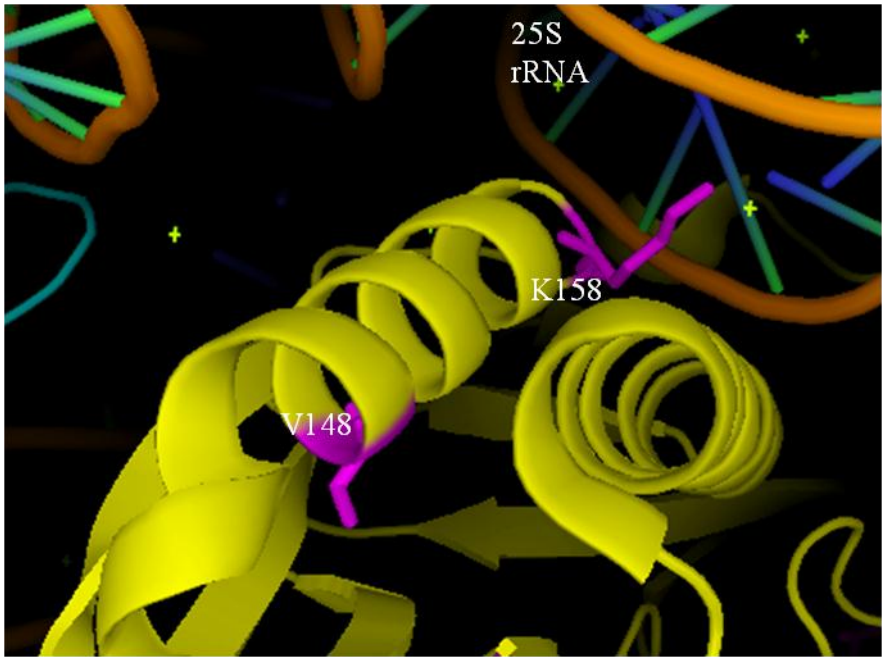

Figure 4: While amino acid V148 faces the interior of the protein, amino acid K158 faces the external environment of the protein and could interact with rRNA. The yellow structure is the Rpl10 protein, while *NMD3 I112T I362T*-suppressed residues of Rpl10 are shown in pink. The orange structure is the phosphate backbone of rRNA, while the nitrogen bases of the rRNA are shown in green and blue.

As shown in Figure 4, the suppressed mutations V148A and K158E mapped to a fairly similar region of Rpl10. V148A involves substituting a nonpolar amino acid for another nonpolar amino acid, both of fairly similar size. The R-group of valine faces the interior of the protein. Because the amino acid substitution is not drastic, and V148A is one of two mutations in PCR8, it could be possible that this particular mutation is not directly affected by *NMD3 I112T I362T*, while the other mutation in PCR 8, K15E, is. The other suppressed mutation in this region of Rpl10 is K158E, which substitutes a positively-charged amino acid with a negatively-charged amino acid. Additionally, the R-group of lysine is shown to be in the vicinity of rRNA, again suggesting the

possibility of electrostatic interaction between the R-group of lysine and the negatively-charged phosphate backbone of rRNA. K158E is the only suppressed mutation in PCR2, a *rpl10* mutant that is fairly dependent on *NMD3 I112T I362T*, thus suggesting that this lysine-rRNA interaction may be important for Nmd3 release.

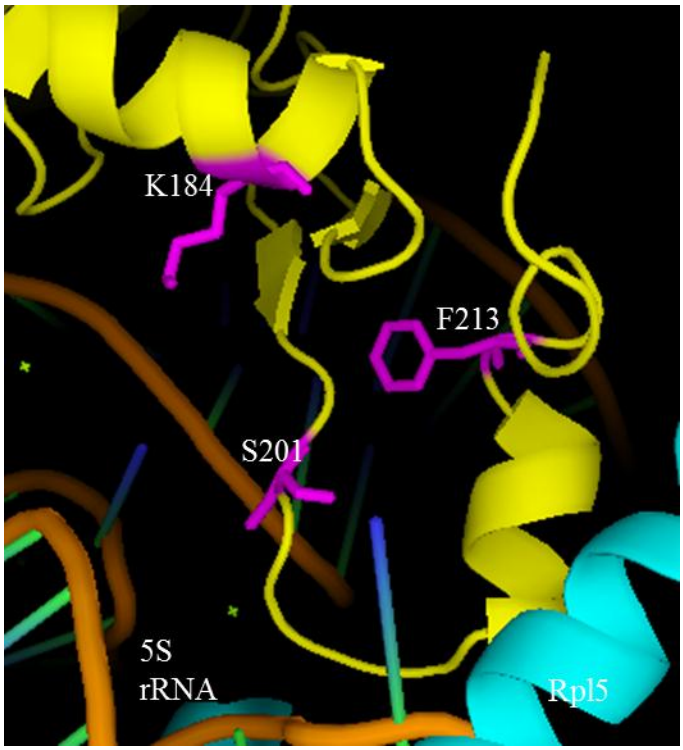

Figure 5: Amino acids K184, S201, and F213 are all near the C-terminus of the protein and could possibly interact with 5S rRNA or possibly Rpl5. The yellow structure is the Rpl10 protein, while *NMD3 I112T I362T*-suppressed residues of Rpl10 are shown in pink. The orange structure is the phosphate backbone of rRNA, while the nitrogen bases of the rRNA are shown in green and blue.

Figure 5 shows that the suppressed mutations K184E, S201F, and F213L all map to a common region of the protein, near the C-terminus of Rpl10. K184E involves the substitution of a positively-charged amino acid for a negatively-charged amino acid. Unlike other cases, there is no rRNA that is very close to this residue, and because K184E is one of four mutations in PCR3, it may be possible that this particular mutation is not one that is suppressed by *NMD3 I112T*

I362T. S201F is a mutation that involves substituting a polar amino acid for a bulky, aromatic amino acid. This mutation would be expected to have an effect on Rpl10 structure, through steric effects and due to the possibility that the R group of serine acts through dipole dipole interactions to interact with the nitrogen bases and possibly hydrogen bond with the bases of rRNA. S201F thus seems to be an important mutation in PCR3 that is likely dependent on *NMD3 I112T I362T*. The mutation F213L substitutes a bulky, aromatic amino acid for a much smaller, nonpolar amino acid. The R-group of phenylalanine faces the interior of Rpl10, and by the steric effects of this mutation substitution, it is possible that this mutation affects the overall structure of the C-terminal region of Rpl10. Additionally, F213L is the only mutation in PCR18, an *rpl10* mutant fairly dependent on *NMD3 I112T I362T*, thus further supporting the fact that this is an important mutation to Rpl10-Nmd3 interaction.

Testing rpl10 Mutants for suppression by TIF6 V192F

Because Tif6 release occurs before Nmd3 release in ribosomal biogenesis, and Tif6 release is a necessary prerequisite for Nmd3 release from the pre-60S subunit, it had to be confirmed whether or not the isolated *rpl10* mutants were only defective in Nmd3 release or whether they were defective in Tif6 release, which would thereby affect Nmd3 release. To determine this, serial dilution tests were performed on AJY 2104 containing wild-type or mutant *RPL10* that was then transformed with 3 different plasmids: an empty vector lacking *TIF6*, a vector containing wild-type *TIF6*, and a vector containing mutant *TIF6 V192F*. *TIF6 V192F* has been shown to have less affinity for the ribosomal subunit and had previously been shown to suppress mutations in the P-site loop of Rpl10, thus suppressing the Tif6 release defect of such mutants.[2] If the *rpl10* mutants were suppressed by *TIF6 V192F* but not wild-type *TIF6*, this would provide

evidence that the *rpl10* mutants were dependent on Tif6 release for proper growth, rather than on only Nmd3 release, which would happen after Tif6 release. The results of the serial dilution assay are shown in Figure 6.

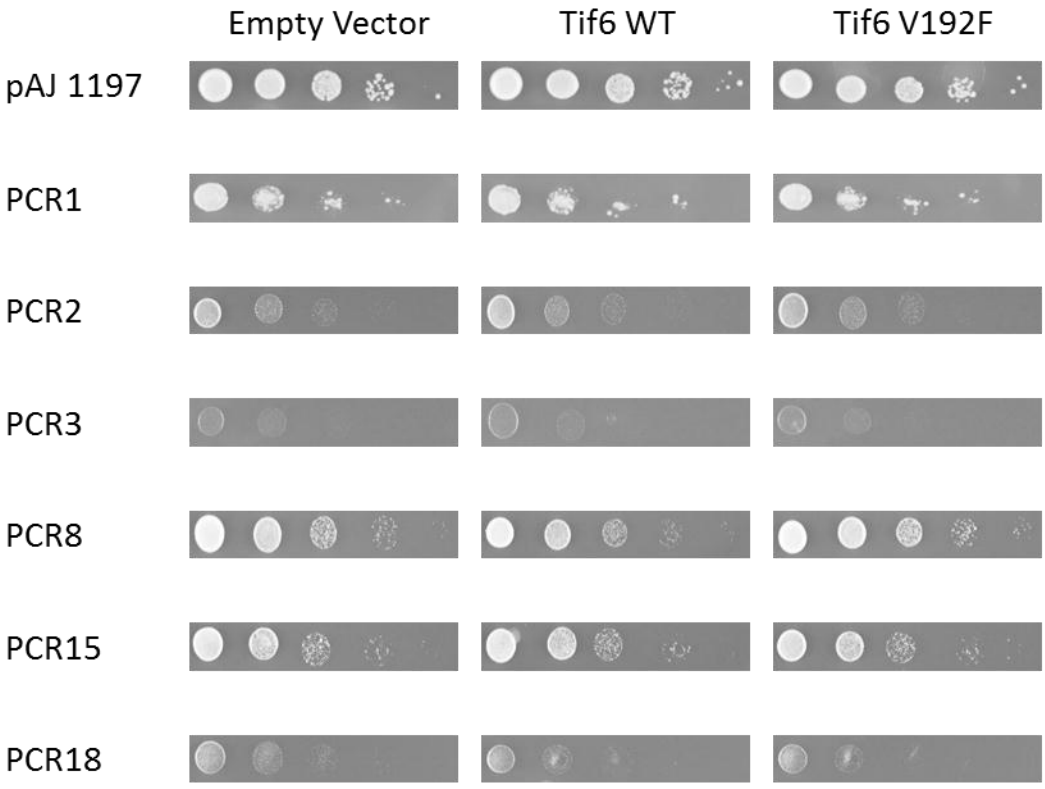

Figure 6: AJY 2104 containing wild-type or mutant *RPL10* was transformed with pAJ 100 (empty vector), pAJ 2240 (*TIF6 V192F*), and pAJ 2665 (*TIF6* WT). Transformants were grown in 2ml ura$^-$leu$^-$galactose media overnight in a 30°C shaker incubator. The OD_{600} of samples was taken the next day to standardize concentrations and successive 10-fold dilutions were done for each spot indicated, from left to right. 4ul of cells were spotted on ura$^-$leu$^-$glucose plates, which were incubated at 30°C for 2 days.

As shown in Figure 6, all of the *rpl10* mutants showed fairly similar growth in the presence of empty vector, wild-type *TIF6*, and *TIF6 V192F*. Thus, it can be concluded that all of the isolated mutants are dependent on *NMD3 I112T I362T* for suppression, and do not have any role in Tif6 release.

Confirmation of Rpl10 Mutant Ribosome Loading

In order to confirm that the *rpl10* mutants were actually loading onto the ribosome, sucrose cushions were performed, as described in the Methods. To counter the issue of galactose-induced *RPL10* overexpression due to AJY 2104 containing genomic galactose-induced *RPL10*, AJY 2104 was transformed with both *rpl10* mutants and the *NMD3 I112T I362T*-containing vector, pAJ 2541, and mutant strains were grown in glucose-containing media so as to shut down genomic Rpl10 production. A positive control to ensure the western blot was working when α-myc antibody was used was wild-type pAJ 1197, while the negative control used was pAJ 2522, which contains untagged Rpl10. Both of these controls did not contain pAJ 2541, so the *RPL10*-containing vectors produced proteins that interacted with wild-type *NMD3*. To confirm that myc-tagged mutant *rpl10* proteins were cosedimenting with ribosomes, the sedimentation of ribosomes was monitored independently using an antibody against a wild-type ribosomal protein, α-rpl8 (results not shown). The results of the α-myc western blots are shown below in Figure 7.

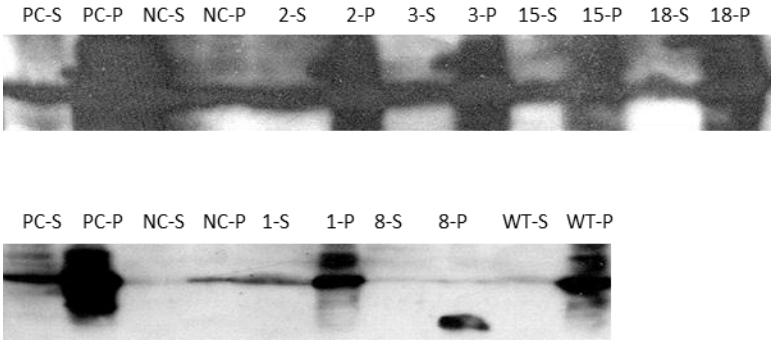

Figure 7: Nitrocellulose membranes were incubated with 2ul α-myc primary antibody and 0.3ul goat-α-mouse secondary antibody. In the samples labeled above PC indicates positive control while NC indicates negative control. Numbers indicate the PCR number of the various *rpl10* mutants and WT indicates pAJ 1197 was used as the *RPL10* vector. The ending of S and P indicate that the sample was taken from the supernatant or pellet, respectively, of the sucrose cushion samples. Positive and negative control samples involved wild-type myc-tagged *RPL10* and untagged *RPL10*, respectively, interacting with wild-type *NMD3*, while the wild-type sample involved myc-tagged *RPL10* interacting with GFP-tagged *NMD3 I112T I362T*.

As can be seen, the *rpl10* mutants are found predominantly in the ribosomal pellet, indicating ribosome association. Considering that the *rpl10* mutants interacting with *NMD3 I112T I362T* support cell growth, this must be the case. The one sample that poses an issue is that of PCR 8, which is shown to have its Rpl10 protein run much lower on the western blot than any other sample. This would suggest that this protein was somehow truncated. The myc-tag must remain on the protein, however, or else no signal would appear for this sample on the western blot. It may be possible that this *rpl10* mutant is more susceptible to proteolysis. Further investigation should be done on PCR 8 to determine the exact cause of why it appears to be a smaller size than every other *rpl10* mutant.

Discussion

The objective of this study was to perform a PCR mutagenesis on *RPL10* in the hopes of determining a region of Rpl10 that is significant for Nmd3 release. Based on the mapping of *RPL10* mutations, there is no one region of Rpl10 that is involved in the release of Nmd3, though some important interactions between Rpl10 and rRNA have been identified, thus suggesting that the mechanism by which Nmd3 is released may be a complex process of accommodating Rpl10 into the ribosome, with structural changes inducing Nmd3 release, all through rRNA interactions. Mutations clustered in two main regions, one near the N-terminus of the protein and one near the C-terminus of the protein. The mutations that were most strongly dependent on *NMD3 I112T I362T* were primarily split between these two regions as well, with the exception of K158E, which, as discussed earlier, may play a role in interacting with rRNA at its location on the 60S subunit.

N-terminal Cluster

The N-terminal mutations that clustered included K15, N23, and V26. As discussed earlier, V26 may not play an important role in Nmd3 release. K15, however can interact with the 25S rRNA backbone electrostatically. Measurements have shown that this residue is 3.4 and 3.5 angstroms away from the phosphate backbone of U-1125 and A-2635 of 25S rRNA, respectively. Making contact with two different rRNA regions of the same rRNA molecule may suggest that these are key intra-ribosomal interactions. Residue N23 may play a role in affecting the overall structure of this region of the protein through interactions with other residues internal to the protein. Work by Jon Dinman indicated that mutations in this region of Rpl10 may play a role in linking

rRNA helices 38 and 39.[9] It may be possible that linking these helices provides more stability to Rpl10 loading onto the ribosome, and possibly indirectly affects rRNA contacts elsewhere that could affect Nmd3 release.

C-terminal Cluster

The C-terminal mutations that clustered included K184, S201, and F213. These residues were all also found in *rpl10* mutants that were strongly dependent on *NMD3 112T I362T*. Though K184 may not have a significant role in Nmd3 release, S201 may interact with 5S rRNA, while F213 may interact internally with the protein. A measurement taken of residue S201 in particular showed that it is only located 3.4 angstroms away from A-64 of 5S rRNA, and this may be a key electrostatic or hydrogen-bonding interaction that is possibly involved in Nmd3 release. Studies of this region of Rpl10 have not been conducted in depth, and this particular region of the protein may warrant further investigation as to its role in Nmd3 release.

Other Mutations

Residue K158 is in position to hydrogen bond with the nitrogen bases of 25S rRNA. In particular, K158 is only 2.6 Angstroms away from C-2852 of 25S rRNA and only 4.3 Angstroms away from A-2851 of 25S rRNA. It is possible that both of these contacts may be important in Nmd3 release. The mutation L103S has no image associated with it because it is present in the P-site loop, for which there is no structure given in the crystal structure of the 60S subunit. From previous studies, the P-site loop, consisting of amino acids 102-112 in Rpl10, has been shown to be significant in Tif6

release.[4] Thus, this mutation would be thought to affect Tif6 release, in turn affecting Nmd3 release.

Regarding A-site and P-site tRNA interactions

Previous work by Voorhees indicated that arginine residues 51 and 56 play a role in interacting with A-site tRNA in bacteria, while previous work by Anne Hofer indicated that residues 102-112 play a role in interacting with P-site tRNA.[4,14] Work by Spahn determined that residue R24 interacts with P-site tRNA in the 60S Subunit.[12] The N-terminal mutations identified in this study are very close to this amino acid residue and thus these mutations may play a role in affecting a key contact between P-site tRNA and Rpl10 that may be involved in Nmd3 release.

Due to the lack of mutants, it cannot be concluded that Rpl10 interactions with A-site and P-site tRNA do not have a role to play in Nmd3 release; however, both warrant further investigation.

Outstanding Issues to be Resolved

Identifying 5FOA-sensitive mutants proved difficult, as replica plating often produced many false-positives: strains that appeared 5FOA-sensitive but in reality were not, due to the strains not being given a chance to lose their URA3 vectors before being plated onto 5FOA-containing plates. This issue was improved by replica plating colonies from ura$^-$leu$^-$glucose plates to leu$^-$ glucose plates and then to leu$^-$5FOA glucose plates; however, while the issue of false-positives being produced was improved, false-positives were still present, and serial dilution tests comparing *rpl10* mutants to wild-type *RPL10* in the presence of *NMD3 I112T I362T* may prove

more effective, instead of streaking for single colonies and comparing the growth rates of mutant strains to a strain containing wild-type *RPL10* to determine which *rpl10* mutants are truly dependent on *NMD3 I112T I362T*.

Despite my efforts to map a specific region of Rpl10 that is required for the release of Nmd3, it remains ambiguous if we can map this function to a particular domain or face of Rpl10, largely because I was unable to generate enough mutants to definitively conclude anything from this study. In order for better deductions to be made based on where mutations mapped in the Rpl10 structure, more 5FOA-sensitive mutants would need to identified and sequenced. In particular, mutants containing only single mutations should be made from *rpl10* mutants such as PCR 3 and PCR 8, that contain multiple mutations. Additionally, site-directed mutagenesis could be used to create more mutants in the C-terminal and N-terminal cluster regions identified in this study to further confirm or reject that importance of these regions in Nmd3 release.

Lastly, it would be wise to use a *NMD3 I112T I362T* vector and *rpl10* mutant vectors that do not contain any tags on them. In this experiment, the *NMD3 I112T I362T* vector contained a GFP tag and the *rpl10* mutant vectors contained 13-myc tags. Tags were necessary in this study for downstream applications such as western-blotting, but could also affect the function of both proteins and thus affect results obtained from this experiment. A serial dilution assay was done comparing the effects of untagged, myc-tagged, and GFP-tagged *NMD3 I112T I362T* on a mutation of *RPL10* known to be suppressed: *rpl10 G161D.* In that test, GFP-tagged *NMD3 I112T I362T* suppressed the mutation relatively well compared to the other vectors used.

However, if the mechanism of Nmd3 release is complex, it would be best to test all identified *rpl10* mutants for suppression by untagged *NMD3 I112T I362T* as well.

Future Work

To continue with this research in the future, it may prove useful, now that two possible regions of Rpl10 that may affect Nmd3 release have been identified, to create more mutants and further investigate if these regions have a role to play in Nmd3 release. Additional mutations in these regions that are suppressed by *NMD3 I112T I362T* would provide more support in making a claim of particular regions of Rpl10 being involved with Nmd3 release.

Appendix

Table 2: Yeast Strains Used in this Study

Strain	Genotype	Source
AJY 2104	matα KanMX::GAL1::RPL10 ade2 ade3 ura3 leu2	Hofer *et al*, 2007

Table 3: Oligonucleotides Used for PCR and Sequencing

Oligonucleotide	Sequence
AJO 525	5'-AGCGGATAACAATTTCACACAGGA
AJO 722	5'-TCAAGTCTTCCTCGGAGATTAGCTTTTGTTCACCGTTAATTAACC

Table 4: Plasmids Used in this Study

Plasmid	Relevant Markers	Source
pAJ 24	LEU2-CEN	Sikorski *et al*, 1989
pAJ 100	URA3-CEN	Sikorski *et al*, 1989
pAJ 755	URA3-CEN NMD3-GFP	This Study
pAJ 1197	LEU2-CEN RPL10-myc	Hofer *et al*, 2007
pAJ 2240	URA3-CEN TIF6[V192F]	Bussiere *et al*, 2011
pAJ 2522	LEU2-CEN RPL10	Bussiere *et al*, 2011
pAJ 2541	URA3-CEN NMD3[I112T,I362T]-GFP	This Study
pAJ 2665	URA3-CEN TIF6	Bussiere *et al*, 2011
pAJ 3001	LEU2-CEN RPL10[N23D]-myc	This Study
pAJ 3002	LEU2-CEN RPL10[K158E]-myc	This Study
pAJ 3003	LEU2-CEN RPL10[V26A,L103S,K184E,S201F]-myc	This Study
pAJ 3004	LEU2-CEN RPL10[K15E,V148A]-myc	This Study
pAJ 3005	LEU2-CEN RPL10[R90G]-myc	This Study
pAJ 3006	LEU2-CEN RPL10[F213L]-myc	This Study

Bibliography

Ben-Shem, Adam, Nicolas Garreau de Loubresse, Sergey Melnikov, Lasse Jenner, Gulnara Yusupova, and Marat Yusupov. "The Structure of the Eukaryotic Ribosome at 3.0 Å Resolution." *Science (New York, N.Y.)* 334, no. 6062 (December 16, 2011): 1524–1529.

Bussiere, Cyril, Yaser Hashem, Sucheta Arora, Joachim Frank and Arlen W Johnson. (2011). *Probing the P site during maturation of the 60S ribosomal subunit.* Unpublished Manuscript.

Hedges, John, Matthew West, and Arlen W Johnson. "Release of the Export Adapter, Nmd3p, from the 60S Ribosomal Subunit Requires Rpl10p and the Cytoplasmic GTPase Lsg1p." *The EMBO Journal* 24, no. 3 (February 9, 2005): 567–579.

Hofer, Anne, Cyril Bussiere, and Arlen W Johnson. "Mutational Analysis of the Ribosomal Protein Rpl10 from Yeast." *The Journal of Biological Chemistry* 282, no. 45 (November 9, 2007): 32630–32639.

Hung, Nai-Jung, Kai-Yin Lo, Samir S Patel, Kara Helmke, and Arlen W Johnson. "Arx1 Is a Nuclear Export Receptor for the 60S Ribosomal Subunit in Yeast." *Molecular Biology of the Cell* 19, no. 2 (February 2008): 735–744.

Johnson, Arlen W, Elsebet Lund, and James Dahlberg. "Nuclear Export of Ribosomal Subunits." *Trends in Biochemical Sciences* 27, no. 11 (November 2002): 580–585.

Lo, Kai-Yin, Zhihua Li, Cyril Bussiere, Stefan Bresson, Edward M Marcotte, and Arlen W Johnson. "Defining the Pathway of Cytoplasmic Maturation of the 60S Ribosomal Subunit." *Molecular Cell* 39, no. 2 (July 30, 2010): 196–208.

Oeffinger, Marlene. “Joining the Interface: a Site for Nmd3 Association on 60S Ribosome Subunits.” *The Journal of Cell Biology* 189, no. 7 (June 28, 2010): 1071–1073.

Petrov, Alexey N., Arturas Meskauskas, Sara C. Roshwalb and Jonathan D. Dinman. "Yeast ribosomal protein L10 helps coordinate tRNA movement through the large subunit". Nucleic Acids Research 36, no. 19 (September 29, 2008): 6187-6198.

Sengupta, Jayati, Cyril Bussiere, Jesper Pallesen, Matthew West, Arlen W Johnson, and Joachim Frank. “Characterization of the Nuclear Export Adaptor Protein Nmd3 in Association with the 60S Ribosomal Subunit.” *The Journal of Cell Biology* 189, no. 7 (June 28, 2010): 1079–1086.

Sikorski, Robert, S. and Philip Heiter. "A System of Shuttle Vectors and Yeast Host Strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae." *Genetics* 122, no. 1(May 1989): 19-27

Spahn, C M, R Beckmann, N Eswar, P A Penczek, A Sali, G Blobel, and J Frank. “Structure of the 80S Ribosome from Saccharomyces cerevisiae--tRNA-ribosome and Subunit-subunit Interactions.” *Cell* 107, no. 3 (November 2, 2001): 373–386.

Tschochner, Herbert, and Ed Hurt. “Pre-ribosomes on the Road from the Nucleolus to the Cytoplasm.” *Trends in Cell Biology* 13, no. 5 (May 2003): 255–263.

Voorhees, Rebecca M, Albert Weixlbaumer, David Loakes, Ann C Kelley, and V Ramakrishnan. “Insights into Substrate Stabilization from Snapshots of the Peptidyl Transferase Center of the Intact 70S Ribosome.” *Nature Structural & Molecular Biology* 16, no. 5 (May 2009): 528–533.

West, Matthew, John B Hedges, Anthony Chen, and Arlen W Johnson. “Defining the Order in Which Nmd3p and Rpl10p Load onto Nascent 60S Ribosomal Subunits.” *Molecular and Cellular Biology* 25, no. 9 (May 2005): 3802–3813.

West, Matthew, John B Hedges, Kai-Yin Lo, and Arlen W Johnson. “Novel Interaction of the 60S Ribosomal Subunit Export Adapter Nmd3 at the Nuclear Pore Complex.” *The Journal of Biological Chemistry* 282, no. 19 (May 11, 2007): 14028–14037.

Zemp, Ivo, and Ulrike Kutay. “Nuclear Export and Cytoplasmic Maturation of Ribosomal Subunits.” *FEBS Letters* 581, no. 15 (June 19, 2007): 2783–2793.

Acknowledgements

I would like to first and foremost thank Dr. Arlen Johnson for allowing me to work in his lab, as well as for his guidance and support throughout this project, from the lab work to the writing of this very thesis. I would also like to thank Richa Sardana, who was always there to answer any questions I had regarding lab techniques, as well as all other members of the Johnson lab for their help throughout this project. Thanks also goes to Dr. Karen Browning for being the second reader of this thesis, and Dr. Shelley Payne for helping teach me how to speak about science at a level that can be understood by people of all majors.