Characterization of New Factors in the 18S Nonfunctional Ribosomal RNA Decay Pathway in S. cerevisiae: A Dissertation

Christopher N. Merrikh
University of Massachusetts Medical School

Let us know how access to this document benefits you.
Follow this and additional works at: https://escholarship.umassmed.edu/gsbs_diss

Part of the Amino Acids, Peptides, and Proteins Commons, Cells Commons, Fungi Commons, Molecular Biology Commons, and the Nucleic Acids, Nucleotides, and Nucleosides Commons

Repository Citation

This material is brought to you by eScholarship@UMMS. It has been accepted for inclusion in GSBS Dissertations and Theses by an authorized administrator of eScholarship@UMMS. For more information, please contact Lisa.Palmer@umassmed.edu.
CHARACTERIZATION OF NEW FACTORS IN THE 18S NONFUNCTIONAL RIBOSOMAL RNA DECAY PATHWAY IN S. CEREVISIAE

A Dissertation Presented

By

Christopher N. Merrikh

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

March 5, 2012

Biochemistry and Molecular Pharmacology Program
CHARACTERIZATION OF NEW FACTORS IN THE 18S NONFUNCTIONAL RIBOSOMAL RNA DECAY PATHWAY IN S. CEREVISIAE

A Dissertation Presented
By
Christopher N. Merrikh

The signatures of the Dissertation Defense Committee signify completion and approval as to style and content of the Dissertation

Dr. Melissa J. Moore, Thesis Advisor

Dr. Nicholas Rhind, Member of Committee

Dr. Sean Ryder, Member of Committee

Dr. Michael Volkert, Member of Committee

Dr. Rachel Green, Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets the requirements of the Dissertation Committee

Dr. Allan Jacobson, Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requirements of the school.

Anthony Carruthers, Ph.D.,
Dean of the Graduate School of Biomedical Sciences
Basic Biomedical Sciences

March 5, 2012
Dedication

I would like to dedicate my thesis to the friends, teachers,

and family who have taken the time to teach me what they know,

and who have offered me their time, love and support.

There are many of you.
Acknowledgements

During the course of graduate school, a large number of people have offered me their wisdom, feedback, and kindness:

First and foremost, my wife, Houra, who has been with me every step of the way, knows me best, and has been my greatest supporter.

I would also like to acknowledge the critical impact that my committee members have had on my progress in the Ph.D program here at UMass. To Mike, Nick, Sean, and Allan: I would not have reached this point without your help.

I would like to offer thanks to Rachel Green who is taking the time to fly to Boston to serve as my outside committee member, and who has enriched my understanding of this project through her own work.

Last, I would like to thank and acknowledge my advisor, Melissa, for being a constant source of inspiration about my project, and science as a whole. Her training and feedback have fundamentally impacted my understanding of molecular biology, and, I think, improved who I am as a person. I will always be grateful for her support and generosity.
Preface

The molecular biology revolution of the 1960s has given rise to an enormous body of literature describing, in great detail, the inner workings of the cell. Over the course of the past 50 years, and countless hours at the bench, biologists have used the implications of basic research to produce vaccines, antibiotics, and other therapies that have improved both the quality and duration of our lives. Despite these incredible advances, basic questions remain unanswered. In even the simplest model organism, hundreds of essential genes have never been studied. Moreover, the central dogma of molecular biology – DNA to RNA to Protein – is understood largely in terms of how the cell functions under ideal conditions. What happens when things go wrong?

This study seeks to characterize one of the cell’s contingency plans – a quality control measure for the eukaryotic ribosome. Today, despite the abundance of ribosomes in all cells, we are only beginning to understand the details of how they function, and the mechanisms that monitor their behavior. Recently, inactivated ribosomes were shown to be destroyed by the cell's own quality control measures, potentially preventing them from harming the cell. This system, dubbed 18S Nonfunctional rRNA Decay, is known to utilize a pair of ribosome-binding proteins to carry out its function. Yet the pathway still functions, albeit more slowly, in the absence of these two proteins, suggesting that other components must exist. The work discussed here is largely concerned with identifying these other factors, characterizing their activities, and determining how the 18S Nonfunctional rRNA Decay pathway impacts the health of the cell.
### Table of Contents

**Title Page**
**Signature Page**
**Dedication**
**Acknowledgements**
**Preface**
**Table of Contents**

**CHAPTER 1: INTRODUCTION**
- Translation ........................................1
- rRNA Synthesis and Maturation ........10
- Ribosome Assembly ..........................14
- Ribosomal Proteins Rack1 and Rps3 ....18
- Translation Surveillance Mechanisms ...22
- 18S Nonfunctional rRNA Decay ..........25
- Translation-Independent Ribosome Decay ....29
- RNA Damage ..................................31

**CHAPTER 2: TURNOVER OF NONFUNCTIONAL RIBOSOMAL RNAs REQUIRES RIBOPHAGY FACTORS, AND THE RIBOSOMAL PROTEIN ASC1**
- Introduction ................................40
- Results ...........................................49
- Materials and Methods ....................101
- Discussion ...................................102

**CHAPTER 3: TESTING THE ENDOGENOUS FUNCTIONS OF 18S NRD**
- Introduction ................................118
- Results .........................................122
- Materials and Methods ...................132
- Discussion ...................................134

**CHAPTER 4: PROSPECTUS**
- The role of Asc1 ..............................141
- The roles of Ubc4 and Doa1 ...............144
- Implications of Ubiquitin Involvement in NRD ....145
- A potential role for Rps3 in NRD ..........145
- The potential function of NRD in the clearance of damaged RNAs........147

**APPENDICES**
- Global ribosome footprinting analysis in the absence of UPF1
- Introduction ................................149
Results.............................................................................................................................. 153
Discussion...................................................................................................................... 176

Bibliography .................................................................................................................. 180
## List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>63</td>
</tr>
<tr>
<td>A1</td>
<td>158-159</td>
</tr>
<tr>
<td>A2</td>
<td>161-164</td>
</tr>
<tr>
<td>A3</td>
<td>166-167</td>
</tr>
<tr>
<td>A4</td>
<td>168-170</td>
</tr>
<tr>
<td>A5</td>
<td>181</td>
</tr>
<tr>
<td>A6</td>
<td>182</td>
</tr>
<tr>
<td>A7</td>
<td>185-188</td>
</tr>
</tbody>
</table>

## List of Figures

1. **Figure 1.1.** Translation initiation .................................................. 2
2. **Figure 1.2.** Translation elongation ....................................................... 6
3. **Figure 1.3.** Translation termination .................................................... 9
4. **Figure 1.4.** Pre-rRNA Processing in *S. cerevisiae*.................................. 11
5. **Figure 1.5.** Rps3 contacts Asc1 via a long C-terminal tail and is situated at the mouth of the mRNA channel opposite Rps30 .................. 19
6. **Figure 1.6.** Structure of Asc1 and the Asc1 heterodimer ..................... 20
7. **Figure 1.7.** Translation-dependent mRNA surveillance mechanisms in *S. cerevisiae* ........................................................................... 25
8. **Figure 1.8.** Models for 18S NRD, NGD, and PDTA ................................. 28
9. **Figure 1.9.** Oxidized mRNAs accumulate in heavier polysome fractions ........................................................................................................... 34
10. **Figure 2.1.** Crystal structures of the eukaryotic ribosome, Rack1, and Dom34:Hbs1........................................... 45
11. **Figure 2.2.** ASC1 mutational analysis schematic .................................... 46
12. **Figure 2.3.** Single timepoint analysis of mutant 18S rRNA levels ............ 51
13. **Figure 2.4.** Mutant 18S:A1492C destabilization in various deletion strains ......................................................... 52
14. **Figure 2.5.** Double deletion of ASC1 and DOM34 is synthetic for 18S NRD .... 54-55
15. **Figure 2.6.** 18S NRD is not detected in the absence of UBC4 .................. 58
16. **Figure 2.7.** DOA1 deletion confers a partial stabilization of mutant 18S rRNAs and is synthetic with ASC1 deletion ........................................... 60
17. **Figure 2.8.** Validation of NRD protein overexpression constructs via western blot ........................................ 66
18. **Figure 2.9.** Effect of NRD protein overexpression on the levels of mutant 18S rRNAs .................................................................................. 68-74
19. **Figure 2.10.** Single timepoint analysis of hbs1∆asc1∆ .................................. 75
20. **Figure 2.11.** 18S NRD still occurs in an hbs1∆asc1∆ strain .......................... 77
21. **Figure 2.12.** Mutant 18S rRNA timecourse analysis in SKI7 deficient strains ......................... 78
22. **Figure 2.13.** 18S NRD timecourse in xrn1∆ ............................................ 80
23. **Figure 2.14.** asc1∆ complementation ................................................... 83
24. **Figure 2.15.** ASC1 mutagenesis ............................................................. 84
Figure 2.16 Mutations of ASC1 shown to affect ribosome binding ......................... 85
Figure 2.17 Analysis of mutant 18S rRNAs in polysome profiles ............................... 89-91
Figure 2.18 Other mutant strains screened for effects on 18S NRD ............................ 97-98
Figure 2.19 Growth rates of single and double mutant strains .................................... 100
Figure 2.20 A model for 18S NRD ............................................................................. 115
Figure 3.1 18S rRNA levels in rps27AΔ and rps27bΔ deletion strains ...................... 124
Figure 3.2 4NQO sensitivity of dom34Δasc1Δ and hbs1Δasc1Δ strains .................... 127
Figure 3.3 CHX sensitivity of dom34Δasc1Δ and hbs1Δasc1Δ strains ....................... 129
Figure 3.4 MMS and TBH sensitivity of dom34Δasc1Δ and hbs1Δasc1Δ strains ......... 141
Figure A1 The 1/x phenotype as demonstrated by the CAN1, and PMA1 mRNAs ......... 121
Figure A.2 Genes for four candidate mRNAs displaying start codon enrichment in the absence of UPF1 or MTT1, or both ............................................................ 122
## List of 3rd Party Copyrighted Material

The following figures were reproduced from journals:

<table>
<thead>
<tr>
<th>Figure</th>
<th>Publisher</th>
<th>License Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1.</td>
<td>Nature Publishing Group</td>
<td>2887250781824</td>
</tr>
<tr>
<td>Figure 1.4.</td>
<td>Eukaryotic Cell</td>
<td>None required for inclusion in a thesis</td>
</tr>
<tr>
<td>Figure 1.5.</td>
<td>Science</td>
<td>2887260625176</td>
</tr>
<tr>
<td>Figure 1.6.</td>
<td>Journal of Molecular Biology</td>
<td>2887260228658</td>
</tr>
<tr>
<td>Figure 2.2A.</td>
<td>Nature</td>
<td>2887260882084</td>
</tr>
<tr>
<td>Figure 2.2B.</td>
<td>Journal of Molecular Biology</td>
<td>2887260228658</td>
</tr>
<tr>
<td>Figure 2.2C.</td>
<td>Science</td>
<td>2887260625176</td>
</tr>
</tbody>
</table>
Chapter 1:
Translation

The central dogma describes the process by which genetic information directs the synthesis of proteins. First, the sequence of a given gene, which is encoded in the form of DNA, is transcribed into RNA. The RNA sequence is then translated to produce protein. The process of mRNA translation is carried out by the ribosome, a macromolecular machine that couples the reading of the mRNA with the linear, ordered assembly of amino acids. This is a high-fidelity process capable of preventing most errors, and also dealing with those that arise. Both canonical translation and translation quality control mechanisms are promoted by a host of transiently associated ribosome binding factors. The complexity of translation allows multiple quality control events to take place, thereby preserving the integrity of the gene expression process and enabling detection of errors that do arise.

Translation Initiation

The process of mRNA translation begins with an initiating phase that involves the binding of an mRNA and its associated proteins to a ribosomal small subunit. The resulting complex undergoes a series of intermediate steps before it becomes competent for large subunit recruitment and proceeds to 80S formation. This process utilizes a large number of proteins including complexes on the mRNA cap, mRNA tail, both ribosomal subunits, and individual initiation factors. A detailed overview of initiation was reviewed by Jackson et al. 2010, and is reiterated briefly below (Fig. 1.1).
At the first step in initiation, a 40S ribosomal subunit is prepared for mRNA binding. Initially, the 40S is bound by three initiation factors: eIF3, which binds on the exterior of the ribosome near the E-site, eIF-1a, which binds the E-site itself, and eIF-1, which binds in the A-
site. This leaves the P-site vacant for the binding of the Met-tRNA as part of the eIF2/tRNA(Met)/GTP ternary complex. Subsequently, eIF5 binds. At this point, a 40S subunit is prepared for interaction with the mature mRNA, and is referred to as a 43S complex, a name based upon its sucrose gradient sedimentation coefficient (Jackson, Hellen, & Pestova, 2010).

The next major initiation complex is comprised of the 43S complex bound to an mRNA. In preparation for translation, pre-mRNAs are subjected to nuclear maturation including the removal of intronic sequences via splicing, the addition of a 7-methyl-Guanine cap, polyadenylation, and the deposition of associated proteins (Matlin et al., 2007; Reed et al., 2002). During translation initiation, the cap is bound by the initiation factors eIF4G, eIF4E, and eIF4A, which together comprise the eIF4F complex. In addition to eIF4F, the cap is bound by eIF4B (von der Haar, Gross, Wagner, & McCarthy, 2004). The protein eIF4A is a DEAD-box helicase that helps resolve secondary structure within the 5` UTR of the mRNA during initiation (Rogers, Richter, Lima, & Merrick, 2001). Its activity is greatly enhanced by both eIF4B and eIF4G, and current work in the field has suggested the involvement of several other DEAD-box helicases, including Ddx3 and Ded1 (Tarn & Chang, 2009). Beyond its role in promoting the activity of eIF4A, eIF4G recruits the 43S complex via its interactions with eIF3 on the ribosome (LeFevbre et al., 2006). A 43S ribosomal small subunit complex bound to a mature mRNA comprises the 48S preinitiation complex.

The small ribosomal subunit of a newly formed 48S complex scans the mRNA sequence in a 5`-3` direction until it reaches an AUG start codon. When the ribosomal P-site is positioned on the AUG, the proper pairing of Met-tRNA to the AUG is recognized by eIF1(Pestova et al., 2002). This process is in contrast to the codon:anticodon pairing that occurs in the A-site during translation's elongation phase. Similar to the A-site proofreading mechanisms discussed below,
the P-site possesses mechanisms that allow faithful identification of proper AUG start codons. This ability is promoted by the initiation factors, especially eIF1, which also discourage the use of improper AUG codons (Donahue et al., 2000). Localization of purines at the -3 and +4 positions relative to a true start codon provides one of the means by which the initiation factors can discriminate between these two possibilities (Pisarev et al., 2006).

Following start codon recognition, eIF5B is recruited along with a large ribosomal subunit. The protein eIF5B first activates the GTPase function of eIF2, triggering the hydrolysis of its bound GTP and the subsequent dissociation of eIF2 (Paulin et al. 2001). After this step, the 60S subunit binds, allowing the eIF5B-mediated dissociation of the remaining eIF proteins, including, as a downstream event, eIF5B itself (Pestova et al. 2007). The resulting complex, comprised of a large and a small subunit positioned at the start codon, now represents an 80S ribosome:mRNA complex capable of making the transition from initiation to translation elongation (Jackson et al., 2010).

**Translation Elongation**

The elongation cycle begins following the clearance of eIF5B from the A-site. Once vacant, the A-site becomes available for insertion of eEF1A-bound aminoacylated tRNAs, which occurs stochastically until a proper codon:anticodon pair is formed (Stark et al. 1997). At this point, eEF1A hydrolyzes its GTP and dissociates, allowing the tRNA to fully insert into the A-site, positioning it in the peptidyl transferase center in a process called accommodation (Noble et al., 2008). As part of the tRNA selection process, the small subunit rRNA also detects proper codon:anticodon pairing via a process known as the kinetic proofreading function of the
ribosome. This proofreading step represents a separate round of selection based upon the proper orientation of the codon and anticodon, and can distinguish between cognate and near-cognate tRNAs with significantly higher fidelity than initial codon:anticodon pairing (Blanchard, Gonzalez, Kim, Chu, & Puglisi, 2004). The process is facilitated by rRNA itself as it surveys the codon:anticodon interaction, predominantly within the first two positions. Three bases in the small subunit RNA – G530, A1492, and A1493 – (using the E. coli numbering system) undergo conformational changes, rotating along the sugar-phosphate axis and forming hydrogen bonds with the tRNA and mRNA backbone. Base G530 interacts with the anticodon at position 2, and with the mRNA at position 3. Base A1492 contacts position 1 of the mRNA’s minor groove, and A1493 contacts position 2, detecting the orientation of the mRNA as a measure of its pairing with the A-site tRNA. Despite the single contact point at position 3 by G530, quality control is almost entirely based on correct codon:anticodon pairing at positions 1 and 2, which permits imperfect pairing at position 3, or the “wobble” position. Mutations in these bases have been found to affect disparate aspects of the ribosome’s kinetic proofreading function: proofreading in the A-site is greatly decreased in the case of A1492 mutation, whereas proofreading in the P-site is affected by A1493 mutations (Ogle et al., 2001; Yoshizawa, et al., 1999).
Figure 1.2. The translation elongation cycle. Following large subunit recruitment, tRNAs are inserted into the A-site soichastically until proper codon:anticodon pairing occurs. Peptidyl transfer then proceeds, followed by ribosome progression to the next codon, and movement of tRNA-peptide from the A-site to the P-site. The cycles then restarts.

If proofreading is successful, the peptidyl transfer reaction can proceed (Pape et al., 1998). The rRNA itself catalyzes the dehydration synthesis reaction at the peptidyl transferase
(PTC) center of the large subunit, covalently linking the C-terminal of the P-site amino acid to the N-terminal of the A-site aa-tRNA (Ban et al., 2000). The mRNA then moves forward through the ribosome by three bases, thereby moving a new codon into the A-site, and moving the A-site and P-site codons into the P-site and E-site, respectively. This causes the tRNA in the A-site to tilt as it maintains contact with the tRNA at its base, without fully transferring its upper portion into the P-site. The process by which the tRNAs fully transfer from A to P to E is called translocation, and is driven by the GTPase EF-G (Rodnina et al., 1997; Valle et al., 2003).

Interestingly, fungi possess an additional elongation factor not present in either prokaryotes or other eukaryotes – eEF3. Work on eEF3 has indicated that it is required for both binding of eEF1:Amino Acid:tRNA to the ribosome and exit of tRNA from the E-site (Triana-Alonso et al., 1995).

Translation Termination

The elongation cycle will continue until the ribosome encounters a termination codon in the A-site. No tRNA codon matches the UAA, UAG, or UGA termination codon. Instead, the release factor protein eRF1 recognizes them, in part, via its NIKS motif. Additional work has demonstrated that the N-terminal region of eRF1 is also involved in stop codon recognition despite its distant location from the mRNA (Ito et al., 2002). The shape of eRF1 resembles that of a tRNA, consistent with its ability to insert into the A-site (Shin et al., 2004) (Fig. 1.3).

The consequences of successful eRF1 binding include the release of the mRNA and ribosomal subunits, hydrolysis of the ester bond linking the C-terminal amino acid in the P-site
with its tRNA, and dissociation of tRNA and nascent peptide (Frolova et al., 2002; Song et al., 2000).

In addition to eRF1, its binding partner, eRF3, is required for canonical translation termination. Though eRF3 is not essential for termination in vitro, as part of its activity in vivo eRF3 greatly stimulates the activity of eRF1. Consistent with the structural similarity of eRF1 to tRNA, eRF3 resembles EF1A. These two proteins form a stable heterodimer and insert together into the A-site (Ito et al., 1998). Also consistent with its homology to EF1a, eRF3 is a guanine nucleotide exchange factor. Following stop codon recognition, eRF3 hydrolyzes GTP, triggering a dramatic conformational change in eRF1 and the subsequent release of the ribosome, mRNA, tRNA, and peptide.

The activities of eRF1 and eRF3 are greatly stimulated by an factor, RliI (mammalian ABCE1) which is also involved in ribosome maturation, and translation initiation (Shoemaker, Eyler, & Green, 2010). This ATPase binds the eRF1:eRF3 to promote the process of termination and subsequent recycling of translation components (Fig. 1.3). Additionally, translation initiation factors eIF3/1/1A are required for recycling to finish (Shoemaker et al., 2010).
Figure 1.3. Translation Termination and Recycling.
**rRNA Synthesis and Maturation**

The eukaryotic ribosome is comprised of four long RNAs totaling 5 kb in length and more than 80 proteins, reaching a total mass of at least 3.3 megadaltons (Ben-Shem, Jenner, Yusupova, & Yusupov, 2010). The sheer size of the ribosome necessitates a complex synthesis and maturation process. rRNA processing alone involves the transcription of a long RNA precursor molecule, numerous cleavage and modification events, and the proper production of a complex three-dimensional structure. This is all coordinated with the binding of a multitude of ribosomal proteins.

The mature ribosome is a high-fidelity machine that possesses multiple independently functioning proofreading measures and quality control mechanisms. In its canonical function described above, the ribosome conducts translation with many initiation, elongation, and termination factors, underscoring the complexity of the translation process. Added to this, many transiently associated proteins promote the selective translation, or degradation, of mRNAs, allowing the ribosome to function as a highly flexible device for the control of gene expression.

**Ribosomal RNA Maturation**

The eukaryotic ribosome consists of two distinct subunits, the 40S and 60S. The 60S contains the 25S, 5.8S, and 5S rRNAs, and the 40S contains the 18S rRNA. In *S. cerevisiae*, these four RNAs are encoded in the genome in the form of two tandem genes that together form a single rDNA repeat (Venema et al., 1999). The first RNA is a long operon called the 35S (Venema, J., Tollervey, 1999). It includes the 18S-5.8S-25S rRNAs plus two spacer regions, the ITS1 and ITS2, which separate the 18S from the 5.8S and the 3’ end of the 5.8S from the 25S,
respectively. The 35S also contains flanking sequences – the 5’ and 3’ ETS. The 5S rRNA is encoded separately. The 35S and 5S rRNAs are transcribed by RNA polymerase I and RNA polymerase III, respectively (Venema, et al., 1999; Bernstein et al., 2004) (Fig. 1.4).

Figure 1.4. Pre-rRNA Processing in *S. cerevisiae*. (Adapted from Bernstein et al. Eukaryotic Cell. 2004)

In the *S. cerevisiae* genome, the rDNA genes are repeated 100 to 200 times, allowing large numbers of rRNAs to be produced in parallel. During log phase growth, an average yeast cell contains 2×10^5 ribosomes and doubles every 90 minutes, indicating a production rate of 2,000 per minute (Warner, 1999). Production at this scale is possible, in large part, due to the
compartmentalization of the rDNA genes and maturation factors within a discrete organelle called the nucleolus. rRNA transcription and maturation, as well as a major portion of the ribosome assembly process, takes place there (Taddei, Schober, & Gasser, 2010).

Transcription of the rRNA occurs slowly, taking as long as 6 minutes. It is followed by the very fast maturation of the rRNA, which occurs on the level of seconds (Kressler, Hurt, & Bassler, 2010). Early in the maturation process, the 3’ ETS of the 35S rRNA is co-transcriptionally removed by the endoribonuclease Rnt1 (Elela et al., 1996). Next, approximately 70 small nucleolar RNAs (snoRNAs) direct the 2’-O-ribose methylation (CH3) and pseudouridylation of the rRNA at various sites. The targets of pseudouridylation and 2’-O-ribose methylation are entirely within the core conserved structure of the rRNA, and are especially prominent at functional rather than scaffolding sites, suggesting they should have an impact on ribosomal function (Bakin, Lane, & Ofengand, 1994). Despite the anticipated importance of the snoRNAs and the implication of snoRNA misregulation in various forms of cancer, determining specific functions for many of these modifications has proven difficult (Esteller, 2011).

Following chemical modification, the 35S rRNA is cleaved at positions A0 and A1, thereby removing the 5’ ETS, which is degraded by the nuclear exosome and the exoribonuclease Rat1. The resulting 33S is cleaved again at position A2 to form the 20S and 27SA2 rRNAs. The former is exported from the nucleus where it is cleaved again, most likely by Nob1, to produce the 18S, a mature small subunit rRNA (Fatica, Tollervey, & Mensur, 2004). Meanwhile, the 27SA2, which contains the 5.8S and 25S rRNAs, is matured independently by the endoribonuclease MRP, Rat1, and the exosome. Downstream cleavage events are mediated...
by the exosome and unidentified endoribonucleases to form the mature 5.8S and 25S (Udem et al. 1973; Venema, et al., 1999).

**snoRNA Structure and Function**

The general purpose of snoRNAs is to bring together an RNA target and the enzymes required for its site-directed chemical modification. Two functional categories of snoRNAs exist – the C/D box snoRNAs, which direct 2’-O-ribose methylation, and the H/ACA box snoRNAs, which direct pseudouridylation. The latter reaction entails the rotation of the uridine across the C3/C6 axis, producing a base that is unable to properly hydrogen bond to other nuclear bases (Charette & Gray, 2000). Both modifications are known to influence the folding and maturation of the ribosomal RNAs, though other functions may exist (Charette & Gray, 2000).

The snoRNA classes are identifiable by sequence. A single snoRNA always encodes at least one set of highly conserved C and D boxes: RUGAUGA (R=purine) and CUGA, respectively. Often, a second set of less-conserved C and D boxes are also encoded, leading to an alternating order of C, D’, C’, D, with the symbol ‘ signifying the less-conserved sequences. A stem-loop structure is formed by inverted repeats at the 5’ and 3’ termini of the snoRNA, bringing the two internal and two terminal C and D boxes together.

H/ACA snoRNAs also form a stem loop structure, though they are generally longer than C/D box snoRNAs and only contain single H and ACA guide sequences. Like the C/D box snoRNAs, the guide sequence is sufficient for nucleolar localization(Holley & Topkara, 2011).

The snoRNAs function via their associated proteins. The proteins 15.5 K, Nop58, and Nop58 bind to C/D boxes, allowing subsequent binding of the enzyme Nop1, which conducts the
2’-O-ribose methylation. A different group of proteins binds the H/ACA snoRNAs: Cbf5, Gar1, Nhp2, Nop10, and the enzyme Cbf5 (human dyskerin), the latter of which actually performs the uridine isomerization reaction (Ganot, Bortolin, & Kiss, 1997; Henrase et al., 1998; Holley & Topkara, 2011). Though individual snoRNAs are not essential, their loss appears to have subtle effects on rRNA folding. Moreover, strains deficient in NOP1 or CBF5 deletions are lethal, suggesting that the complete loss of 2’-O-methyl or pseudouridine formation is harmful for the cell (Bachellerie, Cavaillé, & Hüttenhofer, 2002).

**Ribosome Assembly**

*Production of the 40S and 60S subunits*

The process of ribosome assembly, from rRNA transcription initiation to the completion of translation-competent 40S and 60S subunits, takes less than 10 minutes. The speed of this process is facilitated by the cotranscriptional production of the ribosomal small subunit. Some of the Rps (ribosomal protein small subunit) proteins actually bind and integrate with the 18S portion of the 35S rRNA, forming the initial structure of the small subunits at a very early point in production. Production of the larger 60S subunit is a bit slower, and begins following the A2 cleavage of the 35S rRNA. After this point, the large and small subunit RNAs separate and integration of the large subunit ribosomal proteins begins (Pérez-Fernández, Román, De Las Rivas, Bustelo, & Dosil, 2007).

Biogenesis of the 40S subunit continues with the ordered assembly of UTP-A, UTP-B, and UTP-C complexes, which include ~20 non-ribosomal proteins that must be removed as the
subunit is matured (Pérez-Fernández et al., 2007). The addition and removal of the many ribosome maturation factors make the assembly process greatly resemble a factory with workers performing discrete tasks on an assembly line. After the A0, A1, and A2, cleavage events are complete, yielding the 20S pre-rRNA, the pre-40S composition goes through a major structural change. At this point, non-ribosomal proteins are removed and the complex is transported to the cytoplasm where other factors complete the maturation process, including the cleavage of the 20S rRNA to the mature 18S (Schäfer et al., 2003; Schäfer et al., 2006). Hence, small ribosomal subunits enter the cytoplasm in an immature state, raising the possibility that immature small subunits could potentially attempt initiation.

The 60S subunit maturation process also utilizes a large number of transiently associated factors, though discrete maturation intermediates have not been as easily identified as those for the 40S subunit. Instead, there appears to be a continuous flow of proteins in and out of the pre-60S, with various proteins present at some stages and still others present throughout the assembly process (Kressler et al., 2010).

The 5S complex, which simply includes the 5S rRNA bound to Rrp5, integrates early into the large subunit, just after the cleavage and separation of the 20S and the 27SA2 (Zhang et al., 2007). Downstream, the removal of the Rix1–Ipi3–Ipi1 complex by the 550 kd AAA+ ATPase Rea1 serves to underscore the energy-intensive nature of ribosome assembly. The ring domain of Rea1 includes 6 ATPase domains, which are used to drive the movement of its tail domain in the manner of a lever. The leverage and power expenditure allow the removal of Rix1 and its bound counterparts from the pre-60S subunit (Ulbrich et al., 2009). The resulting complex is nearly ready for export by via the Mex67-Mtr2 pathway (Ansel et al., 2008).
Major alterations in pre-60S composition continue in the cytoplasm. The addition of the 7S rRNA to the pre-60 particle occurs around the time of nuclear export, though incorporation does not appear to be required for the export process itself (Yao et al., 2007). In the cytoplasm, the 7S rRNA is further matured into the 5.8S (Ansel et al., 2008). The last large subunit protein to be added is Rpl10 (Hedges, West, & A.W. Johnson, 2005). Shortly after its incorporation, the 60S reaches maturity.

_Catalytic Functions of the Ribosomal RNA_

Though the RNA serves largely as a scaffold for the binding of ribosomal proteins, it is also catalytic. In its role as a ribozyme, the 25S rRNA directly participates in the transamination reaction that transfers the nascent peptide from the P-site tRNA to the A-site tRNA (Schmeing, Huang, Strobel, & Steitz, 2005). No ribosomal proteins are directly involved, though in bacteria, L27 does appear to be important for the function of the peptidyl transferase center (Maguire, Beniaminov, Ramu, Mankin, & Zimmermann, 2005).

The small subunit rRNA also contributes to the proofreading ability of the ribosome. As discussed above, bases A1492 and A1493 contribute to A-site proofreading by hydrogen bonding to the minor groove of the mRNA. The A-site proofreading mechanisms alone are sufficiently selective that they would allow for an overall error rate of ~ 1 in $10^3$ to $10^4$, a significant proportion of the full ribosomal proofreading ability, which has an error rate of ~ 1 x $10^6$ (Battle et al., 2002; Johansson et al., 2008).
Ribosomal Protein Function

The crystal structure of the eukaryotic ribosome was only recently solved (Ben-Shem et al., 2010; Rabl, Leibundgut, Ataide, Haag, & Ban, 2011). This provided some of the first detailed structural information about the eukaryotic ribosomal proteins, including their location and intermolecular contacts. The eukaryotic ribosome is roughly 40% larger than the prokaryotic ribosome, and contains both RNA expansion sections and 25 proteins not found in eubacteria (Ben-Shem et al., 2010). Also distinguishing the eukaryotic ribosome is the degree of contact between ribosomal proteins. In the prokaryotic small subunit, 5 out of the 20 ribosomal proteins do not make any protein:protein contacts. Conversely, in the eukaryotic small subunit, most of the ribosomal proteins possess long C- or N-terminal tails that extend to distant locations on the subunit, making extensive contact with other proteins. Likewise, many of the eukaryotic ribosomal proteins that have direct counterparts on the prokaryotic ribosome have evolved C- or N-terminal extensions allowing protein:protein interactions (Rabl et al., 2011).

Though we now have a great deal of structural information about the eukaryotic ribosomal proteins, their functions are not well understood. This is due in part to the difficulty inherent in studying essential proteins. Ribosomal proteins also present an additional challenge in that they function as part of a complex that also renders many mutations lethal. In the anecdotal examples of Rps30, Rps9, Rps26, and Rpl10, a PubMed search yields only a handful of papers. Moreover, these studies often simply report that a given ribosomal protein is implicated in a disease, or defines their extraribosomal functions, without providing insight into their mechanism on the ribosome.
Rack1 and Rps3

Despite our general lack of understanding about the functions of the ribosomal proteins, studies have elucidated some of the activities of the proteins Rps3 and Rack1. Rps3 is present on both the prokaryotic and eukaryotic ribosome in essentially the same form (Rabl et al., 2011). It is located at the entrance to the mRNA channel where it functions as an mRNA helicase along with Rps4 and Rps5. These three proteins require the positively charged amino acids that extend into the mRNA channel for their helicase activities (Takyar, Hickerson, & Noller, 2005).

A great deal more information is available for Rack1. This non-essential, eukaryotic-specific, ribosomal protein acts in a wide variety of gene expression pathways and promotes the ubiquitin mediated degradation of proteins (Nilsson, Sengupta, Frank, & Nissen, 2004; Rabl et al., 2011). The name Rack1 refers to both the human protein and the family of homologous proteins; however, the *S. cerevisiae* homologue of Rack1, Asc1, is the primary homologue discussed here.
Figure 1.5. Rps3 contacts Asc1 via a long C-terminal tail and is situated at the mouth of the mRNA channel opposite Rps30. A. Asc1 is shown to the right of the picture in light gray. Rps3 is shown in dark gray with its C-terminal tail contacting Asc1. B. View of the small ribosomal subunit looking into the mRNA channel from the entry point. Rps3 and Rps30 are shown at the entry. Structural data from Rabl et al. 2011, viewed in PyMol.

Like the other Rack1 orthologues, Asc1 is a WD40 repeat protein with a 7-bladed beta-propeller structure (Coyle, Gilbert, & Doudna, 2009) (Fig. 1.2). The founding member of the WD40 family of proteins is the beta subunit of heterotrimeric G-proteins, a signaling molecule. The function of G-beta suggested a potentially similar role for Rack1, and it was accordingly
identified as a scaffolding protein in the PDE4D5- and Src signal transduction pathways. As a scaffold, Rack1 allows the simultaneous binding of multiple proteins, thereby promoting cross-talk between substrates that do not interact directly (McCahill, Warwicker, B., Houslay, & Yarwood., 2002). As part of its role as a scaffold, Rack1, and specifically Asc1, are capable of forming homodimers. Each of the two Rack1 molecules in a given pair may bind discrete partners, thereby promoting interactions between proteins that do not bind each other directly. The ability to simultaneously homodimerize and also bind a second protein allows mammalian Rack1 to promote the Cullin complex mediated ubiquitination of target proteins, leading to their degradation by the 26S proteasome (Liu et al.).

Interestingly, Asc1 and Rps3 interact directly via the long C-terminal tail of Rps3 which is conserved between prokaryotes and eukaryotes (Fig. 1.5) (Rabl et al., 2011). The tail of Rps3 contacts the WD4 domain of Asc1 which is implicated in mediating the dimerization of Asc1, a function critical for the ability of mammalian Rack1 to conduct ubiquitin mediated proteolysis.

![Figure 1.6. Structure of Asc1 and the Asc1 heterodimer. (Yatime et al, J. Mol. Bio. 2011)](image)
Rack1 was initially identified as a ribosomal protein by cryo EM (J. Sengupta et al., 2004). On the ribosome, Rack1 promotes translation via both specific and general mechanisms: during initiation, Rack1 binds Protein Kinase C, leading to the phosphorylation eIF6 on the large subunit. This triggers the loss of eIF6 from the large subunit, thereby promoting 80S complex formation (Ceci et al., 2003). Rack1 also has the capability to promote the translation of specific transcripts by binding to the mRNA-associated factor Scp160.

Though Rack1 clearly functions on the ribosome, it may also have extra-ribosomal functions. Certain mutations in the ASC1 gene partially or severely decrease the association of Asc1 with the ribosome, allowing the study of ribosome dependent, and independent functions. Strains harboring even the most potent of these mutants do not show a complete phenotypic overlap with ASC1 deletion strains, suggesting that Rack1 has functions both on and off the ribosome (Coyle et al., 2009). The functions for which ribosome binding is critical include the translational arrest and mRNA cleavage phenotypes in Nascent Peptide Dependent Translational Arrest (PDTA). In PDTA, Asc1 promotes a form of termination that releases the nascent peptide and triggers mRNA cleavage when the ribosome slows significantly or pauses during translation elongation. By detecting and resolving slow or stalled translation complexes, this system may represent a defense mechanism against improperly made mRNAs (Kuroha et al., 2010). The details of this process are described in the next section on translation surveillance mechanisms.
Translation Surveillance Mechanisms

In *S. cerevisiae*, translation is monitored by several pathways that detect problems with ribosome:mRNA complexes. These pathways theoretically protect the cell from improperly made proteins and components of the translation complexes that are functioning incorrectly. Problems with translation can occur in all organisms, though the mechanistic details of the resolution mechanisms differs between eubacteria and eukaryotes.

Translation Surveillance in Eubacteria

In *E. coli* and *B. subtilis*, mRNAs that lack a termination codon, or mRNAs that are translated by a ribosome which experiences an impediment to progression, are subject to the same type of quality control. In both cases, an RNA molecule called tmRNA resolves the stalled complex, acting on the mRNA, the nascent peptide, and the ribosome. As the name implies, tmRNA possesses structural features of both tRNA and mRNA. The tRNA-like portion of the molecule is aminoacylated with Alanine and bound by both Ef-Tu and an accessory protein, SmbP. In keeping with its structure, the tRNA-like portion of the molecule inserts into the A-site of the ribosome, while the mRNA portion inserts into the mRNA channel (Hallier et al., 2004). Due to the lack of codon:anticodon pairing, a mechanism exists to prevent kinetic proofreading mediated rejection of tmRNA. SmbP fulfills this role, apparently by occupying the A-site and interacting with bases 18S:1492 and 1493 (Nonin-Lecomte et al., 2009). After tmRNA insertion into the ribosome is complete, the nascent peptide is transferred to A-site alanine. tmRNA then extends the nascent peptide using its own internal open reading frame in a process called translational extension. The extra amino acids added to the C-terminal of the nascent peptide are
recognized by various proteases, targeting the entire peptide for degradation (Tu, Reid, Zhang, Moritz, & Simpson, 1995). RNaseR (homologous to yeast Rrp44, a component of the exosome) was also implicated in the degradation of the mRNA, thereby precluding the possibility that a potentially errant mRNA will cause the formation of additional stalled complexes (Ge, Mehta, Richards, & Karzai, 2010).

Beyond the degradation of aberrant mRNAs and their associated peptides, the tmRNA system appears to be important during both heat and cold shock (Fujihara et al., 2002). This suggests that problems with nascent peptide folding can also be resolved by tmRNA.

Despite the versatility of tmRNA, mutations in the rRNA that render the ribosome non-functional are dominant negative. In 1990, the Noller lab demonstrated in bacteria that 16S rRNAs, mutated at G530, would produce small ribosomal subunits that killed the cell (Powers et al., 1990). These mutant subunits accumulate in the cell and also appear in polysomes, suggesting that they can complete initiation and 60S recruitment. Though this finding does not preclude the possibility that tmRNA rescues mutated ribosomes stuck during translation, it suggests that bacteria are unable to remove such ribosomes from the translation pool. As such, they may repeatedly create new stalled complexes, sequestering mRNAs and interfering with normal gene expression (Powers et al., 1993; Muth, 2000).

Translation-dependent mRNA Surveillance in S. cerevisiae

Eukaryotes and archea have not been found to possess tmRNA (Williams, 2000). Instead, protein-based systems have taken its place. Central to the function of the translation complex rescue systems is the heterodimer Dom34:Hbs1, a pair of proteins that closely resembles the
canonical release factors eRF1:eRF3. Both heterodimers structurally resemble EF-Tu:tRNA, consistent with the ability of all three pairs to insert into the A-site (Shoemaker et al., 2010). When Dom34:Hbs1 binds the A-site of a translating ribosome, there are multiple consequences: the P-site tRNA:peptide is released, the ribosomal subunits separate, and the mRNA is released. These events occur in a codon-independent manner, distinguishing the activity of Dom34:Hbs1 from that of either EF-Tu:tRNA, which require the presence of a sense codon in the A-site, or eRF1:eRF3, which requires a stop codon in the A-site (Shoemaker et al., 2010).

Just as the tmRNA system promotes the degradation of the components of a stalled complex, Dom34:Hbs1 promotes the degradation of the mRNA, 18S rRNA, and nascent peptide of stalled complexes. These effects were discovered independently as part of the genetic analyses of Dom34:Hbs1 in three related pathways. First, the No-Go Decay (NGD) pathway was shown to degrade mRNAs with strong secondary structure or rare codons within the ORF. These structures apparently slow translation sufficiently to trigger the activity of Dom34:Hbs1, leading to the endonucleolytic cleavage of the mRNA (Doma et al., 2006). mRNAs encoding multiple lysine or arginine codons are also believed to slow ribosome progression, thereby triggering mRNA cleavage, translation termination, and degradation of the associated peptide. This process is named Nascent Peptide Dependent Translational Arrest (PDTA) (Kuroha et al., 2010). The Dom34:Hbs1 system also has a secondary activity that tmRNA does not: it promotes the degradation of mutant 18S rRNAs that are dominant negative in bacteria via the Nonfunctional 18S rRNA decay pathway (Cole, LaRiviere, Merrikh, & Moore, 2009; LaRiviere, Cole, Ferullo, & Moore, 2006).
Figure 1.7. Translation-dependent mRNA surveillance mechanisms in \textit{S. cerevisiae} and the corresponding references. Each pathway degrades the associated mRNA and is triggered by discrete mRNA mediated impediments: In No-Go Decay, the trigger is strong mRNA secondary structure; in Non-Stop Decay the trigger is an empty A-site; and in Peptide Dependent Translation Arrest the trigger appears to be the translation of codons encoding positively charged amino acids. Dom34:Hbs1 function in each pathway.

18S Nonfunctional rRNA Decay (18S NRD)

The experiments demonstrating the dominant effects of rRNA mutants paved the way for the same experiments in eukaryotes. To accomplish this, a robust system for working with eukaryotic rRNAs was established. Working in \textit{S. cerevisiae}, multiple labs contributed to the development of a plasmid based system for ribosomal RNA expression. Though multiple iterations of the plasmid were created, the two systems relevant to this report are 2 micron
plasmids that encode the 35S rDNA repeat under either a constitutive Pgk1 promoter or a galactose inducible promoter (LaRiviere et al., 2006). Despite the fact that in vivo, the 35S rRNA is transcribed by RNA polymerase I, polymerase II-derived rRNAs support growth even when they are the only rRNAs in the cell (Venema, Dirks-Mulder, Faber, & Raué, 1995).

In order to detect the plasmid-derived rRNAs amid the endogenous rRNAs, sequence tags were inserted near the 5’ ends of the 18S and 25S sequences (Beltrame & Tollervey, 1995; Musters et al., 1989). Importantly, these extra sequences do not appear to affect the ability of the rRNAs to conduct normal translation. The tags can be used for detection via northern blot with little to no background.

Initially, the constitutively expressed rRNA plasmids were tested for dominant negative effects. The dominant effects were not observed in bacteria until the mutant rRNAs reached 20% of the total, indicating that the same might be true in yeast. Yet, in a wild type yeast strain, the low abundance of the rDNA plasmid produced tagged rRNAs at roughly 2% of the total. To compensate for this, the strain YJV100 was used. This strain is a pol I deletion strain that remains viable due to the presence of ~10 chromosomal copies of the rDNA repeat under galactose-inducible promoters. Hence, YJV100 grows on galactose-containing medium and ceases growing on glucose, due to repression of the galactose-inducible promoter. Due to the low endogenous levels of rRNA expression in YJV100, when plasmid-derived rRNAs are expressed, their final concentration represents a significantly higher percentage of the total rRNAs in the cell – roughly 20%. Hence in YJV100, the dominant effect of a given rRNA mutation should be detectable. Moreover, the ability to turn off the expression of chromosome-derived wild type rRNAs makes it possible to grow this strain on plasmid-derived rRNAs alone. As such, it is possible to ask two questions: 1) Is a given rRNA mutation dominant negative? 2) Can cells
grow using a particular mutant rRNA, when it is the only rRNA in the cell? The subsequent experiments in YJV100 demonstrated that cells live when 18S:A1492C and 18S:G530U are present at high abundance, though neither mutant 18S is able to support growth on its own. This suggested that yeast possesses a mechanism for dealing with mutant 18S rRNAs. Follow-up experiments quantified mutant 18S rRNA abundance and found that in both cases, levels were greatly depleted at steady state, suggesting that cells may be degrading the deleterious rRNAs. In order to detect degradation, the same constructs were used in a pulse-chase assay. Following a brief induction, glucose was added and the stability of both the plasmid-derived 18S and 25S rRNAs was monitored over a 2-hour period. Though the wild type 18S and 25S were stable over this period, both of the mutant 18S rRNAs disappeared with a half-life of roughly 75 minutes. This established that nonfunctional rRNA decay (NRD) occurs in yeast (LaRiviere et al., 2006).

In an effort to define the trans-acting factors in NRD, strains lacking DOM34 and HBS1 were tested for their ability to conduct NRD. Results showed that the rate of NRD is diminished by approximately half in the absence of either protein. This effect is not additive when the two deletions are combined, suggesting that both halves of the heterodimer are required. Moreover, the fact that 18S NRD still continues without Dom34 and Hbs1 indicates that other factors are promoting NRD independently (Fig. 1.8) (Cole et al., 2009).
**Figure 1.8.** Models for 18S NRD, NGD, and PDTA (not reflecting unpublished data). A. NRD - Small ribosomal subunits containing a mutant 18S:A1492C rRNA are targeted for degradation via the action of Dom34:Hbs1, and previously unidentified factors. B. NGD - Dom34:Hbs1 promote the cleavage of the NGD substrate mRNA by unidentified nucleases. C. PDTA - mRNA cleavage by unidentified nucleases is promoted by Dom34:Hbs1 and Asc1. Fragments of all substrate RNAs are degraded by Xrn1 and the cytoplasmic exosome.

18S NRD substrates also accumulate in cytoplasmic foci called P-bodies. P-bodies contain translationally silenced mRNAs and a number of RNA degradation factors including decapping enzymes, and the major cytoplasmic 5'-3' exoribonuclease Xrn1. As such, they appear to represent a site used for the sequestration or degradation of nonfunctional ribosomes (Cole et al., 2009).
The discovery that mutant 18S rRNAs localize to P-bodies represents a second overlap between 18S NRD and the NGD pathway. No-Go mRNAs are cleaved in a Dom34:Hbs1 dependent manner and also accumulate in P-bodies (Cole et al., 2009). These overlaps suggested that the NGD and NRD pathways may overlap. By extension, the Nascent Peptide Dependent Translational Arrest (PDTA) pathway may overlap as well. This possibility sets up the hypothesis that all stalled ribosome:mRNA complexes may be subject to degradation of the ribosome, mRNA, and nascent peptide.

**Translation-Independent Ribosome Degradation**

In addition to the translation-dependent 18S NRD pathway discussed above, ribosomes are subject to translation-independent degradation. In yeast, three such pathways have been reported to degrade the large and small ribosomal subunits: 25S NRD, 60S Ribophagy, and 40S Ribophagy (Kraft, Deplazes, Sohrmann, & Peter, 2008; LaRiviere et al., 2006; Ossareh-Nazari et al., 2010). These pathways utilize a number of trans-acting factors that have not been examined for effects in NRD, NGD, NSD, and PDTA.

The 25S NRD pathway selectively detects and degrades mutant 25S:U2585A and 25S:A2451G rRNAs that are part of, apparently immature, 60S subunits (Cole et al., 2009; LaRiviere et al., 2006). These two bases are located in the peptidyl transferase center of the 60S and directly participate in the aminotransfer reaction, suggesting that mutations at this location may decrease the ability of the 60S to function properly. Consistent with this hypothesis, *E. coli* cells incorporate the equivalent mutant rRNAs, 23S:U2585 and 23S:A2451, into mature subunits that demonstrate a highly compromised capacity for producing protein. These mutant rRNAs
ultimately kill the cell via dominant effects when they are expressed at ~20% of the total 25S rRNAs in the cell (Youngman, Brunelle, Kochaniak, & Green, 2004). In yeast, 25S:U2585A rRNAs are not dominant negative. Instead, they are selectively degraded with a half-life of approximately 80 minutes. Unlike the translation-dependent 18S NRD pathway, the application of the translation inhibitor cycloheximide does not decrease the rate at which 25S NRD occurs (Cole et al., 2009). This functional distinction between 25S and 18S NRD suggests that these pathways are distinct. Moreover, localization data indicate that mutant 25S rRNAs collect at the nuclear periphery, suggesting 25S NRD may represent a quality control measure for immature 60S subunits (Cole et al., 2009).

Ribophagy is another translation-independent ribosome turnover pathway. During starvation, cells consume their intracellular organelles, including the peroxisomes, mitochondria, and ribosomes, via the process of autophagy (Kraft et al., 2008). The term “ribophagy” refers to one specific aspect of autophagy that is responsible for the degradation of the large and small ribosomal subunits. As part of ribophagy, both of the ribosomal subunits localize to the vacuole, apparently via discrete mechanisms. Though the proteins responsible for small subunit translocation are unknown, large subunit ribophagy is promoted by Doa1, Ubp3/Bre5, and Cdc48 (MacIntosh et al., 2011; Ossareh-Nazari et al., 2010; Kraft et al., 2008). Moreover, ribophagy requires the general autophagy proteins Atg7 and Pep4 (Ossareh-Nazari et al., 2010). The Doa1/Ubp3/Bre5/Cdc48 group is also involved in the ubiquitin mediated selection of cytosolic proteins for degradation by the 26S proteasome (Ossareh-Nazari et al., 2010). In this capacity, Doa1 functions as an adaptor protein, binding ubiquitin on ubiquitinated proteins and thereby promoting the Cdc48-mediated passage of these target proteins to the 26S proteasome (Ren, Pashkova, Winistorfer, & Piper, 2008). As part of this pathway, Ubp3 functions as a
ubiquitin protease, recycling the ubiquitin tag prior to proteolysis (Doelling et al., 2007). In ribophagy, the 26S proteasome appears to be expendable, suggesting that ribophagy is functionally distinct from these turnover events. One possible function for Doa1/Ubp3/Cdc48 in ribophagy is the promotion of ribosome translocation into the vacuole (Ossareh-Nazari et al., 2010).

Like ribophagy, 25S NRD is promoted by the ubiquitin system (Fujii, Kitabatake, Sakata, Miyata, & Ohno, 2009a). A screen for factors involved in 25S NRD demonstrated that 25S:U2585A rRNAs are stabilized in the absence of Mms1 and Rtt101, two members of the Cullin complex that function as E3 ubiquitin ligases (Fujii, Kitabatake, Sakata, Miyata, & Ohno, 2009b). Though the target of ubiquitination is unclear, western blot data show that at least three ribosomal protein targets are ubiquitinated in an Rtt101 and Mms1 dependent manner.

Ubiquitin has also been implicated in the stability of wild type ribosomes. Research on the essential E3 ubiquitin ligase Rsp5 has demonstrated that expression of dominant negative forms of the protein lead to widespread ribosome turnover (Shcherbik et al., 2011). Similarly, wild type rRNAs in an Rsp5 temperature-sensitive strain are uniformly degraded when the culture is raised to the restrictive temperature. Non-coding RNAs and mRNAs are also degraded, but to a far lesser extent, suggesting that Rsp5 is either directly or indirectly controlling ribosome stability (Shcherbik et al., 2011).

**RNA Damage**

Damaged RNAs may be one of the targets of translation surveillance pathways discussed above. Though the effects of RNA damage were overlooked for many years due to the presumed
ease with which RNAs can be degraded, many reports over the past decade have demonstrated that RNA damage is common. Cellular metabolism in most cell types produces alkylating and oxidizing agents that chemically modify DNA and RNA. In humans, the accumulation of damaged RNAs correlates with the severity of neurological disease, suggesting that RNA damage is a very real threat to the health of our cells (Nunomura et al., 2009; Nonumura et al., 2006). Presumably, RNA lesions may lead to miscoding, ribosome dysfunction, and other translation problems, potentially causing the translational stalling that triggers NGD, NRD, NSD, or PDTA.

**RNA Oxidation**

RNA damage appears to play a role in human disease. Though no study has directly implicated damaged RNA as the causative agent of any illness, oxidized RNAs and decreased protein expression are correlated with diseases such as diabetes and Alzheimer’s disease (Nunomura et al., 2009). Contributing to the oxidation of RNAs in the central nervous system are the high prevalence of oxidation reactions relative to the rest of the body, high concentrations of redox-capable metals, and low concentrations of anti-oxidants (Nunomura et al., 2009). Furthermore, neurons in the central nervous system are bombarded by additional reactive oxygen species produced by the microglia that surround them (Block, Zecca, & Hong, 2007).

Several model systems support the oxidative stress hypothesis of neurological disease. For example, mice doubly deficient for the anti-oxidant enzyme superoxide dismutase 2 (Sod2) have extremely brief lifespans and display muscle weakness and other symptoms corresponding to amyotrophic lateral sclerosis (Nunomura et al., 2009). This matches well with the observation that neurons from patients with Alzheimer’s disease display highly oxidized RNAs including
rRNAs and mRNAs (Anderson, 2004). Testing in human leukocytes, among many other cell lines, indicates that RNA rather than DNA is the major target of oxidation in the cell (Shen, Wu, & Hazen, 2000). *In vitro* experiments in rabbit reticulocyte lysates demonstrated that oxidized mRNAs accumulate in heavier fractions and produce less protein, suggesting that ribosomes may stall on such mRNAs, leading to the accumulation of additional ribosomes (Fig. 1.9) (Shan, Chang, & Lin, 2007). Moreover, oxidized transcripts cause translation errors (Tanaka, Chock, & Stadtman, 2006). Accordingly, in the CNS neurons of Alzheimer’s disease patients, oxidized transcripts and malfunctioning ribosomes are correlated with a relative paucity of protein expression compared to that of healthy individuals (Nunomura et al., 2009).
**Figure 1.9.** Proposed model explaining the observation that oxidized mRNAs accumulate in heavier polysome fractions. A. The canonical translation cycle. Following subunit dissociation, individual subunits will initiate again on the same or a new mRNA. B. Oxidized RNA bases may prevent proper codon:anticodon pairing, impairing translation and leading to additional stalling of upstream ribosomes. This could lead to an accumulation of oxidized mRNAs in heavier polysome fractions.
Hence, endogenously produced, reactive oxygen species may promote disease via RNA oxidation. Though the mechanism by which RNA damage precipitates disease is unknown, the changes in protein level suggest that the cell may lose its ability to control gene expression at the level of translation.

**RNA Alkylation**

Cellular metabolism produces alkylation agents that modify DNA and RNA. Though these agents typically react weakly with the DNA, at physiological concentrations they promote mutagenesis (Taverna & Sedgwick, 1996). Examples of endogenous alkylation agents include S-Adenosylmethionine, which is a common component of the methyl group transfer reactions in bacteria, and nitrosated amines, which can be formed from bile acids. Both examples are O-alkylating agents (Drabløs et al., 2004).

Alkylation damages both DNA and RNA in similar ways. Depending upon the particular agent, alkylation may occur on all oxygen and nitrogen atoms, producing a plethora of damaged species. Though the potential variety is vast, most alkylation damage is in the form of 7-alkylG in both DNA and RNA (Shooter, Howse, Shah, & Lawley, 1974). This modification is, generally speaking, not directly harmful to the cell. However, in DNA, such lesions are quickly removed via base-excision repair, thereby producing apurinic site intermediates that can be cytotoxic (Memisoglu et al., 2000). Alkylated RNAs are also repaired. The recent discovery of the oxidative human RNA demethylases AlkB and AlkBH3 suggests that the ability to clear alkylated RNAs provides a selective advantage for cells (S. Wu et al., 2011). Based upon the theorized ability of damaged RNAs to cause translation problems, it is also possible that the
translation surveillance mechanisms NGD, NSD, NRD, and PDTA may be responsible for the clearance of damaged RNA species (P.A. Aas et al., 2003).

RNA repair may have implications for human health following exogenous RNA alkylation during chemotherapy. To destroy cancer cells, doctors often deliver alkylating agents such as Cisplatin, Oxiplatin, and Temodar (Takimoto & Calvo, 2008). These agents selectively damage fast-growing cells, thereby targeting the cancer cells while generally sparing healthy cells. Despite this partial selectivity, DNA and RNA of healthy cells are also damaged by chemotherapy agents. This raises the possibility that RNA damage may be relevant to the survival of healthy cells and cancerous cells alike. Accordingly, decreased expression of the RNA demethylase AlkB and AlkBH3 in cancer cells leads to increased apoptosis during Cisplatin treatment (S. Wu et al., 2011).

UV Damage to RNA

Ultraviolet light damages RNA, producing photochemical modification, cross-linking, and oxidative damage. These products were originally detected in vitro using RNA from the tobacco mosaic virus, as well as poly(U), and poly(C). Damaged RNAs were enzymatically hydrolyzed and separated by chromatography to detect dimers and modified nucleotides.(Small, Tao, & MP, 1968). Researchers observed that UV treatment produced cyclobutane pyrimidine dimers, uridine hydrate, and cytidine hydrate (Miller & Cerutti, 1968; Singer, 1971). They also demonstrated that single-stranded RNA is more susceptible to UV damage than double-stranded RNA (Pearson & Johns, 1966). Essentially, these findings established that UV should modify RNA in a manner similar to DNA, likely preventing its proper function.
The question of whether the UV exposures used in vitro are relevant for normal physiological conditions has also been addressed. UV-C, corresponding to wavelengths 200-290 nm, was initially used in the RNA damage tests (Gambichler et al., 2006). These wavelengths are typically filtered by the ozone layer, such that it is mainly UV-B and UV-C that reach the earth (Gambichler et al., 2006). UV-B and UV-A have less energy than UV-C but reach the earth in higher amounts and can penetrate farther into tissue. A dose of UV-A/B relevant for human health is 704 ± 188 J/m2, which represents the minimum amount of energy needed to produce a sunburn (Gambichler et al., 2006). This is roughly the amount of energy a person would absorb during an hour of sun exposure in the northern hemisphere (Ambach & Blumthaler, 1993). A dose of physiologically relevant UV-B (specifically 600 J/m2) produces measurable impediments to reverse transcriptase in the 28S rRNA of cultured mammalian cells (Iordanov et al., 1998). Given preferential location of these impediments adjacent to pyrimidine nucleotides, these lesions are likely to be pyrimidine dimers. Hence, normal environmental exposure to UV is likely to produce pyrimidine dimers in RNA.

Evidence for UV-mediated RNA crosslinking has also been reported. Crosslinks between RNA and protein have been reported for many years as a tool to study the ribosome (Möller, Zwieb, & Brimacombe, 1978). Moreover, RNA-RNA crosslinks have been shown to form in E. coli cells after UV-A irradiation. One major group of these cross-links include cytidine - pseudouridine bases (Ramabhadran, Fossum, & Jagger, 1976). UV-cross-linked tRNAs with these cross-links have lower rates of aminoacylation and produce less protein in translation assays (Ramabhadran et al., 1976).
Despite the frequency with which damaged RNAs are formed, it is only recently that the impact of damaged RNAs has garnered much attention. In the past 10 years, an increasing number of reports have demonstrated the turnover of damaged RNAs by unidentified pathways. Three examples in bacteria and plants are discussed below. In each case, cells treated with a genotoxic stressor caused widespread mRNA or rRNA turnover.

In the plant model system *Z. mays*, leaf cells selectively degrade ribosomal RNAs following UV exposure (Casati & Walbot, 2004). Though the process is slow, occurring over the course of 16-24 hours, the selective turnover is clearly demonstrated and correlates with decreased protein expression. The loss of ribosomes is apparently compensated for by the subsequent increase in transcription of ribosomal protein genes.

Likewise, in *A. thaliana*, mRNAs are turned over following UVB exposure (Revenkova et al., 1999). This turnover is measured relative to non-coding RNAs, suggesting that the non-coding RNAs are not degraded as quickly or are not degraded at all. This, in turn, suggests that translation may be required for the selective degradation of damaged RNAs.

To complement the studies of RNA turnover in plants, additional damaging agents in a variety of systems have triggered RNA turnover. In one example, *E. coli* cells damaged via hyperbaric oxygen treatment display widespread ribosome degradation. Here cells were grown in a hyperbaric chamber containing air plus 300 psi nitrogen or 300 psi oxygen. The mortality of the cells grown in the presence of high-pressure oxygen increased dramatically relative to the cells grown in high-pressure nitrogen (Harley, Flaks, Goldfine, Bayer, & Rasmussen, 1980). This
increase in cell mortality correlated with rRNA turnover, indicating that oxidative rRNA damage might contribute to cell death.

In conclusion, RNA damage appears to promote both cell death in a variety of organisms, and human disease. Though the mechanism by which this occurs is not yet clear, it is possible that damaged RNAs sequester translation factors and ribosomes, potentially leading to improper gene expression.
Chapter 2:

Turnover of nonfunctional rRNAs requires ribophagy factors and the ribosomal protein Asc1

Introduction

Production of functional proteins depends upon accurate mRNA translation. Impediments to translation are known to slow, or potentially stall, ribosome:mRNA complexes, subsequently interfering with the formation of full-length peptides. A variety of events have been shown to promote such stalls, including oxidation of the associated mRNA, depurination of the rRNA by biological toxins, mutation of the rDNA, and impairment of ribosomal subunit maturation (Shan et al., 2007; Barbieri et al., 1992; Lariviere et al., 2006; Soudet et al., 2010). The variety of sources suggests that ribosome stalling may not be uncommon. Accordingly, at least four pathways are known to monitor the process of translation for the purposes of resolving stalled translation complexes.

The process of No-Go Decay (NGD) selectively degrades transcripts that have strong secondary structures in the open reading frame or that encode a series of rare codons (Doma et al., 2006). These two features are believed to slow ribosome progression, subsequently triggering the observed endonucleolytic cleavage of the transcript. Non-Stop Decay (NSD) degrades errant mRNAs that lack a termination codon, leading to the formation of a stalled ribosome with the final 3 nucleotides of the mRNA in the P-site (L. N. Dimitrova, Kuroha, Tatematsu, & Inada, 2009; Inada & Aiba, 2005). Such mRNAs are degraded by the exosome in a 3’ to 5’ manner
A third mechanism, Nascent Peptide Dependent Translational Arrest (PDTA) is triggered by mRNAs that encode serial arginine or lysine codons. It was initially hypothesized that PDTA causes ribosome stalling when positively charged lysine or arginine amino acids stick to the negatively charged peptide exit channel (Kuroha et al., 2010). PDTA entails dual consequences – endonucleolytic mRNA cleavage and degradation of the nascent peptide (Kuroha et al., 2010). Stalled complexes also have consequences for the ribosome vis a vis, the Non-Functional 18S rRNA Decay (18S NRD) pathway. This translation-dependent mechanism selectively degrades the 18S rRNA component of 40S subunits believed to be defective for proper codon:anticodon pairing (Cole et al., 2009; LaRiviere et al., 2006). Presumably, ribosomes stalled for a variety of reasons, including those discussed above, may also be subject to 18S NRD.

In keeping with the common theorized triggers, these pathways may share common mechanisms. NRD, NGD, and PDTA are all promoted by the same trans-acting heterodimer, Dom34:Hbs1 (Cole et al., 2009). In vitro experiments have shown that these two proteins insert into the ribosomal A-site, subsequently promoting the codon-independent release of the ribosomal subunits, mRNA, and tRNA-peptide (Shoemaker et al., 2010). Therefore, their activity is ideally suited for the remediation of immobile translation complexes.

Recently, additional trans-acting factors have been identified in NSD and PDTA, suggesting that they may have roles in NRD and NGD. In PDTA, the ribosomal small subunit protein Asc1 (of the Rack1 family) was shown to promote the translational arrest phenotype (Kuroha et al., 2010). Moreover, in both PDTA and NSD, the associated nascent peptide is ubiquitinated, leading to its degradation by the 26S proteasome (Ito-harashima, Kuroha, Tatematsu, & Inada, 2007). In PDTA, this process is carried out by the E2 conjugase Ubc4 and
the E3 ubiquitin ligase Not4 as part of the Ccr4/Not4 complex. In NSD, the E3 ubiquitin ligase Ltn1 fulfills the same role with an unidentified E2 conjugase (Bengtson et al., 2010).

**Rack1**

The *H. sapiens* and *A. thaliana* homologues of Asc1, known by the family name Rack1, have been the subject of numerous studies. Mechanistic analyses of Rack1 which have identified a number of regulatory mechanisms including phosphorylation, sumoylation, and protein:protein interactions (B. Y. Chang, Chiang, & Cartwright, 2001; Dell et al., 2002; Yang & Grégoire, 2006). Figure 2.2 displays the phosphorylated tyrosines and proposed sumoylation sites.

Sumo is a small, ubiquitin-like protein that is covalently attached to the lysine residues of certain proteins that contain consensus sumoylation sequences, as one aspect of the post-translational modification process. Sumoylation has been proposed to alter protein:protein interactions, promote compartmentalization, and modify protein stability (Ullah et al., 2008). The proposed regulation of Rack1 by sumo is based upon the discovery of multiple examples of the sequence ψKxExxSP, called a “phosphor-sumoyl switch” that is predicted to be both phosphorylated on the serine residue and subsequently sumoylated on the lysine residue (Yang & Grégoire, 2006). The discovery of phosphor-sumoyl switches was based initially on the finding that the ψKxE sequence can be sumoylated, and that downstream serine residues can be phosphorylated (Melchior, Schergaut, & Pichler, 2003). Later investigations determined that sumoylation in phosphor-sumoyl switches can be dependent upon serine phosphorylation (Y. Hong et al., 2001).
The anticipated tyrosine phosphorylation sites on Rack1 represent residues conserved between *Arabidopsis*, flies, humans and yeast. In humans, these two residues, Y248, and Y246, are phosphorylated by the protein Src, leading to enhanced Rack1:Src binding. Despite the lack of tyrosine kinases in yeast, it has been suggested that under certain circumstances, serine/threonine kinases may also phosphorylate tyrosine residues. The combined availability of a potential kinase and the conservation of the two known regulatory sites suggest that Y248 and Y246 may alter Rack1 activity in yeast (Fig. 2.2B).
Figure 2.1. Crystal structures of the eukaryotic ribosome, Rack1, and Dom34:Hbs1. A. Hbs1 contacts Rps3 via its N-terminal domain (Adapted from Becker et al. 2011) B. Rack1 forms homodimers via its WD4 domain (Adapted from Sengupta et al. 2004). C. Rps3 interacts with Rack1 via its long C-terminal tail (Adapted from Rabl et al. 2011).
<table>
<thead>
<tr>
<th></th>
<th>YPD</th>
<th>SCD-URA</th>
<th>Invasive Growth</th>
<th>Potassium Disulfite</th>
<th>Rapamycin</th>
<th>Calcofluor White</th>
<th>% RACK1 at Ribosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype</td>
<td>+++</td>
<td>+++</td>
<td>Y</td>
<td>-</td>
<td>+</td>
<td>W</td>
<td>88</td>
</tr>
<tr>
<td>(Loop)Δ</td>
<td>+++</td>
<td>+++</td>
<td>Y</td>
<td>+++</td>
<td>W</td>
<td></td>
<td>89</td>
</tr>
<tr>
<td>(Knob)Δ</td>
<td>+++</td>
<td>+++</td>
<td>Y</td>
<td>-</td>
<td>++</td>
<td>W</td>
<td>88</td>
</tr>
<tr>
<td>K285A</td>
<td>NA</td>
<td>+++</td>
<td>NA</td>
<td>-</td>
<td>++</td>
<td>W</td>
<td>87</td>
</tr>
<tr>
<td>(65-67)Δ</td>
<td>NA</td>
<td>+++</td>
<td>NA</td>
<td>+++</td>
<td>++</td>
<td>W</td>
<td>86</td>
</tr>
<tr>
<td>R102A</td>
<td>+++</td>
<td>+++</td>
<td>Y</td>
<td>-</td>
<td>+++</td>
<td>W</td>
<td>74</td>
</tr>
<tr>
<td>K62A</td>
<td>+++</td>
<td>+++</td>
<td>Y</td>
<td>+</td>
<td>++</td>
<td>B</td>
<td>59</td>
</tr>
<tr>
<td>K87A R90A</td>
<td>+++</td>
<td>+++</td>
<td>Y</td>
<td>++</td>
<td>++</td>
<td>B</td>
<td>56</td>
</tr>
<tr>
<td>R38D K40E</td>
<td>+++</td>
<td>+++</td>
<td>Y</td>
<td>++</td>
<td>+</td>
<td>B</td>
<td>13</td>
</tr>
<tr>
<td>RACK1Δ</td>
<td>+</td>
<td>+</td>
<td>N</td>
<td>++</td>
<td>+</td>
<td>B</td>
<td>---</td>
</tr>
</tbody>
</table>
Figure 2.2. ASC1 mutational analysis schematic. A. The effect of various ASC1 mutations on ribosome binding is displayed (Adapted from Coyle et al. 2010). B. Analysis of ASC1 functional domains, and conserved structures (Adapted from Sengupta et al. 2004).

Protein:protein, and ribosome interaction domains are conserved between Rack1 orthologues. The two main interactions surfaces are on the opposing sides of the planar ring structure. On the ribosome, one plane faces the ribosome, and the other faces the cytosol (Coyle...
et al., 2009). On the yeast Asc1 protein, a long loop faces into the cytosol, and represents one of the asymmetric features of the protein (Fig. 2.1A, C, D). Asymmetric features of WD40 repeat proteins are known to mediate protein binding, suggesting a possible function for the cytosolic loop. An additional asymmetric structure is the ribosome-facing “knob” structure which was initially suspected to mediate ribosome binding, a hypothesis that has since been disproven (Coyle et al., 2009). However, ribosome binding mutants have been developed, leading to the discovery that the interaction of Rack1 with the ribosome is critical for a number of Rack1’s functions, including its role in PDTA (Coyle et al., 2009; Kuroha et al., 2010).

**Ribophagy**

Given the functional overlap between 18S NRD and the mRNA surveillance pathways, general ribosome turnover mechanisms may have implications for translation surveillance. Though endogenous ribosomal degradation pathways are not well understood, a number of proteins have been shown to promote the selective degradation of ribosomes during starvation in a process known as ribophagy (Kraft et al., 2008). Ribophagy represents one specific branch of the autophagy pathway and, therefore, relies on general autophagy proteins. Though the ribophagy proteins responsible for small ribosomal subunit degradation are unknown, several proteins that carry out large subunit degradation have been identified. These include the ubiquitin-binding protein Doa1, which functions as an adaptor protein for the AAA+ ATPase Cdc48. Doa1 is capable of binding to both the ubiquitin tag of ubiquitinated proteins and Cdc48, allowing the Cdc48-mediated passage of the target protein to the 26S proteasome where they are degraded. Indirect evidence in Arabidopsis suggest that the nuclease responsible for the rRNA degradation portion of ribophagy may be the endoribonuclease RNAse T2 (yeast Rny1), which
also appears to affect translation through the cleavage of tRNAs (MacIntosh et al. 2011; Thompson et al., 2009). Interestingly, application of the 26S proteasome inhibitor MG132 did not affect the rate of large subunit ribophagy, suggesting that ribophagy functions via an independent mechanism (Ossareh-Nazari et al., 2010). Accordingly, in ribophagy, both Cdc48 and Doa1 appear to be important in the transport of ribosomes into the vacuole, where degradation events are carried out.

In the current study, we present evidence that a subset of the factors involved in NSD, PDTA, and 60S ribophagy also promote 18S NRD.

**Results**

_18S NRD shares a subset of factors with PDTA_

Recent work on translation surveillance and ribosome degradation pathways has implicated the involvement of several new proteins, raising the possibility that these factors could also have effects on NRD. To test this possibility, we conducted a survey of genes reported to act in PDTA, ribophagy, NSD, and endogenous rRNA turnover pathways, and tested the respective deletion strains for effects on mutant 18S:A1492C rRNA degradation. To expedite the process, we used a single timepoint assay similar to that used by van den Elzen et al. 2010, rather than the time course analyses used in our previous publication (Cole et al., 2009). For this assay, a gene deletion strain from the BY4741 knock-out collection and the isogenic WT strains were transformed with plasmids encoding galactose-inducible wild type 18S, or mutant 18S:A1492C rRNAs, known to be targeted by NRD. Also encoded on the plasmids is a wild type 25S rRNA, which is stable in the cell and can be used to normalize the 18S rRNA levels. Three individual
replicates of each transformed strain were grown to mid-log (OD$_{600} = 0.5$) in synthetic drop-out medium, in the presence of raffinose. rRNA expression was then induced for 90 minutes via the addition of galactose. We then harvested the cultures and subjected total RNA preparations to northern blot analysis. By probing for sequence tags integrated into the plasmid-derived 18S and 25S rDNA genes, we were able to quantify the 18S and 25S rRNA levels in each sample. The 18S:25S rRNA ratios were plotted relative to the levels observed in the isogenic wild type strain.

We first validated the single timepoint assay by comparing the levels of mutant 18S rRNAs in the dom34Δ strain, which is known to produce a 2-fold decrease in the rate of 18S NRD in timecourse assays (Cole et al., 2009) (Fig. 2.3A and 2.3B). At both 45 minutes and 90 minutes after induction of rRNA transcription, we observe levels of mutant 18S rRNAs in the dom34Δ strain roughly 2-fold higher than the levels in the WT strain. This demonstrates that even a partial loss of 18S NRD is detectable using both the timecourse and single timepoint assays (Fig. 2.3A).
Figure 2.3. Mutant 18S:A1492C destabilization in various deletion strains. A. 18S NRD timecourse in WT and dom34Δ strains as previously demonstrated. Quantitation (top) of northern blot analysis (bottom). T0 18S:25S ratio is set to 1 for both strains. B. Single timepoint analysis recapitulates timecourse data. Data for mutant 18S rRNA levels at 45 and 90 minutes post transcriptional induction in WT and dom34Δ cells. 18S rRNA levels are normalized to the WT 25S and displayed relative to levels of WT 18S level observed in the WT strain.
Figure 2.4. Gene deletion strains tested for effects on 18S NRD. Deletion strains transformed with WT or Mutant 18S rRNA constructs were subjected to Single Timepoint Assays. The abundance of WT or Mutant 18S rRNAs in each strain were determined by northern blot. Respective 18S rRNA levels were normalized to WT 25S rRNA levels in the same strain and plotted relative to the normalized, WT 18S rRNA levels in the WT strain.

We then tested a number of strains deficient for PDTA, Ribophagy, and NSD factors for effects on 18S NRD. In two of the three PDTA gene deletion strains, asc1Δ, and ubc4Δ, we observed higher levels of mutant 18S rRNAs. We also observed increased levels in doa1Δ and pep4Δ (Fig. 2.4). Based upon these initial data, we suspected that Asc1, Ubc4, Doa1, and Pep4 might be involved in NRD.
**Asc1 functions in parallel with Dom34**

Due to the observation that ASC1 or DOM34 deletion only leads to a partial effect on PDTA and NRD (Fig 2.4), we suspected that these two proteins might be functioning in parallel. To test this possibility, we constructed an asc1Δdom34Δ strain and analyzed its effects on NRD via a single timepoint assay. The mutant 18S rRNA levels in this strain were indistinguishable from WT 18S levels (Fig. 2.5A). These data suggest that in the absence of either Dom34 or Asc1, the other is still capable of promoting 18S NRD, and that in the absence of both, the loss of NRD is sufficiently severe that 18S NRD might be completely ablated.
Figure 2.5. Double deletion of ASC1 and DOM34 is synthetic for 18S NRD.

WT, dom34Δ, asc1Δ, and dom34Δasc1Δ strains harboring WT (Left) or mutant (Right) 18S rDNA plasmids were assayed via A) single timepoint assay, or B) pulse-chase assay. In both experiments, the 18S:25S rRNA ratio in each lane was determined via northern blot, shown below the graph in A, and to the left of the graphs in B. In the single timepoint assay, the WT 18S:25S ratio in the WT strain is set to 1. In the pulse-chase analysis, the T0 18S:25S ratio (WT or Mutant) in each timecourse is set to 1.

In the asc1Δ and dom34Δasc1Δ strains, mutant 18S rRNAs could potentially attain increased levels via an increase in production or a decrease in the rate of degradation. To distinguish between these two possibilities, we conducted a pulse-chase assay, and monitored mutant 18S rRNA stability over three hours (Figure 2.5B). In keeping with our previous study, our timecourse represents the chase portion of a pulse-chase assay ((Cole et al., 2009; LaRiviere et al., 2006). Here, strains harboring wild type or mutant 18S rDNA plasmid were grown to mid-log in drop-out medium containing raffinose. rRNA transcription was then induced for 45
minutes via galactose addition, and shut off via glucose addition. The first timepoint was collected immediately after transcriptional repression and at 30 minute intervals thereafter. As with the single timepoint assay, levels of the plasmid-derived 18S and 25S rRNAs were determined via northern blot and plotted in the form of the 18S:25S ratio for each data point. Results of the timecourse demonstrate that at T0, mutant 18S rRNA levels are no lower than WT 18S levels. Moreover, the rate of 18S NRD is decreased by roughly 2-fold in the dom34Δ and asc1Δ single deletion strains, consistent with the results of the single timepoint assay. Combining the deletions produces the previously observed synthetic effect, leading to a severe loss of mutant 18S rRNA degradation. Again, these data suggest that Dom34 and Asc1 are both capable of functioning independently to promote 18S NRD, and raise the possibility that mutant 18S rRNAs may be fully stabilized in the dom34Δasc1Δ strain.

In the absence of UBC4, 18S NRD is not detectable

Data from the initial single timepoint assay shows that, in the absence of UBC4, the mutant 18S rRNA level is equivalent that of the WT 18S (Fig. 2.4). Again, to confirm that increased mutant 18S rRNA levels are due to a loss of degradation rather than increased synthesis, we conducted a pulse-chase assay. Given the high mutant 18S levels in this strain, we also decided to increase the resolution of the timecourse assay by extending its duration to 6 hours. Results show that mutant 18S rRNA degradation was not detectable in the ubc4Δ strain over this period, suggesting that it is the loss of degradation that led to the high mutant 18S levels in the single timepoint assay (the same ubc4Δ data are depicted in both Fig. 2.6A and 2.6B). We also considered the possibility that double deletion of UBC4 and either DOM34 or
ASC1 could potentially have a compensatory effect on the rate of 18S NRD, and thereby provide mechanistic insights into the role of UBC4. To this end, we developed both ubc4Δdom34Δ and ubc4Δasc1Δ strains, and ran pulse-chase assays to determine the rate of NRD in each strain (Fig. 2.6A, 2.6B). Again, we failed to observe any loss of mutant 18S rRNAs. This suggests that UBC4 is required for one of the steps in the NRD pathway, and that it therefore operates at a level distinct from both Dom34 and Asc1.
Figure 2.6. **18S NRD is not detected in the absence of UBC4.** A) Wt, dom34Δ, ubc4Δ, and dom34Δubc4Δ strains harboring WT 18S (left graph) or Mutant 18S (right graph) rDNA plasmids were subjected to pulse-chase analysis. The T0 18S:25S ratio for each timecourse was set to 1. Northern blots of representative timecourses are shown on the far left. B) Pulse-chase analysis of Wt, asc1Δ, ubc4Δ, ubc4Δasc1Δ strains performed as in 3A.
The Ribophagy protein Doa1 is epistatic to DOM34, but not ASC1

In addition to the overlap between 18S NRD and the other translation surveillance pathways, we also suspected a functional overlap with the ribosome degradation pathway, ribophagy. In order to test the effects of ribophagy proteins in 18S NRD, we assayed strains deficient for the reported ribophagy genes, ATG7, PEP4, BRE5, UBP3, and DOA1, as well as a the temperature-sensitive strain cdc48-3. Again we used our single timepoint assay to quantify the effects of each gene deletion/mutation on NRD. In the case of the CDC48 ts mutant, we grew cdc48-3 cells harboring both the WT 18S and mutant 18S rDNA plasmids to mid-log phase in the presence of raffinose, at the permissive temperature, 30°. We then added galactose and immediately split the cultures in half, leaving one group at the permissive temperature and transferring the other group to the restrictive temperature, 37°. After a 90-minute incubation, we harvested the cells and completed the northern blot analysis as described above.
Figure 2.7. DOA1 deletion confers a partial stabilization of mutant 18S rRNAs and is synthetic with ASC1 deletion. A) Wt, dom34Δ, doa1Δ, dom34Δdoa1Δ strains harboring Wt 18S (Left) or mutant 18S (Right) rDNA plasmids were subjected to pulse-chase analysis. The T0 18S:25S ratio for each timecourse is set at 1. Northern blots of representative timecourses are shown on the far left. B) Pulse-chase analysis of Wt, asc1Δ, doa1Δ, and asc1Δdoa1Δ were performed as in 4A.

Among this group of ribophagy-deficient strains, only the doa1Δ strain showed increased levels of the mutant 18S, suggesting an incomplete overlap between 18S NRD and ribophagy. The 2-fold effect of DOA1 deletion indicates that Doa1 is not absolutely required for 18S NRD,
similar to the effects observed in the asc1Δ and dom34Δ strains. This parallel suggested that Doa1 could be functioning on one side of the apparently bifurcated NRD pathway, either with Dom34 or with Asc1. To test this possibility, we developed doa1Δdom34Δ and doa1Δasc1Δ strains, and subjected them to pulse-chase analysis. Northern blot analysis showed that the additional loss of DOM34 in the doa1Δ background did not slow 18S NRD any further, suggesting that Doa1 and Dom34 affect a related step (Fig. 2.7A). However, in keeping with this hypothesis, the doa1Δasc1Δ demonstrated a synthetic decrease in the rate of 18S NRD, similar to the effect observed in dom34Δasc1Δ (Fig. 2.7B). Together, these data support the dual branch model for NRD and suggest that Doa1 functions with Dom34.

In addition to DOA1, the ribophagy gene PEP4 also appears to promote 18S NRD. This increases the degree of overlap between ribophagy and 18S NRD established by the involvement of DOA1. However, the loss of ATG7 did not lead to an increase in mutant 18S rRNA levels, implying that auto/ribophagy can be functionally decoupled from 18S NRD (Fig. 2.4).
Table 2.1: Reported Pathway Involvement of Genes Selected for Our Analysis. Columns from left to right: 1. Gene name, 2. Known function annotated on the yeast genome database website 3. Pathways in which a given gene has been shown to be involved.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Known function</th>
<th>POTA</th>
<th>NSD</th>
<th>Ribophagy</th>
<th>RPL18</th>
<th>RPL6A</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>UBC4</td>
<td>E2 Ubiquitin Conjugating Enzyme</td>
<td>x</td>
<td></td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASC1</td>
<td>Euk. ribosomal small subunit protein, WD40 repeat family</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEX2</td>
<td>Interacts with Ubc4, E3 ligase</td>
<td>x</td>
<td></td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UBC5</td>
<td>E2 ubiquitin conjugating enzyme, functionally redundant with Ubc4</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOT4</td>
<td>Non-Stop Decay E3 Ubiquitin ligase</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DOA1</td>
<td>Binds ubiquitin on modified proteins, functions as an adaptor protein for Cdc48</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEP4</td>
<td>Involved in vacuole formation</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UBP3</td>
<td>Ubiquitin protease</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATG7</td>
<td>Promotes autophagy</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRES</td>
<td>Interacts with Not4</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VPS27</td>
<td>Indirect Doa1 interactor, promotes the sorting of proteins to Multivesicular bodies.</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPL1</td>
<td>Cdc48 Adaptor on the ER</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LTN1</td>
<td>E3 Ubiquitin ligase</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPN10</td>
<td>Component of the 26S Proteasome, binds ubiquitin</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UBP6</td>
<td>Ubiquitin specific protease in the 26S Proteasome</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LSN1</td>
<td>Part of the Lsm protein complex, involved in cytoplasmic mRNA degradation</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YCL001W-A</td>
<td>Dom34 parologue (dubious orf), alone or in combination with DOM34 deletion</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YCL001W-B</td>
<td>Dom34 parologue (dubious orf), alone or in combination with DOM34 deletion</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YLR456W</td>
<td>Hbs1 interacting protein</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRE1</td>
<td>Endoribonuclease, RNAse L homologue, (RNAse L Inhibitor, Rli1/ABCE1, binds Dom34:Hbs1.)</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCR1</td>
<td>Binds Rli1</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RN1</td>
<td>tRNA Cleavage, endogenous rRNA degradation in A.thaliana</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Effects of increased Ubc4, Asc1, Dom34, and Hbs1 expression on NRD

As part of our effort to understand the mechanistic function for the 18S NRD pathway, we hypothesized that that some NRD proteins might function in a regulatory manner. In other
pathways, the loss of regulatory proteins can be compensated for by increasing the abundance of downstream factors. Generally, the ability of downstream factors to overcome the lack of an upstream regulator can be attributed to the inherent activity of the downstream proteins. Such activities are stochastically activated, albeit at a low frequency, in the absence of upstream factors. Therefore, by artificially overexpressing the downstream proteins, researchers increase the number of activation events, sometimes overcoming the lack of upstream regulatory proteins. Consistent with this previously used technique, we suspected that overexpression certain NRD proteins might overcome the loss of others, thereby providing insight into their potential regulatory activities.

For these experiments, we developed epitope-tagged clones of the 18S NRD genes DOM34, HBS1, and UBC4, under the control of either the respective endogenous promoters or the strong ADH1 promoter. We also developed an epitope-tagged clone of the ASC1 gene under its own promoter. As a ribosomal protein, ASC1 is already highly expressed, precluding the possibility of finding a stronger promoter. Therefore we used the tagged ASC1 gene under its endogenous promoter for our overexpression studies. We expressed the FLAG-ASC1 in strains already possessing an ASC1 gene, leading to a 2-fold increase in Asc1 abundance. Additionally, we were unable to clone DOA1, and were therefore unable to study the effects of DOA1 overexpression.

We transformed each of the high or low expression plasmids into WT cells, then tested the levels of the tagged proteins via western blot (Fig. 2.8). We detected each protein using primary antibodies that target the epitope tags. As anticipated, our western blot data demonstrated that Dom34, Hbs1, and Ubc4 abundance increases when expressed under the ADH1 promoter. The increase in Dom34 and Hbs1 abundance was roughly 10-fold, whereas
Ubc4 abundance increased by approximately 4-fold. An additional consideration for the validation process is whether ASC1 levels are truly doubled when a second copy of ASC1 is present in the cell. Feedback regulation is a common mechanism for the control of gene expression, raising the possibility that Asc1 levels could remain constant despite the presence of a second ASC1 gene. Western blot data shows that FLAG-Asc1 levels are equivalent when expressed in either WT or asc1Δ strains. Because the WT strain harbors a chromosomal copy of the ASC1 gene, whereas the asc1Δ does not, this experiment serves to indirectly test the feedback regulation of Asc1 and infers that the overall abundance of the Asc1 protein is double when two copies of the ASC1 gene are present. As such, the data suggest that our FLAG-ASC1 construct is a valid high-expression construct. Likewise, due to the ability of our DOM34, HBS1, and UBC4 construct to partially, or fully, complement their respective deletions, we believe that they represent viable tools for our study.

We then used our single-timepoint assay and northern blot analysis to assess the ability of each construct to produce functional protein by testing their abilities to complement the respective deletion strains (Fig. 2.9). For this experiment, we transformed WT, doa1Δ, asc1Δ, dom34Δhbs1Δ, and ubc4Δ strains with an empty vector, or the respective low or high expression construct. We then conducted a second round of transformations in which we delivered to each strain, already expressing the expression plasmids, our WT 18S rDNA plasmid or our 18S:A1492C rDNA plasmid. The resulting strains possessed two plasmids: one NRD protein expression plasmid and one rDNA expression plasmid. This led to six possible combinations per strain: 1) WT rDNA/empty expression vector, 2) WT rDNA/endogenous expression construct, 3) Wt rDNA/high expression construct, 4) Mutant 18S rDNA/empty expression vector, 5) Mutant 18S rDNA/endogenous expression construct, and 6) Mutant 18S rDNA/high expression
construct. Three replicates for each strain/plasmid combination were then subjected to steady-state NRD analysis (Fig. 2.9). As before, each culture was grown to mid-log in synthetic drop-out media in the presence of raffinose. Galactose was then added for 90 minutes and cells were subsequently harvested and flash frozen. Northern blot analysis was used to quantify the ratio of plasmid-derived 18S:25S rRNAs. These ratios were then graphed relative to the wild type 18S levels observed in the WT strain harboring the empty vector control. The data demonstrated that co-expression of FLAG-DOM34, and HBS1-FLAG restored the ability of the dom34Δhbs1Δ strain to conduct 18S NRD. Likewise, our FLAG-ASC1 strain fully complemented asc1Δ. The UBC4-HA construct, however, was only partially capable of complementation in the ubc4Δ strain. This suggests that the c-terminal HA tag may have impaired the function of UBC4, preventing its full activity in NRD.
Figure 2.8. Validation of Overexpression Constructs. Western blot data demonstrate increased expression of Dom34, Hbs1 and Ubc4 under the ADH1 promoter. Plasmid-derived FLAG-Asc1 is present at equivalent levels with and without a second copy of the ASC1 gene.

In parallel with the complementation experiments, we probed the genetic relationships between NRD protein genes by expressing the other, non-respective expression constructs in
each of the deletion strains. In other words, this experiment tested the possibility that overexpression of a given NRD protein might compensate for the loss of another.

To assay each strain for changes in its ability to conduct NRD when another protein was present in high concentration, we also transformed each strain with our WT or mutant 18S rDNA plasmids. This led to the production of four variations for each strain: 1) WT rDNA/empty vector, 2) WT rDNA/high expression construct, 3) Mutant 18S rDNA/empty vector, and 4) Mutant 18S rDNA/high expression construct. Each strain was subjected to the single timepoint assay as before, and analyzed by both western and northern blot. Western blot data confirm that each of the expression constructs successfully produced increased levels of each of the NRD proteins, irrespective of the strain harboring the construct. However, northern blot data suggest that mutant 18S rRNA levels do not change in any of the NRD protein deletion strain, regardless of which overexpression construct is present. This suggests that the NRD proteins cannot be overexpressed as a means of compensating for the loss of the others (Fig. 2.9). However, an alternative possibility is that the degree of difference in expression levels between the endogenous and overexpression promoter constructs, is insufficient to allow NRD proteins to compensate for the loss of others.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Empty Vector</th>
<th>Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>dom34Δ</td>
<td>Wt</td>
<td>Mut</td>
</tr>
<tr>
<td>hbs1Δ</td>
<td>Wt</td>
<td>Mut</td>
</tr>
<tr>
<td>Strain</td>
<td>Wt</td>
<td>Mut</td>
</tr>
<tr>
<td>doa1Δ</td>
<td>Wt</td>
<td>Mut</td>
</tr>
</tbody>
</table>

**graph:**

- **dom34Δ hbs1Δ Strain + ASC1**
  - 18S/Wt: Wt, Mut
  - 18S/Mut: Wt, Mut

- **doa1Δ Strain + ASC1**
  - 18S/Wt: Wt, Mut
  - 18S/Mut: Wt, Mut

**dom34Δ hbs1Δ + UBC4-HA**

- Empty Vector
- UBC4-HA (Low)
- UBC4-HA (High)

**graph:**

- 18S/Wt: Wt, 18S: A1492C
- 18S/Mut: Wt, 18S: A1492C
**Figure 2.9.** Effect of NRD protein overexpression on the levels of mutant 18S rRNAs. Steady state analysis of strains harboring either the empty vector, low-expression plasmid, or the high-expression plasmid and the mutant 18S rDNA plasmid. Levels of mutant 18S and WT 25S rRNAs are analyzed by northern blot for each deletion strain (Left) and quantified in terms of the 18S/25S ratio under each condition (Right).

*HBS1 and DOM34 have distinct genetic relationships with ASC1*

The genetic data suggest that Dom34 and Asc1 function independently in NRD. If our current model is correct, Hbs1 and Asc1 should also function independently. As such, we predicted that 18S NRD would not be detected in an hbs1Δasc1Δ strain. To test this hypothesis, we developed an hbs1Δasc1Δ strain and assayed its ability to conduct 18S NRD by single timepoint 18S NRD assay. We determined the level of mutant 18S rRNAs in each strain as before, and found that mutant 18S rRNAs are still destabilized even in the absence of both HBS1 and ASC1, though only slightly so (Fig. 2.10).
Figure 2.10. Mutant 18S rRNA levels in hbs1Δasc1Δ strains. Steady state analysis of mutant and WT 18S rRNA levels in dom34Δasc1Δ and hbs1Δasc1Δ strains. 18S levels were normalized to the WT 25S levels in each strain and graphed relative to the levels of WT 18S rRNAs observed in the WT strain.
The remaining activity of 18S NRD in the hbs1Δasc1Δ strain, if any, is very low, suggesting that the results of our single timepoint could be due to technical error. Therefore, we increased the resolution of our data by running a timecourse assay in the hbs1Δasc1Δ strain. Unlike previous timecourses, we did not compare the rate of NRD in asc1Δ, hbs1Δ, and hbs1Δasc1Δ strains. Instead, we used a binary assay in which we simply asked whether 18S rRNAs are completely or partially stabilized in this strain. Therefore, we transformed WT and hbs1Δasc1Δ cells with the mutant 18S rDNA plasmid only. We then ran, in triplicate, a 6-hour timecourse as previously described, taking timepoints every hour. Northern blot analysis of the mutant 18S rRNA levels, normalized to WT 25S rRNA levels, indicated that mutant 18S rRNAs are still destabilized in the hbs1Δasc1Δ strain, suggesting that 18S NRD is still active (Fig. 2.11).
Previously reported NRD Factor Ski7 Probably Does Not Promote 18S NRD

In keeping with the data suggesting that HBS1 and DOM34 have distinct genetic relationships with ASC1, we also investigated the possibility that the Non-Stop Decay protein and HBS1 homologue, SKI7, could promote 18S NRD. Previously, Ski7 was implicated in NRD, due to the observation that mutant 18S rRNAs were synthetically stabilized in a ski7Δhbs1Δ
strain (Cole et al., 2009). If this hypothesis is correct, then a double ski7Δdom34Δ strain should display the same phenotype. However, when we examined the rate of NRD via timecourse assay in a dom34Δski7Δ background, we did not observe synthetic stability (Fig. 2.12). Instead, the dom34Δski7Δ strain showed the same mutant 18S stability as the dom34Δ strain alone. Together, these data would initially suggest that SKI7 is epistatic to DOM34, but functions in parallel with HBS1. However, the synthetic sickness observed in hbs1Δski7Δ, together with the lack of any synthetic effect in the dom34Δski7Δ strain, suggests that the loss of 18S NRD in the hbs1Δski7Δ strain is likely due to slow growth, rather than a loss of NRD.

**Figure 2.12.** Mutant 18S rRNA timecourse analysis in SKI7 deficient strains. Timecourse analysis of mutant 18S rRNAs was conducted in dom34Δski7Δ, hbs1Δski7Δ, and the corresponding single deletion strains. Mutant 18S rRNA levels were detected by northern blot. Mutant 18S levels in each strain were normalized to WT 25s levels and the T0 timepoint for each timecourse was set to 1.
Previously Reported NRD Factor Xrn1 Is Not Required for 18S NRD

In a previous report, we presented genetic evidence that Xrn1 is involved in 18S NRD. Though our initial timecourse data suggested that the rate of 18S NRD is not diminished in an xrn1Δ strain, the additional loss of DOM34 led to a dramatic stabilization of mutant 18S rRNAs, potentially indicating that 18S NRD does not occur in this strain (Cole et al., 2009). Despite our consistent observation of this phenotype in multiple repeats of this experiment, the dom34Δxrn1Δ strain yields opposing results when the protocol is altered: under pulse-chase conditions, dom34Δxrn1Δ is synthetically sick, and mutant 18S rRNAs are highly stabilized. Conversely, when raffinose is excluded from the assay, the degree of difference in growth rate between dom34Δxrn1Δ and WT is far less, and mutant 18S rRNAs are degraded at a rate equivalent to the dom34Δ single deletion strain (Fig. 2.13).
Figure 2.13. Mutant 18S rRNA timecourse analysis in xrn1Δ cells in the presence or absence of raffinose. Timecourse analysis of mutant 18S rRNAs was conducted in xrn1Δ cells grown to mid-log in galactose-containing medium (diamonds) or raffinose-containing medium (squares). Transcription was then induced by the addition of galactose in the culture containing raffinose. Glucose was then added to both sets of cultures to shut off transcription. T0 was collected immediately following glucose addition and at the indicated time intervals thereafter.

These data suggest that the loss of XRN1 in a dom34Δ background is not necessarily synthetic with respect to NRD, and may instead lead to mutant 18S rRNA stabilization as an indirect result of strain sickness during growth in raffinose. Moreover, the ability of a
dom34∆xrn1∆ strain to conduct 18S NRD at a fast pace indicates strongly that Xrn1 and Dom34 do not represent all of the major factors in 18S NRD as initially suspected.

Nevertheless, evidence does suggest the downstream involvement of Xrn1: in strain xrn1Δ, a low molecular mass band is bound by the 18S probe, suggesting that at least one mutant 18S degradation intermediate is degraded by Xrn1. This implies that Xrn1 may be involved in the cleanup of mutant 18S rRNAs that were already subjected to 18S NRD.

**Mutagenic analysis of ASC1**

In order to better understand the mechanism by which Asc1 functions in NRD, we developed a number of ASC1 regulatory domain mutants expected to display decreased ability to conduct NRD. We first transformed either the empty vector, WT ASC1 clone, or the mutant ASC1 clones into an asc1Δ strain. The resulting strains were then transformed again with either the WT rDNA or Mutant 18S:A1482C rDNA plasmid. These strains were then subjected to a single timepoint 18S NRD assay as previously described. Abundance of the WT or mutant 18S was determined via northern blot and plotted in terms of the 18S:25S ratio in each strain. This method allowed us to quantify the ability for each mutant ASC1 construct to complement the loss of the chromosomal ASC1 gene. The mutants we tested include mutations of the ribosome-facing “knob”, the cytosolic “loop”, the knob’s central lysine residue, K282A, either one or both of the conserved tyrosine residues Y232, Y250, or Y232 Y250 (Fig. 2.2B). We also tested the ribosome binding mutants R38D/K40E, and D109Y (Fig. 2.16). For each of the mutations we tested, we failed to observe any loss of complementation ability. However, loss of the ribosome-facing Knob structure appears to have actually improved the ability of Asc1 to promote NRD.
Moreover, the Y232F mutation also appears to promote the ability of Asc1 to conduct 18S NRD, regardless of the presence or absence of the Y250F mutation (Fig. 2.15). This suggests that phosphorylation of the two conserved tyrosine residues is not required, however phosphorylation of Y232 may play an inhibitory role. Likewise, though neither the loop or knob structures appear to be required for NRD, the knob may have an inhibitory function.

Despite the observation that the ribosome binding mutations affect the ability of Asc1 to promote PDTA, the same mutations do not appear to negatively impact the ability of Asc1 to conduct NRD (Kuroha et al., 2010). This could indicate that PDTA is functionally distinct from 18S NRD.
Figure 2.14. Complementation of asc1Δ. Strain asc1Δ harboring either an empty vector, ASC1, ASC1 with the intron and snoRNA124 removed, or FLAG-ASC1 without the intron, was also transformed with the mutant 18S rRNA plasmid and subjected to single timepoint analysis. WT cells harboring the same empty vector or ASC1 plasmid was transformed with the mutant 18S rDNA plasmid and subjected to the same single timepoint analysis. Mutant 18S rRNA levels were normalized to WT 25S levels.
Figure 2.15. Structure based functional analysis of ASC1. asc1Δ cells transformed with either the empty vector, wild type ASC1 gene, or mutant forms of ASC1 suspected to promote protein:protein interactions, or to function as phosphorylation sites, were also transformed with the mutant 18S rDNA plasmid. Cells harboring both plasmids were subjected to single timepoint analysis.
Figure 2.16. Effect of ASC1 ribosome binding mutations on mutant 18S abundance. asc1Δ cells were transformed with either the empty vector, wild type ASC1 gene, or mutant forms of ASC1 that have been shown to negatively affect the association of Asc1 with polysomes, as well as the mutant 18S rDNA plasmid. Doubly transformed cells were subjected to single timepoint analysis.
Our model suggests that we could gain additional information about the behavior of mutant 18S:A1492C rRNAs, and by extension, 18S NRD, by looking at mutant 18S rRNA abundance in the polysome profiles of NRD deficient strains. We know that the loss of NRD proteins leads to a higher mutant 18S rRNA abundance, however whether this effect would be seen in monosomes or polysomes was unknown. Our model suggests that signal in the polysome fractions should be determined by the rate of ribosome run-off rather than the efficiency of the NRD pathway. This expectation is due to the fact that our model for NRD does not predict recycling of mutant 18S-containing small subunits following a stall. Otherwise, at higher concentrations, recycled mutant 18S-containing subunits could re-initiate on polysomes and raise levels within these fractions. Hence, we expected to see that in NRD deficient strains, the mutant 18S signal would increase in monosome fractions, but not the polysome fractions.

To test our model, we repeated the original polysome experiment, using dom34Δ, asc1Δ, and dom34Δasc1Δ strains. Briefly, we transformed WT, dom34Δ, asc1Δ, and dom34Δasc1Δ strains with the mutant 18S:A1492C rDNA plasmid, and grew these cells to mid-log in the presence of raffinose. We then induced mutant 18S transcription via the addition of galactose for 90 minutes, in keeping with our steady state assay. We then added cycloheximide to our cultures to a final concentration of 100 mg/ml, harvested our cells on ice, and prepared cell lysates. Individual lysates were layered onto 5-50% sucrose gradients and spun in an ultracentrifuge. Individual fractions were then collected and subjected to northern blot analysis. After quantifying the total mutant 18S abundance in all fractions of the polysome profile, we observed an overall increase in mutant 18S signal in the three mutant strains. Hence, our experiments are ostensibly compatible with the previous single timepoint and timecourse assays. Moreover, the
major increase in signal seen in the mutant strains occurs exclusively with the monosome and monosome plus half-mer fractions, thereby supporting our model (Fig. 2.17B). This increase appears to be specific for the mutant 18S rRNAs as the methylene blue stain does not show a detectible increase in wild type 18S rRNAs in the corresponding fractions.

One portion of our data set does not fit with our model: in the asc1∆ data set, there is a slight increase in mutant 18S signal within the polysome fractions. This may be explained by the general large subunit recruitment defect previously described in ac1∆ strains. This defect is apparently manifested in the polysome profile in the form of an increase in half-mer peaks behind each discrete polysome peak. Further experiments are needed to determine whether the increase in 18S abundance in the polysome pool is a general or specific effect. Likely, the proper experiment is to simply look at WT 18S rRNAs for changes in abundance within the same group of strains.

Mutant 18S rRNA enrichment in monosomes is not retained under ribosome run-off conditions (Preliminary Data).

Our model for 18S NRD suggests that mutant 18S-containing 80S ribosomes become stuck at AUG start codons. If this is correct, then the mutant 18S signal should be selectively retained under ribosome run-off conditions. To test whether this is correct, we set up an experiment that compared the monosome 18S rRNA signal in WT and dom34∆asc1∆ strains under ribosome run-off or standard conditions. We expected to observe that in both cases the mutant 18S rRNA abundance would not diminish, though the overall monosome and polysome fractions would be greatly reduced, signifying run-off has occurred.
To accomplish this, we split mid-log phase WT or dom34Δasc1Δ cultures in half. For each pair of cultures, we treated one with cycloheximide (CHX), according to the standard polysome protocol, and the other with DMSO. The lysate from CHX treated cells was maintained on ice for 20 minutes, while the run-off lysate was incubated at 30°C for 20 minutes. Each extract was layered on top of a separate 5-50% sucrose gradient, subjected to ultracentrifugation for 3 hours, and separated into fractions.

Preliminary data (N=1 for each of the 4 cultures) demonstrated that the original accumulation of the mutant 18S in the monosome fraction was lost during run off (Fig. 2.17C, middle). Interestingly, a loss of approximately 80% was consistent between both the wild type and the dom34Δasc1Δ strains. Also, this loss appears to have been selective for the mutant 18S, as the methylene blue staining did not show any gross perturbation in 18S:25S ratio in any of the strains, either with, or without CHX treatment. Still more, the run-off dependent loss of mutant 18S signal was accompanied by a dramatic increase in the abundance of a sub-18S molecular weight band within the 40S pool in both strains. In the dom34Δasc1Δ strain, this low-mass band appeared at higher abundance than in the wild type strain. This increase correlates with the greater abundance of mutant 18S rRNA, originally in the monosome fraction. This suggests that the mutant 18S rRNAs may have been selectively degraded in lysates, even in the absence of both Dom34 and Asc1. Yet, to be certain that these effects are specific, WT 18S rRNA abundance should be tested under run-off conditions.
C.
Figure 2.17. Polysome profile analysis in NRD deficient strains. A. WT cells expressing mutant 18S rRNAs was grown to mid-log in galactose, then harvested and fractionated on
5-50% sucrose gradients as in a typical polysome analysis. Total RNA from individual fractions was purified and subjected to northern blot analysis. The A260 trace was recorded and plotted above the corresponding northern blot lanes. B. WT, dom34Δ, asc1Δ, and dom34Δasc1Δ strains harboring mutant 18S rDNA plasmids were grown to mid-log in galactose, then harvested and subjected to polysome analysis as above. Fractions were taken at shorter intervals than in 1A to increase the resolution. C. Replicates of the analysis in 1B are shown. Additional polysome analysis of WT and dom34Δasc1Δ cells not treated with CHX, and incubated at 30 degrees following lysis is shown in the WT run off and dom34Δasc1Δ run off graphs/northern blots. D. Mutant 18S rRNA signal normalized to the 25S rRNA signal in each lane was quantified and graphed. E. The average, normalized, 18S signal in monosomes or polysomes was determined for each strain and plotted on the chart.

Implications of Rps3 involvement in NRD

The genetic data establishing a role for Asc1, and Dom34 on opposing halves of the NRD pathway raised the question of how, or if, both branches could be coordinately regulated. The possibility that both branches of NRD are regulated by an upstream factor is supported by the polysome profile run-off data (Fig. 2.17C). At the time this research was being conducted, the crystal structures of both the eukaryotic ribosome and Dom34:Hbs1 bound to the eukaryotic ribosome, had not been reported. Yet, as of the writing of this thesis, both structures have been solved, leading to the discovery that a single ribosomal protein, Rps3, binds both Hbs1, and Asc1 (Becker et al., 2011; Ben-Shem et al., 2010; Rabl et al., 2011; Yatime, Hein, Nilsson, & Nissen, 2011). The long, unstructured, c-terminal tail of Rps3 extends across the ribosome, where, on the
tetrahymena ribosome, it makes extensive contact with WD4 repeat number 4 of Rack1. The previously described function of WD4 in mediating the dimerization of Rack1 suggests that the Rps3 c-terminal tail may be regulating Rack1 by preventing its dimerization. Rps3 and Hbs1 make contact via the long “ball and chain” domain of Hbs1. This n-terminal region extends across the ribosome to make contact with Rps3 (Becker et al., 2011).

The Ben-Shem structure of the eukaryotic ribosome led to another important discovery about Rps3. In addition to contacting Hbs1 and Asc1, Rps3 is positioned at the entrance to the mRNA channel of the small subunit, suggesting that is ideally positioned to sense a decrease in ribosome translocation rate along the mRNA. As such, Rps3 is an excellent candidate for sensing ribosome stalling, and responding by regulating the activities of both Dom34:Hbs1, and Asc1.

To probe the potential regulatory activity of the Rps3 c-terminal tail on Asc1, we attempted to remove the final 46 codons of RPS3 which encode the unstructured portion of its c-terminal tail. To accomplish this, we first developed a plasmid-based truncated RPS3 construct which includes both the upstream and downstream regions of RPS3. We then amplified, as a single 4kb product, the entire truncated RPS3 gene, plus a downstream LEU2 promoter/LEU2 ORF/LEU2 3’ UTR which is encoded on the plasmid. To provide a source of homology on the 3’ end of the PCR product, we added 75 bp of homology to the 3’UTR of RPS3. This PCR product should be capable of integrating at the RPS3 locus, leading to the loss of the wild type RPS3 gene, and subsequently incorporating both the truncated RPS3 gene and LEU2 gene. Following transformation of 5-20 µg of this construct, approximately 25 colonies appeared on leucine drop out media. The RPS3 locus from 10 colonies were sequenced and shown to encode the Leu2 gene in the correct orientation, and a non-truncated RPS3 gene. To ensure that the appearance of the wild type RPS3 gene was not the result of a technical error, we verified that RPS3 is
truncated in the actual PCR product used for transformation. The successful integration of the PCR product, and confirmation that RPS3 is truly truncated on it, suggests that technical errors are not preventing truncation of RPS3 on the chromosome. Instead, we propose that our inability to truncate the RPS3 indicates that it is lethal for the cell.

To test whether the apparent lethality of a truncated RPS3 gene could be due to the loss of Asc1 regulation, we transformed an asc1Δ cell with the truncated RPS3 construct PCR. Again, among ten colonies capable of growing on SC-Leu media, we did not detect any cells with the truncated RPS3 gene. Another possibility not yet tested, is whether the apparently lethal effects caused by Rps3 tail truncation are brought about by removal of regulation of both Asc1 and of Hbs1. This could be easily tested by attempting the truncation of RPS3 in an hbs1Δasc1Δ strain.
As part of our candidate survey for factors affecting 18S NRD, we examined deletion strains deficient for a variety of factors we suspected to be involved in NRD. Among these genes were the putative DOM34 paralogues, YCL001W-A and YCL001W-B. This pair of dubious ORFs are homologous to the N and C-terminal portions of DOM34, respectively, and could theoretically produce truncated forms of the Dom34 protein. However, we did not observe any significant increase in mutant 18S rRNA levels in the absence of either YCL001W-A or YCL001W-B alone, or in strains doubly deficient for either YCL001W gene and DOM34 (Fig 2.18A). These data argue against a role for the YCL001W genes in NRD.

We also suspected that the RNA endonuclease Rny1 might represent the nuclease in NRD, based upon the reported activity of its homologue Rnase T2 in endogenous rRNA turnover in Arabidopsis, and the observed specificity of Rny1 for rRNA in yeast (Thompson et al., 2009). Yet, again, we failed to observe any increase in mutant 18S rRNA levels in an rny1Δ strain (Fig. 2.18B).

As a third possibility, we noted the recent discovery that mammalian ABCE1, the homologue of yeast Rnase L Inhibitor, Rli1, binds mammalian Dom34:Hbs1 and promotes ribosomal subunit dissociation (Pisareva et al., 2011). This suggested that Rli1 might promote NRD. By extension, we also suspected that the yeast homologue of the promiscuous RNA endo/exonuclease RnaseL, which is repressed by Rli1, might represent the nuclease in 18S NRD. Blast analysis of the mammalian RnaseL amino acid sequence against the *S. cerevisiae* genome suggests that the closest homologue of RnaseL is Ire1. Though the essential nature of Rli1 precluded the possibility of including it in our survey, we were able to test an ire1Δ strain and a
strain deficient for the Rli1 binding partner, Hcr1, for effects on NRD. In both cases, we did not observe any negative effect on mutant 18S rRNA levels, suggesting that Ire1 and Hcr1 are not involved in NRD (Fig. 2.18B).

The involvement of the ubiquitin binding protein Doa1 in 18S NRD suggested that ubiquitin mediated proteolysis could be important for the pathway. To test this possibility, strains deficient for proteasome components, including Rpn10, Ubp3, and Ubp6, were tested for effects on NRD. None of these strains displayed increased levels of mutant 18S rRNAs, suggesting partial impairment of the proteasome does not negatively affect NRD, and arguing against its involvement (Fig. 2.18B).
A.

B.
C.

**Figure 2.18.** Several genes suspected to promote 18S NRD do not affect mutant 18S rRNA levels. A. Deletion of the putative DOM34 parologue genes YCL001W-A and YCL001W-B do not lead to an increase in the level of the mutant 18S, either alone or in combination with dom34Δ. B. Deletion of a variety of candidate genes, including the genes for the endoribonucleases Rny1 and Ire1, or the proteasome proteins Ubp3 and Rpn10, do not lead to a significant increase in mutant 18S levels. C. deletion of VPS27 does not affect levels of mutant 18S rRNAs.

*Slow Growth Correlates with a Loss of 18S NRD*

Slow growth is known to correlate with decreased translation (Petroulakis et al., 2004). It is therefore possible, that slow growing strains may have fewer translating ribosomes, and therefore, a compromised mechanism for the detection of mutant 18S rRNAs. To test the possibility that the loss of 18S NRD in different deletions strains is an indirect effect of slow growth, we calculated the growth rates of each strain in synthetic complete media containing
either raffinose or galactose. Though certain strains have longer doubling times relative to the wild type strain, we suspect that these differences are sufficiently minimal that they are not the cause of the loss of NRD. In the case of dom34Δasc1Δ, we do not observe synthetic slow growth relative to asc1Δ. Also, in the case of doa1Δasc1Δ, after an initial adjustment period, we observe only a mild synthetic slow growth phenotype (Fig. 2.19). In the ubc4Δ strain, which is completely deficient for 18S NRD, no slow growth is observed at all.

Conversely, three strains show severe slow growth which correlates with a loss of NRD: ctk1Δ, hbs1Δski7Δ, and, when grown in raffinose, dom34Δxrn1Δ. In the case of dom34Δxrn1Δ, altering the carbon source triggered an increase in growth rate that subsequently reduced the degree of difference in growth rate relative to WT. Under these conditions, the 18S NRD defect disappeared, suggesting that growth rate rather than loss of NRD contributed to mutant 18S stabilization.

The strains ctk1Δ and hbs1Δski7Δ also exhibit strong growth defects and apparent loss of 18S NRD. However, this slow growth (>240 min) and loss of NRD is still apparent in hbs1Δasc1Δ, even during growth in galactose. This precludes the possibility of decoupling slow growth from the rate of 18S NRD in this strain. Likewise, the high mutant 18S levels in ctk1Δ correlate with extremely slow growth in raffinose (>8 hours), suggesting that the loss of NRD in this strain may be an indirect consequence of slow growth.
Figure 2.19: Growth rate analysis of strains in galactose and raffinose.

Doubling times of various strains growing in SC medium containing 2% raffinose or 2% galactose. Cells growing in SC media for 24 hours were diluted to low density, then monitored via OD600 reading during their progression to high density. Three cultures/strains were used for growth rate calculations. Note: the initial doubling time of strain doa1Δasc1Δ is shown in black; however, this time decreases after 36 hours, leading to a 240 ± 17 min. doubling time shown in gray. Cells growing at the faster rate were used in timecourse and single timepoint analyses.
Materials and Methods

Single Timepoint Assay:

Strains harboring pSC40-Wt or pSC40-18S:A1492C plasmids were grown at 30° in SD-uracil, in the presence of 2% raffinose, to mid-log phase (OD$_{600}$ = 0.5). Galactose was added to a final concentration of 2% to induce rRNA transcription, and cells were incubated for an additional 90 minutes. OD$_{600}$ was monitored during this time and pre-warmed medium was added as necessary to maintain OD$_{600}$ = 0.5. Cells were then harvested by centrifugation and flash frozen on dry ice/EtOH.

Pulse-chase Analysis:

Pulse-chase analysis was performed as previously described (Lariviere et al. 2006). Strains harboring pSC40-Wt or pSC40-18S:A1492C plasmids were grown at 30° in SD-uracil with 2% raffinose to mid-log phase (OD$_{600}$ = ~0.5). Galactose was added to a final concentration of 2% in order to induce rRNA transcription, and cells were incubated 90 minutes. Pre-warmed glucose was then added to a final concentration of 2% and timepoint T0 was immediately harvested. Samples were then flash frozen on dry ice/EtOH. Pre-warmed SD-ura with 2% glucose was added as necessary to maintain an OD$_{600}$ of 0.5 for the duration of the timecourse. Timepoints were taken at specified intervals.

Northern Blot Analysis:

For each sample, 2 µg total RNA was separated on a 1% agarose-formaldehyde gel and transferred to a nitrocellulose membrane as previously described (Brown et al., 2004). Total
RNA was detected via staining with Methylene blue stain (Molecular Research). Membranes were hybridized with 32P-end-labeled probes FL125 (anneals to plasmid derived 18S rRNA) and FL126 (anneals to plasmid derived 25S rRNA) for 12 hr in ExpressHyb (BD Biosciences). Signals were visualized using a Typhoon Phosphorimager (Molecular Dynamics) and quantified via ImageQuant software (GE Lifesciences).

**Discussion**

*Crosstalk between 18S NRD and other Pathways*

Though the functional overlap between translation surveillance pathways has been recognized for some time, the extent of these similarities is unclear. Based upon the similar function of the PDTA and NRD pathways, as well as the common involvement of Dom34, we suspected that the PDTA factors Asc1, Ubc4, and Not4 might also function in NRD. To test this possibility we assayed the levels of mutant 18S:A1492C rRNAs in asc1Δ, ubc4Δ, and not4Δ deletion backgrounds and observed a selective increase in mutant 18S rRNA levels in the asc1Δ and ubc4Δ strains. We interpret this increase as an indication that Asc1 and Ubc4 are involved in NRD. These findings suggest a greater overlap between PDTA and NRD than previously observed. The lack of effect observed when the E3 ubiquitin ligase, Not4, is absent, suggests either that the mechanisms by which NRD and PDTA function may not overlap entirely, or that the potential degradation of the nascent peptide associated with an 18S:A1492C-containing ribosome does not influence 18S rRNA degradation. This latter possibility is not entirely unexpected given that the nascent peptide degradation and mRNA cleavage phenotypes in PDTA have not yet been shown to influence each other.
Though the data on Not4 are negative, the partner of Not4, the E2 ubiquitin conjugase Ubc4, is likely to be part of the 18S NRD pathway. Loss of UBC4 conferred a defect in NRD so severe that no measurable mutant 18S rRNA half life was detected over 6 hours, suggesting that Ubc4 may be essential for NRD. The activity of Ubc4 in the cellular ubiquitin system also sets up the expectation that ubiquitin modification is likely to play an important part in 18S NRD. This suggestion is consistent with several reports describing a role for ubiquitin in NSD, PDTA, and ribophagy.

To further investigate the effects of the PDTA genes on NRD, we combined the ASC1 and DOM34 deletions. This led to a strong synthetic stabilization of mutant 18S rRNAs, suggesting that Asc1 and Dom34 function independently in 18S NRD. This finding suggests a simple model for 18S NRD in which Asc1 and Dom34 promote parallel branches of the pathway.

The reported activities of the Rack1 family, to which Asc1 belongs, suggest that *S. cerevisiae* Asc1 may function as part of the cell’s ubiquitin modification systems. When mammalian Rack1 takes on its homodimeric conformation, it becomes competent for its interaction with the Cullin complex. Together Rack1:Rack1:Cullin ubiquitinates the protein Hif1, leading to its proteolysis by the 26S proteasome (Lu et al.). Recently, the dimerization activity of Asc1 was also described, suggesting that its activity in ubiquitin modification pathways could also be conserved. If ubiquitination of a target protein occurs during NRD, the target of that modification is, as yet, unknown. Presumably, ribosomal protein on an 18S:A1492C-containing ribosome would be a logical candidate.
Given the similarity inherent in the ribosome turnover phenotypes in NRD and ribophagy, we also suspected that ribophagy proteins might function in the 18S NRD pathway. Interestingly, only factors known to affect large subunit ribophagy have been discovered. Nevertheless, we tested the possible overlap with ribophagy by analyzing mutant 18S rRNA levels in strains deficient for the ribophagy genes BRE5, UBP3, and DOA1. Loss of DOA1 conferred a partial stabilization of mutant 18S rRNAs, suggesting that Doa1 functions in NRD. The disparity between the large subunit specificity of Doa1 in ribophagy and effects on the small subunit rRNA in NRD suggests that either Doa1 is acting in a different fashion in NRD than it does in ribophagy, or that Doa1 promotes 18S NRD through the involvement of the large subunit. In support of the latter hypothesis, three lines of evidence have already suggested that large subunit recruitment is needed for degradation of 18S:A1492C rRNAs: 1) these mutant 18S rRNAs have been shown to appear in monosomes and polysomes, indicating that such small subunits are capable of recruiting a large subunit; 2) the NRD proteins Dom34:Hbs1 insert into the A-site; 3) addition of cycloheximide inhibits 18S NRD, but not the distinct, Dom34-independent process, 25S NRD (Lariviere et al., Cole et al.). Together, these findings suggest that it would not be unlikely for Doa1 to act in NRD via an interaction with the large subunit.

To elucidate the placement of Doa1 within the NRD pathway, we crossed strain doa1Δ with either dom34Δ or asc1Δ. We then conducted mutant 18S rRNA timecourse analyses in the resulting doa1Δdom34Δ or doa1Δasc1Δ strains, and observed a synthetic decrease in the rate of decay exclusively in the doa1Δasc1Δ strain. These data suggest that Doa1 functions with Dom34, and not with Asc1, thereby supporting our initial model and suggesting that Doa1 functions with Dom34. Interestingly, in this configuration, WD40 repeat proteins are present on
both sides of the pathway, raising the possibility that Doa1 and Asc1 could function in a similar fashion.

In keeping with the theme of ubiquitin involvement in NRD, Doa1 possesses two ubiquitin binding domains, again raising the possibility that Doa1 and Asc1 could promote the ubiquitin modification of a target protein as part of their functions in NRD.

Additionally, though the potential synthetic interactions of pep4Δ with the other NRD proteins was not examined, the partial loss of NRD in the absence of this gene suggests that it may be a part of one of the two branches of the NRD pathway. Consistent with this is the observation that both Doa1 and Pep4 are involved in ribophagy, suggesting Pep4 may be functioning with Doa1 in NRD.

**Involvement of Hbs1 in NRD**

One model for 18S NRD provides an explanation for the different phenotypes exhibited by dom34Δasc1Δ and hbs1Δasc1Δ strains: Hbs1 may regulate the activity of Dom34 by promoting its function in NRD, making it less important for NRD than Dom34. A previously published *in vitro* study has established this regulatory activity for Hbs1, noting that Hbs1 promotes the activity of Dom34, but is not required for the Dom34-mediated release of the ribosomal subunits from stalled translation complexes (Pisareva et al., 2011). Supporting this is a second study by Shoemaker et al. 2011, which also demonstrates that Hbs1 is dispensable for ribosome recycling. As such, our model suggests that in the absence of both Hbs1 and Asc1 proteins, Dom34 continues to promote 18S NRD.
**Involvement of Ski7 in NRD**

To clarify the apparent role for Ski7 in NRD, we tested the genetic relationship of DOM34 or HBS1 with SKI7, by combining the respective deletions. Though we observed a synthetic effect in the hbs1Δski7Δ strain, this effect did not repeat in the dom34Δski7Δ strain. The simplest interpretation of these data is that in 18S NRD, Ski7 functions in parallel with Hbs1 and serially with Dom34. The high degree of structural homology between Ski7 and Hbs1 would suggest that Ski7 is capable of binding Dom34 in place of Hbs1. However, this interpretation seems unlikely for a number of reasons. First, despite the publication of several global protein:protein interaction databases, Dom34 has not been reported to bind Ski7. Second, the deletion of DOM34 has the same effect on NRD as an HBS1 deletion (Cole et al., 2009). If Ski7 can substitute for Hbs1, then loss of HBS1 or SKI7 should produce a partial effect on NRD that is less severe than the effect observed when DOM34 is deleted. A third problem with the above-mentioned conclusion is that, if it were true, then loss of SKI7 and DOM34 should produce the same slow growth phenotype as double deletion of both SKI7 and HBS1, but this is not the case. Fourth, if Ski7 promotes NRD without biding to Dom34, then the deletion of Dom34 and ASC1 should not completely shut off NRD because Ski7 would still be able to act. This is also not the case – 18S NRD is not detected in dom34Δasc1Δ.

As such, another explanation seems necessary. I propose instead that Hbs1, functioning without Dom34, may be functionally redundant with Ski7 in Non-Stop Decay. If this were the case, then Non-Stop Decay would continue, in an Hbs1-mediated manner, in a ski7Δ strain, potentially explaining why SKI7 deletion had little effect on NSD in the BY4741 background (Inada et al., 2005) whereas in the W303 background, loss of SKI7 decreased the rate of NSD (van Hoof et al. 2002)(van Hoof A, Frischmeyer PA, Dietz HC, 2002). In a strain doubly
deficient for SKI7 and HBS1, Non-Stop Decay would be severely diminished. The complete loss
of Non-Stop decay could potentially cause cell sickness, leading to the observed slow growth
phenotype and indirectly producing the deleterious effects we see on 18S NRD.

Mutagenic Analysis of Asc1

Though we were unable to identify structural features of Asc1 required for 18S NRD, the
apparent gain of function produced by the knob deletion and Y232F mutations suggest that these
sites may be responsible for the repression of Asc1 activity in NRD. Furthermore, the fact that
the ASC1 ribosome binding mutations were fully capable of complementing the asc1Δ strain for
its ability to conduct NRD suggests that Asc1 may function in an ribosome independent manner
in NRD. Alternatively, these mutations might simply decrease ribosome binding affinity without
abolishing it entirely. Such residual binding activity could be sufficient for NRD.

The ability of mammalian and yeast Rack1/Asc1 to form homodimers suggests that this
is another means by which Asc1 might be regulated in NRD (Fig. 2.1). In mammalian cells,
Ser146, which is located on WD repeat 4 (WD4), is known to be phosphorylated, leading to
Rack1 homo-dimerization. Once formed, homodimeric Rack1 facilitates the interaction between
the cullin complex and their substrate, Hif-1alpha, which is subsequently ubiquitinated by cullin
and degraded by the 26S proteasome (Y. V. Liu et al., 2007). In yeast, Ser146 is not conserved,
initially preventing us from developing a dimerization-deficient Asc1 mutant. Nevertheless,
following the completion of our Asc1 mutagenesis experiments, the crystal structure of
dimerized Asc1 was published (Yatime et al., 2011). The structure revealed that, like its
mammalian homologue, Asc1 dimerizes via its WD4 domain. This interaction requires major
restructuring of the beta-pleated sheets that comprise WD4, allowing for a high degree of
protein:protein contact between both proteins. The Nilsson structure also implicated the highly conserved residue, His147, as a molecular switch, likely capable of promoting, or preventing, dimerization. As such, future studies of 18S NRD should consider mutation of His147 a major priority in the investigation of Asc1 function.

The implication that Rack1 dimerization is required for its ability to promote the ubiquitination of target proteins provides an appealing mechanism by which Asc1 might act in NRD. In hypothesis, Asc1 might interact with, for example, Ubc4, to promote the ubiquitination of a ribosomal protein, thereby promoting the identification of the corresponding 40S subunit as a NRD substrate.

**Polysome Profile Analyses**

Previously, it was demonstrated that mutant 18S rRNAs appear in both the monosome and polysome fractions of a polysome profile in wild type yeast. Though the signal for the mutant 18S is low in the wild type strain due to deletion via the 18S NRD pathway, it is still detectable. The relative distribution of mutant 18S signal was shown to be very low in the polysome fractions, and high within the 40S and monosome fractions. The abundance in the 80S fraction suggests strongly that 40S subunits are capable of recruiting a large subunit. This is consistent with the fact that Dom34:Hbs1 binds the large subunit during NRD. Yet, a model is needed to explain the uneven distribution of mutant 18S rRNAs between mono and polysomes.

One model that could explain this distribution proposes that mutant 18S-containing 40S subunits stall immediately following the completion of translation initiation, right at the shift to elongation phase. This is based upon the observation that base 18S:A1492 is needed for kinetic
proofreading in the A-site, an event which is not known to occur prior to translation elongation phase (Yoshizawa et al., 1999). As such, even mutant 18S-containing ribosomes should have an intact P-site, capable of conducting codon:anticodon pairing between the Met-tRNA and the AUG start codon during initiation. Only after the recruitment of the large subunit, and transition to elongation phase, would the ribosome require the use of the mutant A-site. It is at this point, the first attempted A-site mediated codon:anticodon pairing, that the 18S:A1492 mutation could reasonably be expected to cause translation problems via the creation of a translational stall.

This model sets up the expectation that most of the mutant 18S rRNA signal should appear in the fractions corresponding to a full ribosome plus a half-mer, thereby accurately explaining the data. If correct, the model suggests that a mutant 18S-containing 80S complex could initiate on a polyribosome. By stalling on the start codon, it would prevent the formation of additional 80S ribosomes, leading to a net loss of ribosomes on the transcript as the downstream ribosomes run off. This would have the effect of converting a poly-ribosome into a monosome. Moreover, by blocking the start codon, no other 40S subunits would be able to complete initiation via the recruitment of the large subunit. Instead, all upstream 40S subunits would be expected to stall behind the stalled ribosome. The combination of downstream ribosome run-off, and the blockade of initiating 40S subunits, would promote the conversion of mutant 18S-containing 80S ribosomes into monosomes plus a half-mer. The only mutant 18S signal expected to be present in the polysome fractions would represent mutant 18S-containing small subunits that had very recently initiated on an existing polysome. Hence, mutant 18S signal is expected be highest within the 1.5-mer fractions and much lower, but evenly distributed, across the polysome fractions. Within the 40S pool there should also be mutant 18S signal, though the abundance of this signal is not addressed by our model. Overall, this model accurately describes the polysome
profile data, while taking into account the known role and activity of the 40S subunits that contain mutant 18S:A1492C rRNAs.

In addition to generally supporting our data, these polysome experiments suggest that shutting down 18S NRD via DOM34 and ASC1 double deletion, yields its major effects on mutant 18S abundance within the pool of translating ribosomes. This is an important distinction due to the fact that the mutant 18S signal could have conceivably increased exclusively within the free 40S pool. Quantification of the signal within the free 40S fractions is difficult because there is no 25S with which to normalized these signals. This means that loading errors, among other problems, could easily disrupt the quantification process in these fractions. Hence, our data do not exclude the possibility that mutant 18S rRNA abundance could increase in free 40S pool. However, the indication that NRD proteins do yield their effect on the translation pool contributes significantly to our understanding of this process.

An additional consideration not apparent from this study is that the enrichment of mutant 18S:A1492C in monosomes could be specific to this mutation. In the case of an endogenously produced stalled ribosome, the offending ribosome could conceivably be present at any location on the transcript. For example, much of our data suggest that PDTA, NSD, and NGD are all functionally related, and indeed, could even represent the same pathway. As such, the fact that offending string of lysine or arginine codons in PDTA transcripts is in the middle of the transcript serves to underscore the fact that both Asc1 and Dom34 are still capable of acting on these transcripts (Kuroha et al). Therefore, the enrichment of 18S:A1492C rRNAs in the monosome fraction of polysome profiles is likely to be specific for this particular mutant. Therefore, the important finding here is not that mutant 18S signal is enriched in the monosome fraction, but rather the fact that it is enriched within the pool of translating ribosomes.
Unlike the polysome profile data set, the run-off data may offer additional insights into NRD beyond the confirmation of our model. Assuming for a moment, that the run-off data set were to be repeated successfully, and also that this effect is selective for the mutant 18S, as suggested by the methylene blue stain, this data set may imply the activity of a NRD factor upstream of Dom34 and Asc1. The selective degradation of mutant 18S rRNAs in NRD indicates that a mechanism exists to discriminate between wild type and mutant 18S rRNAs. The ability of Dom34:Hbs1 to bind to the ribosome, and the presence of Asc1 on the ribosome, suggest that these factors could be responsible this discrimination event, or, that this step occurs prior to their involvement. The possibility that discrimination occurs down-stream of Dom34 and Asc1 activity is not supported by the data, due to the fact that wild type 18S rRNAs are not subject to Dom34 and Asc1-mediated degradation. Moreover, the discrimination step is highly unlikely to exist in parallel to Dom34 and Asc1, due to the fact that, in the absence of both proteins, 18S NRD does not occur. A parallel discrimination event would bypass the need for Dom34:Hbs1 and Asc1 involvement, which, as demonstrated by the lack of detectible NRD in dom34Δasc1Δ, is clearly not the case. Therefore, the discrimination step must be conducted by Dom34:Hbs1 and Asc1, or, it must occur upstream of them. Given the run-off data (though it is preliminary), we observe evidence that that discrimination between wild type and mutant 18S rRNAs still exists in the absence of both Dom34 and Asc1. This suggests that discrimination occurs upstream of Dom34 and Asc1. One candidate for the upstream discrimination factor is Rps3, which is discussed below.
**NRD Protein Overexpression Studies**

Our overexpression studies attempted to detect redundant roles for the known 18S NRD proteins or their potential ability to regulate each other. Although complementation data suggested that these constructs represent valid tools for overexpression, the lack of ability of any of the proteins to functionally compensate for the loss of others has three possible explanations: 1) The endogenous levels of each NRD protein may be saturating; 2) 18S NRD proteins may not regulate the others; or 3) the overexpression constructs do not produce a sufficient increase in protein level to produce a detectable change in phenotype. This latter possibility is credible, at least in the case of the UBC4 and ASC1 overexpression constructs, for which we observed a only a 4-fold or 2-fold increase, respectively. The theoretical 2-fold increase for ASC1 is based upon the fact ASC1 is simply present in a second, identical copy during “overexpression,” which could lead to an additional 200k copies of Asc1, according to the O'Shea and Weissman GFP-tag database (Huh et al., 2003). In the case of the high expression UBC4-HA construct, we observed only a 4-fold increase over the levels produced by the endogenous UBC4 promoter construct. This increase in expression is 2.5-fold less than the 10-fold increase produced by high expression DOM34 and HBS1 constructs. This disparity is not entirely surprising, given that, under its endogenous promoter, Ubc4 has been reported to reach 14k copies per cell, whereas Dom34 and Hbs1 are present at roughly 2-3k copies. This suggests that, when switched to the ADH1 promoter, all three genes are expressed at the level of the Adh1 protein, which would represent a larger change for Dom34 and Hbs1 than for Ubc4. Hence, the UBC4 gene might be more effectively overexpressed under a stronger promoter.

With respect to Dom34 and Hbs1 overexpression, the simplest explanation for their lack of ability to compensate for the loss of other NRD proteins is that they may be saturating, even at
low abundance. This would explain why, even at 10 times the normal abundance, they failed to promote NRD in the absence of Asc1. If this were not the case, then the increased concentration of these two proteins would likely have led to an improved rate of detection and degradation of mutant 18S rRNAs.

*Implication of ESCRT complex involvement in NRD*

The major pathway for the breakdown of cellular components is mediated by the lysosome. In yeast, proteins from the cell surface and other compartments arrive at the lysosome via invagination of the membrane or via direct transfer across the endosome membrane. Cell surface receptors contained in endosome membranes are selectively incorporated into smaller endosomes by an additional invagination process. As the outer endosome membrane becomes filled with smaller internal bodies, it becomes a multivesicular body (MVB). Three protein complexes produce MVBs: the Endosomal Sorting Complex Required for Transport (ESCRT) I, II, and III. The protein Vps27 initially recruits the ESCRT-I complex, which then recruits ESCRT-II, which, in turn, is believed to recruit ESCRT-III. The process of MVB formation and lysosome-mediated protein degradation was reviewed by Hurley et al. and is summarized below (Hurley, 2008).

The monoubiquitination and polyubiquitination of protein targets lead to their incorporation into MVBs via activity of Vps27:Hse1 (Hirano et al., 2006). This heterodimer binds both ubiquitin and ESCRT-I to promote the recruitment process. Furthermore, Vps27:Hse1 also recruits the E3 ubiquitin ligases and deubiquitinating enzymes that function, in some capacity, to promote this process. Together, Vps27 and the Tom proteins sequester ubiquitinated
protein cargo into defined areas along the outside of the endosome membrane. These areas are then coated in clathrin, leading to their subsequent incorporation into the MVB. Downstream, the acidification of the lysosome contributes to the degradation of the encapsulated materials (Hurley, 2008).

The MVB system may be involved in 18S NRD. The initial clue suggesting this connection came from genetic data indicating Doa1 involvement in NRD. Doa1 functions in at least two major pathways that involve the ubiquitin-mediated selection of target proteins for degradation by the 26S proteasome or their sequestration into MVBs (Qiu et al., 2010; Ren et al., 2008). Moreover, Doa1 is also involved in the transport of wild type ribosomes into the vacuole during ribophagy (Ossareh-Nazari et al., 2010). The established overlap between 18S NRD and ribophagy supports the possibility that ribosomes may move into a membrane-bound compartment, such as an MVB, in a Doa1-dependent manner as part of 18S NRD. However, our data suggest that the Vps27 protein is not required for this process (Fig. 2.18C). This argues against a role for MBVs in NRD, raising the possibility that another membrane-bound compartment is used, or that sorting into MVBs can occur in a Vps27-independent manner.

**A Model for 18S NRD**

The genetic evidence for Ubc4, Doa1, and Asc1 involvement in NRD allows us to partially outline their locations in the pathway (Fig. 2.20). First, Dom34 and Asc1 appear to function independently at the same level, thereby establishing parallel branches at their step of the pathway. Given the suggestion that Doa1 is epistatic to Dom34, we have grouped them together. *In vitro* data suggest that Hbs1 has a stimulatory effect on Dom34 but is not essential for its activity (Pisareva et al., 2011). Hence, we have placed Hbs1 above Dom34. In the case of
Doa1, our data do not indicate whether it functions upstream or downstream from Dom34; hence our model is ambiguous about these two possibilities.

**Figure 2.20.** A model for 18S NRD. A. Pathway diagram including Ubc4 and an anticipated, associated, E3 ubiquitin ligase and Rps3. The activities of both Rps3 and the E3 ligase are inferred based upon known physical interactions, but have not been demonstrated in NRD. B. Diagram of the ribosome displaying schematically the interactions between Hbs1, Rps3, and Asc1.

The severe decrease in 18S NRD activity in the absence of UBC4 suggests that Ubc4 is required at its level in the pathway. While it is clear that Ubc4 functions at a level distinct from
Dom34, Doa1, and Asc1, the data, again, do not allow us to establish order. For the purposes of simplicity, we have placed it upstream, though multiple other possibilities exist.

In an effort to gain additional insight into the order of the pathway, and potential functional redundancy within the system, we overexpressed different NRD proteins in the absence of others. We expected that functionally redundant proteins might compensate for the loss of others, or that certain proteins might, when overexpressed, overcome the loss of an upstream regulator. To test this possibility, we developed high expression ADH1 promoter fusion constructs for DOM34, HBS1, and UBC4. Because the endogenous ASC1 promoter is already highly efficient, we simply expressed FLAG-ASC1 under its own promoter. For each of these four proteins, we observed partial or full complementation in the respective deletion backgrounds, but no evidence that they are capable of compensating for the loss of other NRD proteins. This suggests that endogenous NRD protein levels may be saturating, or that these constructs did produce quantities of protein sufficient to detect dosage compensation (data not shown).

The two major ambiguities inherent in our data set allow for four possible configurations of the 18S NRD pathway: Ubc4 could function above or below the Asc1/Dom34 branches, and the two branches either converge again at the end opposite Ubc4, or they remain separate for the remainder of the pathway.

The dual branch model for NRD also raises the possibility that the Asc1 and Dom34 branches could be coordinately regulated by an upstream factor. In consideration of this possibility, we note that both Asc1 and Hbs1 directly interact with Rps3, which in turn directly interacts with the mRNA at the entrance to the mRNA channel. As such, Rps3 is ideally
positioned to function as a sensor of ribosome translocation along the mRNA, and could potentially function as a regulator of both branches of the NRD pathway. Interestingly, the tail of Rps3 directly interacts with WD4 of Asc1, the WD40 repeat involved in Asc1 dimerization, thereby suggesting a potential mechanism by which Rps3 could regulate Asc1.
Chapter 3:

Testing the endogenous functions of 18S NRD

Introduction

To date, 18S NRD has been shown to degrade synthetic, mutant rRNAs expressed from plasmids. Though it is conceivable that yeast may naturally develop an 18S:A1492, 18S:1493, or 18SG530U mutation, the existence of the complex NRS system suggests that a more general trigger for translational stalls is likely to exist. One likely function is the detection of improperly or incompletely made small subunits. A second possibility is that NRD/NGD deletes partially degraded or damaged mRNAs. In rare cases, NRD may also ameliorate the toxic effects of ribosome-inactivating toxins released by microorganisms.

18S NRD serves as a quality control measure for translating ribosomes

The production of ribosomes is complex and requires the involvement of a large number of assembly factors, nucleases, small accessory RNAs, and chemical modifications. All of this takes place quickly and requires the correct localization and transfer of the pre-ribosomal subunits. The complexity of this process implies that even minor errors may contribute to the production of a nonfunctional ribosome. This suggests that mutations in ribosome assembly proteins could lead to widespread ribosome/translation problems. In a recent publication, Soudet et al. demonstrated that defects in 20S maturation promote the formation 20S-containing, but
otherwise mature, small ribosomal subunits that attempt to conduct translation initiation. These subunits appear in polysome profiles predominantly in the monosome fraction, and appear to cause widespread problems with translation as evidenced by altered polysome profiles and slow growth (Soudet et al.). The loss of Dom34 and Hbs1 in this strain background has the surprising effect of relieving the slow-growth phenotype. Despite the observation that Dom34 and Hbs1 promote cell growth when ribosome assembly is altered due to ribosomal protein haploinsufficiency, in this instance, the opposite appears to be true (Bhattacharya et al.). Here, the authors propose that a mild decrease in the rate of 20S processing leads to the accumulation of incompletely synthesized small subunits, which attempt translation. These immature small subunits are apparently degraded by Dom34:Hbs1 even though, given additional time, they might reach full maturation. Hence, in this case, the deletion of these immature small subunits by Dom34:Hbs1 appears to be detrimental to the cell.

In a second example, Dom34:Hbs1 were also shown to promote the growth of cells deficient for one copy of the Rps6A gene (Bhattacharya, McIntosh, Willis, & Warner, 2010). The deletion of one copy of a ribosomal protein gene is anticipated to lead to a decrease in the corresponding protein, potentially leading to a defect in ribosome production. The slow growth of such strains supports this hypothesis. Interestingly, the loss of Dom34 or Hbs1, which normally does not affect the growth of WT cells, decreases the growth rate of RPS6A haploinsufficient cells, suggesting that the NRD pathway is somehow active in the protection of the cell against the deleterious effects of this ribosome production defect.

Data discussed below supports the Soudet et al 2010, and Bhattacharya et al. 2010 data sets by providing direct evidence of rRNA degradation during ribosomal protein
haploinsufficiency. It also contributes to the identification of additional endogenous roles for 18S NRD, including protection from genotoxic stress and translation inhibitors.

**18S NRD may protect the cell from damaged RNAs**

Damaged RNAs represent a theoretically common impediment to canonical translation, potentially leading to their removal from the cell by NRD/NGD. Cells continually produce endogenous alkylating and oxidizing agents via cellular metabolic processes, creating a constant source of hazardous RNA-damaging chemicals. The discovery in mammalian cells of the oxidative RNA demethylases, AlkB and AlkBH3, suggests that the alkylation of RNA has negative implications for the health of the cell (Drabløs et al., 2004). Oxidized mRNAs show apparently normal polysome association; however, at levels corresponding to the degree of oxidation, they lead to the formation of incompletely synthesized peptides (Tanaka et al., 2006). These truncated peptides suggest that oxidized mRNAs cause translational stalling, the theorized trigger for NRD/NGD.

In *Arabidopsis*, wild type plants display widespread mRNA turnover following UV exposure, a phenotype lost in strains lacking the RPS27A gene. Loss of this gene also triggers sensitivity to the alkylating agent MMS, which is capable of forming adducts on both DNA and RNA. These phenotypes are reminiscent of the mRNA degradation events observed in NGD, suggesting Rps27A might act in NGD or 18S NRD (Revenkova et al., 1999). In yeast, the RPS27 genes encode a pair of highly similar copies of the small subunit protein Rps27, which is located on near the mRNA channel. This location suggests that Rps27 is potentially able to directly bind the associated mRNA, as well as the rRNA, making it suitable as a sensor of translation stress or
potentially as a nuclease, though its structure does not imply this activity. Similar to the yeast genes, in A.thaliana, there are four Rps27 genes: -A, -B, -C, and Ψ. These genes differ at the same amino acid shown to be variable in yeast, with the exception of Ψ, which is divergent at an adjacent residue. In yeast, Rps27A is apparently the closest orthologue to the Arabidopsis Rps27A. This suggests that a yeast rps27aΔ strain might display defects for NRD and NGD, whereas an rps27bΔ strain might not.
18S NRD may protect the cell from eukaryotic ribosome-targeting antibiotics

Cycloheximide (CHX) is a ribosome-inactivating toxin produced by the bacterium Streptomyces griseus. It specifically targets the eukaryotic ribosome, essentially locking the large and small subunits together (Baliga et al., 1969, Obrig et al., 1971). Even at low concentrations, CHX is cytotoxic to most eukaryotic cells. Due to the opposing activities of CHX and Dom34:Hbs1, we theorized that CHX-bound ribosomes might be resolved by Dom34:Hbs1, either by releasing them or by degrading them via the 18S NRD pathway. If correct, we anticipated that cells deficient for NRD would be killed at a lower concentration of CHX.

Results

Ribosomes in RPS27B deficient cells are targeted for degradation by Dom34, correlating with faster cell division.

The discovery that 18S NRD may function to delete incompletely synthesized ribosomes occurred indirectly, as part of the search for new 18S NRD factors. As of 2009, Dom34 and Hbs1 were the only known 18S NRD factors. The continued activity of the 18S NRD pathway in the absence of both proteins implied the existence of additional factors, prompting us to investigate new candidate genes. This search led us to examine the potential role for RPS27A and RPS27B, two factors that were implicated in a NGD-like process in Arabidopsis (Casati et al.). To test the involvement of the RPS27 genes in NRD, we assayed the two deletion strains for effects on NRD using a single timepoint assay and northern blot analysis. We also tested rps27aΔdom34Δ and rps27bΔdom34Δ strains in order to detect potential synergistic effects. Our results demonstrated that mutant 18S rRNAs were not stabilized in the absence of either RPS28A
or RPS27B. Surprisingly, the stabilizing effect on mutant 18S rRNA seen in the absence of DOM34 was slightly diminished in strains lacking either RPS27A or B. This effect was stronger in the dom34Δrps27bΔ strain than in the dom34Δrps27aΔ strain (Fig 3.1.)
Figure 3.1. 18S rRNA levels in rps27AΔ and rps27bΔ deletion strains. Strains rps27aΔ or rps27bΔ were crossed with dom34Δ and subjected to single timepoint analysis. 18S/25S ratios
were plotted, and representative northern blot data are shown below (A). The single rps27aΔ and rps27bΔ strains were subjected to timecourse analysis. 18S/25S ratios were plotted and T0 ratios for each timecourse were normalized to 1 (B).

The data displaying WT 18S rRNA levels in the rps27aΔ/bΔ strains provide some insight into the mutant 18S data set. Surprisingly, in the single timepoint data set, WT 18S rRNAs were dramatically destabilized in the rps27bΔ strain, but not the rps27aΔ strain. This destabilization correlates with slow growth and reported sensitivity of the rps27bΔ strain to various sources of genotoxic stress (Jelinsky, Estep, Church, & LD, 2001). This strong destabilization was significantly, but partially, relieved by the additional deletion of DOM34. This suggests that Dom34 may be subjecting many of the small subunits in these cells to 18S NRD. Timecourse analysis of WT 18S rRNAs in this strain suggests that WT 18S rRNAs only represent 50% of the total seen in WT or rps27a cells, but that this population is stable. This agrees with the hypothesis that a large percentage of the 18S rRNAs in the rps27bΔ strain are degraded.

18S NRD deficient cells are sensitive to 4-nitroquinoline

To test whether NRD protects the cell from damaged RNAs, we compared the growth of NRD-deficient and wild type strains during application of genotoxic chemicals thought to damage RNA. Specifically, we applied the same genotoxic chemicals used in the Genomic Phenotyping database prepared by the Samson lab to a variety of strains (Said, Begley, Oppenheim, Lauffenburger, & Samson, 2004). These chemicals include 4-nitroquinoline-N-oxide (4NQO), a UV mimetic that also produces oxidative damage; an oxidizing agent, tert-Butyl hydroperoxide; and the alkylating agent MMS. We theorized that certain chemical lesions
may lead to translational stalling, thereby triggering their resolution by the NRD/NGD pathways. As such, the loss of NRD proteins would be expected to promote strain sensitivity to certain chemicals.

To assay our deletion strains, we chose to conduct halo assays, an experimental system in which a lawn of cells is plated out, and then a disk of paper containing a defined quantity of a chemical agent is placed in the center of the plate. The drug then diffuses from the disk, creating a concentration gradient on the plate. The quantity of each damaging agent applied to the disk is titrated to produce a zone of growth inhibition that is easily measured. Different strains may show variable areas of growth inhibition or “halos,” thereby providing a quantifiable measure of their sensitivity to the damaging agent.

It was previously reported in the high throughput Genomic Phenotyping database that both dom34Δ and hbs1Δ strains are sensitive to oxidative damage by tert-butyl hydroperoxide (Said et al., 2004). Strain asc1Δ was also reported to be sensitive to MMS and 4NQO. Like the Samson lab, we observed that asc1Δ is sensitive to 4NQO; however, we did not detect any sensitivity to MMS. Likewise, for the dom34Δ and hbs1Δ strains, we did not observe the reported sensitivity to any of the damaging agents. The differences between these two data sets suggest that differences between the W303 strain background used in the Samson data versus the BY4741 strain background used in our lab may be affecting the outcome of the experiments.

Nevertheless, we continued to test our hypothesis by examining the effects of damaging agent treatment on the double mutants dom34Δasc1Δ, in which 18S NRD is not detectable, and hbs1Δasc1Δ, which still conducts NRD, albeit at a slower rate than either of the corresponding single deletions. We found that for 4NQO treatment, dom34Δasc1Δ showed synthetic sensitivity
whereas hbs1Δasc1Δ did not. This correlates with the apparently complete shut-off of NRD observed in dom34Δasc1Δ, but not in hbs1Δasc1Δ (Fig. 3.2).

**Figure 3.2.** 4NQO sensitivity of dom34Δasc1Δ and hbs1Δasc1Δ strains. Both single and double deletions strains were subjected to halo assays in the presence of 25 µg 4-nitroquinoline-n-oxide which was placed onto the watman paper disk. The relative area of growth inhibition for each strain was plotted.
18S NRD-deficient cells are sensitive to translation inhibitor Cycloheximide (CHX).

Given that CHX prevents translocation of the eukaryotic ribosome by essentially locking the large and small subunits together, we theorized that CHX-treated ribosomes would resemble NRD targets. As such, the ability of the NRD pathway to resolve stalled ribosome complexes should protect cells from the activity of this drug. To test this hypothesis, we conducted halo assays in strains deficient for DOM34, ASC1, or both genes, and compared the area of growth inhibition to that of the isogenic wild type strain (Fig. 3.3). Strains deficient for DOM34 or HBS1 did not display any sensitivity to CHX in halo assays, although deletion of ASC1 led to a ~40% increase in halo area. Both dom34Δasc1Δ and hbs1Δasc1Δ strains showed synthetic sensitivity to CHX, consistent with the synthetic effect in NRD previously observed in both strains. In these strains, loss of NRD correlated with CHX sensitivity. However, this correlation does not always apply: strain ubc4Δ showed no sensitivity to CHX despite its apparent complete lack of ability to conduct 18S NRD. This difference may reflect differences in the level at which Dom34, Asc1, and Ubc4 function in NRD.
Figure 3.3. CHX sensitivity of dom34Δasc1Δ and hbs1Δasc1Δ strains. The dom34Δasc1Δ, hbs1Δasc1Δ strains, and corresponding single deletion strains were subjected to halo assay in the presence of 7.5 µg cycloheximide placed onto the watman paper disk. The relative area of growth inhibition for each strain was plotted.
Our doa1Δ and ubc4Δ strains also showed a CHX sensitivity equivalent to the WT strain, consistent with the effect seen in dom34Δ and hbs1Δ strains. Given the genetic evidence that Doa1 is epistatic to Dom34, this is not surprising. Also, based on previous results, it would certainly stand to reason that synthetic sensitivity to CHX would be observed in doa1Δasc1Δ, but not in doa1Δdom34Δ. Again, this hypothesis is based upon the previously established synthetic loss of NRD in doa1Δasc1Δ but not doa1Δdom34Δ. These possibilities have not yet been tested.

The sensitivity of NRD deficient strains to alkylating and oxidative damaging agents was also tested (Fig. 3.4). The halos of the assayed strains were highly similar, suggesting that the tested strains are not sensitive to damage by MMS or tert-butyl hydroperoxide.
Figure 3.4. MMS and TBH sensitivity of dom34Δasc1Δ and hbs1Δasc1Δ strains. The dom34Δasc1Δ strains, hbs1Δasc1Δ strains, and corresponding single-deletion strains were subjected to halo assays in the presence of either 5µl Methylmethanesulfonate or 1.5 µl of 70% tert-butyl hydroperoxide pipetted directly onto the watman paper disk. The relative area of growth inhibition for each strain was plotted.
Materials and Methods

Single Timepoint Assay:

Strains harboring pSC40-Wt or pSC40-18S:A1492C plasmids were grown at 30° in SD-uracil, in the presence of 2% raffinose, to mid-log phase (OD$_{600}$ = 0.5). Galactose was added to a final concentration of 2% to induce rRNA transcription, and cells were incubated for an additional 90 minutes. OD$_{600}$ was monitored during this time and pre-warmed medium was added as necessary to maintain OD$_{600}$ = 0.5. Cells were then harvested by centrifugation and flash frozen on dry ice/EtOH.

Pulse-chase Analysis:

Pulse-chase analysis was performed as previously described (Lariviere et al. 2006). Strains harboring pSC40-Wt or pSC40-18S:A1492C plasmids were grown at 30° in SD-uracil with 2% raffinose to mid-log phase (OD$_{600}$ = ~0.5). Galactose was added to a final concentration of 2% in order to induce rRNA transcription, and cells were incubated 90 minutes. Pre-warmed glucose was then added to a final concentration of 2% and timepoint T0 was immediately harvested. Samples were then flash frozen on dry ice/EtOH. Pre-warmed SD-ura with 2% glucose was added as necessary to maintain an OD$_{600}$ of 0.5 for the duration of the timecourse. Timepoints were taken at specified intervals.

Northern Blot Analysis:

For each sample, 2 µg total RNA was separated on a 1% agarose-formaldehyde gel and transferred to a nitrocellulose membrane as previously described (Brown et al., 2004). Total
RNA was detected via staining with Methylene blue stain (Molecular Research). Membranes were hybridized with 32P-end-labeled probes FL125 (anneals to plasmid derived 18S rRNA) and FL126 (anneals to plasmid derived 25S rRNA) for 12 hr in ExpressHyb (BD Biosciences). Signals were visualized using a Typhoon Phosphorimager (Molecular Dynamics) and quantified via ImageQuant software (GE Lifesciences).

**Halo Assays:**

WT and the corresponding mutant cells were grown to mid-log (~OD 0.5) in YPD medium, then spun down to collect the cells, and resuspended in 150 ul YPD medium. Cells were then spread evenly on plates consisting of exactly 25 ml of YPD/Agar, and set at 30° for one hour until dry. Sterile whatman paper disks were then placed in the center of the plate, and drugs were added in the indicated quantities. Cells were then grown for ~16-32 hours until a clear zone of growth inhibition was established. Plates were then digitally photographed, and the area of growth inhibition was measured using software. Damaging agent amounts added to whatman paper disks are as follows: 5µl Methylmethanesulfonate, 1.5 µl of 70% tert-butyl hydroperoxide, 7.5 µg cycloheximide, 25 µg 4-nitroquinoline-n-oxide.
Discussion:

These experiments test a number of proposed functions for the 18S NRD pathway, including the turnover of immature ribosomes, and degradation of damaged RNAs. The initial experiments on the Rps27 proteins was initiated based upon the suggestion that a subset of the Arabidopsis Rps27 proteins could promote a NGD like process. Though we do not observe evidence of any effects on 18S NRD, the dramatic lack of wild type 18S rRNAs in the rps27bΔ background, and the subsequent, partial restoration of their levels when DOM34 is deleted, are in keeping with the conclusions of Bhattacharya et al. This group demonstrated that DOM34 and HBS1 promote cell growth when certain small subunit ribosomal protein genes are absent from the cell. This indirectly implies that Dom34:Hbs1 may recognize improperly assembled small subunits. The translation-dependent nature of 18S NRD also suggests that such improperly assembled, but otherwise wild type ribosomal subunits may be attempting translation initiation. Our other work, demonstrating a selective increase in NRD substrates in the monosome fraction also suggests that immature small subunits that attempt initiation may successfully proceed to recruit a large subunit recruitment prior to triggering the effects of Dom34:Hbs1. These suggestions are both supported by the data presented by Soudet et al. which demonstrates that immature, but otherwise wild type small subunits can attempt initiation, leading to their degradation in a Dom34 dependent manner. Unlike the Soudet et al. dataset which deals with an analogous, but distinct situation, in the rps27bΔ background, Dom34 in its normal function, appears to be rescuing the cell from the detrimental effects of incompletely or improperly made small subunits. This helps establish the previously theorized role for Dom34:Hbs1 as a final round of quality control for wild type ribosomes.
Our data set also includes a preliminary investigation of the effects of genotoxic chemicals on cell growth in the absence of 18S NRD. Though we observe a significant, detrimental effect of these chemicals on cell growth, the NRD pathway only appears to have a mild protective effect on cells. It is currently unknown whether 4NQO directly damages RNA, though the chemical structure of RNA is sufficiently similar to that of DNA that it would not be unreasonable to expect that it produces similar lesions on RNA. Nevertheless, a thorough investigation of the effects of 4NQO on RNA is necessary before any credible claims can be made about the mechanism by which Dom34, Hbs1 and Asc1 protect the cell from 4NQO. However, it is worth noting that there has never been any published work reporting the detection of a role for Dom34 in DNA damage repair, and that, conversely, its role in RNA metabolism is quite clear. Moreover, unpublished data from Roy Parker's lab and Rachel Green's lab argue against changes in the transcriptional and translational profile of cells in the absence of DOM34. This suggests that indirect effects of DOM34 deletion on the expression of DNA damage repair genes are minimal. In light of these data, the possibility that NRD protects the cell from 4NQO-damaged RNAs remains credible. Future testing of the ubc4Δ strain should serve to further test this possibility.

Finally, our data also suggest that 18S NRD may counteract the detrimental effects of cycloheximide on the cell. Dom34, Hbs1, and Asc1 may or may not directly release ribosomal subunits that have stopped during elongation due to the activity of CHX, however this is certainly a possibility. Alternatively, these three proteins could simply act on wild type ribosomes, thereby increasing the pool of free ribosomal subunits which may be limiting at increasing concentrations of CHX.
Ribosomal processing defects as tools for studying NRD:

The Soudet paper demonstrated the Dom34:Hbs1-mediated degradation of 20S-containing small subunits, suggesting that the strains mutant for 40S production could potentially provide important new information about 18S NRD. Specifically, the data implies that the subcellular localization of small ribosomal subunits in maturation deficient strains, would correspond to the location where NRD takes place. Perhaps this compartment is simply P-bodies, as previously observed, or perhaps P-bodies are a downstream location. One alternate possibility is that these 20S rRNAs could be present in the vacuole in the same manner observed in ribophagy. Another appealing possibility is that these strains could be used to look for spontaneous suppressors. Presumably, the up-regulation of 18S NRD proteins could lead to faster growth, allowing for a selection mechanism. Alternatively, it might be possible to use an analogous process to find large subunit defects that led to an analogous, translation-dependent process for the large subunits. Along these same lines, the Bhattacharya paper suggests that RplX haploinsufficient might also represent an equally facile tool.

Implications of 18S NRD in protecting the cell from antibiotics and damaging agents:

Though we are not the first group to discover that NRD protein deficient strains are sensitive to genotoxic stress, we are the first to combine these previously decoupled deletions to show that they produce a synthetic effect. The sensitivity to 4NQO observed in dom34Δasc1Δ suggests that Dom34:Hbs1, could potentially be directly protecting the cell from 4NQO mediated RNA damage. Though 4NQO should also damage DNA, Dom34:Hbs1 has not been shown to have activity within the nucleus. Moreover, it’s well characterized activities in NRD,
NGD, and NSD suggest that its synthetic effect with ASC1 deletion in protection from 4NQO likely represents a defect in an RNA degradation pathway. Yet, barring direct evidence that 4NQO damages RNA, and that it is the RNA damage, rather than DNA damage, that is the causative agent behind the increase in cell death in dom34Δasc1Δ, we cannot draw any hard conclusions regarding the mechanism by which NRD protects the cell from 4NQO.

The sensitivity disparity between dom34Δasc1Δ and hbs1Δasc1Δ is also interesting. One potential explanation for this difference is the continued activity of Dom34 in the absence of Hbs1. In vitro, the loss of Hbs1 can be compensated for by increasing the concentration of the Dom34 homologue, Pelota (Pisareva et al., 2011). This suggests that the same may be true in vivo, potentially leading to a compensatory up-regulation of the DOM34 gene or simply residual activity by the Dom34 protein.

Also of interest is the fact that NRD deficient cells are not susceptible to increased killing by oxidative or alkylating damaging agents. This lack of effect suggests that 18S NRD may not be capable of detecting or degrading oxidized or alkylated RNAs. Instead, NRD may only function to detect specific chemical modifications such the bulky lesions produced by 4NQO.
Chapter 4:
Prospectus

When this phase of the 18S NRD project was initiated, the role of Dom34:Hbs1 in NRD and NGD had already been identified. Despite their involvement, it was clear that other proteins contribute to 18S NRD, due to the continued degradation of mutant 18S:A1492C rRNAs in the absence of either protein. We were particularly interested in finding the other NRD proteins because we wanted the ability to completely shut off the pathway. We anticipated that NRD deficient cells might show phenotypic effects, such as sensitivity to chemicals that damage nucleic acids. The identification of such phenotypes could potentially allow us to identify endogenous functions and targets of the NRD pathway. To find the additional NRD proteins, we tested a number of candidate gene deletion strains for loss of 18S NRD.

Initial attempts to find new 18S NRD factors focused on the two dubious ORFs that represent the original DOM34 gene on chromosome XIV. These two ORFs, YCL001W-A and -B, are homologous to the 5` and 3` portions of the DOM34 gene, suggesting that they might function in 18S NRD. To test this possibility, we examined mutant 18S:A1492C stability via timecourse and single timepoint assays, in strains ycl001w-AΔ, ycl001w-bΔ, and in the double mutant strains ycl001w-AΔdom34Δ, ycl001w-bΔdom34Δ. We did not observe any stabilization of the mutant 18S rRNAs in the single ycl001WΔ strains, or any synthetic effect when they were combined with dom34Δ, arguing against any role for these two genes in NRD.

We also tested the role of two ribosomal small subunit proteins, Rps27a and Rps27b, for effects in NRD. These two proteins were implicated in a NGD like process in plants, suggesting that they might be involved in NRD/NGD in yeast (Casati et al., 2004). Though we did not
observe any stabilization of mutant 18S rRNAs in the absence of either protein, we did notice that WT 18S rRNAs were low in abundance in the rps27bΔ strain, which has a slow growth rate. Further loss of DOM34 in this strain led to a partial rescue of the WT 18S rRNA levels, but also made the cells grow slower still. These data support the conclusions of Bhattacharya et al. which indicated the same genetic interaction between DOM34 and RPS27B, as well as the synthetic sickness of the rps27bΔdom34Δ strain.

A simple model that could explain this data: In the absence of RPS27B, many of the cell's 40S subunits could be improperly/incompletely manufactured. These immature small subunits could be detected by Dom34, via the 18S NRD pathway, leading to their clearance from the cell, and decreasing cell sickness due to translation problems. A recent report by Soudet et al. 2010, described a similar role for Dom34 in the clearance of incompletely manufactured small subunits which attempt initiation. Our data on RPS27B supports the Soudet et al. data set, providing additional evidence that one endogenous function for Dom34, and by extension, Hbs1, is the deletion of improperly made small subunits. Interestingly the phenotypic rescue of the rps27bΔ strain by DOM34 deletion, was partial, just as we had seen before in our 18S NRD assays. This again suggested that a second, as yet unidentified protein, was deleting the 18S rRNA component of small subunits.

**The role of Asc1 in NRD**

We later learned of the involvement of Asc1 (Rack1) in a NRD related process - PDTA. The previously described involvement of Dom34 in PDTA, as well as the newly discovered role for Asc1, suggested that Asc1 was also likely to be involved in 18S NRD. Accordingly, our
results show that Ascl promotes 18S NRD, and that it acts in parallel with Dom34. The lack of NRD observed in the absence of both protein suggests that Ascl represents the other half of the 18S NRD pathway.

Ascl is a eukaryote specific ribosomal protein involved in a multitude of processes in the cell. In a number of pathogenic organisms, Rack1 function is required for virulence. It is also involved in general translation, the selective translation of a number of different transcripts, ubiquitin mediated proteolysis, signal transduction, and the small subunit rRNA turnover we observe in 18S NRD. The activities of Ascl in translation and its localization to the ribosome suggest that it would function while ribosome-bound in 18S NRD. Despite our expectation, ASC1 mutations shown to decrease the association of Ascl with the ribosome in polysome profiles, had no negative effect on NRD. In fact, single timepoint analysis suggests that these mutations could even promote Ascl activity. These data suggest that Ascl functions off the ribosome in NRD.

We gathered additional clues about the mechanism of Ascl activity by mutating two other putative regulatory domains - deletion of the ribosome facing "knob" and the Y to F mutation of the two proposed sumoylation sites. The knob deletion was shown to be irrelevant for ribosome binding activity, suggesting that decreased ribosome association is not the reason for this potential gain of function activity. However, the decrease in mutant 18S rRNA levels observed in the presence of the two ASC1 tyrosine mutants suggests that regulation of Ascl by sumoylation, or another ubiquitin like modifier, may occur.

Studies on the mammalian homologue of Ascl, Rack1, indicated the ability of Rack1 to dimerize (Yatime et al., 2011). However, the residues responsible for Rack1 dimerization are not
conserved in yeast, precluding our ability to test the effects of Asc1 dimerization mutants on 18S NRD. Following the completion of this work, the existence of Asc1 homodimers was confirmed, and two crystal structures were determined (Yatime et al., 2011). Based upon these structures, the homodimerization process was proposed to be mediated by, or require, His147. As such, the future testing the ability of ASC1:H147 mutant to promote NRD will likely provide important information about the mechanistic basis of Asc1 involvement.

The roles of Ubc4 and Doa1 in NRD

The initial data suggesting Asc1 involvement in NRD set up the expectation that one of the previously reported Asc1 binding partners could facilitate Asc1’s activity in NRD. We therefore looked for factors known to physically associate with Asc1 and screened a number of them for effects on NRD. This led to the discovery of Ubc4, and Doa1, two Asc1 binding factors implicated in PDTA and Ribophagy. The identification of these two proteins strengthened the growing connections between PDTA, NRD, and the other translation surveillance pathways NGD, and NSD. All of these pathways are facilitated by Dom34:Hbs1, and growing evidence suggests that the associated nascent peptides are also targeted for degradation via the ligation of a ubiquitin tag. Hence, what is true for one pathway is often true for the others. This has led to the suggestion that these pathways may, to a large extent, be coupled.

The implication that Doa1, which specifically affects large subunit ribophagy, is involved in small subunit rRNA turnover suggests that Doa1 may also exert its influence on 18S NRD via interaction with the large subunit. This is certainly not unreasonable given the requirement for large subunit recruitment prior to 18S:A1492C detection, as well as the A-site binding activity of Dom34:Hbs1. Moreover, the Bhattacharya et al. dataset suggest that Ubc4, Asc1, and Doa1 all
promote cell growth, specifically during large subunit ribosomal protein haploinsufficiency. Hence, it is possible that certain NRD proteins could function through large subunit interactions.

In considering 60S involvement in NRD, it is important to note that a translation dependent 25S rRNA decay pathway, analogous to 18S NRD, has not yet been detected. It is entirely possible that such a pathway exists. An equally intriguing possibility, given what we know about Asc1, Doa1, and Ubc4, is that the large subunit might be degraded via 18S NRD when it becomes paired with a nonfunctional small subunit. In the future, it will be important to determine if this occurs.

Similar to the possibility that a mutant 18S-containing small subunit could prompt the degradation of the associated large subunit, it is also possible that a mutant 18S containing small subunit could promote the degradation of the entire complex, including the nascent peptide, mRNA, large subunit, and tRNA. Moreover, ribosomes upstream of a stalled 80S complex would eventually encounter the stalled ribosome, leading to the production of additional stalled complexes (Akimitsu, Tanaka, & Pelletier, 2007). This suggests that a single ribosome stalling event could promote the NRD-mediated degradation of all of the associated upstream ribosomes. Among this group of potential secondary targets, the degradation of the associated tRNA is a particularly appealing. One previous report demonstrated that Dom34:Hbs1 triggers tRNA-peptide release rather than the separate release of the two which normally occurs during cannonical termination (Shoemaker et al., 2010). If the nascent peptide is subject to selective degradation as part of 18S NRD the way it is in PDTA and NSD, this mechanism could also clear the associated tRNA from the cell. (Conversely, this also suggests that tRNA-mediated translational stalling could be physiologically relevant, and that Dom34:Hbs1 might be responsible for the deletion of such a molecule.) As such, a global translation complex
degradation mechanism would preclude the need to evolve independent quality control devices capable of distinguishing between an mRNA/tRNA/rRNA/ribosomal protein/peptide mediated stall. Hence, the data suggesting significant overlap between NRD, NGD, PDTA and NSD, pathways certainly seem to make logical sense in that it suggests a relatively simple means for dealing with improperly functioning translation complexes. Nevertheless, several lines of evidence have demonstrated that NSD, NDG, and NRD can all be decoupled, indicating that discreet detection and degradation mechanisms do exist (Pisareva et al., 2011; D. Schaeffer & Hoof, 2011). Together, these observations would seem to suggest that an indiscriminate global translation complex degradation process is unlikely, and that instead, combinations of translational stall induced tRNA/mRNA/rRNA/nascent peptide/ribosomal protein degradation events may overlap in more subtle ways.

Following our analysis of Asc1, we tested the roles of Ubc4 and Doa1 in NRD, by crossing the two respective deletion strains to either dom34Δ or asc1Δ, which appear to represent separate halves of the NRD pathway. Timecourse assays reveals that in the absence of UBC4, 18S NRD is not detectible over a 6 hour period, and that the additional loss of DOM34, or ASC1, does not alter this apparent complete stabilization. The loss of mutant 18S degradation in the ubc4Δ strain suggests that Ubc4 is essential for 18S NRD. Loss of DOA1 however, produced a partial stabilization of mutant 18S rRNAs, suggesting that it functions in parallel with other factors. The synthetic stabilization observed in doa1Δasc1Δ but not doa1Δdom34Δ, indicates that Doa1 functions with Dom34, and in parallel with Asc1. The phenotypes were similar to the rate of NRD, and degree of mutant 18S stabilization, observed in the dataset comparing the rate of NRD in dom34Δ, asc1Δ and dom34Δasc1Δ. As such, we developed a model for 18S NRD in which Dom34:Hbs1 functions with Doa1 in one branch of the 18S NRD pathway, opposite Asc1.
Ubc4 either functions in both branches of the pathway, or at a different level where the NRD pathway exists as a single branch. Interestingly, under this configuration there are WD40 repeat proteins on both sides of the pathway, raising the possibility that Doa1 and Asc1 could have similar functions.

**Implications of Ubiquitin Involvement in NRD**

Though we established a general framework by which the NRD proteins promote the degradation of mutant 18S rRNAs, we did not determine the mechanistic basis for their involvement. However Asc1, Doa1 and Ubc4 have all been the subject of other studies, allowing us to speculate on the means by which they could promote NRD. With the exception of Dom34 and Hbs1, all of the NRD proteins have been shown to function as part of the cellular ubiquitin systems. Rack1 promotes ubiquitin mediated proteolysis in human cells, Doa1 possesses two ubiquitin binding domains, and Ubc4 directly ligates ubiquitin onto protein targets in its role as an E2 ubiquitin conjugase (Pashkova et al., 2010; Ruan et al., 2009; Stoll, Brzovic, Davis, & Klevit, 2011). The target(s) of Ubc4-mediated ubiquitination event(s) are currently unknown, though ribosomal proteins are obvious candidates. One possibility is that Asc1 itself is ubiquitinated. Future characterization of the 18S NRD pathway should include the identification of the target proteins. The method used in Fujii et al. 2009 could be instrumental in this regard. Briefly, they expressed FLAG-tagged ubiquitin in WT cells, and cells deficient for an E3 ubiquitin ligase implicated in ribosomal protein ubiquitination in 25S NRD. They isolated ribosomes from cells expressing WT or mutant 25S rRNAs and ran western blots against FLAG in order to detect ubiquitinated ribosomal proteins. Using this method, they identified two
distinct bands at roughly 40 and 50 kd. Mass spectrometry analysis could potentially identify such proteins in an analogous experiment to identify ribosomal proteins that are ubiquitinated during 18S NRD (Fujii, Kitabatake, Sakata, Miyata, & Ohno, 2009).

The role of Ubc4 as an E2 conjugase, is well established, suggesting that it functions in the same role in NRD. Ubc4, like other E2 conjugases, binds a number of E3 ubiquitin ligases, including Pex2, Not4, and the essential protein Rsp5. We did not observe any loss of 18S NRD in the absence of NOT4, or PEX2, increasing the likelihood that Rsp5, as the only remaining E3 ligase reported to interact with Ubc4, is indeed the E3 that works with Ubc4 in NRD. Previous studies have already implicated Rsp5 in affecting ribosome stability. Though the Scherbik et al. 2011 dataset indicated that Rsp5 promotes ribosome stability, which would argue against a role for Rsp5 in ribosome destabilization, their data do not preclude the possible that Rsp5 could also function to destabilize ribosomes in NRD by ubiquitinating a different target on the ribosome. It is possible to test the involvement of Rsp5 in NRD in two ways: 1) Assay the ability of UBC4 RING or HECT binding mutants to complement ubc4Δ for its ability to conduct NRD (Stoll et al., 2011). 2) Test the effects of a dominant negative Rsp5 protein on the steady state levels of mutant or WT 18S rRNAs over time (Shcherbik et al., 2011).

A potential role for Rps3 in NRD

Though the 18S NRD pathway includes parallel, independently functioning branches, it is possible that both branches could be coordinately regulated. The recently released crystal structures of the eukaryotic ribosome, and the bound Dom34:Hbs1 heterodimer, have provided
insight into the potential role for Rps3, which touches both Hbs1 and Asc1 (Becker et al., 2011; Ben-Shem et al., 2010). Rps3 is positioned at the mouth of the mRNA channel, and interacts directly with the mRNA. Given these three interactions, Rps3 is perfectly situated for sensing ribosome translocation along the mRNA, and to regulate both Asc1 and Hbs1. Moreover, it appears that the length of mRNA sequence present downstream of the P-site of a stalled ribosome determines whether or not Dom34:Hbs1 will dissociate the subunits (Pisareva et al., 2011). The addition of more than 9 nucleotides will simultaneously position the mRNA 5′ end in the vicinity of Rps3, and lead to the inhibition of Dom34:Hbs1 mediated dissociation of the ribosomal subunits. (Importantly, thought the effect of downstream mRNA length on subunit dissociation is known, its effect on rRNA degradation is not.) This raises the possibility that normal and Non-Stop mRNAs could be distinguished by Rps3, leading to the activation or inhibition of Dom34:Hbs1-mediated ribosomal subunit dissociation. Whether Rps3 actually possesses these activities remains to be seen. To test the possible regulation of Asc1 by Rps3, we attempted to remove the long c-terminal tail of Rps3. Despite the detection of recombination by our truncated RPS3 construct at the RPS3 locus, and confirmation that our PCR product encodes the truncated form of the RPS3 gene as expected, all of the recombinant strains still possessed the wild type RPS3 gene. This suggests that truncation of the RPS3 tail is harmful to the cell. We also attempted this truncation in an asc1Δ strain to avoid the possibility that misregulation of Asc1 in a truncated RPS3 strain was leading to toxicity. Again, we were not able to truncate the RPS3 gene, raising the possibility that its c-terminal tail is necessary for stability of the small subunit. In the future, an alternative approach could be attempted: instead of truncating RPS3, the N-terminal domain of Hbs1, or the WD4 wedge of Asc1 could be removed. These truncations would have the effect of ablating contacts with Rps3 and should not affect cell
viability. However, the major alterations in the structures of Asc1 and Hbs1 could simply lead to widespread misfolding and/or proteolysis. Hence these experiments would serve primarily to allow a positive result to disprove the Rps3 interaction hypothesis.

**The potential function of NRD in the clearance of damaged RNAs**

Though the 18S NRD pathway is incompletely characterized, we were able to reduce the activity of the off the NRD pathway to undetectable levels by deleting both DOM34 and ASC1, or by deleting UBC4. This allowed us to begin testing our hypothesis that one of the functions of 18S NRD is to rid the cell of damaged RNAs. We hypothesized that chemically damaged bases in an mRNA could lead to translational stalling, necessitating intervention by NRD/NGD, and leading to the removal of the mRNA. As such, we expected that NRD deficient strains would show sensitivity to genotoxic chemicals shown, or expected to damage RNA. Our results showed that dom34Δasc1Δ cells were synthetically sensitive to 4-nitroquinoline, but unexpectedly, they were not sensitive to alkylation, or oxidation. Though 4-nitroquinoline may be capable of damaging DNA, protein, and lipids, in addition to RNA, the established activity of Dom34 on translation surveillance suggests that the synthetic sensitivity seen in its absence is due to damaged translation components that would otherwise be remediated by NRD/NGD/NSD. Further studies are needed to confirm and characterize the effects of 18S NRD on the clearance of 4-nitroquinoline-damaged RNAs.

As a whole, these studies serve to update our understanding of the 18S NRD pathway in terms of the factors that promote it, and the degree to which NRD overlaps with the other ribosome degradation and translation surveillance pathways. We now know that ubiquitin
ligation is likely to play a part in small subunit degradation, as it does in 25S NRD, ribophagy, and the degradation of aberrant nascent peptides. Our model for 18S NRD suggests a regulatory role for an additional protein, Rps3, which could function as a sensor of ribosome stalling, and as a regulator of both branches of the NRD pathway. We also know that the Ribophagy pathway has implications for NRD, raising the possibility that NRD substrates may localize to the vacuole. Finally, we offer preliminary evidence that NRD could function as a detection and degradation mechanism that rids the cell of damaged RNAs. These data offer tantalizing clues about a number of functions for NRD, and suggest a direction for future studies on general ribosome function, and translation quality control.
Appendix:

Global ribosome footprinting analysis in a upf1Δ strain

Introduction

In eukaryotes, translation is monitored by the Nonsense Mediated Decay (NMD) pathway which serves as a post-transcriptional mechanism for regulating gene expression, and also as a quality control device that degrades mRNAs encoding a termination codon in an unfavorable context (Amrani et al., 2006). For example, DNA mutations or transcription errors may produce premature termination codons (PTCs) upstream of an mRNA's standard termination codon. Such transcripts are detected by NMD. Following detection, such transcripts are translationally silenced, then degraded (Muhlrad et al. 1999). In its role as a modifier of gene expression, NMD may also repress the translation of up to 3-10% of the transcripts produced in S. cerevisiae (He et al., 2003; Lelivelt et al., 1999). This regulatory activity of NMD occurs both directly, and also indirectly, by modulating the translation of regulatory factor mRNAs. Together, these activities of NMD regulate the expression of a large percentage of the yeast genome, and also protects the cell from the potentially deleterious effects of truncated peptides that would otherwise be produced by PTC containing transcripts (Rebbapragada et al., 2009).

NMD is promoted by a number of proteins including Upf1, Upf2, and Upf3, as well as the Smg proteins (suppressor of morphological defects on genitalia) Smg1, Smg5, Smg6, and Smg7 (Czaplinski et al. 1995; Kashima et al., 2006; Singh et al., 2008). The Upf proteins represent the functional core of the NMD machinery. All three proteins, Upf1, Upf2, and Upf3
are essential for NMD in yeast (Culbertson et. al, 2003; Czapinski et al., 1995; Leeds et al., 1991). Smg1 phosphorylates Upf1 at several different locations, altering its activity, and creating binding sites for Smg5:Smg7 (Okada-Katsuhata et al., 2011). These events precipitate two major downstream consequences for the mRNA: 1) the Smg6-mediated endonucleolytic cleavage of the mRNA, and 2) the deadenylation, decapping, and Xrn1-mediated exonucleolytic decay of the mRNA. These two outcomes may not be decoupled (Nicholson et al., 2010).

In yeast, NMD is activated or repressed on a given mRNA through a competition between Upf1, and Poly(A) binding protein (PABP) (Singh et al., 2008). During canonical translation termination, the release factors eRF1:eRF3 bind the ribosomal A-site, recognize the stop codon, and promote ribosome release. When a PTC is present, the downstream sequence previously representing the 3’ portion of the open reading frame becomes incorporated into the 3’ UTR, and increases the distance between the terminating ribosome and PABP. This is thought to lead to a decrease in the interaction between PABP and eRF3, and allow for increased association between eRF3 and Upf1. This has the effect of stimulating NMD (Singh et al., 2008).

NMD also has effects on the ribosome and general translation. In vitro, the Upf proteins have been shown to promote the downstream reinitiation of ribosomes that reach a PTC. Conversely, Upf1 has been shown to repress translation of a PTC containing mRNA, presumably as an intermediate step before mRNA degradation (Ghosh et al., 2010). In a third study, Upf1 appears to be important for the effective translation of non-abberant transcripts (Muhlrad & Parker, 1999).

Substrate definition is of particular interest to the field due to the observed effects of the NMD proteins on both PTC containing and wild type transcripts. Examples of both wild type and
PCT containing transcripts have been shown to increase in abundance when NMD is ablated (Johansson et al., 2007). The effects on certain wild type genes may in fact represent indirect effects of UPF1 loss rather than loss of degradation by NMD (He et al., 2003; Johansson et al., 2010). In yeast, UPF1 loss can yield indirect effects on translation through an increase in the level of Alr1 protein which serves as a Mg++ transporter. The added Alr1 proteins cause an increase in cytoplasmic Mg++ concentration, thereby affecting canonical termination by the ribosome (Johansson et al., 2010).

Given the ability of NMD to regulate gene expression both directly and indirectly, it is not surprising that the NMD machinery has been implicated in a variety of genetic diseases, and certain forms of cancer (Frischmeyer et al., 1999; Ionov et al., 2004). In human cells, NMD plays a part in regulating the levels of the spliceosomal protein SC35 (Sureau et al., 2001). If SC35 levels are increased, this splicing regulator promotes its own alternative splicing, leading to the formation of an SC35 transcript that contains a PTC. This splice variant is then targeted by NMD, bringing down the levels of the protein, and restoring the normal splicing pattern (Sureau et al. 2001). Certain reports have also indicated that the protein Tdp-43, a gene which is implicated in both alternative splicing and the onset of amyotrophic lateral sclerosis (ALS), is also regulated by this mechanism (Dreumont et al., 2010; Sureau et al., 2001). In certain forms of cancer, a properly functioning NMD system can sometimes promote oncogenesis (Karam et al., 2008). In the example of gastric cancer, a large number of germ line mutations in the gene encoding the tumor suppressor protein E-cadherin, can lead to the formation of nonsense codons. The resulting transcripts are targeted for degradation by NMD, suggesting that NMD could be inhibited as a form of therapy (Karam et al., 2008). Accordingly, drugs that promote the read through of PTCs, including the aminoglycoside antibiotics, are expected to represent good
candidates for bypassing NMD mediated quality control, and thereby treating certain illnesses (Floquet et al., 2011).

The literature has demonstrated effects of the NMD pathway on both PTC containing and normal transcripts, leading to physiologically relevant outcomes in both humans and yeast. The ability of Upf1 to affect ribosome translocation, or to repress translation of certain mRNAs, also suggests that Upf1 has functions outside of its promotion of mRNA degradation. These subtle, variegated effects on ribosome function have made it difficult to clearly define the full behavior of Upf1. Here we investigate for the first time, the effects of UPF1 loss on global ribosome positioning in *S. cerevisiae*.

The UPF1 family of helicases also includes the protein Mtt1, which has been shown to bind to eRF1:eRF3 and to associate with polysomes, similar to the observed behavior of Upf1 (Czapinski et al., 2000). When overexpressed, Mtt1 suppresses nonsense read through, suggesting again that like Upf1, Mtt1 may have a role in termination (Czapinski et al., 2000). Moreover, mtt1Δ cells are sensitive to hygromycin B, which negatively affects the proofreading function of the ribosome, potentially allowing for misincorporation of tRNA into the P-site, and triggering additional proofreading functions that could halt ribosome elongation (Czapinski et al., 2000). Despite these similarities between Mtt1 and Upf1, no mRNAs have been shown to either increase or decrease in abundance in the absence of MTT1, arguing against a role in mRNA decay. To date, its function in the cell has not yet been full determined. Below we present an investigation into the effects of Mtt1 on global ribosome position in *S. cerevisiae*. 
Results:

In the absence of UPF1, a subset of genes display both decreased overall ribosome occupancy with preferential start codon occupancy. (Preliminary Data)

To elucidate the effects of Upf1 on translation with greater clarity, we conducted global ribosome footprinting in *S. cerevisiae*. Global ribosome footprinting allows us to determine the position of a large number of the ribosomes in the cell, leading to the production of a highly accurate depiction of the translational profile for a given strain. We developed a global ribosome footprinting model for a WT, upf1Δ, and mtt1Δ strains, allowing us to identify disparities between the ribosome positions in these strains. These data were produced using an N of 1 for each of the three strains tested, and have yet to be repeated. As such, this data set should be regarded as preliminary evidence.

To accomplish this, wild type and upf1Δ cultures were grown to mid-log in YPD medium and treated with 100 µg/ml cycloheximide (CHX). Cells were then harvested by vacuum filtration on a nylon membrane. Dry cells were then resuspended in a small volume of lysis buffer containing CHX, and flash frozen in liquid nitrogen. Frozen cell suspensions were then lysed on a Retsch ball mill for 3 min at 15 Hz, in canisters cooled in liquid nitrogen. 70 units of RNase1 was added, and lysates were incubated at room temperature for 1 hour. RNAses digestion serves to cleave cellular RNAs, including the mRNA fragments exposed between translating ribosomes (Ingolia et al., 2009). This collapses polysomes into monosomes, and produces the roughly 30 nucleotide mRNA fragments representing the "footprint" of each ribosome in the cell (Ingolia et al., 2009). RNase-treated lysates were then layered on 5-50%
sucrose gradients, and spun at 35k rpm for 2:40 at 4°C. Sucrose gradients were separated into 0.5 ml fractions on a fraction collector, and simultaneously analyzed in real-time by UV-vis. A$_{260}$ measurements were graphed with the corresponding fractions to allow for the identification of individual ribosomal subunits and 80S monosomes within the collected fractions. To isolate ribosome footprints, only the fractions corresponding to the monosome peaks were pooled and subjected to phenol:chloroform extraction and ethanol precipitation. Total RNA preps were then passed over a YM-10 microconcentrator column to decrease the sample size. The resulting samples were then subjected to RNaseH digestion using oligos complementary to rRNA fragments previously observed to be at high abundance in previous libraries. Following another phenol:chloroform extraction to remove the enzyme and buffer, samples were separated on 15% acrylamide gels and stained with Sybr Gold. Samples at the 28-30 nt range were excised and the RNAs were extracted from the gel piece via overnight incubation at room temperature in aqueous solution in the presence of RNase inhibitor. The gel pieces were removed by passing the sample over a Corning Spin-x column, leading to isolation of the final RNA samples. T4 polynucleotide kinase was used to remove phosphate from the 3` end of sample RNAs, and also to transfer phosphate from ATP to the 5` end of the RNAs in the sample. 1 ul from each pre-treated RNA sample was separately treated with radiolabelled phosphate, and separated on a 15% acrylamide gel along with defined quantities of radiolabelled oligo. These oligos were used as a standard to measure the amount of RNA in 1ul of each sample. From each sample, 1 ng of phosphatased and kinased RNA was used as input into Applied Biosystems Small RNA Expression Kit. This protocol entails the ligation of DNA linker oligos onto the sample RNAs, their reverse-transcription, and subsequent bar-coding to allow for multiplexing in subsequent sequencing reactions. Samples were sequenced on an Applied Biosystems SOLiD machine,
yielding 10-100 million reads. Data output in the form of bed files was then aligned to the *S.
cerevisiae* genome and uploaded into the UCSC genome browser. By comparing the histograms
derived from the bed files, we were able to directly observe the ribosome occupancy on RNAs
derived from any location in the genome.

We first attempted to validate our data set by looking for anticipated differences and
similarities between the WT and upf1Δ data sets. First, we examined the UPF1 ORF to look for
loss of ribosome occupancy in the upf1Δ strain. As anticipated, we observe ribosome occupancy
in WT and mtt1Δ data sets, but not in the upf1Δ data set, suggesting that our data set accurately
reflects dramatic differences in gene expression, and does not produce detectable non-specific
signal. We also examined the ALR1 gene which is reported to increase in expression in the
absence of upf1Δ. In keeping with a previous report, we also observe a strong increase in
ribosome occupancy in ALR1 ORF, again supporting the validity and sensitivity of our assay
(Johansson et al., 2010). We then examined a number of candidate genes on each chromosome to
establish the overall similarity between the three data sets. We observe that for most genes, the
general pattern of ribosome occupancy is highly similar. When zooming the view to an entire
chromosome, the two datasets are indistinguishable for many large regions. Next, we examined
the ribosome occupancy at the CAN1 gene, which encodes a PTC in our strain - BY4741/S288c.
This *can1* allele is *can1-100*. Again, as anticipated, we observe a major difference in signal
pattern within the CAN1 ORF in the upf1Δ data set, suggesting that the loss of UPF1 is
specifically affecting a known PTC containing gene. Together, these data suggest that our data
set is valid, and that only a subset of known genes, including a known NMD substrate, are
subject to regulation by Upf1.
To detect patterns in ribosome occupancy, as well as alterations in these patterns in the absence of UPF1 or MTT1, we visually scanned each data set using the UCSC genome browser. Genes displaying differential patterns between the strains were identified and binned according to general pattern differences. The clearest pattern of change in the upf1∆ data set was dubbed "1/x" due to the resemblance the occupancy pattern bears for the graph of the equation $y=\frac{1}{x}$, in which a curved line asymptotically approaches both the $y$ and $x$ axes. The genes displaying the 1/x phenotype, also display dramatically decreased overall signal. What signal remains for these genes is predominantly at the start codon. This pattern suggests that these genes may not be translated properly in the absence of UPF1. The 1/x genes are displayed in Table A1.
Figure A1. The 1/x phenotype as demonstrated by ribosome occupancy profiles of the CAN1 gene. A. Graph of y=1/x. This shape is similar to that of the histogram for the upf1Δ data set for the CAN1 gene. B. Ribosome occupancy histogram for CAN1 in upf1Δ (top), WT (middle), and mtt1Δ (bottom). The size and orientation of the CAN1 open reading frame is depicted at the top.
C. Simplified approximation of the occupancy profiles of the CAN1 gene in upf1Δ (top), WT (middle), and mtt1Δ (bottom) strains. D. Ribosome occupancy of the REI1 gene demonstrating the 1/x phenotype in the upf1Δ and mtt1Δ strains (left). Occupancy approximations are shown on the right.

<table>
<thead>
<tr>
<th>SSH1</th>
<th>POP6</th>
<th>MDM34</th>
<th>PRM5</th>
<th>GAA1</th>
<th>LYP1</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAN1</td>
<td>MUP1</td>
<td>ERG4</td>
<td>CBR1</td>
<td>YPS1</td>
<td>PSD1</td>
</tr>
<tr>
<td>YBR13W</td>
<td>ERG25</td>
<td>PMA1</td>
<td>SSM4</td>
<td>NHA1</td>
<td>YNL165</td>
</tr>
<tr>
<td>YOL007C</td>
<td>YGR106C</td>
<td>STF2</td>
<td>PHO90</td>
<td>PEP3</td>
<td>YGP1</td>
</tr>
<tr>
<td>UTP23</td>
<td>CRH1</td>
<td>YGR012W</td>
<td>YJL200C</td>
<td>MSS51</td>
<td>TOM70</td>
</tr>
<tr>
<td>PHO89</td>
<td>PCM1</td>
<td>MSB2</td>
<td>ATG27</td>
<td>FRE1</td>
<td>TCB2</td>
</tr>
<tr>
<td>CHS2</td>
<td>SPF1</td>
<td>YGR017W</td>
<td>KRE9</td>
<td>YLR241W</td>
<td>ALG11</td>
</tr>
<tr>
<td>HXT3</td>
<td>GEA2</td>
<td>POP6</td>
<td>HAL5</td>
<td>EXG1</td>
<td>DSE4</td>
</tr>
<tr>
<td>PHT2</td>
<td>YEL001C</td>
<td>YGR031W</td>
<td>KAR2</td>
<td>BUD5</td>
<td>ENB1</td>
</tr>
<tr>
<td>ROT2</td>
<td>HEM14</td>
<td>GSC2</td>
<td>AVT1</td>
<td>FKS1</td>
<td>TAT2</td>
</tr>
<tr>
<td>NUG1</td>
<td>CHO1</td>
<td>TFC4</td>
<td>GEL1</td>
<td>ORM2</td>
<td>YOL007C</td>
</tr>
<tr>
<td>ERV46</td>
<td>CAJ1</td>
<td>MUP1</td>
<td>HOC1</td>
<td>SUR4</td>
<td>TCB1</td>
</tr>
<tr>
<td>YBR159W</td>
<td>FTR1</td>
<td>ERG25</td>
<td>MSN1</td>
<td>YLR413</td>
<td>YOR093C</td>
</tr>
<tr>
<td>PCM1</td>
<td>UBP3</td>
<td>YGR106C</td>
<td>YKL207W</td>
<td>GAB1</td>
<td>LEU9</td>
</tr>
<tr>
<td>SPF1</td>
<td>FET5</td>
<td>CRH1</td>
<td>LOS1</td>
<td>ERO1</td>
<td>SEC63</td>
</tr>
<tr>
<td>YEL001C</td>
<td>CAF16</td>
<td>YHL017W</td>
<td>ZRT3</td>
<td>YML125</td>
<td>DIP5</td>
</tr>
<tr>
<td>A1</td>
<td>A2</td>
<td>A3</td>
<td>A4</td>
<td>A5</td>
<td>A6</td>
</tr>
<tr>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>HEM14</td>
<td>STE2</td>
<td>MRP4</td>
<td>RRN3</td>
<td>VAN1</td>
<td>RBD2</td>
</tr>
<tr>
<td>CHO1</td>
<td>SMX2</td>
<td>LAG1</td>
<td>YKL077W</td>
<td>TSL1</td>
<td>YPL221W</td>
</tr>
<tr>
<td>CAJ1</td>
<td>HSP12</td>
<td>ERG11</td>
<td>YKL075C</td>
<td>OST6</td>
<td>TPK2</td>
</tr>
<tr>
<td>FTR1</td>
<td>SMC1</td>
<td>PPA1</td>
<td>LHS1</td>
<td>YML018C</td>
<td>PET20</td>
</tr>
<tr>
<td>UBP3</td>
<td>YFR016C</td>
<td>DAP2</td>
<td>YKL065C</td>
<td>HXT2</td>
<td>PEP4</td>
</tr>
<tr>
<td>FET5</td>
<td>YFR018C</td>
<td>MSC7</td>
<td>DCW1</td>
<td>ERG5</td>
<td>TIM50</td>
</tr>
<tr>
<td>CAF16</td>
<td>SHE10</td>
<td>NCP1</td>
<td>PSR1</td>
<td>FET3</td>
<td>CWC27</td>
</tr>
<tr>
<td>STE2</td>
<td>VRG4</td>
<td>KIC1</td>
<td>YII012W</td>
<td>SWP1</td>
<td>SUR1</td>
</tr>
<tr>
<td>YFR018C</td>
<td>TPN1</td>
<td>TOM71</td>
<td>SMF3</td>
<td>ERG25</td>
<td>KTR6</td>
</tr>
<tr>
<td>VRG4</td>
<td>YGL139W</td>
<td>LSM12</td>
<td>YLR050c</td>
<td>PFK2</td>
<td>SSN3</td>
</tr>
<tr>
<td>STT3</td>
<td>OLE1</td>
<td>ECM14</td>
<td>ERG3</td>
<td>ZRC1</td>
<td>YME1</td>
</tr>
<tr>
<td>MDM34</td>
<td>MIG1</td>
<td>WSS1</td>
<td>YLR065C</td>
<td>SCW10</td>
<td>YPR028</td>
</tr>
<tr>
<td>PMA1</td>
<td>SCW11</td>
<td>YIL130W</td>
<td>MEF1</td>
<td>GAS1</td>
<td>YPR085</td>
</tr>
<tr>
<td>MSB2</td>
<td>STT3</td>
<td>STH1</td>
<td>RAX2</td>
<td>EGT2</td>
<td>RPN7</td>
</tr>
</tbody>
</table>

Table A1. A comprehensive list of genes initially suspected to show the 1/x phenotype. For some of these genes, the signal to noise ratio is low due to low ribosome occupancy, raising the possibility of false positives.
The group of genes displaying the $1/x$ phenotype predominantly encode proteins that localize to the plasma membrane, mitochondria, and golgi apparatus. Many of these proteins are exported from the cell via the secretory pathway, suggesting a possible link between them. This list is reported in Table A1. Two examples showing a strong $1/x$ phenotype are Can1, and Pma1 (Fig. A1).
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Function (Copied from SGD)</th>
<th>Localization</th>
<th>Additional Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crh1</td>
<td>Chitin transglycosylase that functions in the transfer of Chitin to beta(1-6) and beta(1-3) glucans in the cell wall, similar and functionally redundant to Utr2; localizes to sites of polarized growth; expression induced by cell wall</td>
<td>Cellular bud neck</td>
<td></td>
</tr>
<tr>
<td>Yor107c</td>
<td>Involved in chitin synthesis</td>
<td>Cellular bud neck</td>
<td>Vacuole</td>
</tr>
<tr>
<td>Chs2</td>
<td>Chitin synthase II, involved in chitin synthesis. Localization regulated by Cdk1 during mitosis. Involved w/contractile ring</td>
<td>Cellular bud neck</td>
<td></td>
</tr>
<tr>
<td>Fant1</td>
<td>Essential N-acetylglucosamine-phosphate mutase; converts GlcNAc-6-P to GlcNAc-1-P, which is a precursor for the biosynthesis of chitin and for the formation of N-glycosylated mannosidases and glycosylphosphatidylinositol anchors.</td>
<td>Nucleus</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>Ybr139w</td>
<td>Serine carboxypeptidase</td>
<td>Vacuole</td>
<td></td>
</tr>
<tr>
<td>Fet5</td>
<td>Multicopper oxidase, integral membrane protein with similarity to Fet3p; may have a role in iron transport</td>
<td>Vacuole membrane (integral)</td>
<td></td>
</tr>
<tr>
<td>YGR106C</td>
<td>Endoplasmic reticulum protein that functions, together with other assembly factors, in assembly of the V0 sector of the vacuolar ATPase (V-ATPase); null mutation enhances the V-ATPase deficiency of a vma21 mutant impaired in ER retrieval</td>
<td>Vacuole membrane</td>
<td>ER membrane</td>
</tr>
<tr>
<td>Caf1G</td>
<td>Part of evolutionarily-conserved CCR4-NOT regulatory complex; contains single ABC-type ATPase domain but no transmembrane domain; interacts with several subunits of Mediator</td>
<td>Cytoplasm</td>
<td></td>
</tr>
<tr>
<td>Ubp3</td>
<td>Regulate anterograde and retrograde transport between endoplasmic reticulum and Golgi compartments; inhibitor of gene silencing; cleaves ubiquitin fusions but not polyubiquitin</td>
<td>ER</td>
<td></td>
</tr>
<tr>
<td>YEL001C</td>
<td>Putative protein of unknown function; green fluorescent protein (GFP)-fusion localizes to the ER; YEL001C is non-essential; null mutant displays increased levels of spontaneous Rad52p foci</td>
<td>ER</td>
<td></td>
</tr>
<tr>
<td>Erv46</td>
<td>&quot;ER vesicle #46&quot; Protein localized to COPI-coated vesicles, forms a complex with Erv41p, involved in the membrane fusion stage of transport. ER, integral to ER membrane, golgi membrane...</td>
<td>ER membrane</td>
<td>Golgi membrane</td>
</tr>
<tr>
<td>Ybr155w</td>
<td>Microsomal beta-keto-reductase; contains oleate response element (ORE) sequence in the promoter region; mutants exhibit reduced VLCFA synthesis, accumulate high levels of dithydrophosphoglycerol, phytosphingosine and medium-chain ceramides. ER, integral to membrane.</td>
<td>ER membrane</td>
<td></td>
</tr>
<tr>
<td>Sst3</td>
<td>Subunit of the oligosaccharyltransferase complex of the ER lumen, which catalyzes asparagine-linked glycosylation of newly synthesized proteins; forms a subcomplex with Ost5p and Ost8p and is directly involved in catalysis</td>
<td>ER membrane</td>
<td></td>
</tr>
<tr>
<td>Ygr106c</td>
<td>Endoplasmic reticulum protein that functions, together with other assembly factors, in assembly of the V0 sector of the vacuolar ATPase (V-ATPase); null mutation enhances the V-ATPase deficiency of a vma21 mutant impaired in ER retrieval</td>
<td>ER membrane</td>
<td>Vacuole membrane</td>
</tr>
<tr>
<td>Rot2</td>
<td>Glucosidase II catalytic subunit required for normal cell wall synthesis; mutations in rot2 suppress tor2 mutations, and are synthetically lethal with rot1 mutations.</td>
<td>ER Lumen</td>
<td>Mitochondrion</td>
</tr>
<tr>
<td>Spf1</td>
<td>P-type ATPase, ion transporter of the ER membrane involved in ER function and Ca2+ homeostasis; required for regulating Hmg2p degradation; confers sensitivity to a killer toxin (SMK7) produced by Picnia farinosa K1. Goi1, ER, mitochondrion.</td>
<td>ER</td>
<td>Mitochondrion</td>
</tr>
<tr>
<td>Erg25</td>
<td>C4 methylsterol oxidase, catalyzes the first of three steps required to remove two C4 methyl groups from an intermediate in ergosterol biosynthesis; mutants accumulate the sterol intermediate 4,4-dimethylysterol</td>
<td>ER</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Location</td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Vrg4</td>
<td>Golgi GDP-mannose transporter; regulates Golgi function and glycoprotein synthesis</td>
<td>Mitochondrion</td>
<td></td>
</tr>
<tr>
<td>Ssh1</td>
<td>Mitochondrial matrix chaperone.</td>
<td>Mitochondrion</td>
<td></td>
</tr>
<tr>
<td>Mdm34</td>
<td>Mitochondrial component of the ERN5 complex that links the ER to mitochondria and may promote inter-membrane exchange as well as coordinating mitochondrial DNA replication and growth</td>
<td>Mitochondrial outer membrane</td>
<td></td>
</tr>
<tr>
<td>Rot2</td>
<td>Glucosidase II catalytic subunit required for normal cell wall synthesis; mutations in rot2 suppress tor2 mutations, and are synthetically lethal with rot1 mutations. ER, ER lumen, mitochondrion.</td>
<td>Mitochondrion</td>
<td></td>
</tr>
<tr>
<td>Spf1</td>
<td>P-type ATPase, ion transporter of the ER membrane involved in ER function and Ca^{2+} homeostasis, required for regulating Hsp26 degradation, confers sensitivity to a killer toxin (SMKT) produced by Pichia fermenta KK1. Golgi, ER, mitochondrion.</td>
<td>Mitochondrion</td>
<td></td>
</tr>
<tr>
<td>Utp23</td>
<td>Essential nuclear protein, component of processome for 40S ribosomal subunit.</td>
<td>Mitochondrion</td>
<td></td>
</tr>
<tr>
<td>Hem14</td>
<td>Protoporhyrinogen oxidase, a mitochondrial enzyme that catalyzes the seventh step in the heme biosynthetic pathway, converting protoporhyrin IX to protoporphyrin IX, inhibited by diphenyl ether-type herbicides. Mitochondrial inner membrane.</td>
<td>Mitochondrion inner membrane</td>
<td></td>
</tr>
<tr>
<td>Cho1</td>
<td>Phosphatidylserine synthase, functions in phospholipid biosynthesis; catalyzes the reaction CDP-diacylglycerol + L-serine = CMP + L-1-phosphatidylserine, transcriptionally repressed by myo-inositol and choline.</td>
<td>Mitochondrion outer membrane</td>
<td></td>
</tr>
<tr>
<td>Utp23</td>
<td>Essential nuclear protein, component of processome for 40S ribosomal subunit.</td>
<td>Nucleus</td>
<td></td>
</tr>
<tr>
<td>Nup1</td>
<td>GTPase that associates with nuclear 60S pre-ribosomes, required for export of 60S ribosomal subunits from the nucleus. Prenosome large subunit precursor.</td>
<td>Nucleus</td>
<td></td>
</tr>
<tr>
<td>Pop6</td>
<td>Subunit of both RNase MRP, which cleaves pre-rRNA, and nuclear RNase P, which cleaves tRNA precursors to generate mature 5’ ends.</td>
<td>Nucleus</td>
<td></td>
</tr>
<tr>
<td>Pcm1</td>
<td>Essential N-acetylglucosamine-phosphate mutase, converts GlcNAc-8-P to GlcNAc-1-P, which is a precursor for the biosynthesis of chitin and for the formation of N-glycosylated mannoproteins and glycosylphosphatidylinositol anchors.</td>
<td>Nucleus</td>
<td></td>
</tr>
<tr>
<td>Caj1</td>
<td>Nuclear type II J heat shock protein of the E. coli onaj family, contains a leucine zipper-like motif, binds to non-native substrates for presentation to Ssa3p, may function during protein translocation, assembly and disassembly. Mitochondrial genome maintenance abnormal. Protein folding involved.</td>
<td>Nucleus</td>
<td></td>
</tr>
<tr>
<td>Gene</td>
<td>Function and Reported Localizations</td>
<td>Localization</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>---------------------------------------------------------------------------------------------------</td>
<td>-----------------------------------</td>
<td></td>
</tr>
<tr>
<td>Hxt3</td>
<td>&quot;Hexose transporter&quot; Low affinity glucose transporter of the major facilitator superfamily, expression is induced in low or high glucose conditions.</td>
<td>Plasma membrane</td>
<td></td>
</tr>
<tr>
<td>Flr1</td>
<td>High affinity iron permease involved in the transport of iron across the plasma membrane; forms complex with Fet3p; expression is regulated by iron.</td>
<td>Plasma membrane</td>
<td></td>
</tr>
<tr>
<td>Ste2</td>
<td>Receptor for alpha-factor pheromone, seven transmembrane-domain GPCR that interacts with both pheromone and a heterotrimERIC G protein to initiate the signaling response that leads to mating between haploid a and alpha cells</td>
<td>Plasma membrane</td>
<td></td>
</tr>
<tr>
<td>Pma1</td>
<td>Plasma membrane H+/ATPase, pumps protons out of the cell; major regulator of cytoplasmic pH and plasma membrane potential; part of the P2 subgroup of cation-transporting ATPases.</td>
<td>Plasma membrane, Mitochondrial membrane</td>
<td></td>
</tr>
<tr>
<td>Erg25</td>
<td>C-4 methyl sterol oxidase, catalyzes the first of three steps required to remove two C-4 methyl groups from an intermediate in ergosterol biosynthesis; mutants accumulate the sterol intermediate 4,4-dimethylzymosterol.</td>
<td>Plasma membrane, ER</td>
<td></td>
</tr>
<tr>
<td>Msb2</td>
<td>Mucin family member involved in the Cdc42p- and MAP kinase-dependent filamentous growth signaling pathway; also functions as an osmosensor in parallel to the Sho1p-mediated pathway; potential Cdc28p substrate.</td>
<td>Plasma membrane, integral</td>
<td></td>
</tr>
<tr>
<td>Mup1</td>
<td>High affinity melhionine permease, integral membrane protein with 13 putative membrane-spanning regions; also involved in cysteine uptake.</td>
<td>Plasma membrane, integral</td>
<td></td>
</tr>
<tr>
<td>Vrg4</td>
<td>Golgi GDP-mannose transporter; regulates Golgi function and glycoprotein modification.</td>
<td>Golgi, Mitochondrion</td>
<td></td>
</tr>
<tr>
<td>Ssh1</td>
<td>Mitochondrial matrix chaperone.</td>
<td>Golgi, Mitochondrion</td>
<td></td>
</tr>
<tr>
<td>Erv46</td>
<td>&quot;ER vesicle #46&quot; Protein localized to COPI-coated vesicles, forms a complex with Env41p; involved in the membrane fusion stage of transport. ER, integral to ER membrane, golgi membrane.</td>
<td>Golgi membrane, ER membrane</td>
<td></td>
</tr>
<tr>
<td>YFR018C</td>
<td>Putative protein of unknown function</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table A2. The 1-x genes annotated by function and reported localizations of the respective protein product. Note: Some of these genes should not be on this list. Initial characterization was
overly sensitive, leading to a number of false positives, due to a certain individual's lack of experience with the program.

To examine the possibility that l/x genes may be directly, rather than indirectly, regulated via Upf1, we cross referenced the list of l/x genes to the previously published list of mRNAs known to immunoprecipitate with the Upf1 protein, and the list of mRNAs that increased/decreased in abundance in the absence of Upf1 (He et al., 2003; Johansson et al., 2007; Maderazo et al., 2003). An annotated lists of the cross referenced genes are reported in Tables A3, A4, A5, and A6.
Comparison to the dataset reported in He et al. Mol. Cell, Vol 12, 1439-1452, December 2003. Table 2.

<table>
<thead>
<tr>
<th>RNA Type</th>
<th>Description</th>
<th>Gene Name</th>
<th>upf1Δ</th>
<th>CM Footprinting Data:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsense Containing</td>
<td>YEL063C</td>
<td>CAN1</td>
<td>9.7</td>
<td>Hit</td>
</tr>
<tr>
<td></td>
<td>YOR128C</td>
<td>ADE2</td>
<td>42.2</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>YDR007W</td>
<td>TRP1</td>
<td>18.0</td>
<td>--</td>
</tr>
<tr>
<td>Pre-mRNAs</td>
<td>YJR021C</td>
<td>MER2</td>
<td>10.1</td>
<td>Hit</td>
</tr>
<tr>
<td></td>
<td>YDL115C</td>
<td>Iwr1</td>
<td>7.2</td>
<td>Hit</td>
</tr>
<tr>
<td></td>
<td>YGL251C</td>
<td>MER3</td>
<td>5.7</td>
<td>--</td>
</tr>
<tr>
<td>uORF-containing</td>
<td>YOR302W</td>
<td>CPA1</td>
<td>5.0</td>
<td>Hit</td>
</tr>
<tr>
<td></td>
<td>YLR233C</td>
<td>EST1</td>
<td>14.0</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>YJL023C</td>
<td>PET130</td>
<td>7.3</td>
<td>Hit</td>
</tr>
<tr>
<td></td>
<td>YOL055C</td>
<td>THI20</td>
<td>8.0</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Potential spike difference in mtt1Δ</td>
</tr>
<tr>
<td>Leaky Scanners</td>
<td>YCR007C</td>
<td>2.4</td>
<td>--</td>
<td>Equivalent, but signal spike in 5’ UTR in Upf1</td>
</tr>
<tr>
<td></td>
<td>YHL050C</td>
<td>2.9</td>
<td>--</td>
<td>Very low signal but one is in intron.</td>
</tr>
<tr>
<td></td>
<td>YIL107C</td>
<td>PFK26</td>
<td>2.8</td>
<td>--</td>
</tr>
<tr>
<td>Plus 1</td>
<td>YIL009C-A</td>
<td>EST3</td>
<td>3.9</td>
<td>Hit</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Signal increase in upf1Δ, and possibly in mtt1Δ.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Start codon enrichment upf1Δ</td>
</tr>
<tr>
<td></td>
<td>YILWTY3-1</td>
<td></td>
<td>6.5</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Very low signal.</td>
</tr>
<tr>
<td>Polycistronic</td>
<td>YLR315W</td>
<td>Nkp2</td>
<td>3.0</td>
<td>Hit</td>
</tr>
<tr>
<td></td>
<td>YLR317W</td>
<td>3.1</td>
<td>--</td>
<td>Very similar, some shifted peaks between strains.</td>
</tr>
<tr>
<td></td>
<td>YMR181C</td>
<td>2.9</td>
<td>--</td>
<td>Equivalent.</td>
</tr>
<tr>
<td></td>
<td>YMR182C</td>
<td>RGM1</td>
<td>4.4</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5’ UTR signal in upf1Δ</td>
</tr>
<tr>
<td>Pseudogenes</td>
<td>YCR099C</td>
<td>RGM1</td>
<td>3.7</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>YCR100C</td>
<td></td>
<td>4.8</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No signal in any strain</td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>Gene</th>
<th>Transposable Elements</th>
<th>Value</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>YIL164C</td>
<td></td>
<td>16.3</td>
<td>Very low signal</td>
</tr>
<tr>
<td>YIL165C</td>
<td></td>
<td>9.6</td>
<td>No signal in any strain</td>
</tr>
<tr>
<td>YIR043C</td>
<td></td>
<td>12.5</td>
<td>No signal in any strain</td>
</tr>
<tr>
<td>YDLWTAU1</td>
<td></td>
<td>7.0</td>
<td>No signal in any strain</td>
</tr>
<tr>
<td>YJLWTY4-1</td>
<td></td>
<td>4.4</td>
<td>No signal in any strain</td>
</tr>
<tr>
<td>YJLWTY4-1</td>
<td></td>
<td>8.5</td>
<td>No signal in any strain</td>
</tr>
<tr>
<td>YLRCDelta27</td>
<td></td>
<td>4.5</td>
<td>No signal in any strain</td>
</tr>
<tr>
<td>YNLWSigma2</td>
<td></td>
<td>3.7</td>
<td>No signal in any strain</td>
</tr>
</tbody>
</table>

**Table A3.** Comparison to the dataset reported in He et al. Mol. Cell, Vol 12. 1439-1452, December 2003. Table 2. Transcripts reported to display altered mRNA levels in the presence or absence of UPF1 were cross referenced with the current Global Ribosome Footprinting dataset and annotated subjectively according to the observed alterations. CM Dataset refers to the dataset produced by me in this experiment.
<table>
<thead>
<tr>
<th>Gene name</th>
<th>Up/Down</th>
<th>Regulated (AJ)</th>
<th>CM Data Set:</th>
</tr>
</thead>
<tbody>
<tr>
<td>SET7</td>
<td>Up</td>
<td>Overall similar. BIG loss of signal spike at start codon.</td>
<td></td>
</tr>
<tr>
<td>CAN1</td>
<td>Up</td>
<td>Differential occupancy. Major difference.</td>
<td></td>
</tr>
<tr>
<td>REG1</td>
<td>Up</td>
<td>Loss of 5’ ORF signal spike</td>
<td></td>
</tr>
<tr>
<td>TRP1</td>
<td>Up</td>
<td>No major difference</td>
<td></td>
</tr>
<tr>
<td>RIB7</td>
<td>Up</td>
<td>Minor difference</td>
<td></td>
</tr>
<tr>
<td>SSN2</td>
<td>Up</td>
<td>Decrease in Mtt1. Low signal overall.</td>
<td></td>
</tr>
<tr>
<td>SPR6</td>
<td>Up</td>
<td>Slight decrease in Upf1</td>
<td></td>
</tr>
<tr>
<td>CIS1</td>
<td>Up</td>
<td>Slight decrease in Upf1</td>
<td></td>
</tr>
<tr>
<td>DLS1</td>
<td>Up</td>
<td>Major increase in signal. Also 5’ UTR signal</td>
<td></td>
</tr>
<tr>
<td>AQY2</td>
<td>Up</td>
<td>Low signal, but different.</td>
<td></td>
</tr>
<tr>
<td>HAP5</td>
<td>Up</td>
<td>Minor difference</td>
<td></td>
</tr>
<tr>
<td>BUD4</td>
<td>Up</td>
<td>Major decrease. Start codon spike decreased.</td>
<td></td>
</tr>
<tr>
<td>TRM5</td>
<td>Up</td>
<td>Potential signal increase.</td>
<td></td>
</tr>
<tr>
<td>OAZ1</td>
<td>Up</td>
<td>Minor difference</td>
<td></td>
</tr>
<tr>
<td>CTL1</td>
<td>Up</td>
<td>Low signal, but different.</td>
<td></td>
</tr>
<tr>
<td>AMD2</td>
<td>Up</td>
<td>Major spike in 5’ UTR</td>
<td></td>
</tr>
<tr>
<td>SLX1</td>
<td>Up</td>
<td>3’ UTR signal</td>
<td></td>
</tr>
<tr>
<td>PCK1</td>
<td>Up</td>
<td>Small increase. Also a signal spike.</td>
<td></td>
</tr>
<tr>
<td>YBP1</td>
<td>Up</td>
<td>Signal decrease, change in position. Upf1 looks like mtt1. Both different than Wt.</td>
<td></td>
</tr>
<tr>
<td>APL3</td>
<td>Up</td>
<td>Major increase in signal.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Up</td>
<td>Low signal, but loss of start codon spike. Decreased signal. Mtt1 = Wt</td>
<td></td>
</tr>
</tbody>
</table>
SHU2 Up Potential increase in signal in upf1. Mtt1=Wt
Up Increase in signal. No changes in profile.
ADE2 Up Increase in signal. Subtle changes in profile.
REC107 Up Signal in intron. Major increase in signal.
Up 5’ UTR signal. Possible decrease in number of tags. Mtt1 looks different.
Up Almost no signal, but a couple tags in upf1 and mtt1.
Up Very low signal in all 3. None in mtt1.
Up Increase in signal Upf1, not Mtt1.
GSC2 Up Differential occupancy upf1. Signal increase and loss of spike in upf1, mtt1
HRT1 Up Increase in signal Upf1, not Mtt1.
Up Major increase in signal in upf1. Moderate increase mtt1.
Up Major increase in signal in upf1, not in mtt1.
Up Increased signal upf1, possibly mtt1. Buildup at stop codon.
Up Probably not a hit, but there's plenty of signal. All should be equal.
ALR1 Up Major increase in signal in ORF and some in the 5’ UTR
Up Increase in upf1 not Mtt1. May be for YOR012W though (overlapping orf)
CTL1 Up Low signal, but different.
PEX10 Down 5’ UTR Signal
DAL5 Down 5’ UTR signal
PEX4 Down Increased signal
PEX28 Down 5’UTR signal and Start codon spike
CPA1 Down Increased signal
JLP1 Down Increase
Down Increase in signal
SPR6 Down No differences but interesting 5’ UTR Signal.
Overall similar levels/patterns btw strains.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Down</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>TRP1</td>
<td>Potential decrease in signal. Low expression for Wt/mtt1. Hard to tell.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low signal. None in Wt/mtt1. Small signal Upf1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low signal, increase in Upf1, possibly Mtt1</td>
<td></td>
</tr>
<tr>
<td>ARN2</td>
<td>Decrease in signal in upf1.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Potential increase in upf1 and mtt1. 3’ UTR signal.</td>
<td></td>
</tr>
<tr>
<td>FRT2</td>
<td>Decrease in signal in Upf1. Wt same as Mtt1.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low signal but decrease in Upf1.</td>
<td></td>
</tr>
<tr>
<td>PIN2</td>
<td>Low signal but decrease in Upf1.</td>
<td></td>
</tr>
<tr>
<td>CIS1</td>
<td>Increase in Upf1.</td>
<td></td>
</tr>
</tbody>
</table>

**Table A4.** mRNAs previously shown to be up/down regulated (Maderazo et al., 2003) in the absence of UPF1, and which also show a differential phenotype in the absence of UPF1 in terms of ribosome footprinting. Alr1 and Can1, which are known to be affected by NMD are shown in bold. "CM data set" refers to primary data reported here.
Table A5. mRNAs previously shown to be up regulated (Maderazo et al. 2003) in the absence of UPF1, but do not show a differential phenotype in the absence of UPF1 in terms of ribosome footprinting. CM dataset refers to data produced in the current data set.
MOG1    NDR012W    YLL025W    NJR012W    HMRA2    NKL034C  
FAR7    YDR438W    YEA6    YPL257W    VBA2    NGR042W  
THI21    gEL01_1    AAD14    YJL213W    YIR042C    SPO1  
YOL106W    NCL011C    NER017W    PAU3    NGL040C    YNRCTAU3  
YLR164W    GPX1    SNF11    YER185W    YHL048C-A    NPR004C  
AYT1    YGLWDELTA6    YOL159C    YDR459C    PDR8  
YGR066C    YNL063W    YAL063C-A    YBLWDELTA1    YPL277C  
YPRCTAU3    YEL023C    KAR5    YHL037C    YOL162W  

Table A6. mRNAs previously shown to be down regulated (Maderazo et al., 2003) in the absence of UPF1, but do not show a differential phenotype in the absence of UPF1 in terms of ribosome footprinting.

Another trend in signal alterations between the two data sets is start codon enrichment. Among the few genes that noticeably displayed strong start codon enrichment, several were linked to metabolism and ribosome synthesis, suggesting that Upf1 could potentially control the rate of cell growth through these genes. Two representative candidate genes showing start codon
enrichment in the upf1Δ background are depicted in Figure A1. A comprehensive list of these genes is reported in Table A1. Interestingly, mtt1Δ and upf1Δ data sets show similar changes in ribosome occupancy in the form of start codon enrichment in Hxt3, Rei1 (which overlaps with an antisense transcript encoding the YBR266C gene), Ubx7, Rsc3, YDR179W, and Cft1 mRNAs.
Figure A2. Genes for four candidate mRNAs displaying start codon enrichment in the absence of UPF1 or MTT1, or both. A. HXT3 (oriented 3’ to 5’), B. REI1 (5’ to 3’), C. UBX7 (3’ to 5’), and D. YDR179W (5’ to 3’). Some genes in this category could also be considered to be in the 1/x category.
Gene Name:  Annotated Function:

CFT1  RNA-binding subunit of the mRNA cleavage and polyadenylation factor; involved in poly(A) site recognition and required for both pre-mRNA cleavage and polyadenylation, 51% sequence similarity with mammalian AAUAA-binding subunit of CPSF

HEM1  5-aminolevulinate synthase, catalyzes the first step in the heme biosynthetic pathway; an N-terminal signal sequence is required for localization to the mitochondrial matrix; expression is regulated by Hap2p-Hap3p

HXT3  Low affinity glucose transporter of the major facilitator superfamily, expression is induced in low or high glucose conditions

REI1  Cytoplasmic pre-60S factor; required for the correct recycling of shuttling factors Alb1, Arx1 and Tif6 at the end of the ribosomal large subunit biogenesis; involved in bud growth in the mitotic signaling network

ROT2  Glucosidase II catalytic subunit required for normal cell wall synthesis; mutations in rot2 suppress tor2 mutations, and are synthetically lethal with rot1 mutations

RSC3  Component of the RSC chromatin remodeling complex; essential gene required for maintenance of proper ploidy and regulation of ribosomal protein genes and the cell wall/stress response; highly similar to Rsc30p

UBX7  UBX (ubiquitin regulatory X) domain-containing protein that interacts
with Cdc48p

YPRO85C  Putative protein of unknown function; subunit of the ASTRA complex
(Rvb1p, Rvb2p, Tra1p, Tti1p, Tti2, Asa1p and Tra1p) which is part of the chromatin
remodeling machinery. Essential.

YDR179W  Putative protein of unknown function

**Table A7.** Genes displaying strong start codon enrichment in the absence of UPF1 or MTT1.

---

**Deletion of MTT1 does not affect ribosome occupancy for most genes**

To detect redundant roles for Mtt1 and Upf1, we also conducted global ribosome footprinting in an mtt1Δ strain, in parallel with the WT and upf1Δ strains. Cells were prepared in an identical fashion and subjected to visual analysis in parallel with the WT and upf1Δ data sets. This data set was highly similar with the other two data sets, essentially reiterating the WT dataset with the exception of four mRNAs: CFT1, REI1, UBX7, and YRD179W. These data are included below.

---

**Discussion:**

The previously published studies examining the changes in global mRNA levels in the absence of UPF1, and the direct association of UPF1 with various mRNA transcripts, produced partially overlapping data sets (Maderazo et al, 2003, He et al. 2003). Likewise, our data set
demonstrates that the loss of UPF1 leads to specifically altered ribosome occupancy profiles in several genes identified as NMD targets, as well as several targets not previously known to be affected by Upf1. The genes displaying altered ribosome occupancy do not fully overlap with either of the two previous data sets, suggesting that UPF1 deletion may cause changes in ribosome behavior both directly, as with NMD targets, and indirectly. Moreover, Upf1 may function as a modifier of ribosome function independent of its function in mRNA degradation. Interestingly, the CAN1 gene, can1-100, which is known to contain a PTC, also shows the 1/x phenotype when UPF1 is absent, suggesting that the Upf1 promotes ribosome occupancy of Can1, despite its additional promotion of Can1 mRNA degradation via NMD. On the surface this would seem to be contrary to the previously proposed hypothesis that Upf1 discourages the translation of NMD targets as part of its role in NMD.

Of particular interest is the link between the 1/x genes. What common features link them, and is the 1/x phenotype a direct or indirect result of UPF1 deletion? There are certain connections between them, evident in common functions shared amongst different subsets of genes. These include chitin synthesis, glucose metabolism, membrane association, mitochondrial localization, and ER/golgi localization. The latter two categories suggest that Upf1 may regulate a subset of proteins that are sent to the secretory pathway.

One particularly interesting finding is that translation of CPSF, the cleavage and polyadenylation signal factor is apparently altered in the absence of the Upf1 protein. Given the strong data supporting a kinetic competition model between Upf1 and PABP, it is interesting to note this additional connection between Upf1 and poly(A) tail formation. Based on this finding, it is certainly possible that CPSF levels may be regulated by Upf1, increasing the number of mechanisms by which NMD could modulate gene expression.
Among the simplest explanations is the possibility that many of the genes display an altered ribosome occupancy pattern in the absence of Upf1 due to increased Mg++ concentration (M. J. O. Johansson & Jacobson, 2010). For the 1/x genes previously reported to represent NMD targets, it will be interesting to elucidate the mechanism by which Upf1 promotes the normal translation pattern. It is somewhat counterintuitive that in the presence of Upf1, the PTC containing NMD target CAN1 mRNA actually displays increased ribosome occupancy downstream of the PTC. One possible explanation for this is that Upf1 is promoting re-initiation downstream of the PTC, as recently described (Ghosh et al., 2010). This possibility is supported by the Can1 occupancy data in the upf1Δ strain, in that the signal downstream of the PTC is greatly diminished. The function of Upf1 in re-initiation could also serve to explain the higher levels of ribosome occupancy upstream of the PTC in the WT strain. If this is correct, then the explanation for the 1/x phenotype in the Can1 gene in the upf1Δ data set, is simply that ribosomes are translating the mRNA normally, using the PTC as the stop codon.

The mtt1Δ strain showed few differences in ribosome occupancy compared to the WT strain. The lack of effect on Can1 argues against a role for Mtt1 in NMD. Moreover, the general lack of effect on ribosome positioning in nearly every gene in the genome, also argues against a general function for Mtt1 in translation. However, the ribosome occupancy profiles for CFT1, REI1, UBX7, and YRD179W mRNAs in the area of the start codon suggest that Mtt1 could be regulating, either directly or indirectly, the translation of certain genes. Interestingly, these are the only four genes for which a difference in profile was noted for the mtt1Δ data set, and they appear to coincide in all four cases with differences in the upf1Δ data set. This suggests that Mtt1 and Upf1 may have overlapping functions. Interestingly, Cft1, and Rei1 are involved with mRNA maturation and ribosome maturation, respectively. Likewise, Ubx7 interacts with Cdc48,
a protein known to be involved in ubiquitin mediated proteolysis, and ribosome degradation. The protein product of YDR179W is uncharacterized, however, the data suggest that involvement in translation would not be unlikely. These data, if repeated, suggest that a more thorough investigation of CFT1, REI1, UBX7, and YRD179W in either the upf1Δ or mtt1Δ strains is warranted.
Bibliography


Inada, T., & Aiba, H. (2005). Translation of aberrant mRNAs lacking a termination codon or with a shortened 3’-UTR is repressed after initiation in yeast. The EMBO journal, 24(8), 1584-95.


