

**Computational Phylogenetics Reveals Conserved Motifs in Ribosomal
RNA that Link Ribosome Biogenesis with Cell Cycle and Autophagy in
Eukaryotes**

By

Stephen M. Doris

B.S., University of Massachusetts Amherst, 2001

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This dissertation by Stephen M. Doris is accepted in its present form by the Department of Molecular Biology, Cell Biology, and Biochemistry as satisfying the dissertation requirement for the degree of Doctor of Philosophy.

Date _____

Susan A. Gerbi, Ph.D., Advisor

Recommended to the Graduate Council

Date _____

Albert E. Dahlberg, M.D., Ph.D., Reader

Date _____

Kimberly L. Mowry, Ph.D., Reader

Date _____

Tricia R. Serio, Ph.D., Reader

Date _____

John L. Woolford Jr., Ph.D., Reader
Carnegie Mellon University

Approved by the Graduate Council

Date _____

Sheila Bonde
Dean of the Graduate School

Stephen M. Doris
185 Meeting Street
Department of Molecular Biology, Cellular Biology & Biochemistry
Brown University
Providence, RI 02912
(401) 863-1665
Stephen_M_Doris@Brown.edu

Educational Training:

Undergraduate

University of Massachusetts, Amherst, MA
B.S. Biochemistry and Molecular Biology, 1998-2001

Graduate

University of Massachusetts, Amherst, MA
M.S. Biochemistry 2002-2003
Mentor: Jennifer L. Pinkham, Ph.D. (deceased)
Thesis: Yap1p activates transcription of *SOD2* through hydrogen peroxide sensing in *Saccharomyces cerevisiae*

Brown University, Providence, Rhode Island
Ph.D. Molecular Biology, Cell Biology, and Biochemistry 2003-present
Mentor: Susan A. Gerbi, Ph.D.
Thesis: Computational phylogenetics reveals conserved motifs in ribosomal RNA that link ribosome biogenesis with cell cycle and autophagy in eukaryotes

Professional Appointments:

1994–1997 New England Aquarium, Boston, MA - Education Dept., Educator
1998–1999 University of Massachusetts, Amherst, MA – Department of Chemistry, Technician
1999–2001 University of Massachusetts, Amherst, MA – Teaching Assistant, 4 semesters
2004–2005 Brown University, Providence, RI - Teaching Assistant, 3 semesters

Honors:

2002 Teaching Fellowship, University of Massachusetts, Department of Biochemistry – Assistant Lecturer for Advanced Biochemistry
2007 Predoctoral Fellowship, The National Science Foundation RI-EPSCoR (Experimental Program to Stimulate Competitive Research)

Awards:

2006 Travel Award Recipient, RNA Society, The Seventh Annual Ribosome Synthesis Meeting
2007 Travel Award Recipient, RNA Society, International Meeting on Ribosomes: Form and Function

Publications:

Langseth, S.X., Pezza, J.A., Raupp Yamamoto, R., Doris, S.M. and Serio, T.R. (2008) The NatA acetyltransferase couples Sup35 prion complexes to the [PSI⁺] phenotype. *Mol Biol Cell* **20**: 1068-80.

Doris, S.M., Beamesderfer, J.N., and Gerbi, S.A. (2009) Identification of Ribosomal RNA Conserved Nucleotide Elements in the Phylogenetic Domains of Life. (In preparation)

Doris, S.M., Schroeder, E.A. and Gerbi, S.A. (2009) Defects in early ribosome biogenesis stimulate autophagy and cell cycle arrest in *Saccharomyces cerevisiae*. (In preparation)

Meeting Abstracts:

Doris, S.M. and Gerbi, S.A. (2008) Identification of *cis*-Acting Conserved Nucleotide Elements in Eukaryotic Ribosomal RNA. Thirteenth Annual Meeting of the RNA Society. Berlin, Germany (poster 625)

Doris, S.M., Beamesderfer, J.N. and Gerbi, S.A. (2008) Identification of Eukaryotic Ribosomal RNA Binding Proteins Using the Yeast Three-Hybrid Screen. Thirteenth Annual Meeting of the RNA Society. Berlin, Germany (poster 614)

Doris, S.M., Schroeder, E.A. and Gerbi, S.A. (2008) An Analysis of Eukaryotic Ribosomal RNA and Ribosomal Protein L32 in Ribosome Maturation and Cell Cycle. Thirteenth Annual Meeting of the RNA Society. Berlin, Germany (poster 678)

Doris, S.M. and Gerbi, S.A. (2007) Are there *cis*-acting elements in rRNA for nuclear export of nascent ribosomes? Willhelm Bernard Meeting. St. Andrews, Scotland, (invited platform talk #41)

Doris, S.M. and Gerbi, S.A. (2007) Are there *cis*-acting elements in rRNA for nuclear export of nascent ribosomes? EMBO Conference on Nuclear Structure and Dynamics. Montpellier, France (invited platform talk T32)

Doris, S.M. and Gerbi, S.A. (2007) Are there *cis*-acting elements in rRNA for nuclear export of nascent ribosomes? International Meeting on Ribosomes: Form and Function. Falmouth, MA pg. 79, (invited speaker)

Doris, S.M., Ware, V.C. and Gerbi, S.A. (2006) Are there *cis*-acting elements in rRNA for nuclear export of nascent ribosomes? 7th International Conference of Ribosome Synthesis. Airlie House, Warrenton, VA pg. 69 (poster)

Teaching Activities:**Teaching Assistant, University of Massachusetts Amherst, Spring 2001-Spring 2002**

Biochemistry 526- Advanced Topics in Biochemistry, Course Chair: Robert Zimmerman, PhD
Responsibilities included leading biweekly lab sections, grading lab notebooks and quizzes.

Teaching Assistant, University of Massachusetts Amherst, Fall 2002

Biochemistry School of Nursing, Course Chair: Richard O'Brien, PhD

Introduction to topics in biochemistry

Responsibilities included grading weekly quizzes, preparing exam questions, weekly review sessions.

Teaching Assistant, Brown University, Fall 2004

Bio 231- Analysis of Development, Course Chair: Krisit Wharton, PhD

Responsibilities included preparing and directing pre-laboratory seminar, leading weekly reviews of primary literature reading assignments, grading exams.

Teaching Assistant, Brown University Medical School, Winter 2005

Biochem 130A- Intro Medical Genetics, Course Chair: Umadevi Tantravahi, PhD

Responsibilities included writing and grading weekly quizzes, grading exams, holding weekly review sessions

Teaching Assistant, Brown University Medical School, Spring 2005

Biochem 130B- Intro to Medical Biochemistry, Course Chair: Al Dahlberg MD, PhD

Responsibilities included writing and grading weekly quizzes, grading exams, holding weekly review sessions.

Collaborator(s):

Philipp Milkereit - University of Regensburg, Regensburg, Germany

Pierre-Emmanuel Gleizes - University of Toulouse III, Toulouse, France

Vassie C. Ware - Lehigh University, Bethlehem, PA

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Chapter 1: Introduction

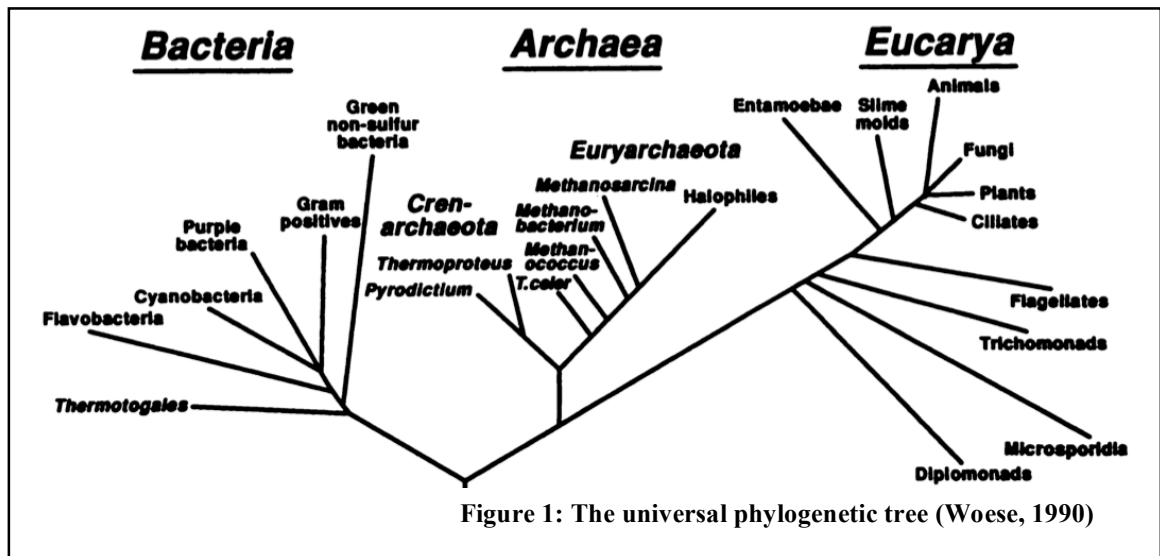
A necessary event in the evolution of the cell was the invention of a system for storing and extracting biological information. Through the process of translation, stored genetic information is converted into protein, and ribosomes are the site of protein synthesis. During translation, a ribosome combines with the correct amino acid bearing tRNA by a mRNA templated mechanism, and amino acids are sequentially linked together by a peptidyl transferase reaction. The basic mechanisms of translation appear to be fully conserved in all forms of life.

Ribosomal RNA and the universal tree of life

Molecular phylogenetic studies use genotype rather than phenotype to understand how organisms are related to one another. Genotypic information (i.e., nucleic acid sequence) is superior to using phenotypic patterns because it can be precisely interpreted and measured. In contrast, phenotypic traits are often subjective relying on the biologist's eye to declare phenotypes as "similar." Classifying phenotypic traits is especially challenging in the microbial world due to their simplistic morphologies.

Ribosomal RNA sequences are currently the most widely used molecular phylogenetic tool (Olsen and Woese, 1993). The major advantages to using rRNA are (i) the molecule is present in the entire phylogenetic spectrum; (ii) it has retained a universal function; (iii) a constrained function slows mutations; (iv) its large size gives ample sequence information (Woese, 1987). A comprehensive phylogenetic analysis of the 16S rRNA sequence revolutionized our understanding of how life is evolutionarily related. These data showed that life on Earth divided into three primary groups termed domains:

Eukarya, Archaea, and Bacteria (Figure 1) (Woese et al., 1990). This major finding eliminated “prokaryote” as a phylogenetic taxon, once believed to be a primary grouping. Indeed, the Archaea and Bacteria are no more similar to one another than either is to Eukarya (Woese, 1987).



The 16S rRNA has become the standard in studying ancestral relationships between organisms. Despite having a high degree of conservation (30-40% between Archaea and Bacteria), there are significant differences between rRNA sequences (Woese, 1987). The strongest phylogenetic signals in rRNA are those sequences that remain constant in one domain of life but are degenerate or rare in the other two domains, and are termed ribosomal signatures (Roberts et al., 2008). In a recent study by Woese and colleagues, 69 distinguishing single nucleotide ribosomal signatures were identified between bacterial and archaeal 16S rRNA (Roberts et al., 2008). These ribosomal signatures are responsible for breaking the tree of life into the three main branches.

An important direction in the evolutionary studies of rRNA is the merger with other scientific disciplines to begin to experimentally address the function of domain-specific rRNA sequences. The identification and characterization of eukaryotic ribosomal signatures is the primary focus of this work.

Protein synthesis

The ribosome is a large ribonucleoprotein (RNP) complex found ubiquitously in nature and abundant in all growing cells. Ribosomes function in a crucial step in the expression of protein-coding genes; they coordinate and catalyze the translation of nucleotide information in mRNA templates into the amino acid polymers of a functional protein. All ribosomes consist of two RNP complexes, termed subunits, of unequal size each with a specialized function in translation. The small ribosomal subunit is responsible for the selection of the correct aminoacyl bearing tRNA during mRNA decoding and the large subunit contains the peptidyl transferase activity where peptide bond formation between the peptidyl-tRNA and aminoacyl-tRNA occurs (Rodnina et al., 2007).

In eukaryotes, translation of mRNA starts with recruitment of the small subunit as a 43S pre-initiation complex that scans from the 5' end of the mRNA to the initiation AUG codon. This is followed by recruitment of the 60S subunit, and the 80S ribosome is formed with initiator-tRNA in the P-site. The joining of the rRNAs in the small and large subunits form three tRNA binding sites: The A-site contains the newly selected amino acid bearing tRNA about to be incorporated into the nascent polypeptide chain, the P-site

contains the growing peptidyl-tRNA, and the E-site holds the deacylated tRNA before it is ejected from the ribosome.

During the translation cycle in bacteria, an incoming aminoacyl-tRNA molecule enters the A-site in a complex with EF-Tu and GTP and interacts with the codon. The correct codon-anticodon pairing activates the GTPase center, GTP is hydrolyzed, and structural rearrangements occur in the rRNA to orient the peptidyl-tRNA and aminoacyl-tRNA for the peptidyl transferase reaction, which occurs in the peptidyl transferase center (Steitz, 2008). After the transferase reaction, there remains a deacylated-tRNA in the P-site and a peptidyl-tRNA in the A-site lengthened by one amino acid. During translocation, elongation factors drive the movements of the tRNAs resulting in a peptidyl-tRNA in the P-site and a deacylated tRNA in the E-site. Translocation also moves the mRNA by one codon in the 3' direction thereby exposing a new codon in the A-site. The elongation of the polypeptide continues until a stop-codon is reached and the nascent peptide is released.

Most of the information regarding translation by the ribosome has been elucidated using bacterial and archaeal ribosomes. It is likely that the fundamental mechanisms of translation are the same in eukaryotes since the core ribosome structure appears to be conserved in the three primary domains of life (Clark et al., 1984; Spahn et al., 2001). However, the eukaryotic ribosome is much larger in both rRNA (Gerbi, 1996) and protein content (Dresios et al., 2006). Two outstanding questions in the ribosome field

are: What is the functional role of these additional eukaryotic components? And how do eukaryotic ribosomes assemble inside cells?

Eukaryotic ribosome biogenesis

Transcription, rRNA processing, and early assembly

Given their central role in protein synthesis, the efficient and accurate production of a ribosome is a vital task for all cells; therefore, a thorough understanding of how this is accomplished is a key feature in understanding basic cellular mechanisms.

A growing yeast cell devotes the majority of its resources toward making new ribosomes. Ribosome biogenesis utilizes the three major RNA polymerases, and accounts for the majority of the RNA in the cell and 90% of all splicing events (Warner, 1999). RNA Pol I synthesizes the pre-ribosomal RNA, and each pre-rRNA must associate with 70 different species of snoRNA, 150 assembly factors, and 80 ribosomal proteins (Lewis and Tollervey, 2000). This massive amount of factor localization and RNP assembly drives the formation of a specialized subnuclear compartment, the nucleolus, which is the primary site of ribosome biogenesis. Once assembled the pre-ribosomes must be transported from the nucleolus, their site of synthesis, to the cytoplasm, their site of function.

In yeast, ribosome biogenesis begins when RNA pol I synthesizes the 35S precursor transcript encoding the 18S, 5.8S, and 25S rRNA separated by two internal transcribed

spacers (ITS1 and ITS2 flank 5.8S rRNA) and two external spacers (5' ETS and 3' ETS), which are removed during rRNA processing (Figure 2). The 18S rRNA will be incorporated into the small subunit and the 25S and 5.8S along with the RNA pol III derived 5S rRNA will form the large subunit. At the 5' end of the nascent rRNA a large RNP complex forms, termed the small subunit processome, and this contains U3 snoRNA (Dragon et al., 2002), which is essential for formation of 18S rRNA (Hughes and Ares, 1991; Savino and Gerbi, 1990). The earliest cleavage event occurs in the 3' ETS producing the 35S pre-rRNA prior to RNA Pol I transcription termination (Kufel et al., 1999). Soon after RNA Pol I termination, cleavages at sites A₀, A₁, and A₂ proceed removing all of the 5' ETS, and separate the 20S pre-rRNA (the precursor to 18S rRNA of the small subunit) from the 27S pre-rRNA (the precursor for 5.8S and 25S rRNA of the large subunit). The 20S pre-rRNA is rapidly exported to the cytoplasm where final cleavage at site D produces mature 18S rRNA (Vanrobays et al., 2001). In contrast to the processing of the 20S pre-rRNA, 27S pre-rRNA reaches its mature form in the nucleolus through a series of ordered endo- and exonucleolytic cleavages (Raué, 2004). The synthesis of mature rRNA also includes nucleotide modification. Modification is primarily carried out by site-specific snoRNAs that target enzymes to rRNA through RNA-RNA hybridization for pseudouridine formation and nucleotide methylation (Decatur and Fournier, 2002).

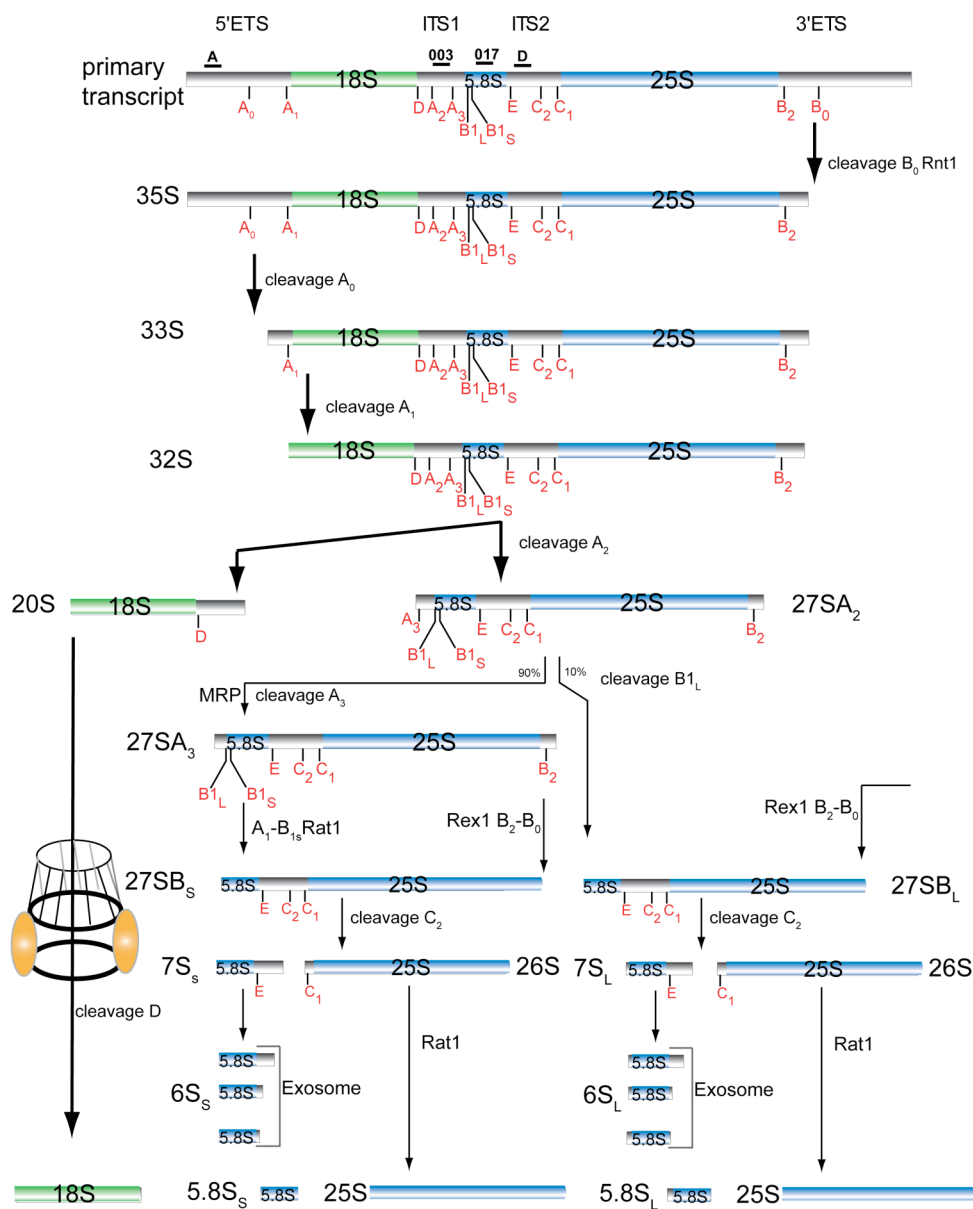


Figure 2. Pre-rRNA processing in *Saccharomyces cerevisiae* adapted from Raue, 2004.

In contrast to pre-rRNA processing, far less is understood about the pre-ribosome assembly pathway. Recent advances in affinity purification of pre-ribosomal intermediates and their analysis by mass spectrometry have identified more than 150 non-ribosomal factors, which transiently associate with the developing ribosome (Tschochner and Hurt, 2003). The 90S pre-ribosome contains the 35S precursor RNA that gives rise to the 40S and 60S subunits. Recently, the 90S pre-ribosome was isolated from yeast cells and analysis of its protein constituents revealed that this early ribosome contained mostly 40S synthesis factors (Grandi et al., 2002) but generally lacked factors previously identified in pre-60S ribosomes (Harnpicharnchai et al., 2001). These results suggested a model that pre-60S maturation factors join their developing subunits after cleavage at site A₂. This observation is analogous to assembly in *E. coli*, where assembly is influenced by the 5'-3' synthesis of the rRNA. As different pre-ribosomes were isolated from the yeast nucle(o)us the number of pre-ribosomal intermediates expanded (Bassler et al., 2001; Harnpicharnchai et al., 2001; Saveanu et al., 2001). These studies set the framework for subsequent proteomic analyses and the initial ordering of the pre-ribosomal network (Fatica et al., 2002). In the pre-ribosomal network, the 90S ribosome is formed in the dense fibrillar component of the nucleolus and gives rise to the early, middle and late pre-ribosomal particles that ultimately mature into cytoplasmic subunits (Figure 3) (Dez and Tollervey, 2004). With this pre-ribosomal network in place, the next major challenge in the field is to determine the precise molecular function and substrate of the 150 assembly factors.

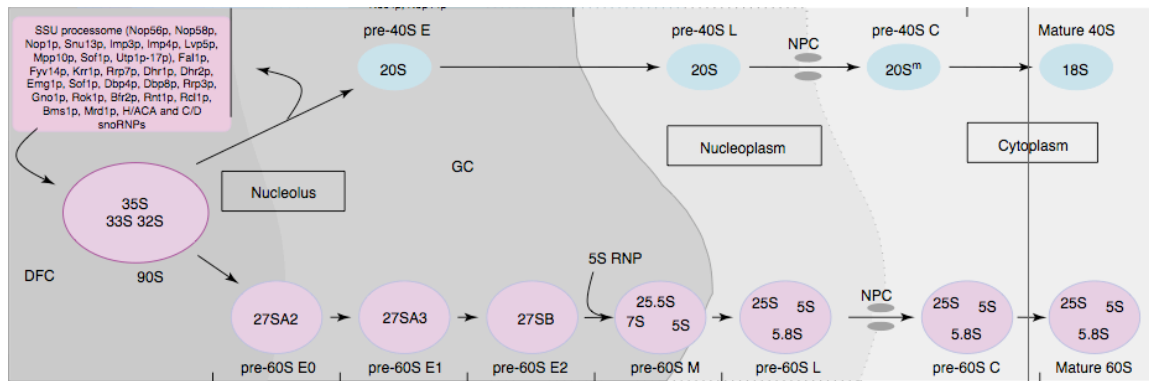


Figure 3. The pre-ribosomal network. Early (E), middle (M), late (L), cytoplasmic (C), dense fibrillar component (DFC), granular component (GC), nuclear pore complex (NPC) (Dez and Tollervey, 2004).

Once attaining the correct conformation, pre-ribosomes must be shuttled from the nucleus to the cytoplasm to function in translation. It has proven difficult to identify *bona fide* export factors because defective transport may be a result of improper assembly. During nuclear export, pre-ribosomes are ushered through the nuclear pore complex- the only known gateway between the nucleus and cytoplasm (Tran and Wente, 2006). Pioneering studies on ribosome trafficking using microinjection into *Xenopus* nuclei showed nuclear export to be a unidirectional, energy consuming, and a saturable process that requires functional nuclear pore complexes (Bataillé et al., 1990). The export of both small and large subunits depends on the activity of the karyopherin receptor CRM1/Xpo1p (Gadal et al., 2001; Ho et al., 2000; Thomas, 2003; Trotta et al., 2003), a member of the evolutionarily conserved importin- β family. CRM1/Xpo1p is not known to recognize RNA substrates directly but rather recognizes a conserved nuclear export peptide sequence (NES) stabilized by Ran-GTP (Weis, 2002). In budding yeast, Nmd3p was the first ribosomal nuclear export factor identified that is essential for export of the large

subunit (Gadal et al., 2001; Ho and Johnson, 1999). The role of Nmd3p in recruiting CRM1/Xpo1p to the large subunit is conserved in yeast, frogs, and humans (Thomas, 2003; Trotta et al., 2003). More recently, two other nuclear receptors were identified as 60S export factors; the Mex67p/Mtr2p general RNP export receptor (Yao et al., 2007) and a novel receptor, Arx1p, which links the pre-60S subunit to the proteins of the nuclear pore (Bradatsch et al., 2007). Together these data show that multiple pathways exist in parallel to ensure efficient export of this large RNP complex.

It is also becoming increasingly clear that the cell requires a mechanism for the intranuclear transport of ribosomes. Evidence for this came from genetic screens that identified the Noc proteins in *S. cerevisiae* (Milkereit et al., 2001; Milkereit et al., 2003). In the proposed model Noc1p and Noc2p bind to an early pre-66S particle in the nucleolus, then upon receiving an unknown cue (a potential quality control check point), Noc2p changes its binding partner from Noc1p to Noc3p with a coordinated movement of the pre-66S particle from the nucleolus to the nucleoplasm (Milkereit et al., 2001). A Noc4p-Nop14p complex is proposed to have a similar role in 40S trafficking (Milkereit et al., 2003).

Deletion analysis of Nmd3p suggests that it has many sites of interaction with the 60S ribosomal subunit (Hedges et al., 2006). The Mex67p/Mtr2p general export factors contain two loop structures that appear to have a specific role in 60S export and not mRNP export and *in vitro* can form a complex with 5S rRNA (Yao et al., 2007).

Although these key components of the large subunit export pathway have been identified,

the mechanism of nuclear export and translocation through the nuclear pore remain major unanswered questions. Furthermore, the binding determinants of Nmd3p, Arx1p, and Mex67p/Mtr2p to the 60S subunit are unknown.

Ribosomal proteins

Due to their low molecular weight and high sequence similarity, the presence of ribosomal proteins (r-proteins) in the pre-ribosomal network remains ambiguous. Therefore, far less is understood about the stepwise assembly of ribosomal proteins in eukaryotic assembly. Recently, the Milkereit group comprehensively depleted each 40S subunit ribosomal protein in yeast and catalogued the resulting phenotype (Ferreira-Cerca et al., 2005). Their work showed that ribosomal proteins take on active roles in ribosome assembly directing pre-rRNA cleavage, nuclear export, and function. Furthermore their genetic experiments strongly suggested r-proteins act either early or late in rRNA processing implying that assembly of ribosomal proteins onto maturing rRNA is hierarchical. Indeed, data support a hierarchical ribosome assembly landscape; kinetic labeling of newly synthesized ribosomal proteins revealed that some proteins associate late in biogenesis (Kruiswijk et al., 1978). Furthermore, using a combination of r-protein genetic depletions followed by pre-40S immunoprecipitation, it was demonstrated that Rps15 association is dependent on the presence of Rps5 (Ferreira-Cerca et al., 2007). These data obtained *in vivo* from yeast support the principles established in *E. coli* that assembly of the small ribosomal subunit is cooperative and hierarchical (Agalarov et al., 2000; Held et al., 1973). Current tools are now in place to begin to construct the first eukaryotic *in vivo* assembly map.

SPECIFIC AIMS

The ribosome is required for protein synthesis and is essential for life. Creation of ribosomes is a vital activity of all cells, consuming a large proportion of cellular resources suggesting that the process of ribosome biogenesis is a focus for coordinate regulation with other aspects of cell physiology. Therefore, it is important to understand this crucial machine integral to basic cellular mechanisms.

The focus of this work is in understanding the structure of eukaryotic ribosomal rRNA, how rRNA has uniquely evolved in the eukaryotic domain, and its functions in the cell.

I. Identification of Conserved Nucleotide Elements in Eukaryotic Ribosomal RNA

II. The Role of Eukaryotic Conserved Nucleotide Elements in Early Ribosome Biogenesis

III. Eukaryotic Ribosome Biogenesis Promotes Cellular Proliferation and Inhibits

Autophagy

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Chapter 2: Identification of Ribosomal RNA Conserved Nucleotide Elements in the Eukaryotic Domain of Life

This project was shared with undergraduate student Julia N. Beamesderfer.

ABSTRACT

A detailed understanding of ribosomal RNA (rRNA) structure is essential to understanding its function in the cell. Here we present a new comparative analysis of eukaryotic rRNA and define discrete nucleotide motifs that display a high sequence and structural conservation within the entire eukaryotic domain of life. Our methods use a broad and comprehensive phylogenetic kingdom sample population, identification of conserved tetranucleotide seeds based on structural position, and a hierarchical compilation and validation of candidates using the ARB software package. For this analysis we developed the database server CORD (The Complete Organismal rRNA Database) based on the 12,506 high quality rRNA sequences in the SILVA database for each of the three primary phylogenetic domains (Eukarya, Bacteria, and Archaea). From these data, we present the identification of 42 eukaryotic conserved nucleotide elements (eCNEs) in the large subunit rRNA. Each eCNE is ≥ 8 bases in length and is highly conserved in sequence in at least 90% of all eukaryotic organisms in the CORD database. Furthermore, we show in structure-based interdomain comparisons that eCNEs can be eukaryotic-specific, showing high degeneracy in Bacteria and/or Archaea and thus serve as a phylogenetic signal. Homology modeling showed that several eCNE structures base-pair and cluster in the folded molecule. The implications of eCNEs on the evolution, structure, and function of the large subunit are discussed. This work serves as a foundation for understating eukaryotic-specific features in the ribosome.

INTRODUCTION

Ribosomes are macromolecular ensembles of RNA and protein. Early studies on the structure of the ribosome showed that the rRNA forms a core secondary structure that can be found in diverse taxa from the bacterium *E. coli* to the vertebrate *X. laevis* (Gerbi, 1996). These observations have had profound effects on biology. First, it emphasized that throughout evolution the fundamental process of translation has remained functionally unchanged (Woese, 2002). Secondly, these allow for comparative analysis of any rRNA primary sequence by position and the deduction of a secondary structure model (Gutell, 1996). And, it shows that rRNA sequence variation can serve as the ultimate molecular tool in deducing phylogenetic relationships (Olsen and Woese, 1993; Woese, 1987).

Although broadly considered universally conserved, sequence variation in rRNA has been paramount to understanding the taxonomy of life on the planet. Pioneering work by Carl Woese and colleagues has established the Universal Phylogenetic Tree using covariation analyses of 16S/18S rRNA (Woese et al., 1990). This tree of life proposed a three domain system: Bacteria, Archaea, and Eukarya. Published nearly two decades ago, this tree generally remains unchanged today. These data eliminate earlier groupings of Archaea and Bacteria into one kingdom and thereby dismiss the usage of “prokaryote” as a phylogenetic term.

In addition to variations in the primary sequence of rRNA that established the universal phylogenetic tree, there exist characteristic RNA structures present uniquely in one

domain of life but absent in the other two. Collectively these variations in primary and secondary structures within each domain of life are termed “ribosomal signatures” and are responsible for the delineation of the Bacterial and Archaeal lineages (Roberts et al., 2008; Woese, 1987). The accumulation of sequence and structural signature data has proven crucial to understanding the evolution of the translation machinery and the categorization of phyla. An interesting question that arises from these data is whether these signatures impart unique function(s) to the ribosome tailoring it for the unique physiology of that domain. However, this question remains largely unexplored.

The archaeal and bacterial ribosomes are the prevailing model systems for understanding protein synthesis and the translation cycle. Recently this knowledge has been significantly extended with the landmark achievements of obtaining high resolution X-ray crystal structures; this includes the 70S ribosome associated with mRNA and the three tRNA binding sites occupied solved at 2.8 Å (Selmer et al., 2006). It is now increasingly apparent that the ribosome is a ribozyme, with rRNA underlying all aspects of protein synthesis (Steitz, 2008).

Comparatively, an equivalent understanding of ribosomes in a eukaryotic system is far less understood. Cryo-electron microscopy of the eukaryotic 80S ribosome from yeast at 15 Å resolution has revealed that the core of the ribosome is generally similar in all domains of life (Spahn et al., 2001). However, eukaryotic ribosomes are larger than bacterial and archaeal ribosomes due to additional ribosomal proteins (Dresios et al., 2006) and rRNA expansion segments (Gerbi, 1996). The precise function of these

eukaryotic ribosomal features remain largely unknown, but are speculated to participate in additional tertiary and quaternary interactions needed for assembly, function, and regulation of eukaryotic ribosomal operations (Chandramouli et al., 2008; Dresios et al., 2006; Spahn et al., 2001).

A striking feature of the eukaryotic ribosome is its maturation pathway, which is vastly more complex than the bacterial system (Hage and Tollervey, 2004). Most notable are the involvements of small nucleolar rRNA needed for processing and covalent modification of the rRNA precursor (Gerbi and Borovjagin, 2004), and the 170 assembly factors needed for forging the two ribosomal subunits (Fromont-Racine et al., 2003).

Understanding how this complex machine assembles *in vivo* remains a major challenge in biology and will require a combination of disciplines to precisely define the assembly pathway.

Here we present an analysis of eukaryotic rRNA structure by combining a new computational phylogenetic method with structural modeling. We applied multiple rRNA sequence positional alignments based on secondary structure across each eukaryotic kingdom. These data identified contiguous stretches of ≥ 8 nucleotides that show high conservation across eukaryotic phyla. These conserved motifs were tested using a distance matrix analysis in a new database CORD (Complete Organismal rRNA Database) that has been filtered to contain only full length 23S-like sequences, organized by phylogenetic domain, and is restricted to single organisms. The results of this analysis

led to the identification of 42 eukaryotic conserved nucleotide elements (eCNEs) in 28S rRNA.

RESULTS

The CORD database

In order for a multisequence rRNA alignment to be accurate, nucleotide sequence must be compared by homologous position. This is a major challenge due to naturally occurring deletions and insertions in rRNA (e.g., expansion segments). Therefore, all sequences in the alignment must have positional identity requiring each sequence to be partially or completely folded. Recently, the SILVA database was initiated to provide the most comprehensive resource of up to date rRNA sequences from Bacteria, Archaea and Eukarya (Pruesse et al., 2007). This powerful database holds 547,521 rRNA sequences aligned by secondary structure. Alignments in SILVA are based on previous alignment data sets that established an RNA folding algorithm enabling new sequences to be structurally aligned by various computational parameters (Pruesse et al., 2007). Because of insertions or deletions in the eukaryotic 28S rRNA molecule and the variability in their lengths, a structural alignment of 28S rRNA represents 150,000 alignment positions. In other words, each position in the alignment represents a nucleotide present or absent in a sequence in the database. Each sequence in the SILVA database must pass several rounds of quality inspection, and sequence files with ambiguities or that cannot be structurally aligned are rejected. SILVA represents one of the most highly curated taxonomically diverse rRNA data sets.

The SILVA large ribosomal subunit (LSU) Ref database uses 1900 bases as a length minimum for curated sequences. This is problematic for testing conservation of bases in

the 3' end of the molecule as yeast 25S rRNA is ~3400 bases in length and mouse 28S rRNA is ~4700 bases. Our motivation to create a new rRNA database was to allow a comprehensive evaluation of nucleotide element conservation in the entire 23S-like molecule in a given phylogenetic domain. To this end, the 12,506 sequences in the complete SILVA LSU Ref database were catalogued by phylogenetic domain into three position tree servers (PT_Servers) using the ARB software package (Ludwig et al., 2004). Several stringent filters were then used to produce rRNA data sets containing only complete rRNA sequences as stipulated by four parameters. All sequence data containing the term “partial” or “shotgun” in their abstracts were eliminated. The resulting databases were then refined further by testing for the presence of the universally conserved sarcin-ricin loop (SRL). The SRL makes for an ideal filter for creating a complete sequence LSU database as (i) it is a 14 nucleotide element universally conserved from ancient mitochondrial rRNA through rat 28S rRNA (Chan et al., 1983) (ii) it is the 3'-most conserved LSU rRNA structure (Gerbi, 1996), and (iii) none of our candidate conserved nucleotide elements were downstream of the SRL (Figure 3). Therefore, accessions that did not contain the SRL at least 70% conserved (i.e., 4 mismatches) in sequence and structure were eliminated.

The SILVA Ref database allows for multiple sequence entries for a single species. To prevent any bias in our analyses that test for the number of organisms that contain a test sequence, all duplicated sequences were eliminated such that the final data sets contain only one full length rRNA sequence per organism. This filter had the most profound affect on the bacterial database, which contained many duplicated sequences (e.g., 160

for *E. coli*). These refined data sets contain complete sequences of rRNA from single organisms organized by primary phylogenetic domains. We refer to this database as the Complete Organismal rRNA Database (CORD). The CORD data set is compatible with the phylogenetic tree software package ARB. All further analyses were done using the CORD data set (Table I).

Generally, the study of rRNA conservation has focused on covariance of single nucleotides to derive phylogenetic relationships (Woese, 1987) or on compensatory base changes in helical regions to deduce secondary structures (Gutell, 1996). Given the biological complexity of eukaryotic rRNA, we reasoned that motifs of consecutive conserved nucleotides could serve as recognition sites, such as for assembly factor binding, and/or code for an energetically efficient RNA folding landscape. In theory, such elements would be selected for in eukaryotic evolution and become highly invariant.

Eukaryotic conserved nucleotide element identification

We tested for the existence of eukaryotic conserved nucleotide elements (eCNEs) in rRNA, imposing a minimum of 8 contiguous bases that are ~90% identical in sequence in 90% of the organisms in CORD. The analysis is hierarchical, starting at the kingdom level with the identification of highly conserved seeds in three independent clusters (Figure 1). Those data are compiled and tested at the domain level. The general method for eCNE identification is outlined in Figure 2.

The process of eCNE identification starts with a 28S rRNA positional alignment with one representative organism from each of the six major eukaryotic kingdoms (Opisthokonta, Amoebozoa, Plantae, Chromalveolata, Rhizaria, Excavata) (Figure 1A). Conserved seeds are defined as ≥ 4 bases that are 100% conserved in sequence and position within the kingdom-level cluster. The cluster analysis and seed detection is conducted in triplicate using a different set of six organisms representing each eukaryotic phylogenetic kingdom (in each cluster a different subphylum was used). The seed data from each kingdom-level cluster are compiled and aligned by position into a single domain-level set. The 5' and 3' boundaries of the candidate CNEs are identified at positions when a seed is ≥ 8 bases in length or where seeds structurally superimpose forming a minimum of 8 bases (Figure 2, Domain cluster). CNE candidates are tested in ARB using a distance matrix probe matching function and are deemed eCNEs if they meet the criteria of being highly conserved in primary sequence in $\geq 90\%$ of the organisms in the CORD database.

The eukaryotic conserved nucleotide elements

The sequence of the CNE candidates and their homologous coordinates in *E. coli* are shown in Table II. In determining if a candidate CNE was an eCNE, a minimum number of mismatches was determined based on CNE length, and the percent conservation tested ranged from 82-90%. The candidate CNE was then tested for the number of organisms that contain the element in a highly conserved format using a distance matrix analysis in ARB. A candidate was deemed eCNE if $> 90\%$ of the eukaryotic organisms in the CORD database contain the sequence (Table II). eCNEs range in length from 8-52 bases and are distributed in all six domains of the LSU (Figure 3). The conservation properties of the

eCNEs were tested by evaluating multiple contiguous stretches of nucleotides from yeast 25S rRNA. These control sequences were chosen based on the characteristics of eCNEs; each tested sequence ranges from 8-19 bases in length, are distributed throughout the six domains of 25S rRNA, and are structurally diverse. None of these test sequences display the conservation of eCNEs, suggesting that eCNE motifs are not a common feature in rRNA (Table III).

Evaluating domain specificity of eCNEs

The eCNEs were evaluated for their cross-domain conservation in Archaea and Bacteria (Table IV). The sarcin-ricin loop serves as a positive control in this analysis as it is a known universally conserved nucleotide element (Chan et al., 1983). Our methods identified eCNE42, which is the sarcin-ricin loop. Degeneracy of a sequence element cannot be measured directly in ARB because increasing the mismatch allowance leads to a loss in positional identity of the element and the server will report on non-homologous regions. To overcome this, we used a modification of the CNE identification strategy outlined in Figure 2. The 42 eCNEs were positionally aligned to the 23S rRNA from organisms representing each major phylogenetic kingdom of the archaeal (3 kingdoms, Figure 1B) and bacterial (11 kingdoms, Figure 1C) lineages. This clustering analysis was done in triplicate comparing each of the 42 eCNEs with its homologue in 31 diverse bacteria and 13 diverse archaea from CORD (Table IV). The results of these data are summarized in Table V and presented as a conservation gradient. The table reports on the number of organisms in Bacteria and Archaea that contain the eCNE in a degenerate state at $\leq 50\%$, $\leq 60\%$, and $\leq 70\%$ conserved in primary sequence. Nine eCNEs (30, 41, 27,

34, 15, 22, 37, 11, and 19) fit our criteria of being eukaryotic specific elements; they are highly conserved (~90%) in all Eukarya but degenerate in Archaea and Bacteria (Figure 4). We also identified five universally conserved CNEs (uCNEs; 2, 5, 23, 32, and 42) and two CNEs common only to Archaea and Eukarya (aeCNEs; 21 and 28).

eCNEs in the large subunit

All of the eCNE sequences mapped on the secondary structure are found in the universally conserved core structure of the large subunit and are not located in expansion segments (Gerbi, 1996). This permits a molecular model of the eCNE tertiary interactions in the 50S subunit based on the refined 2.4 Å structure of the 50S subunit of the archaeon *H. marismortui* (PDB no. 1S72) (Klein et al., 2004). These structures reveal that eCNEs are found within the functional core of the ribosome, at the subunit interface, and exposed on the back surface (Figure 5A). This suggests that eCNEs are potentially multifunctional in the ribosomes. In the next step of the modeling, we mapped the universally conserved eCNEs (uCNEs; 2, 5, 23, 32, and 42), and found that they do not physically interact and are distributed in the 23S structure. (Figure 5B). Two uCNEs (uCNE42 and uCNE23) have known functions in translation, forming the SRL and P-loop structures, respectively. In contrast to the uCNEs, the eCNEs that are highly degenerate in the bacterial and archaeal domains generally cluster in the folded 23S rRNA molecule.

DISCUSSION

A combination of techniques including computational phylogenetics and structural modeling were used to test if conserved elements (contiguous bases) exist in eukaryotic 28S rRNA. Based on the source database SILVA, we developed a modified database CORD. The data in CORD contain only complete (full length) sequence files and each entry is for a different organism. Having single organisms is essential when measuring conservation in an entire domain, as multiple accessions per organisms would bias the result. In our analysis we set the minimum limit for a nucleotide element at eight contiguous bases with no maximum. The logic for choosing eight was to define elements that contain unique and adequate biological information to code for a protein recognition motif and/or an RNA secondary structure. In the initial kingdom-cluster analysis, we tested for conserved seeds that were 100% conserved between eukaryotic kingdoms. Using 100% at this stage of the analysis is essential, as it will ultimately define the 5' and 3' terminal nucleotides of the element.

The minimal seed in the kingdom cluster was half the length (4 nt) of our minimal target element (8 nt). Testing for seeds that are half the length of the target sequence helps to eliminate sequencing errors and/or population polymorphisms in a larger element. Furthermore, conducting the seed analysis in triplicate addresses the possibility of polymorphisms or sequencing errors present in a tetranucleotide seed. Candidate sequence elements produced from a triplicate kingdom analysis are then tested in CORD and deemed an eCNE if the element is highly conserved in most species within the

domain. Our method was functional, as we were able to identify the sarcin-ricin loop as a universally conserved nucleotide element (uCNE), as previously reported (Chan et al., 1983). Of the 44 candidate CNE sequences, the majority (42) were confirmed in the CORD data set. This strongly suggests that the diverse organisms used in the kingdom cluster accurately report on the rRNA identity for the domain as a whole. Using this logic, we designed clusters of organisms for the domains Archaea and Bacteria and used these clusters to measure eCNE domain specificity. In summary, we have defined 42 elements in 28S rRNA that are highly conserved in sequence and position in all eukaryotes.

eCNE function in translation

The distribution of the eCNEs on a 25S rRNA secondary structure is shown in Figure 3 and show that some of the eCNEs localize to regions that are functionally important in rRNA. The region of rRNA that is generally referred to as the GTPase center is composed in part by eCNE 15, 16, and 17 in domain II of 25S rRNA. Interestingly, eCNE15 is highly degenerate in the other two domains of life and is solvent exposed (Figure 5C). A second region of rRNA that activates GTP hydrolysis is eCNE42 (the SRL) in domain VI. The GTPase center is involved in hydrolysis of elongation factor bound GTP during translation. In eukaryotes, ribosomal proteins L12 together with P0/P1/P2 assemble onto the GTPase center in domain II. This is also the site for elongation factor binding (Thompson, 1996). The elongation phase of the eukaryotic translation cycle requires a third and unique elongation factor (EF-3) not found in the bacterial system (Triana-Alonso et al., 1995). The requirement for an additional factor

suggests a difference in the elongation phase in eukaryotes. It is interesting to speculate that eCNE15 could serve as a binding site for EF-3.

The bridges between the small and large ribosomal subunit are essential for translation, facilitating the rotation of the small subunit during translocation (Frank and Agrawal, 2000). Of the eight helices that make conserved RNA-RNA bridges in 80S ribosomes (Chandramouli et al., 2008), five of them are formed by eCNEs in the large subunit (Table VI). One of the best-characterized RNA bridges is the structurally conserved helix 69 (h69) in 23S rRNA. Helix 69 forms an RNA-RNA bridge with helix 44 in 16S rRNA (bridge B2a) and contacts A site and P site tRNAs (Selmer et al., 2006; Yusupov et al., 2001). *In vivo* analysis of h69 mutants display an increase in stop-codon read-through (O'Connor and Dahlberg, 1995) and *in vitro* Δ h69 ribosomes cannot form a 70S ribosome in the absence of tRNA and are deficient in peptide release (Ali et al., 2006). In the eukaryote *S. cerevisiae*, h69 contains two covalent base modifications that are absent in *E. coli* h69 (Decatur and Fournier, 2002). Deletion of the small nucleolar RNAs (snoRNAs) that base-pair with the rRNA and guide covalent modification of h69 in eukaryotes alters the structure of 25S rRNA, increases stop-codon read-through, and decreases rRNA stability (Liang et al., 2007). eCNE26 is the longest eCNE (52 nt) and it encodes h69 and extends from it.

The active site of peptide bond formation is the peptidyl transferase center (PTC) found in domain V of 23 rRNA (Beringer and Rodnina, 2007; Steitz, 2008). Interestingly eCNEs cluster in this region (eCNEs 31, 35, 37, 38, 40, 41). Two of these eCNEs (41 and

37) are degenerate in Bacteria and Archaea suggesting that these elements may fine tune peptide bond formation in eukaryotes. The A-loop structure is formed by eCNE39, and the P-loop structure is formed by uCNE32 and both are essential components of the PTC. The A-loop, P-loop and PTC are extensively modified by snoRNAs (Decatur and Fournier, 2002). Deletion of the snoRNAs that modify these regions lead to the production of ribosomes containing structurally altered rRNA, defects in protein synthesis (Baxter-Roshek et al., 2007; King et al., 2003), and in translational fidelity (Baxter-Roshek et al., 2007). Given these observations, a potential function for eCNEs is to base pair with snoRNAs during rRNA maturation.

eCNE in ribosome assembly

In vitro analysis of ribosome assembly established that protein addition to the rRNA is hierarchical, where a set of primary RNA binding proteins initiates assembly and produces binding sites for secondary proteins (Held et al., 1973). Studies on the folding pathway of rRNA showed that in most cases, the regions of 16S rRNA that contact primary binding proteins fold first (Adilakshmi et al., 2008). eCNEs serve as contact points for five of the 23S rRNA primary binding proteins: L11 and L10 (eCNE15, eCNE16, eCNE17), L24B (eCNE5), L23 (eCNE23, eCNE21), and L3 (eCNE41). However, not all rRNA folding is dependent on protein. Studies with 16S rRNA showed that all helical secondary structures can form in the absence of proteins (Moazed et al., 1986), and some regions can adopt near native tertiary structure in the absence of ribosomal proteins in less than 20 ms (Adilakshmi et al., 2005; Adilakshmi et al., 2008).

In eukaryotes, most steps in rRNA folding occur in the nucleolus and the nuclear surveillance machinery monitors this process. RNAs that do not fold efficiently are recognized by the TRAMP complex and destroyed by the RNase activity of the Exosome (Houseley et al., 2006). One possibility is that in order to avoid kinetic traps inherent to folding an RNA (Herschlag, 1995), eukaryotes have evolved eCNEs as kinetically efficient folding modules serving to code for the most energetically efficient rRNA folding landscape.

eCNE in evolution

Because ribosomal RNA contains a universally conserved core structure, it is believed that the ribosome formed before life differentiated into branches. At some pivotal point in evolution, the RNA world transitioned into a protein-based world but maintained an RNA-based language in the ribosome. Upon splitting into three domains, the ribosomes within these lineages maintained unique rRNA characteristics or signatures (Roberts et al., 2008; Woese, 1987). At the root of the tree, these unique features became fixed and remained constant throughout evolution. Here we trace the evolutionary path of 28S rRNA through the study of eukaryotic conserved nucleotide elements (eCNEs). eCNEs act as a “molecular stopwatch” in eukaryotic evolution being formed at the base of the eukaryotic tree and remaining invariant throughout. The invariant nature of eCNEs highlights their importance in eukaryotic life. It seems likely that eCNEs evolved with the basic functions of the eukaryotic cell.

Conclusion

Understanding the eukaryotic ribosome from synthesis to function is a modern frontier in basic biology (Doudna and Rath, 2002). Compared to the other two lineages, eukaryotic ribosomes require an extensive maturation and transport pathway. It will be interesting to test if eCNEs play a role in the maturation process. Given that the nucleus is a defining morphological feature of eukaryotes, eCNEs may play a role in nuclear export of nascent ribosomes. Preliminary work suggests that eCNE6 may mediate nuclear export of the large subunit (see Appendix I). Furthermore, characterizing proteins that interact with eCNEs will help in the elucidation of their functions. We carried out yeast three-hybrid screens and identified Sec65 (a eukaryotic specific component of the signal recognition complex) and Cms1 (a yeast protein involved in DNA replication) as eCNE binding proteins (see Appendix III). A powerful test for eCNE function *in vivo* is mutagenesis. However, no system exists to study the assembly and transport of mutant eukaryotic ribosomes co-expressed with the wild-type population. To this end, we developed a new system to study mutant populations of ribosomes *in vivo* by engineering RNA affinity tagged ribosomes (Appendix II). Exploring eCNE function is a promising direction for unraveling unique features of the eukaryotic ribosome.

MATERIALS AND METHODS

Sequence alignments

All sequence alignments for the 23S-like molecule were obtained using the alignment tool in ARB. For each alignment a filter was employed using the longest molecule in the alignment. Candidate conserved nucleotide elements were tested in the CORD data set using the probe match function in ARB. All candidates were manually tested for insertions and deletions.

Database construction and server construction

Ribosomal RNA data were obtained from the SILVA Ref database and filtered as explained in the text to create the Complete Organismal rRNA Database (CORD). Organisms in CORD were organized into sequence phylogenetic trees and individual Position Tree Servers were constructed using ARB.

Homology modeling

Homologous sequence positions were obtained using the ARB sequence aligner tool matched to *S. cerevisiae* (J01355) for modeling onto secondary structures (Cannone et al., 2002) and *H. marismortui* (X13738) for three-dimensional modeling. Structure data were input into MacPyMol (2006 DeLano Scientific LLC) to generate models.

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Table I. CORD database construction

	Eukarya	Archaea	Bacteria
Total sequences in SILVA	4874	196	7436
Removal of partial and shotgun sequences	632	142	4302
Presence of sarcin-ricin loop	529	141	4209
Removal of species duplication	443	87	918

Table II. Confirmation of eukaryotic conserved nucleotide elements

5' Position (E. coli)	Sequence 5' to 3'	Length (nt)	# Mismatches (% Conservation)	% Eukaryotes Containing Candidate	eCNE
193	uuaagcauu	10	1 (90%)	76%	-----
208	agcggagaaaagaaa	16	2 (88%)	85%	-----
241	aguaacggcgag	12	2 (83%)	96%	eCNE1
425	gcuaaaaua	8	1 (88%)	99%	eCNE2
440	gagaccgauagc	12	2 (83%)	91%	eCNE3
453	aacaagua	8	1 (88%)	98%	eCNE4
475	ugaaaaga	8	1 (88%)	97%	eCNE5
562	cccgucuuagaacacggaccaaggaguc	28	3 (89%)	96%	eCNE6
668	ggaccgaaag	11	2 (82%)	97%	eCNE7
741	cugacgugcaaaucg	15	2 (87%)	98%	eCNE8
773	uagggcgaaagacu	15	2 (87%)	98%	eCNE9
800	uaguagcugguu	12	2 (83%)	99%	eCNE10
815	uccgaaguuuc	11	2 (82%)	98%	eCNE11
829	caggauagc	9	1 (89%)	99%	eCNE12
904	uucucaacu	10	1 (90%)	98%	eCNE13
977	gggaugaacc	10	1 (90%)	93%	eCNE14
1057	cgguggcc	8	1 (88%)	98%	eCNE15
1079	ccgcuaaggagug	13	2 (85%)	99%	eCNE16
1093	guaacaacucaccu	14	2 (86%)	100%	eCNE17
1125	gaaaauugga	9	1 (89%)	99%	eCNE18
1251	cagaucuuggu	11	2 (82%)	99%	eCNE19
1263	guaguagc	8	1 (88%)	99%	eCNE20
1377	gaaaggga	8	1 (88%)	97%	eCNE21
1617	caggucuccaag	12	2 (83%)	99%	eCNE22
1671	ucggcaaa	8	1 (88%)	98%	eCNE23
1687	guaacuucg	9	1 (89%)	96%	eCNE24
1826	gugauuucugcccagugcuc	20	2 (90%)	99%	eCNE25
1897	guaaacggcgggaguaacuauagacucucuuaagguagccaaaugccucguca	52	6 (88%)	97%	eCNE26
1951	uaauuagugacg	12	2 (83%)	99%	eCNE27

Table II. *Continued*

5' Position (E. coli)	Sequence 5' to 3'	Length (nt)	# Mismatches (% Conservation)	% Eukaryotes Containing Candidate	eCNE
1964	gcaugaauggau	12	2 (83%)	100%	eCNE28
1989	acuguccc	8	1 (88%)	100%	eCNE29
2025	aaggggaacgg	10	1 (90%)	98%	eCNE30
2048	agcggggaaagaagaccguugagcuugacuc	33	4 (88%)	98%	eCNE31
2247	gcuggggcggc	11	2 (82%)	99%	eCNE32
2279	gguguccua	9	1 (89%)	97%	eCNE33
2390	cgauccuu	8	1 (88%)	99%	eCNE34
2427	cagaaaaguaccacaggauaacuggcuguggc	35	4 (89%)	98%	eCNE35
2465	gccaaagcguucauagcgacg	20	2 (90%)	98%	eCNE36
2487	gcuuuuugau	10	1 (90%)	100%	eCNE37
2498	cuucgaugucggcucuuccuac	23	3 (87%)	99%	eCNE38
2548	ggauuguuacccc	13	2 (85%)	100%	eCNE39
2567	agggaaacgugagcuggguuagaccgucgugagacagguu	40	4 (90%)	98%	eCNE40
2608	guuuuacccuac	12	2 (83%)	99%	eCNE41
2654	aguacgagaggaac	14	2 (86%)	100%	eCNE42

Table III. Control sequences

Ribosomal Domain	E. coli Position	Sequence (5' to 3')	Length (nt)	Mismatches Allowed	% Organisms with Candidate
I	343	cgaggagugcgg	12	2 (83%)	0%
II	1022	ucagacaccacaaaa	15	2 (86%)	30%
III	1501	ggagaugg	8	1 (88%)	25%
IV	1800	auggucagaaagugauguu	19	2 (89%)	12%
V	2093	gugaagagacauagaggg	18	2 (89%)	17%
VI	2815	auuuguauacgacuuag	17	2 (88%)	3%

Table IV. Phylogenetic conservation of eCNEs

eCNE #	% Organisms with CNE ≥ ~90% Conserved		% Organisms with CNE ≤ ~70% Conserved		% Organisms with CNE ≤ ~60% Conserved		% Organisms with CNE ≤ ~50% Conserved	
	Archaea	Bacteria	Archaea	Bacteria	Archaea	Bacteria	Archaea	Bacteria
eCNE1	31%	94%	69%	3%	15%	0%	0%	0%
eCNE2	92%	94%	8%	6%	0%	3%	0%	0%
eCNE3	100%	61%	0%	39%	0%	6%	0%	0%
eCNE4	62%	65%	31%	35%	23%	23%	8%	3%
eCNE5	92%	97%	8%	3%	0%	3%	0%	3%
eCNE6	0%	0%	46%	100%	0%	100%	0%	45%
eCNE7	31%	26%	69%	74%	23%	3%	8%	0%
eCNE8	8%	0%	54%	100%	31%	97%	8%	84%
eCNE9	31%	48%	15%	42%	0%	6%	0%	3%
eCNE10	23%	19%	77%	81%	8%	39%	0%	0%
eCNE11	8%	3%	92%	97%	77%	74%	54%	48%
eCNE12	15%	0%	85%	100%	77%	97%	46%	68%
eCNE13	8%	0%	38%	84%	15%	52%	0%	16%
eCNE14	0%	0%	23%	84%	23%	29%	8%	0%
eCNE15	0%	0%	100%	100%	77%	100%	0%	65%
eCNE16	15%	3%	85%	97%	0%	16%	0%	6%
eCNE17	54%	19%	0%	55%	0%	0%	0%	0%
eCNE18	46%	23%	54%	77%	38%	45%	23%	26%
eCNE19	8%	0%	92%	100%	69%	84%	38%	77%
eCNE20	77%	0%	23%	100%	0%	58%	0%	0%
eCNE21	100%	3%	0%	97%	0%	71%	0%	45%
eCNE22	8%	0%	92%	100%	77%	90%	0%	32%
eCNE23	100%	94%	0%	6%	0%	3%	0%	0%
eCNE24	85%	100%	15%	0%	0%	0%	0%	0%
eCNE25	0%	0%	69%	55%	8%	3%	0%	0%
eCNE26	0%	0%	0%	0%	0%	0%	0%	0%
eCNE27	0%	0%	100%	100%	92%	100%	15%	0%
eCNE28	100%	10%	0%	90%	0%	29%	0%	0%
eCNE29	100%	61%	0%	39%	0%	10%	0%	0%
eCNE30	0%	0%	100%	100%	100%	100%	92%	97%
eCNE31	0%	0%	69%	90%	0%	52%	0%	3%
eCNE32	92%	90%	8%	10%	0%	0%	0%	0%
eCNE33	15%	0%	85%	100%	69%	90%	15%	48%
eCNE34	0%	0%	100%	100%	92%	94%	0%	23%
eCNE35	0%	0%	8%	42%	0%	0%	0%	0%
eCNE36	0%	0%	100%	3%	23%	0%	0%	0%
eCNE37	0%	0%	100%	100%	77%	100%	31%	71%
eCNE38	62%	6%	23%	0%	0%	0%	0%	0%
eCNE39	15%	0%	85%	100%	0%	52%	0%	0%
eCNE40	77%	0%	8%	0%	0%	0%	0%	0%
eCNE41	0%	0%	100%	100%	100%	100%	92%	97%
eCNE42	100%	100%	0%	0%	0%	0%	0%	0%

V. Interdomain conservation gradient

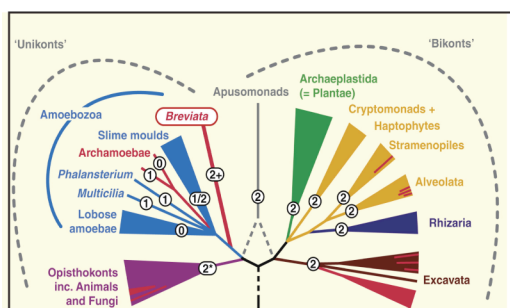
		Archaea			
		≤50%	≤60%	≤70%	≥90%
Increasing Conservation ↓	Bacteria				
	≤50%	eCNE30, eCNE41			
	≤60%		eCNE27, eCNE34	eCNE15, eCNE22, eCNE37	
	≤70%			eCNE11, eCNE19	eCNE21, eCNE28
	≥90%				eCNE2, eCNE5, eCNE23, eCNE32, eCNE42
		Increasing Conservation →			

Table VI. eCNE subunit bridges in the 60S ribosome

Bridge[*]	Helix	eCNE
b1a	h38	eCNE13
b2a	h69	eCNE26
b2b	h66	---
b2c/b2d	h67	eCNE25 eCNE28
b3	h71	eCNE26 eCNE27
b3b	h64	eCNE29
b4	h34	---
b5b	h62	---

* Bridge nomenclature as per Chandramouli (Chandramouli et al., 2008)

A. EUKARYA



Cluster I

Classification	Organism Name	Accession
Opisthokonta	<i>Saccharomyces cerevisiae</i>	J01355
Amoebozoa	<i>Physarum polycephalum</i>	V01159
Archaeplastida (Plantae)	<i>Arabidopsis thaliana</i>	X52320
Chromalveolata	<i>Tetrahymena thermophila</i>	X54512
Rhizaria	<i>Bigelowiella natans</i>	DQ158858
Excavata	<i>Trypanosoma brucei</i>	X14553

Cluster II

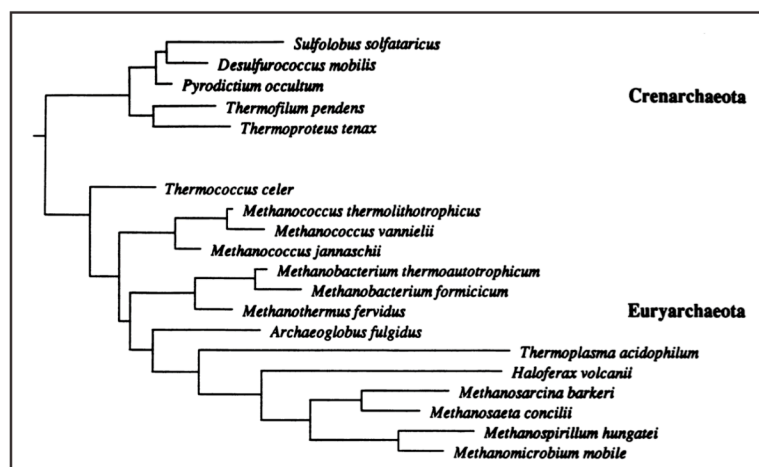
Classification	Organism Name	Accession
Opisthokonta	<i>Mus musculus</i>	X00525
Amoebozoa	<i>Entamoeba histolytica</i>	X65163
Archaeplastida (Plantae)	<i>Chlorella ellipsoidea</i>	D17810
Chromalveolata	<i>Guillardia theta</i>	AF289037
Rhizaria	<i>Bigelowiella natans</i>	DQ158858
Excavata	<i>Trichomonas vaginalis</i>	AF202181

Cluster III

Classification	Organism Name	Accession
Opisthokonta	<i>Capsaspora owczarzaki</i>	AY724688
Amoebozoa	<i>Dictyostelium discoideum</i>	X00601
Archaeplastida (Plantae)	<i>Palmaria palmata</i>	Y11506
Chromalveolata	<i>Phytophthora megasperma</i>	X75631
Rhizaria	-----	-----
Excavata	<i>Giardia muris</i>	X65063

Figure 1A. (legend following)

B. ARCHAEA



Cluster I

	Organism Name	Accession
Euryarchaeota	<i>Haloarcula marismortui</i>	X13738
	<i>Methanosphaera stadtmanae</i>	CP000102
	<i>Archaeoglobus fulgidus</i>	M64487
Crenarchaeota	<i>Sulfolobus solfataricus</i>	M67495
Korarchaeota	<i>Candidatus Korarchaeum cryptofilum</i>	CP000968

Cluster II

	Organism Name	Accession
Euryarchaeota	<i>Picrophilus torridus</i>	AE017261
	<i>Methanocaldococcus jannaschii</i>	L77117
	<i>Thermococcus celer</i>	M67497
Crenarchaeota	<i>Thermofilum pendens</i>	X14835
Korarchaeota	<i>Candidatus Korarchaeum cryptofilum</i>	CP000968

Cluster III

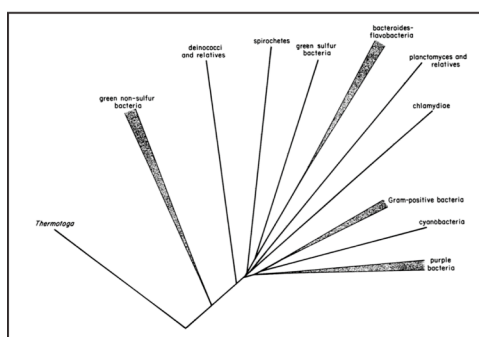
	Organism Name	Accession
Euryarchaeota	<i>Methanospirillum hungatei</i>	M81323
	<i>Methanococcus maripaludis</i>	BX950229
	<i>Methanopyrus kandleri</i>	AE010356
Crenarchaeota	<i>Desulfurococcus mobilis</i>	X05480
Korarchaeota	-----	-----

Figure 1B. (legend following)

C. BACTERIA

Cluster I

	Organism Name	Accession
Proteobacteria	<i>Escherichia coli</i>	J01695
Cyanobacteria	<i>Prochlorococcus marinus</i>	AE017126
Deinococcus-Thermus	<i>Deinococcus geothermalis</i>	CP000358
Thermotogae	<i>Thermotoga maritima</i>	M67498
Chlamydiae	<i>Chlamydia muridarum</i>	AE002160
Bacteroidetes	<i>Bacteroides fragilis</i>	CR626927
Chloroflexi (Green Non-Sulfur)	<i>Dehalococcoides ethenogenes</i>	CP000027
Chlorobi (Green Sulfur)	<i>Chlorobium limicola</i>	CP001097
Gram-Positive	<i>Streptomyces ambofaciens</i>	M27245
Spirochaetes	<i>Borrelia burgdorferi</i>	M88330
Planctomycetes	<i>Blastopirellula marina</i>	X07408



Cluster II

	Organism Name	Accession
Proteobacteria	<i>Agrobacterium tumefaciens</i>	AB102733
Cyanobacteria	<i>Nostoc punctiforme</i>	CP001037
Deinococcus-Thermus	<i>Thermus thermophilus</i>	AY379548
Thermotogae	<i>Petrotoga mobilis</i>	CP000879
Chlamydiae	<i>Waddlia chondrophila</i>	AF042496
Bacteroidetes	<i>Porphyromonas gingivalis</i>	AE015924
Chloroflexi (Green Non-Sulfur)	<i>Roseiflexus castenholzii</i>	CP000804
Chlorobi (Green Sulfur)	<i>Chloroherpeton thalassium</i>	CP001100
Gram-Positive	<i>Bacillus subtilis</i>	K00637
Spirochaetes	<i>Treponema denticola</i>	AE017226
Planctomycetes	<i>Rhodopirellula baltica</i>	BX294148

Cluster III

	Organism Name	Accession
Proteobacteria	<i>Bordetella pertussis</i>	X68323
Cyanobacteria	<i>Synechocystis</i> sp. PCC 6803	BA000022
Deinococcus-Thermus	-----	-----
Thermotogae	<i>Fervidobacterium islandicum</i>	Y15790
Chlamydiae	<i>Chlamydomonas abortus</i>	U76710
Bacteroidetes	<i>Flavobacterium psychrophilum</i>	AM398681
Chloroflexi (Green Non-Sulfur)	<i>Chloroflexus aurantiacus</i>	CP000909
Chlorobi (Green Sulfur)	<i>Pelodictyon luteolum</i>	CP000096
Gram-Positive	<i>Clostridium botulinum</i>	X65602
Spirochaetes	<i>Leptospira interrogans</i>	X14249
Planctomycetes	-----	-----

Figure 1 C. (legend opposite)

Figure 1. Kingdom level phylogenies and diagrammatic tree organization: Organisms used in the kingdom-level cluster alignments represent all major phylogenetic kingdoms and are divided into three clusters. Three kingdoms (Eukarya-Rhizaria, Archaea-Korarchaeota, Bacteria-Planctomycetes) contained only one species with full length rRNA sequence and were not included in the third cluster. Diagrammatic trees were derived based on the previously reported (A) Eukaryotic tree (Simpson and Roger, 2004) (B) Archaeal tree (Olsen and Woese, 1993) and (C) Bacterial tree (Woese, 1987).

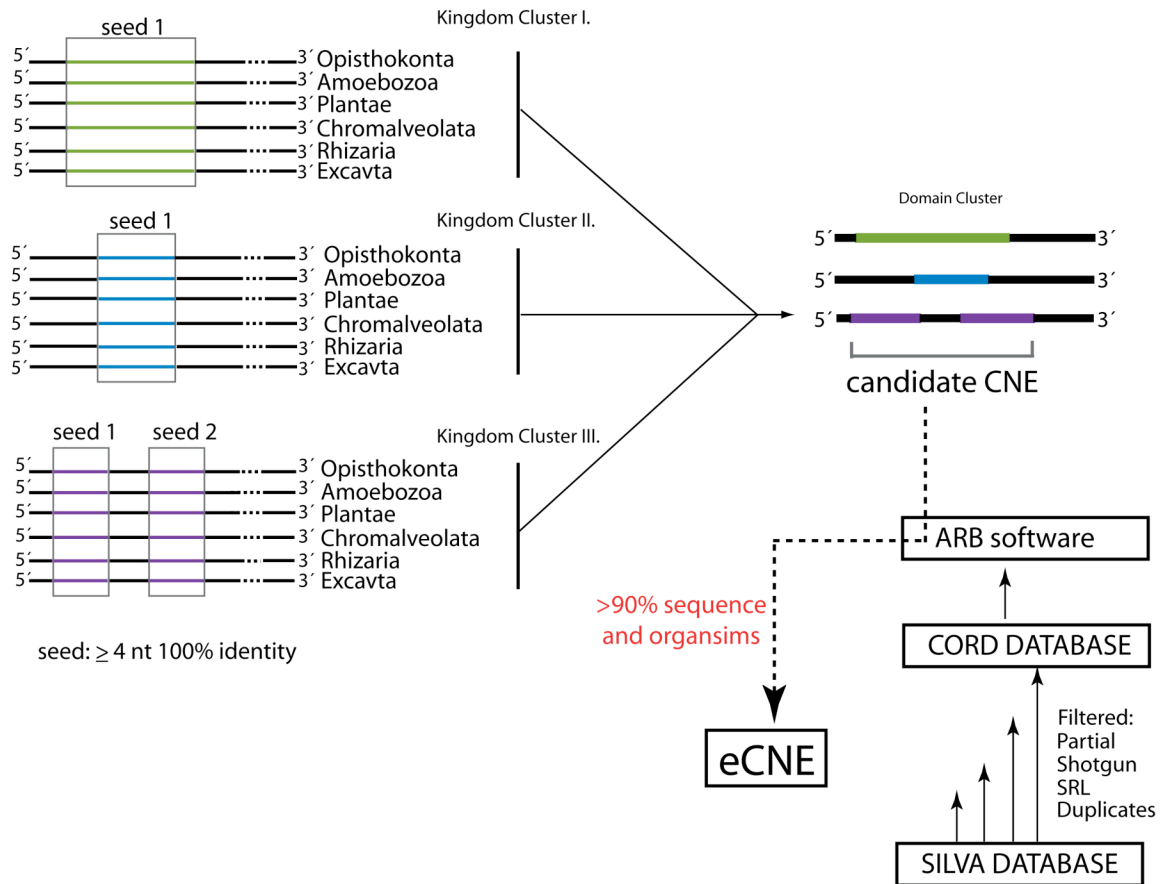


Figure 2. Schematic overview of eCNE identification: A representative organism from each of the major eukaryotic kingdoms was aligned in a kingdom cluster using 28S rRNA. Seed regions (≥ 4 nucleotides) were identified if they were 100% conserved between kingdoms. Seeds reporting on three kingdom clusters were compiled and positionally aligned to generate candidate conserved nucleotide elements (≥ 8 nucleotides). Elements are tested for domain conservation in the CORD database using a distance matrix function. Candidates that are highly conserved (~90%) in >90% of the organisms in CORD are confirmed as eukaryotic conserved nucleotide elements (eCNEs).

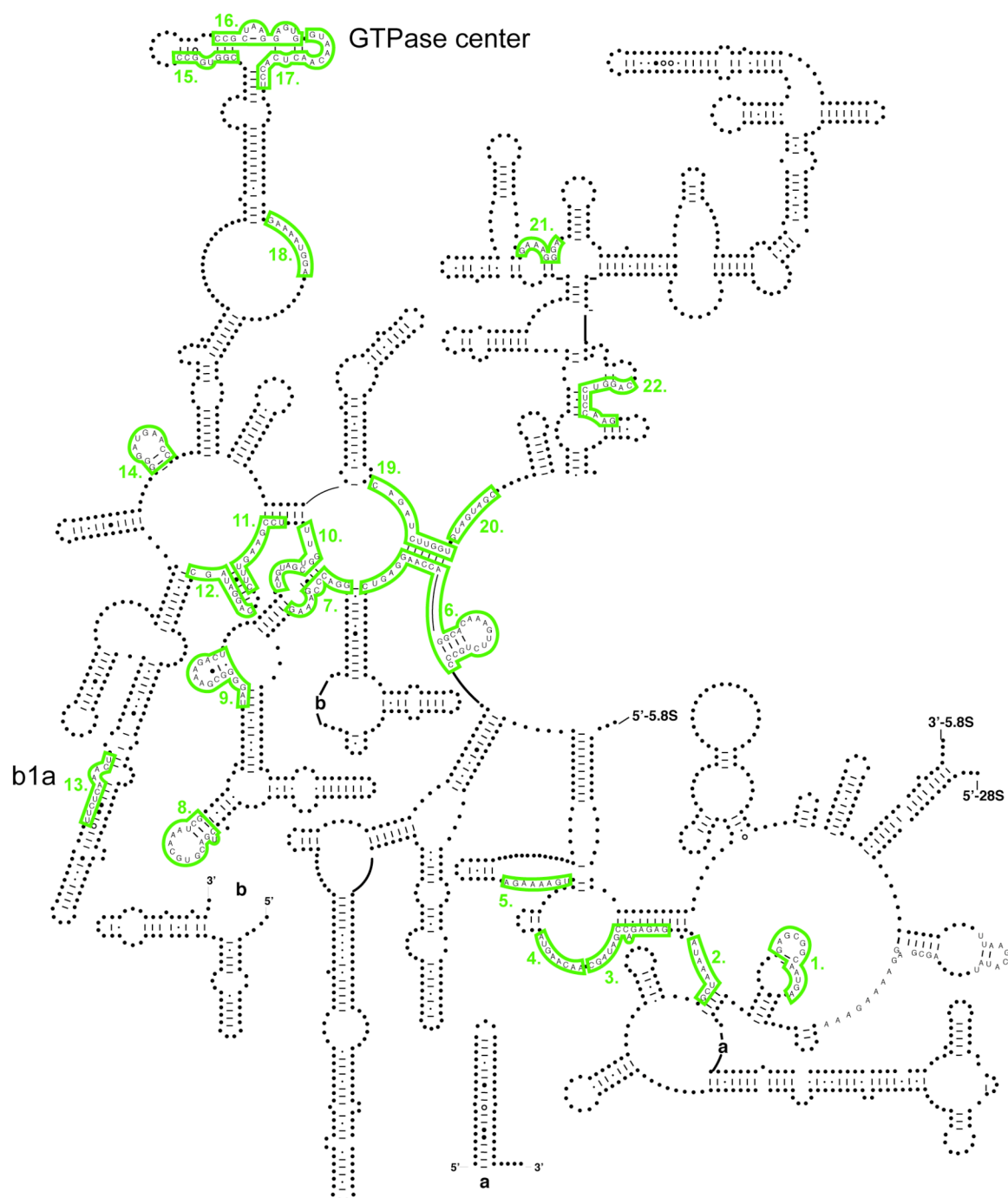


Figure 3A (*legend to follow*)

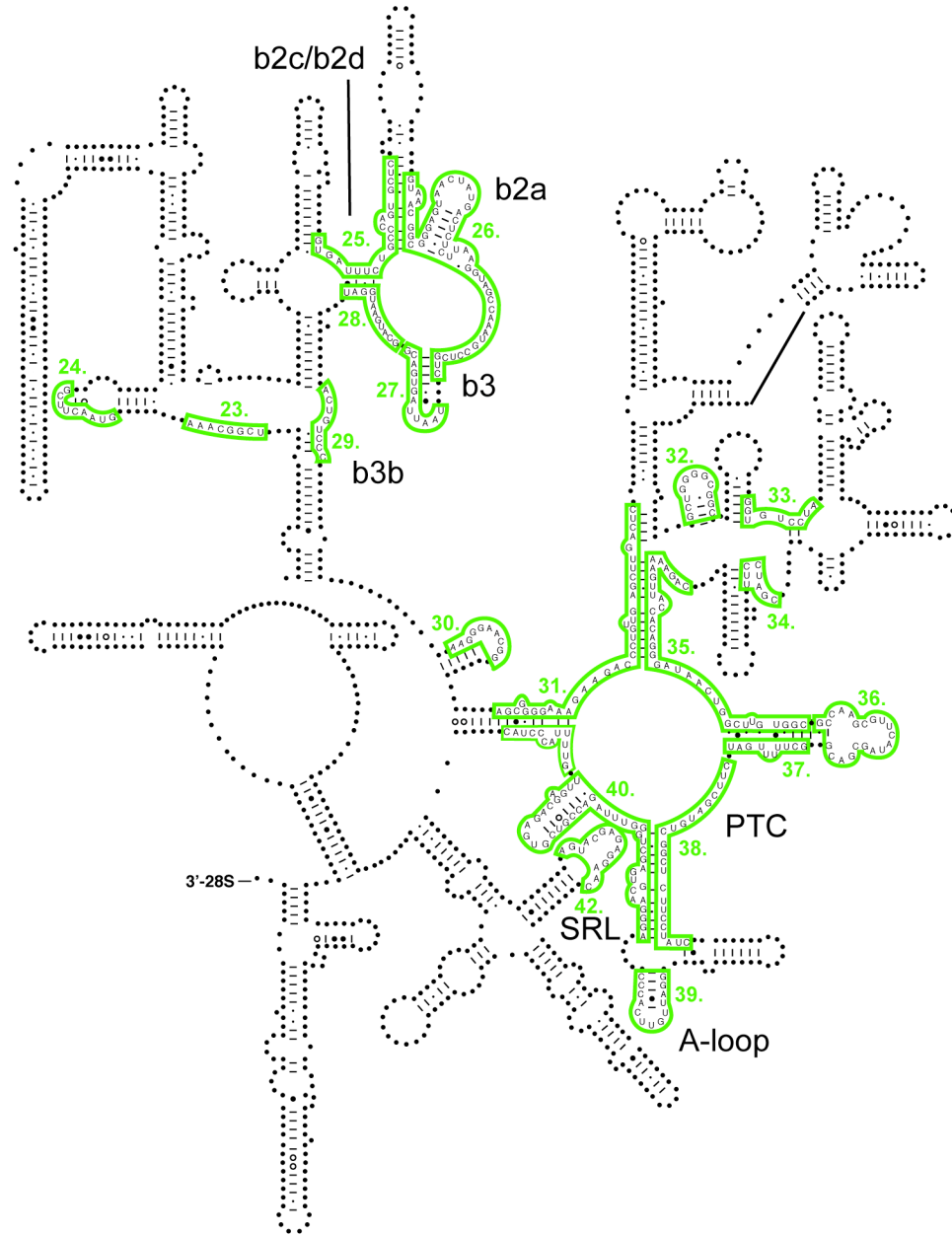


Figure 3. Distribution of eCNEs in the yeast 25S rRNA secondary structure. The 42 eCNEs sequence and secondary structure are boxed in green on a 25S rRNA secondary structure map. (A) The 5' half of 25S. (B) The 3' half of 25S. RNA-RNA bridges b2a, b2c/b2d, b3 and b3b are labeled, and are formed by CNEs 26, 25 and 28, 27 and 29 respectively. In addition, The GTPase center, the peptidyl transferase center (PTC), the A-loop, and the sarcin-ricin loop (SRL) are all comprised of eCNEs.

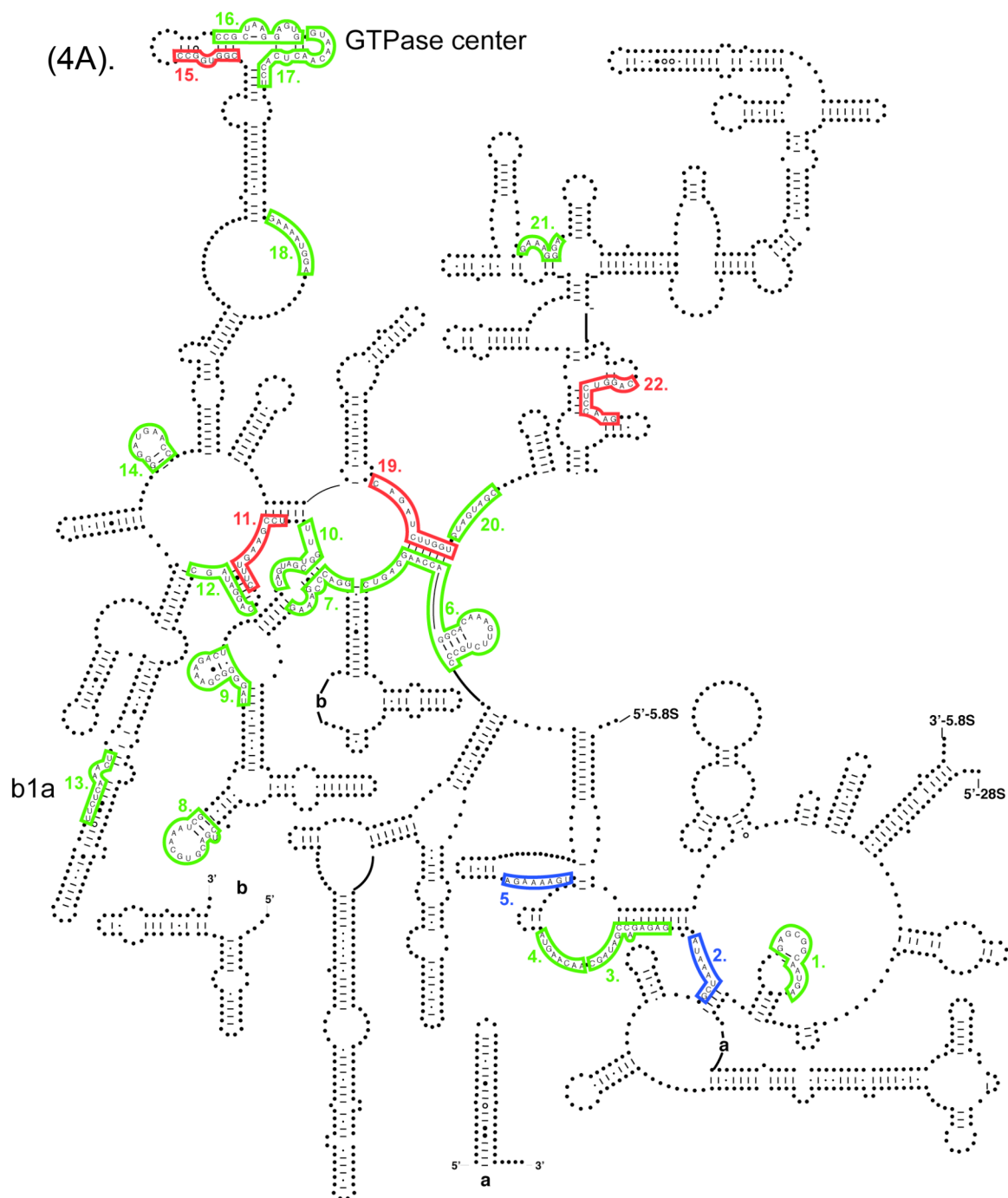


Figure 4A (*legend to follow*)

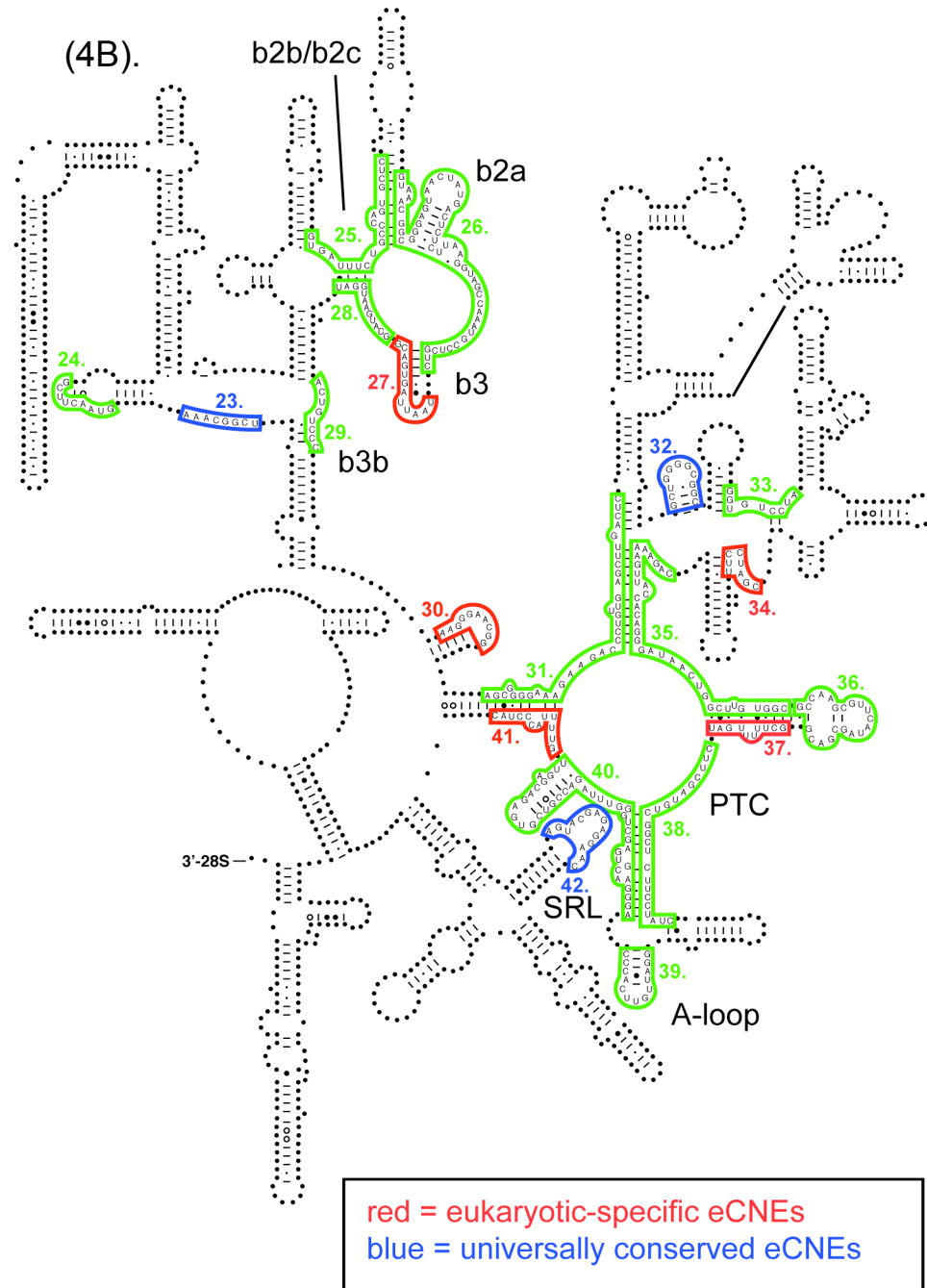


Figure 4. Distribution of eukaryotic-specific and universally conserved eCNEs. The 5' portion of yeast 25S rRNA is shown in A, and the 3' portion in B. In both panels A and B eukaryotic-specific eCNEs are boxed in red, and universally conserved CNEs in blue. All remaining eCNEs are boxed in green.

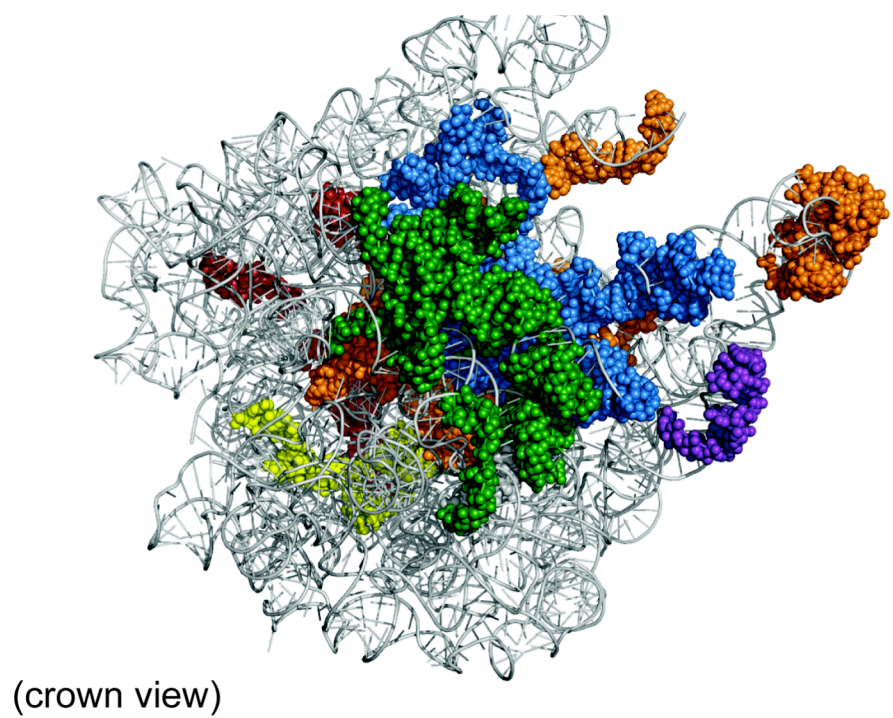
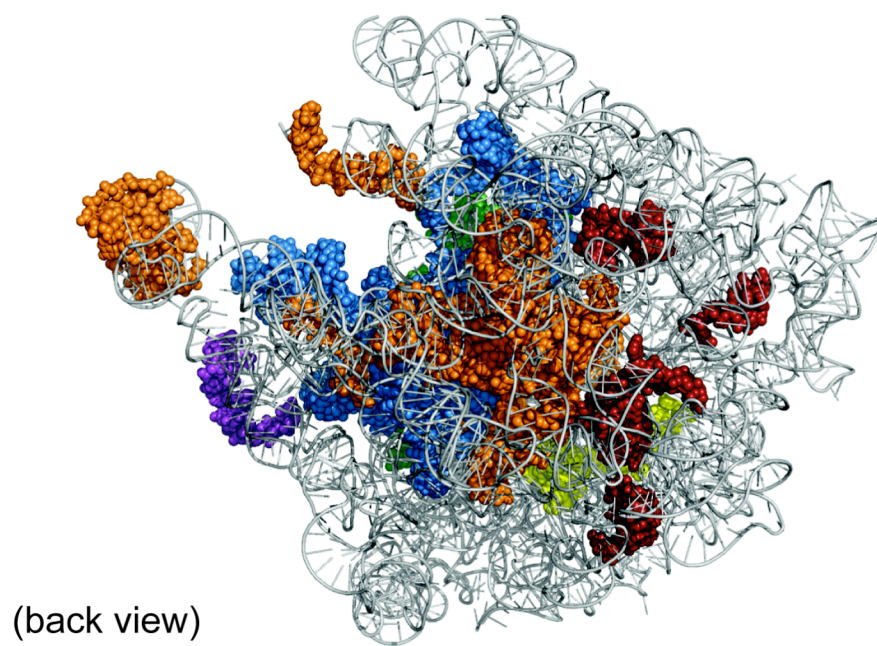


Figure 5A. (*legend following*)

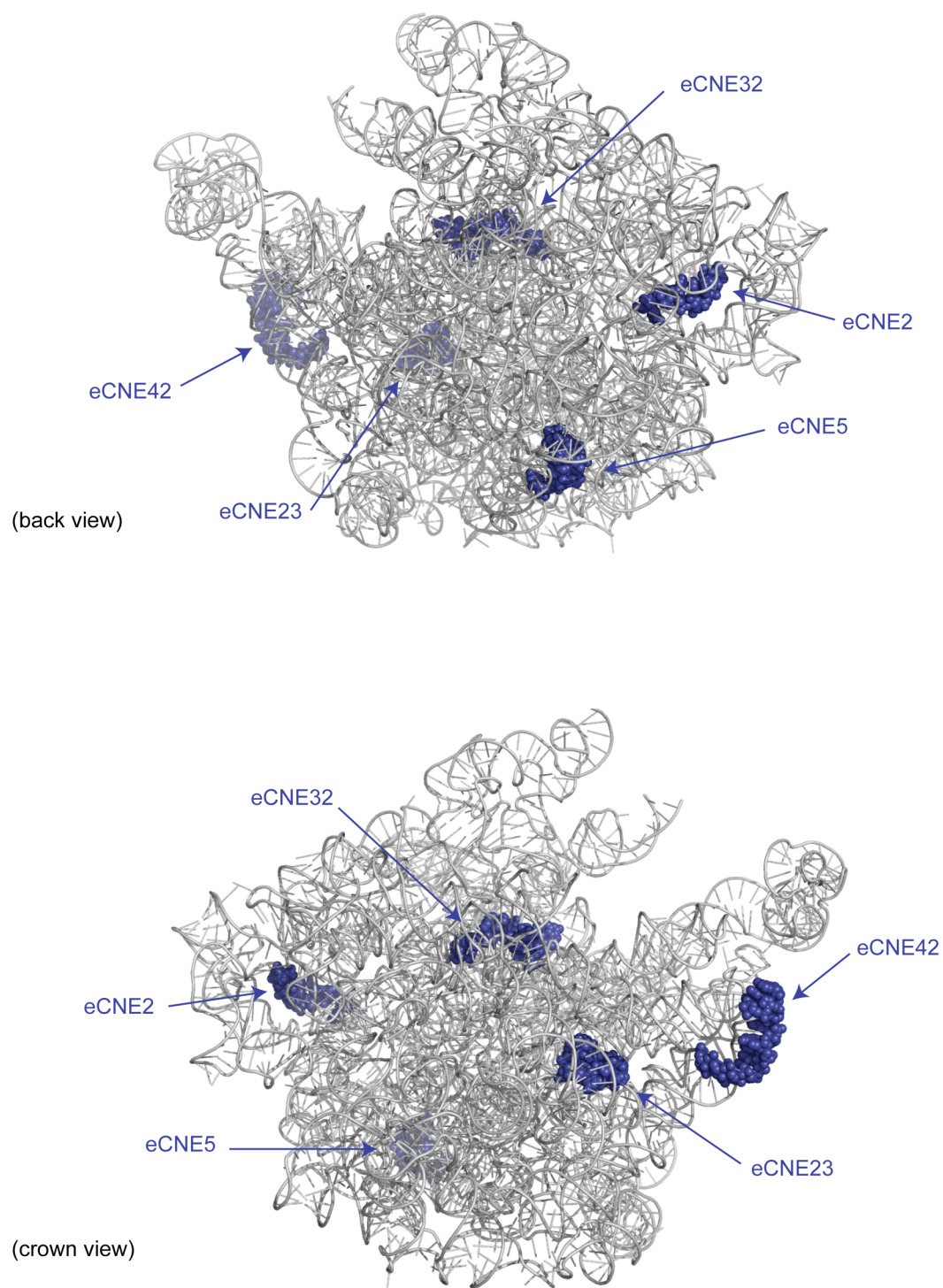


Figure 5B. *(legend following)*

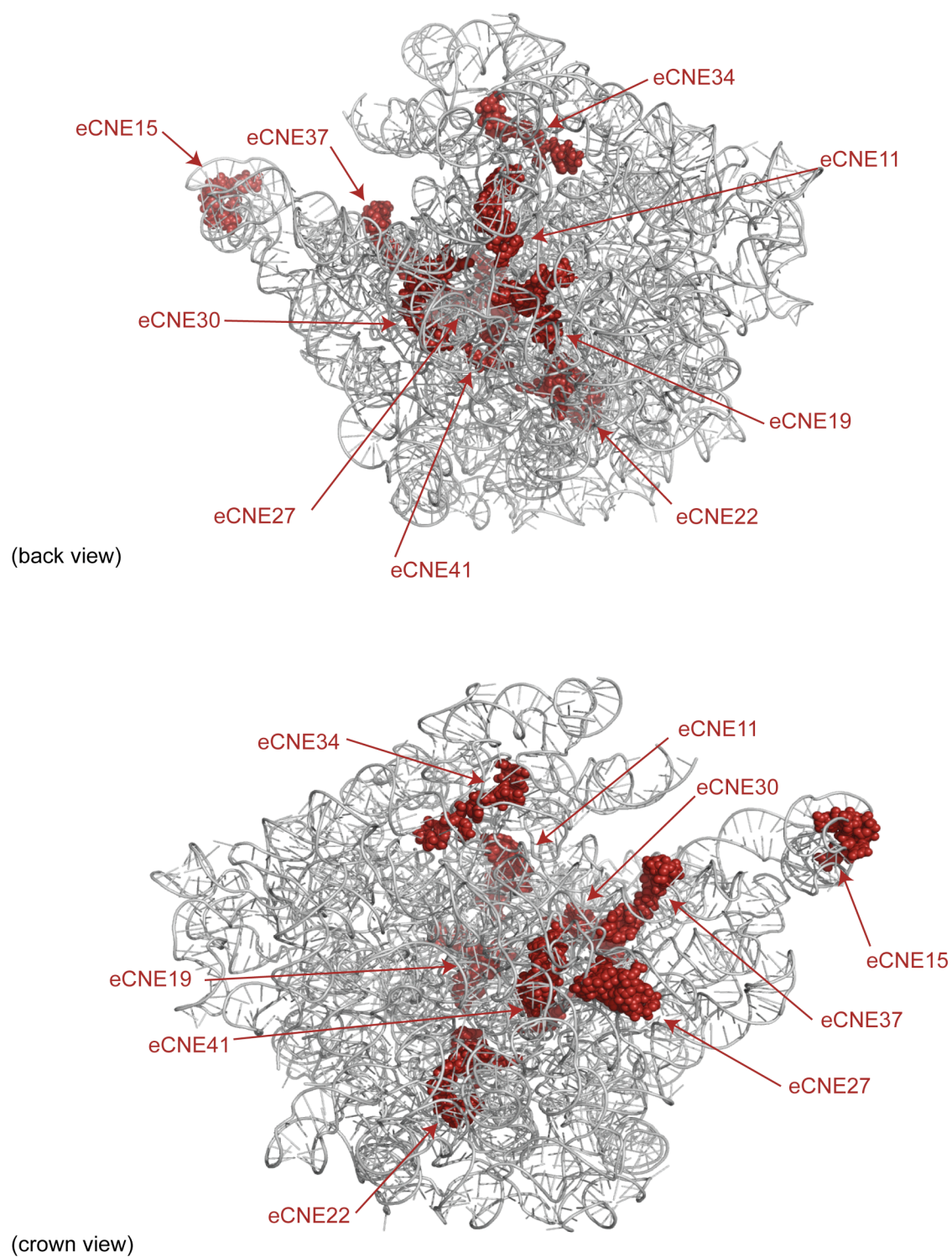


Figure 5C. (*legend opposite*)

Figure 5: Modeling the eCNEs in the 50S ribosomal subunit: The three-dimensional X-ray crystal structure of the 50S subunit from *H. marismortui* shown in back view (solvent exposed) and crown view (subunit interface) with the eCNE homologous displayed as space-filled colored atoms and the remaining rRNA in gray. (A) The 42 eCNEs colored by domain of the ribosome: domain I (red), domain II (orange), domain III (yellow), domain IV (green), domain V (blue), domain VI (purple). (B) The eCNEs that are universally conserved in all three phylogenetic domains colored blue. (C) The eCNEs that are highly degenerate in Bacteria and Archaea colored red.

Chapter 3: Ribosomal Protein L32 and Eukaryotic Conserved Nucleotide Element Six (eCNE6) Are Needed for Biogenesis of Ribosomal Subunits

This project was done with the assistance of undergraduate student Elizabeth A. Schroeder.

ABSTRACT

Recent phylogenetic studies on ribosomal RNA have identified eukaryotic conserved nucleotide elements (eCNEs) in the large subunit. To begin to address the function of eCNEs *in vivo*, we determined that ribosomal protein L32 associates with two eCNEs (eCNE6 and eCNE14) in Domain II of the large subunit rRNA. To assess their functional importance, we disrupted the eCNE6 and L32 interaction in budding yeast *Saccharomyces cerevisiae* and in the oocytes of the frog *Xenopus laevis*. We show that L32 is required for the export of the large subunit and unexpectedly the small subunit. Disruption of the eCNE6 structure inhibits pre-rRNA processing and the primary 40S transcript accumulates in the nucleus. Similarly, genetic depletion of L32 causes a severe inhibition in post-transcriptional pre-rRNA processing and a rapid accumulation of the 35S transcript. Surprisingly, depletion of L32 leads to a rapid down regulation of cytoplasmic 40S and 60S ribosomal subunits. This unique phenotype is the first example of a ribosomal protein needed for pre-rRNA processing and cytoplasmic stability of both ribosomal subunits. We conclude that the L32-eCNE interaction is essential to normal rRNA processing and subunit assembly. These data provide evidence for a sensor between nuclear biogenesis and cytoplasmic degradation of ribosomes.

INTRODUCTION

Ribosomes, the cellular factories for protein synthesis, are crucial for all forms of life. Eukaryotic ribosome biogenesis is a highly orchestrated process (Fatica and Tollervey, 2002; Gerbi and Borovjagin, 2004) and a massive consumer of cellular energy (Warner, 1999). The production of ribosomes requires the three major RNA polymerases. RNA Polymerase I produces the polycistronic 40S/35S pre-rRNA (*X. laevis*/*S. cerevisiae*) which is processed into mature 18S, 5.8S, 28S/25S rRNAs. RNA Polymerase II synthesizes the ~78 ribosomal proteins (r-proteins), and RNA Polymerase III synthesizes 5S rRNA. The assembly of the ribosome employs several hundred non-ribosomal factors: ~200 small nucleolar RNAs (snoRNA) for guiding internal cleavages, folding, and covalent modification of the rRNA (Decatur and Fournier, 2002; Gerbi and Borovjagin, 2004; Kiss, 2001) and ~170 protein assembly factors (Tschochner and Hurt, 2003). The massive amount of factor localization and RNP assembly drives the formation of a specialized subnuclear compartment, the nucleolus, which is the primary site of ribosome biogenesis (Gerbi et al., 2003). The net outcome of ribosome biogenesis is two cytoplasmic ribosomal subunits. The 60S large subunit (LSU) is composed of three rRNAs (28S/25S, 5.8S, 5S) complexed with 47 r-proteins. The small 40S subunit (SSU) contains 18S rRNA and 32 r-proteins. The 80S ribosome directs mRNA translation and is the largest known ribozyme (Nissen et al., 2000).

When considering the universal conservation of rRNA (Ware et al., 1983) and ribosome structure (Ban et al., 2000; Spahn et al., 2001), one would not anticipate the bacterial and

eukaryotic ribosome assembly pathways to be vastly different. But, in contrast to its *E. coli* counterpart (Traub and Nomura, 1968), adding r-proteins to rRNA *in vitro* does not reconstitute a functional *S. cerevisiae* ribosome. A handful of chaperone proteins and RNA helicases assist subunit assembly in *E. coli*, but none are essential (Culver, 2003; Williamson, 2003). In contrast, biogenesis of the eukaryotic ribosome requires the formation of several pre-ribosome intermediates, and the majority of the assembly factors are essential in yeast (Venema and Tollervey, 1999), thus explaining the difficulty in reconstituting eukaryotic ribosomes *in vitro*. Many of the eukaryotic r-proteins may need to assemble cotranscriptionally onto the pre-rRNA and/or require assembly factors. Pioneering work in HeLa cells and yeast identified the three major pre-ribosomes: the earliest pre-ribosome sediments at 90S and this large particle containing pre-rRNA is processed in the nucleolus giving rise to pre-40S and pre-60S subunits (Trapman et al., 1975; Udem and Warner, 1972; Vaughan et al., 1967; Warner et al., 1972). Despite their identification 40 years ago, only recently have pre-ribosomes been isolated from the yeast nucleus and their protein components analyzed (Harnpicharnchai et al., 2001; Saveanu et al., 2001). Proteomic mass spectrometry analyses has allowed for the initial ordering of the pre-ribosomal network (Fatica et al., 2002; Grandi et al., 2002; Milkereit et al., 2003; Nissan et al., 2002; Schäfer et al., 2003). These studies demonstrate that pre-ribosomes change their protein components and structure both spatially and temporally during maturation.

The ~170 assembly factors can be grouped into two categories; the energy utilizing factors (NTPases) that actively remodel RNA-RNA, RNA-protein, or protein-protein

interactions, and the non-energy consuming factors, which likely act as chaperones to prevent thermodynamic and kinetic problems inherent to folding an RNA (Herschlag, 1995). Putative RNA helicases are the largest known class of energy utilizing ribosome assembly factors (de La Cruz et al., 1999; Kressler et al., 1999). Since the pre-rRNA is modified by ~70 snoRNPs (that base-pair with the pre-rRNA), RNA helicase activities are likely required to mediate these interactions during rRNA maturation. The essential yeast gene *DBP4*, a putative RNA helicase, was originally identified as a high-copy suppressor of U14 mutants, a snoRNA that guides both methylation and cleavage of pre-18S rRNA (Liang et al., 1997). Mutagenesis or genetic depletion analysis of *DBP4* shifts the distribution of U14 from free snoRNP to pre-ribosomes suggesting helicase activity is needed to remove U14 from the 90S particle (Kos and Tollervey, 2005). These studies imply that pre-rRNA remodeling by helicase(s) is essential to pre-rRNA processing, modification and rRNA folding (Decatur and Fournier, 2002).

Genetic studies in yeast have also demonstrated the requirement for GTPases and AAA-ATPases in ribosome assembly and export. The AAA-type ATPase Rix7p is required for proper pre-rRNA processing and transport of the pre-60S particle from the nucleus (Gadal et al., 2001). A requirement for AAA-ATPases implicates protein unfolding or dissociation as mechanisms involved in ribosome assembly (Brown, 2001). Given that AAA-ATPases are established molecular motors for generating movement along a track (Vale, 2000), they may also function as molecular motors for unidirectional movement during export of the large rRNP.

A third class of energy utilizing biogenesis factors is the GTPases which are implicated in nucleolar, nucleoplasmic, and cytoplasmic ribosome assembly and trafficking (de La Cruz et al., 2004; Johnson, 2004). GTP hydrolysis drives conformational switches in the ribosome during the translation cycle (Nilsson and Nissen, 2005; Wintermeyer and Rodnina, 2000). A potentially similar GTPase mechanism could also transduce rRNP assembly/disassembly. For example, the cytoplasmic maturation of newly exported ribosomes employs two GTPases, Lsg1p and Elf1p, which remove proteins from ribosomes in the cytoplasm thus allowing them to recycle back to the nucleus (Hedges et al., 2005; Kallstrom et al., 2003; Senger et al., 2001; West et al., 2005). The molecular functions of the majority of the identified essential assembly factors are unknown, but are postulated to stabilize native rRNA structure, recruit other assembly factors, and/or ribosomal proteins (de La Cruz et al., 2004; Venema and Tollervey, 1999). The preliminary studies on ribosome assembly suggest that many structural rearrangements occur within the maturing rRNP particle. This complex process may ensure that nuclear ribosome biogenesis meets the cytoplasmic demands of a growing cell as efficiently as possible, which in growing yeast cells averages 2,000 ribosomes per minute (Warner, 1999).

Ribosomal RNA processing initiates cotranscriptionally (Granneman and Baserga, 2005), but most events occur after transcription termination (Raué, 2004). Although the pre-rRNA species have been catalogued (Figure 1), how assembly factors and native r-proteins interact with pre-rRNA to bring about the precise structural conformations required for cleavages remains predominantly unknown. A requirement for r-proteins in

rRNA processing has been comprehensively analyzed for the synthesis of 18S rRNA and the resulting 40S subunit in yeast (Ferreira-Cerca et al., 2005). Each 40S r-protein was genetically depleted and their contribution to rRNA processing and nuclear export was evaluated, demonstrating that individual r-proteins of the SSU (Rps) could play a unique role in the processing pathway. For example, some Rps were required for early cleavage of the initial 35S precursor (e.g., Rps13) whereas others were needed for late cytoplasmic site D cleavage (e.g., Rps20). A role in RNA processing is not a general feature for all Rps proteins, as some can be depleted and rRNA is synthesized and exported normally, suggesting that these essential genes may play unique roles in ribosome function (e.g., Rps7). In tissue culture, homologous human Rps function similarly in rRNA processing. In this system, siRNA mediated knockdown of Rps were used to assess rRNA processing defects (Robledo et al., 2008). The observation that r-proteins can be classified as acting early or late in rRNA processing implies that assembly of Rps onto maturing rRNA is hierarchical. Indeed, data support a hierarchical ribosome assembly landscape; kinetic labeling of newly synthesized r-proteins revealed that some proteins associate late in biogenesis (Kruiswijk et al., 1978). Furthermore, using a combination of r-protein genetic depletions followed by pre-40S immunoprecipitation, it was demonstrated that Rps15 association is dependent on the presence of Rps5 (Ferreira-Cerca et al., 2007). These data obtained *in vivo* from yeast support the principles established in *E. coli* that assembly of the small ribosomal subunit is cooperative and hierarchical (Agalarov et al., 2000; Held et al., 1973).

Less is understood about the assembly of the large subunit (LSU) as compared to the small subunit assembly pathway. In yeast, there are 46 r-proteins in the mature LSU, and very few of them have been analyzed for their precise role in rRNA processing and/or ribosome function. The majority of r-proteins of the LSU (Rpl) analyzed by mutagenesis or depletion revealed they are all needed for processing of 35S and 27S (L16: Moritz et al., 1990; L3: Rosado et al., 2007; L25: van Beekvelt et al., 2001; L30: Vilardell and Warner, 1997). However, data obtained from HeLa cells showed that 35S and 27S defects are not a general consequence of Rpl depletion as some Rpl can act early and others act late (Robledo et al., 2008). This suggests that 60S assembly, like 40S, occurs in a hierarchical and cooperative manner.

Recently, we have defined eukaryotic conserved nucleotide elements (eCNEs) in large subunit rRNA that show perfect conservation in all Eukarya (Chapter 2). In this study, we report that Rpl32 (L32) contacts two eCNE structures (eCNE6 and eCNE14) in 25S/28S rRNA. These observations coupled to the fact that L32 lacks a bacterial homologue (Dresios et al., 2006) suggest that L32 plays a specialized function in eukaryotic ribosomes; however, the role of L32 in the cell is poorly understood. Here we show that L32 is required for the nuclear export of the small and large subunits. When eukaryotic cells are depleted of L32 or when eCNE function is blocked, early steps in rRNA processing are inhibited causing a build up of the 35S/40S precursor transcript. Thus, L32 and eCNEs are essential features in eukaryotic ribosomes.

RESULTS

Modeling the L32 interactions with eukaryotic conserved nucleotide elements

High-resolution crystal structures of the archaeal large ribosomal subunit from *Haloarcula marismortui* allow deep insight into the final conformation of this RNP (Ban et al., 2000; Klein et al., 2004). We took advantage of these data and modeled the tertiary conformation of eukaryotic conserved nucleotide elements (eCNEs) in large subunit rRNA. eCNEs are ≥ 8 nt stretches found in the universal core structure of the 23S-like molecule that display a $\geq 90\%$ sequence identity in all sequenced Eukarya (Chapter 2). We reason that these highly conserved motifs are critical to eukaryotic rRNA, but their function in ribosomes is unexplored. We observed in the crystal structure (PDB No. 1S72) that ribosomal protein L32e contacts the eCNE6 and eCNE14 archaeal homologues as well as helices 25 and 46. eCNE6 comprises helix 25.1, half of helix 26, and ends at the top of helix 27; eCNE14 forms helix 40 (Figure 2, helix numbering as per Leffers et al., 1988). The L32e protein in Archaea is homologous to Rpl32 in yeast (nomenclature as per (Mager et al., 1997) and RpL32 in flies and the amino acid sequence is evolutionarily conserved (Figure 3, and see Materials and Methods). Therefore, we used the archaeal structure to predict the protein-RNA contact points in eukaryotes (Figure 4). The major difference between the L32 orthologues is an N-terminal domain of 108 amino acids absent in eukaryotes (Figure 3). Important to the modeling studies, the archaeal specific N-terminal domain does not contact rRNA in the crystal structure. The L32 protein contains alpha and beta structure and these structured domains are solvent exposed and primarily contact the RNA backbone of helix 46

(Figure 4A and B). Additionally, L32 is among the group of ribosomal proteins that contain unstructured extensions piercing into the core of the LSU (Figure 4A). It is the extended region of L32 that potentially contacts eCNE6 and eCNE14 in eukaryotes (Figure 4B). About half of the contacts between L32e and rRNA are ionic interactions between basic amino acid residues and the phosphate backbone of the RNA and some base specific interactions (Figure 2 and 3) (Klein et al., 2004). Given that L32 is predicted to bind two eCNE motifs and lacks a bacterial homologue, we hypothesize that L32 plays a critical function in the eukaryotic ribosome.

***RPL32* is essential for cell growth**

To address the function of L32 *in vivo*, we engineered a haploid yeast strain in which the endogenous *RPL32* promoter (a single copy gene) is replaced with the galactose inducible/glucose repressible *GALI* promoter and tagged the expressed protein with the HA epitope at the N- or C-terminus (Figure 5). After confirming targeted integration, strains were grown on solid medium containing glucose or galactose and their phenotypic properties were evaluated globally. The *GAL::RPL32* strains showed a significant growth defect on glucose medium demonstrating that *RPL32* is essential for normal cell growth (Figure 6). We then measured the growth rates of the strains in galactose liquid medium and found that the *GAL::RPL32* and the *GAL::RPL32-HA* strains grew like the parental strain suggesting that the ectopic expression or tagged protein does not interfere with ribosome assembly or function (Figure 6A). Tagging the protein on the N-terminus however resulted in impaired growth (Figure 6A). Therefore, all future experiments were conducted in the *GAL::RPL32* or *GAL::RPL32-HA* strains. To examine the depletion

kinetics of L32 mRNA and protein levels, the *GAL::RPL32-HA* strain was cultured in galactose to exponential growth phase, shifted to glucose, and samples were analyzed for 24 hours (Figure 7). The L32 mRNA levels are rapidly depleted in glucose as no mRNA can be detected by the 2 hr time point (Figure 7A). This is consistent with the rapid response of the previously reported *GALI-RPL1* where transcription of L1 is no longer detected 30 min after shifting to glucose (Deshmukh et al., 1993). After 6 hr in glucose, the majority of the L32 protein has been depleted and is undetectable by 24 hr (Figure 7B and C) compared to the *RPL32-HA* control strain, which maintained a constant level of L32 throughout the carbon source shift (Figure 7B).

L32 and eCNE6 are required for normal assembly and export of both subunits

L32 is required for cell growth (Figure 6) and contacts the eukaryotic-specific sequences encoding eCNE6 and eCNE14 (Figure 4). We tested if this interaction plays a role in the nuclear export pathway using established export reporter assays. Rpl25 assembles early onto newly synthesized rRNA (el-Baradi et al., 1985) and this has been used to monitor nuclear export of 60S in yeast using a Rpl25-GFP fusion protein (Hurt et al., 1999). A Rps2-GFP fusion has similarly been used to monitor export of the small 40S subunit (Dez et al., 2006). The requirement for L32 in nuclear export of 60S and 40S subunits was monitored in *GAL::RPL32* strains transformed with Rpl25-eGFP or Rps2-GFP. After *RPL32* expression is repressed, a predominantly nuclear signal is observed for both the 60S and 40S subunit compared to the cytoplasm by 12 hr of L32 depletion (Figure 8). Thus, L32 is required in the ribosome nuclear export pathway. This result could be a

consequence of defective ribosome biogenesis and/or L32 could be directly involved in nuclear transport.

To test if L32 functions in pre-rRNA processing (Figure 1), RNA was isolated from wild-type and L32-depleted cells and pre-rRNA levels were analyzed by Northern blotting to detect the processing intermediates. Depletion of L32 results in a build-up of the primary 35S pre-rRNA by 4 hr, which continues to accumulate at 6 hr (Figure 9) and no production of the 23S pre-rRNA as a result from premature A₃ cleavage (Granneman and Baserga, 2004; Venema and Tollervey, 1999). Furthermore, a severe decrease in mature small subunit (18S) and large subunit (5.8S and 25S) rRNAs are detected (Figure 9). This demonstrates that L32 is needed for the biogenesis and cytoplasmic accumulation of both 40S and 60S ribosomal subunits.

We next sought to address if eCNE6, a potential binding site for L32, also plays a role in pre-rRNA maturation. To block the function of eCNE6, an antisense morpholino oligo (Mo) against the eCNE6 sequence was tested for its ability to specifically hybridize to its complement in *Xenopus laevis* 28S rRNA. *Xenopus* oocytes were injected with ³²P-UTP and purified labeled rRNA containing 28S and 18S rRNA was added to an RNase H reaction with antisense eCNE6 DNA oligo alone or increasing amounts of Mo. In the absence of Mo, 28S rRNA is cleaved at the position of DNA/RNA hybridization, thereby diminishing the amount of intact 28S rRNA and 18S rRNA is unaffected (Figure 10, lane 2 arrow). As the concentration of Mo increases, DNA oligo directed cleavage by RNase H is inhibited and 28S rRNA is protected (Figure 10, lanes 4 and 5). Having shown the

Mo anneals to its complement *in vitro*, the experiment was then conducted in the nucleus of the oocyte. To determine if eCNE6 is needed for proper pre-rRNA processing, ^{32}P -UTP alone or with Mo was microinjected into the nucleus of stage V/VI oocytes and allowed to react. In the absence of Mo, the ^{32}P -UTP is incorporated into newly synthesized rRNA and processed normally (Figure 11B, lane 1). When the Mo is introduced into the nucleus, transcription of the 40S primary transcript appears normal, but is not efficiently processed (Figure 11B, lane 2). The inhibition of early pre-rRNA processing from Mo injection appears specific, as 40S increases in a dosage-dependent manner (lane 3). Thus eCNE6 and L32 are both required for early steps in ribosome biogenesis.

DISCUSSION

The recent achievements of high-resolution crystal structures of the ribosomal subunits allow a thorough examination of a mature conformation of the rRNA and r-protein structures. The rRNA forms a series of base-paired stems interrupted by unpaired loops that come together in three dimensions and are stabilized by the r-proteins. The r-proteins can contact multiple domains and are responsible for the structural integrity of the ribosome (Klein et al., 2004). Despite these advancements in our understanding of ribosome structure and function, the process of assembling r-proteins and rRNA into a functional ribosomal particle *in vivo* remains poorly understood.

Ribosomal proteins can be multifunctional *in vivo* as active players in all ribosomal processes including rRNA processing, pre-ribosomal assembly, transport, and function. Additionally, r-proteins can have cellular functions independent from the ribosome including chromatin assembly, cell cycle regulation and apoptosis (Warner and McIntosh, 2009). In yeast, a comprehensive analysis of the small subunit r-proteins provides strong evidence that r-proteins have adopted unique roles in biogenesis (Ferreira-Cerca et al., 2005). In this study, we focused on the analysis of a large subunit protein, L32, in yeast. Several observations highlighted the potential significance of L32. First, it is among the class of ribosomal proteins that have a structured domain on the surface of the ribosome with an extended loop that interacts with helices in the core of rRNA (Figure 4A). The polypeptide extensions of r-proteins that penetrate the ribosome are likely critical to the architecture of the ribosome. This is supported by mutagenesis experiments, which show

that r-protein extensions are necessary for accurate translation (e.g. RpS3; Meskauskas and Dinman, 2007) and site D cleavage in 20S pre-rRNA (Rps14; Jakovljevic et al., 2004). Secondly, phylogenetic analyses showed that L32 is not a universal protein, since L32 homologues are only found in Eukarya and Archaea but are absent in Bacteria. (Dresios et al., 2000). Eukaryotic L32 is significantly smaller than the archaeal counterpart as it lacks the ~110 amino acid N-terminus found in archaeal L32e thus reducing its molecular weight by ~45% (Figure 3). The N-terminal truncation may be of structural importance as yeast cells with an N-terminal HA tag fusion display a growth defect compared to a C-terminal fusion (Figure 6A). Interestingly, the L32 extended loop domain enters into the RNA interior of the ribosome and contacts two eukaryotic conserved nucleotide elements (eCNE6 and eCNE14) (Figure 2). The eCNE sequences are highly conserved in all known eukaryotic rRNA (Chapter 2). Given the unique structure of eukaryotic L32 and the fact that it coordinates two eCNE motifs implies that L32 evolved a specialized function in eukaryotic ribosomes.

In this report, we focus on defining the role of L32 in the biogenesis of eukaryotic ribosomes. A yeast strain conditionally expressing L32 (*GAL::RPL32*) could not grow on non-permissive medium showing that L32 is essential for cell growth (Figure 6B). Depleting L32 from logarithmically growing cells is efficient as L32 levels decrease by 4 hours and no protein is detected in cells after 24 hours of growth in glucose. A consequence of L32 depletion is an increase in the 35S primary transcript (Figure 10). This block in rRNA processing is rapid as the 35S rRNA build up is detected within 4 hours of shifting to glucose-based medium, suggesting the processing defect is a direct

consequence of L32 depletion. This is a novel molecular phenotype for a large ribosomal protein as all depletion studies to date have shown that depletion of any large subunit protein allows for the synthesis of 18S rRNA and the export of pre-40S subunit. The 35S pre-rRNA is upstream of nuclear export thus can explain the nuclear retention of the 60S and 40S ribosomal subunit export reporters (Figure 8).

It has been proposed that r-protein assembly and rRNA processing are functionally coupled (Ferreira-Cerca et al., 2005 and references therein). Given that L32 is needed for 35S processing, it would strongly indicate that L32 assembles onto 35S pre-rRNA and is a component of the 35S rRNA-containing 90S pre-ribosome. This is a new finding, as L32 was not identified in the proteomic analysis of the 90S pre-ribosome (Grandi et al., 2002). As noted by the authors, due to their low molecular weight and high sequence homology ribosomal proteins are difficult to analyze by mass spectrometry or Edman analysis, thus explaining why L32 was missed.

An outstanding question in the eukaryotic ribosome biogenesis field is- how does a large subunit assembly factor influence early steps in both 40S and 60S rRNA processing? As the newly synthesized rRNA emerges, a large complex dubbed the small subunit processome (Fournier and Maxwell, 1993) assembles as a terminal ball on the 5' ETS of nascent pre-rRNA. This large rRNA processing complex contains U3 snoRNA and a multitude of assembly factors (Dragon et al., 2002; Gallagher et al., 2004). Perhaps some r-proteins are *bona fide* components of the SSU processome (missed by proteomics as noted above), and their depletion prevents proper assembly onto nascent rRNA and hence

interferes with early rRNA processing of both subunits. Our results using an antisense oligo rule out this model. Morpholino oligos (Mo) act by hybridizing to their complementary sequence and disrupt local RNA structure. The direct nuclear injection of an antisense Mo against the 5' end of eCNE6 (helix 25.1, Figure 2) causes the primary transcript (40S) to accumulate in a morpholino dosage-dependent manner (Figure 11B) and a relative decrease in 28S and 18S rRNA. Due to the fact that eCNE6 transcription is downstream of SSU processome loading (Osheim et al., 2004), the Mo would not inhibit early processome assembly. Instead our data suggest that the eCNE6 structure in the 28S rRNA coding region must fold correctly for pre-rRNA processing to proceed. This is not a general principle in rRNA processing since deletion of helix E20_1 in yeast 25S rRNA is neutral to ribosome biogenesis and cell growth (Jeeninga et al., 1997). The results obtained in *Xenopus* oocytes blocking eCNE6, an L32 binding site, mimics the molecular phenotype of L32 depletion in yeast, where both inhibit the earliest steps in pre-rRNA processing. The depletion of L32 is the first example of a large subunit protein that upon depletion abrogates all posttranscriptional rRNA processing steps. This supports a model whereby the cell monitors the correct assembly of early rRNA binding proteins such as L32 and folded rRNA structures such as eCNE6, before activating rRNA processing- a potential quality control mechanism in ribosome biogenesis (van Beekvelt et al., 2001).

Depletion of L32 resulted in a remarkable decrease in mature 25S rRNA and 5.8S rRNA and 18S rRNA (Figure 9). The decrease in mature rRNA can be explained in part by the build up of the common 35S precursor, but does not fully explain the relative severity in down regulation in the highly stable mature rRNA in the cytoplasm. One possibility is

depletion of L32 causes the abortive assembly of 60S subunits (Moritz et al., 1990). This model proposed that if a component of a ribosomal subunit is missing, the other components of that subunit (proteins and rRNA) are unstable and selectively degraded. However, upon depletion of L32, we detect an immediate down regulation of the pre-existing 25S rRNA (Figure 9). Although abortive assembly is likely influencing the accumulation of newly made 60S subunits, it cannot completely explain the diminished steady-state levels of 25S rRNA 4 hr post-L32 depletion, nor can it explain the concomitant decrease in mature 18S rRNA.

Upon L32 depletion we observed ~60% decrease in mature 60S and 40S ribosomes (Figure 9). Given that the half-life of a ribosomal subunit is several days (Kraft et al., 2008), an active turnover mechanism is likely responsible for this rapid down regulation. Recently, a new pathway in yeast has been described for the selective degradation of mature ribosomes termed ribophagy (Kraft et al., 2008). This process involves the import of cytoplasmic subunits into the vacuole for turnover when yeast are starved of essential nutrients. We speculate that ribophagy is stimulated when early rRNA processing is completely blocked. This possibility is supported by the fact that many steps of ribosome biogenesis and rRNA processing are regulated by the nutritional state of the cells (Rudra, 2004). Further studies are needed to understand if there are cytoplasmic surveillance mechanisms monitoring ribosome biogenesis in the nucleus and how these processes might be functionally linked.

MATERIAL AND METHODS

Yeast Strains and Culturing

Yeast transformations were performed as described by Elble (Elble, 1992). Cells were cultured in rich (YP) medium supplemented with 2% glucose (YPD) or 2% galactose (YPG) and 0.004% adenine (Sherman et al., 1986). For genetic selection, cells were plated on solid YPG and grown until confluent and replica plated onto YPG containing G418. Or cells were selected on synthetic yeast nitrogen base medium (S) containing 2% galactose supplemented with 0.004% required amino acids, 0.004% adenine, and lacking uracil. The *S. cerevisiae* strains used in this study were derived from strain W303-1a (*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*). The yeast strains engineered in this study contain various modifications of the genomic *RPL32* locus tagged with the GAL1 promoter and/or the 3xHA epitope using PCR mediated one-step gene conversion (Longtine et al., 1998). LSY100 (*GAL::RPL32*) was created by PCR amplification of an integration cassette using pFA6a-KanMX6-pGAL1 as template with oligonucleotide primers that contain 5' homology to nucleotides ⁻544- ⁻504 upstream of the *RPL32* ORF and 3' homology to nucleotides ⁺3-⁺43 within the ORF, with ⁺1 being the A of the ATG start codon. Strain LSY200 (*RPL32-3XHA*) encodes a C-terminal 3X-HA tagged L32 fusion protein using pFA6a-HIS3MX6-3XHA as template with primers containing 40 base-pairs of homology to ⁺350-⁺390 in the *RPL32* ORF (deleting the endogenous stop codon) and ⁺⁺1-⁺⁺40, where ⁺⁺1 refers to the first nucleotide after the TAA stop codon. Strain LSY102 (*GAL::RPL32-3XHA*) was made by PCR amplifying the C-terminally tagged 3xHA L32 gene and his5⁺ marker using LSY200 genomic DNA as

template and integrating the DNA into strain LSY100. All *RPL32* strains were confirmed genetically. For L32 depletion experiments, cells were grown to mid-log phase in YPG at 30° C, washed in sterile water, and resuspended in YPD medium pre-warmed to 30° C. Cells were kept in constant logarithmic growth by monitoring OD₆₀₀ and diluting with pre-warmed medium.

Structural modeling and alignments

The eukaryotic L32 protein and eCNE nucleotide homologues were identified in the archaeon *H. marismortui* as follows. The amino acid sequences of L32 from *D. melanogaster* (AE014297.2), *S. cerevisiae* (CAA96731), and *H. marismortui* (P12736) were aligned pairwise by the CLUSTALW server (<http://align.genome.jp/>). For ribosomal RNA analysis, the *S. cerevisiae* 25S (J01355) and *H. marismortui* 23S rRNA (X13738) sequences were aligned by secondary structure using the software package ARB (Ludwig et al., 2004), and the homologous eCNE coordinates in *H. marismortui* 23S rRNA were identified. Together these data were used to synthesize three-dimensional models using the X-ray crystal structure of the *H. marismortui* 50S ribosomal subunit (PDB No. 1S72) (Ban et al., 2000). Structure data was input into MacPyMol (2006 DeLano Scientific LLC), and contact points between rRNA and L32 were identified.

Yeast RNA analysis

RNA extractions were done using the method of hot-acidic phenol extraction. For mRNA detection, 10 µg of total RNA was resolved through 8% polyacrylamide gel containing 8

M urea and electroblotted onto supercharged Nytran membrane (Schleicher and Schuell, Keene, NH). A ^{32}P end labeled oligo probe was used to detect L32 mRNA (give sequence) and blots were exposed to a phosphor storage screen (Amersham) and imaged on the Typhoon 9410. For analysis of ribosomal RNA processing, RNA was extracted from 10 OD₆₀₀ equivalents of cells and 10 µg of total RNA was resolved on formaldehyde containing 1.2% agarose MOPS gel that was stained with ethidium bromide and imaged. RNA was denatured in 0.01 N NaOH 3 M NaCl prior to capillary transfer to Nytran membrane. The sequences of the probes are referenced in Figure 10.

Immunoblot analysis

Cells corresponding to 1 OD₆₀₀ equivalents were centrifuged, washed, resuspended in 100 µL SDS-PAGE sample buffer and stored at -80°C. Samples were boiled and 0.1 OD₆₀₀ equivalents of cells were resolved on a 12% SDS-PAGE or a 4-12% gradient gel (Invitrogen). Protein was transferred to a nitrocellulose membrane using the bufferless Invitrogen iBlot system according to manufacturer's instructions or by wet transfer. Blots were subsequently probed with anti-HA antibodies to detect L32 (Roche, 3F10) or anti-Pgk1 antibodies (Molecular Probes, Eugene, OR) as a loading control. Quantification of protein signals was measured using ImageQuant software (Amersham).

Fluorescence Microscopy

Yeast strain LSY102 was transformed with plasmids L25-GFP (pAJ908) or S2-GFP (pAJ1374). Precultures grown in YPG were used to inoculate SG-Ura medium and cultured until mid-log growth, washed in sterile water, and shifted to SD-Ura. Cells were

maintained in constant logarithmic growth by monitoring OD₆₀₀ and diluting with fresh pre-warmed media. Cells were washed once in PBS and mounted onto glass slides in glycerol. Data were collected on a Zeiss LSM 510 confocal microscope at 63X magnification.

Oocyte Microinjection and RNA analysis

Stage V/VI oocytes from *X. laevis* (Nasco, Fort Atkinson, WI) were obtained surgically, defolliculated by incubation in 2 mg/ml collagenase (Sigma-Aldrich), and washed in buffer OR2 as previously described (Borovjagin and Gerbi, 1999). Oocytes were injected with ³²P-UTP for *in vivo* labeling of rRNA or coinjected with an morpholino oligo (GeneTools) complementary to *Xenopus* 28S eCNE6 (nt 966-990, number according to Ajuh et al., 1991). Total RNA was isolated using the Gentra tissue isolation kit according to the manufacturer's instructions. The RNA was electrophoresed on a 1.2% agarose formaldehyde gel and electroblotted onto Nytran Plus membrane (Schleicher and Schuell, Keene, NH) and exposed to a phosphoimaging plate.

***In vitro* RNase H assay**

Stage V/VI oocytes were injected with ³²P-UTP for *in vivo* labeling of rRNA as described above. Purified labeled RNA from five whole oocytes equivalents was added to a 50 µl RNase H reaction (New England Biolabs). Each reaction was mixed with either RNase H, 500 pmol of antisense eCNE6 DNA oligo, 500 pmol of eCNE6 antisense morpholino oligo (Mo), or increasing amount of Mo. Reactions were incubated for 1.25 hours at 37°C and stopped by the addition of RNA gel loading buffer and heated at 65° C. RNase

H cleavage products were analyzed on a denaturing agarose gel containing formaldehyde and electroblotted onto Nytran Plus membrane (Schleicher and Schuell, Keene, NH) and analyzed by phosphorimage analysis.

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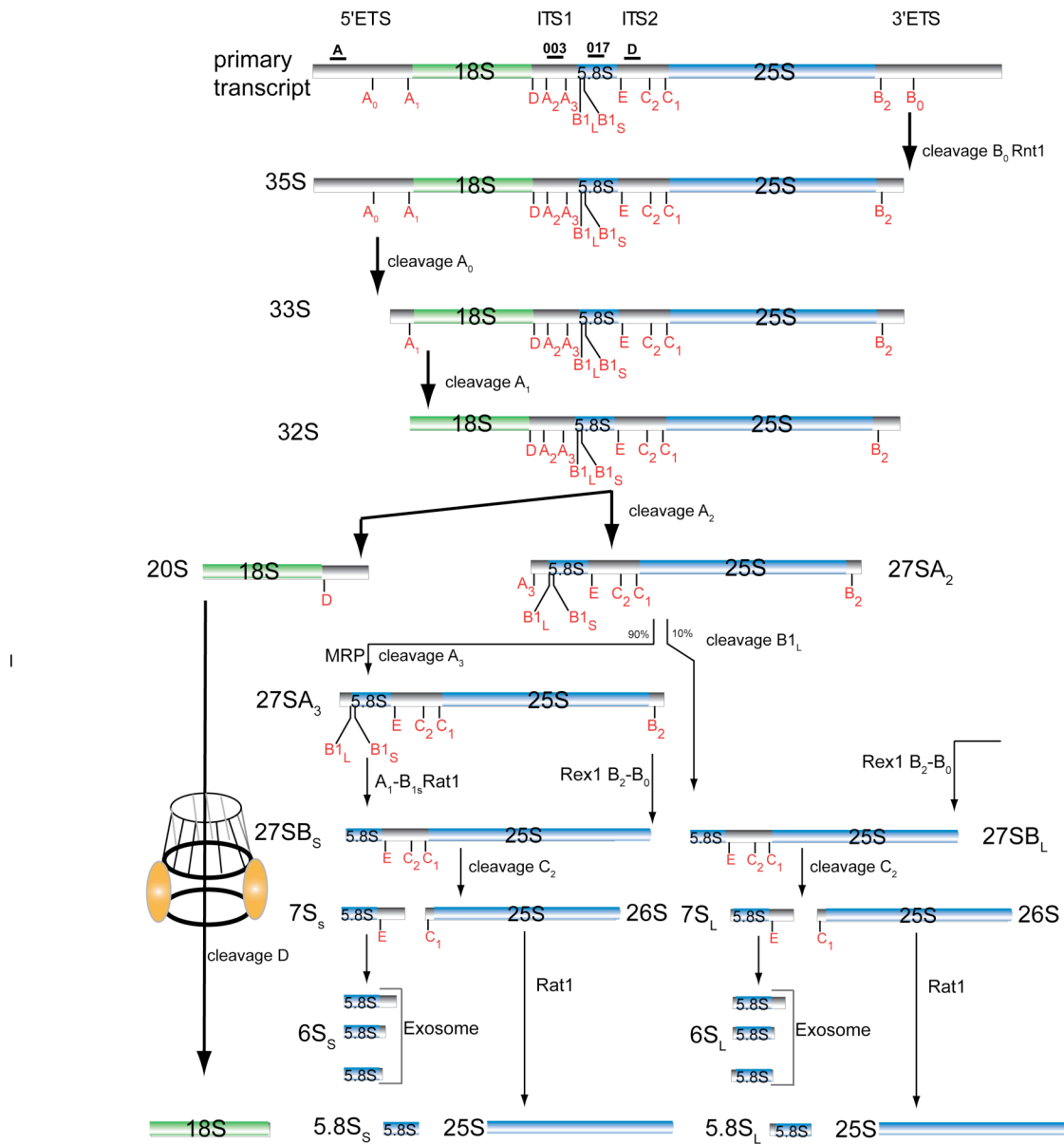


Figure 1. Ribosomal RNA processing in the yeast *Saccharomyces cerevisiae*: The primary transcript is shown first with the spacer sequences (ETS, external transcribed spacer ITS, internal transcribed spacer) in gray, small subunit in green, and large subunit in blue. Site D cleavage occurs after nuclear export. Processing of 25S and 5.8S occurs in the nucleolus and are exported mature rRNA. The large subunit pathway produces two versions of 5.8S- short (5.8S_s) and long (5.8S_L). Oligo probes used in Northern blot analysis are pictures above the primary transcript (A, 003, 007, and D). Figure adapted from H. Raué (Raué, 2004).

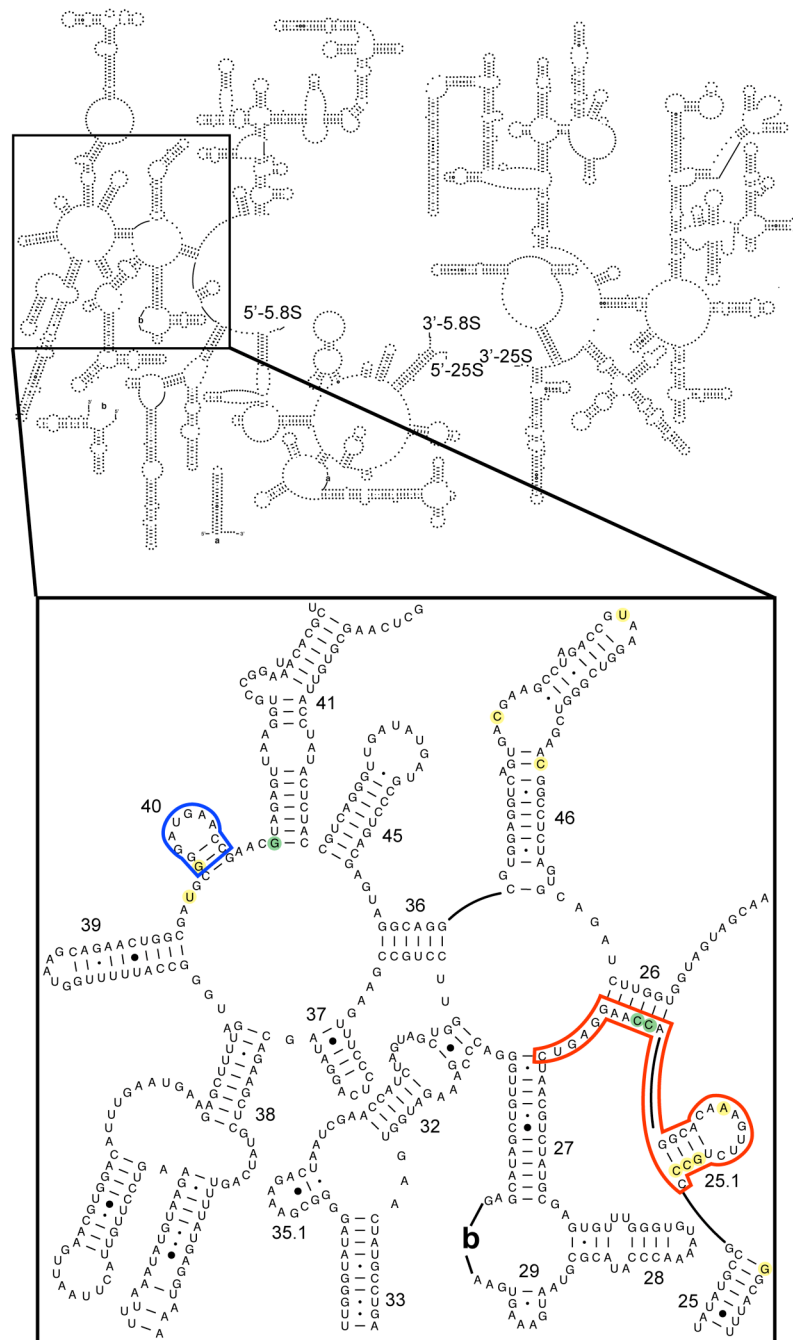


Figure 2. Secondary structure of the Rpl32 binding sites include eukaryotic conserved nucleotide elements 6 and 14 (eCNE6 and eCNE14): Yeast 25S rRNA secondary structure with the region of the L32 binding sites boxed (top, adapted from <http://www.rnaicmb.utexas.edu>). The nucleotides of eCNE6 (red) forms helix 25.1 and part of helix 26. eCNE14 (blue) is helix 40 (numbering according to Leffers et al., 1988). L32 also contacts part of helix 25 and helix 46. Base specific contacts are highlighted green and back bone contacts yellow (bottom, magnified view).

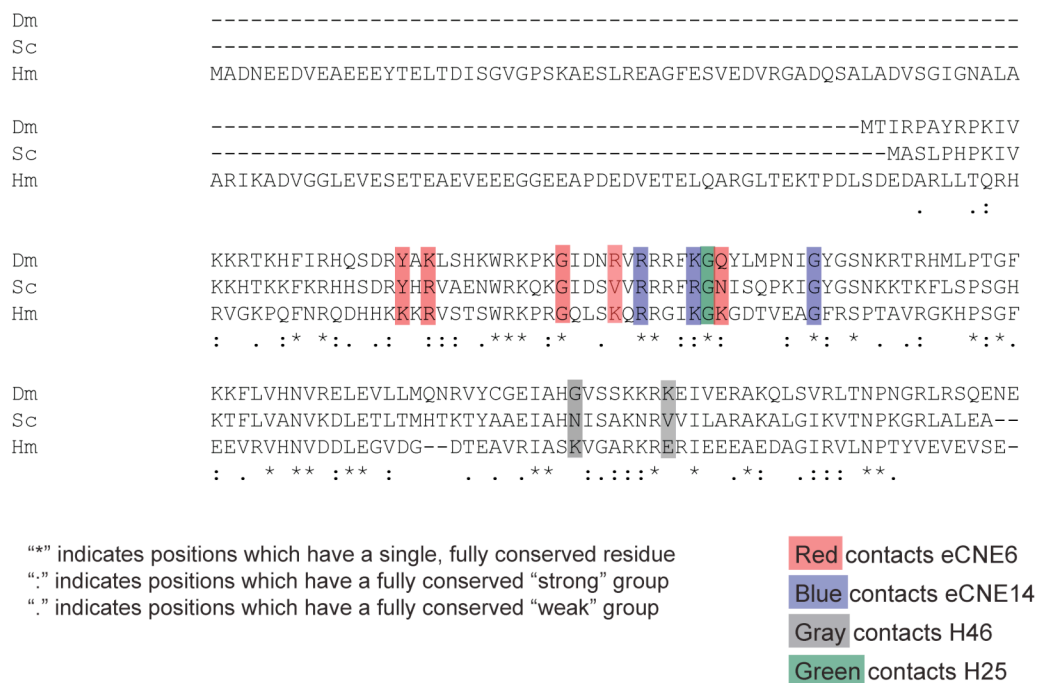


Figure 3. Evolutionary comparison of Rpl32 to L32e: Amino acid conservation of Rpl32e in Archaea and Rpl32 in Eukarya. Amino acids that contact rRNA are colored based on contact points in the 50S crystal structure (PDB No. 1S72). Red contact eCNE6, blue contact eCNE14, grey contact helix 46 and green contact helix 25. *Drosophila melanogaster* (Dm), *Saccharomyces cerevisiae* (Sc), *Haloarcula marismortui* (Hm).

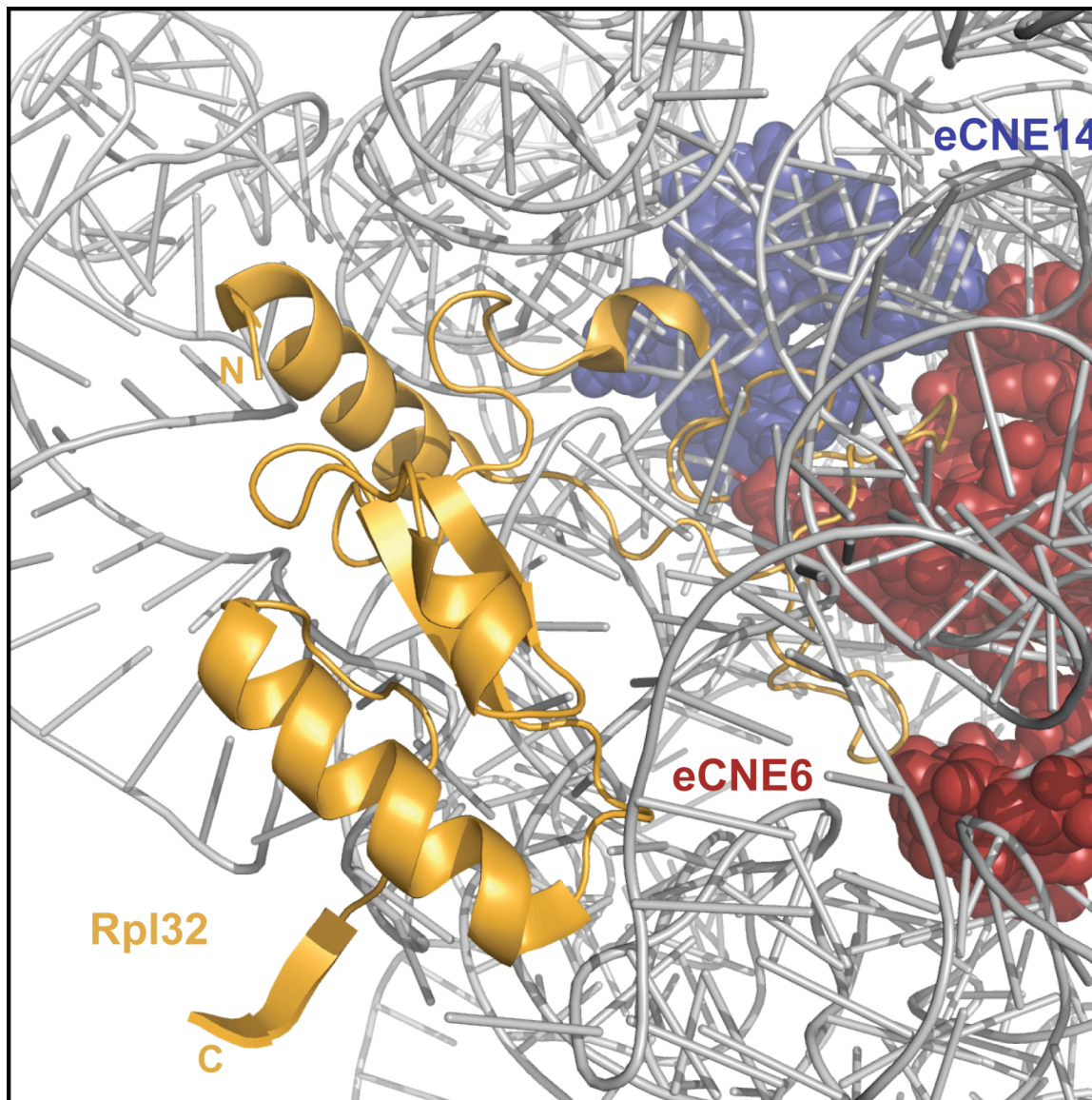
A.

Figure 4A: (*legend opposite*)

B.

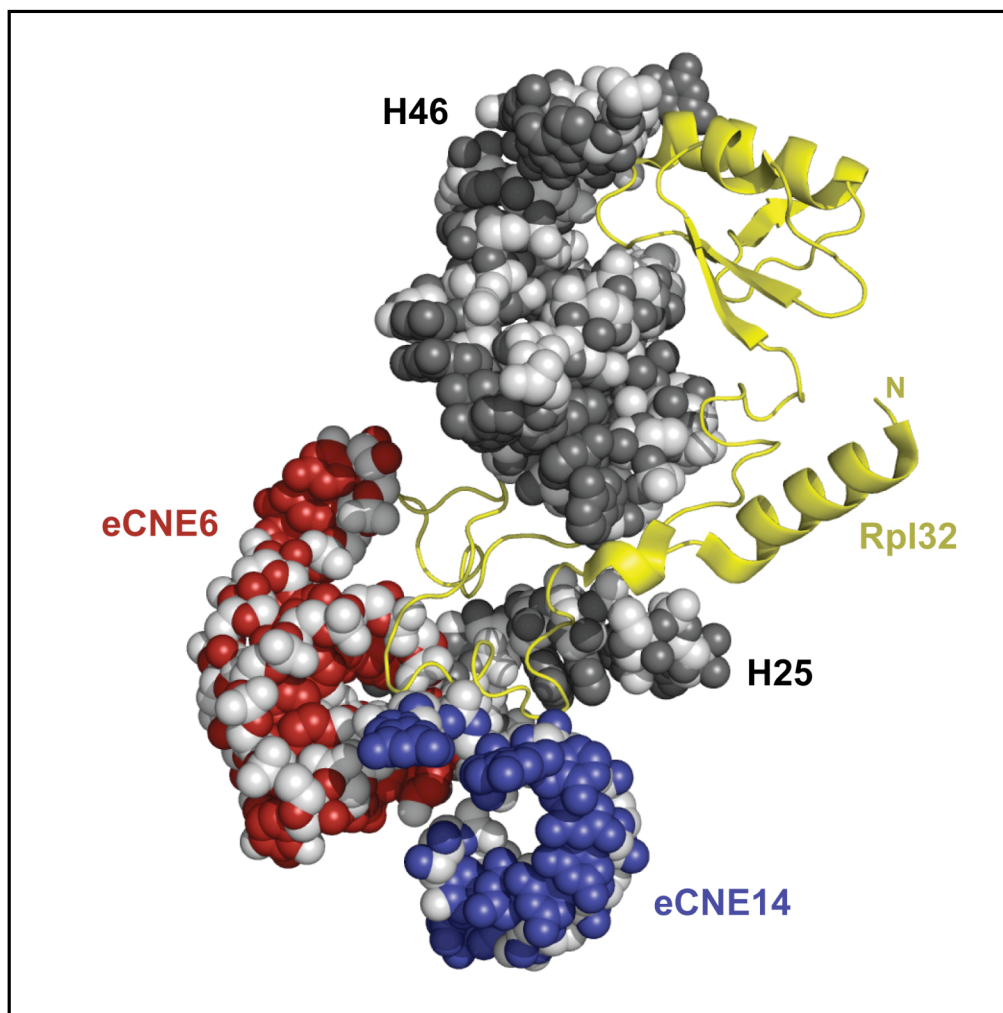


Figure 4. Homology modeling L32 and its contacts to RNA: Rpl32 (yellow ribbon structure) modeled from the *H. marismortui* crystal structure (PDB No. 1S72) contains N- and C-terminal alpha helices and parallel beta-sheets at its C-terminus. (A) The structured region of the protein is exposed to solvent at the surface of the ribosome with an unstructured domain that penetrates into Domain II of rRNA contacting eCNE6 (red) and eCNE14 (blue) homologues. (B) The protein contacts four RNA helices and coordinates the structures of helix 25 (dark grey), eCNE6 (red), eCNE14 (blue) and helix 46 (dark grey) (RNA shown as space filled atoms). The sugar-phosphate backbone of the RNA helices are colored light-grey.

W303-1a Chr II

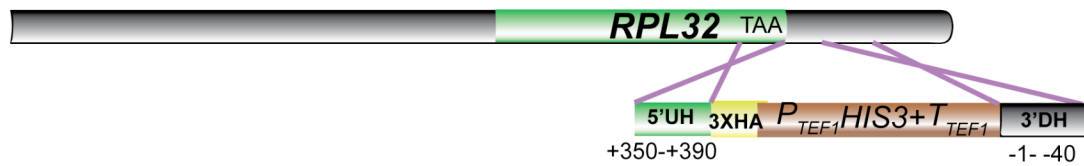
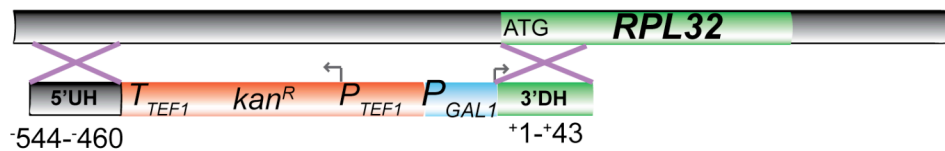
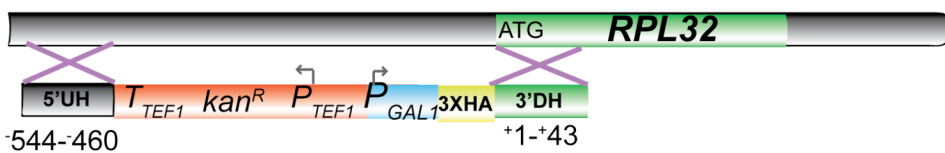
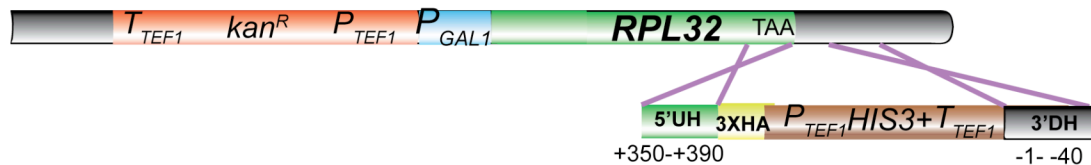
*RPL32*-3xHA*GAL::RPL32**GAL::3xHA-RPL32**GAL::RPL32*-3xHA

Figure 5. Schematic for engineering *RPL32* tester strains: The *RPL32* structural gene is located on Chromosome II in *S. cerevisiae* (parent strain W303-1a Chr II, top bar). A series of four different recombinogenic DNA cassettes were synthesized to tag *RPL32* with three copies of the HA epitope (*RPL32*-3xHA), knock-in the *GAL1* promoter (*GAL::RPL32*) or a combination (*GAL::3xHA-RPL32*, *GAL::RPL32*-3xHA) by homologous recombination directed by upstream (UH) and downstream (DH) homologous sequence. Recombinants were selected on medium lacking histidine (SG-His) or containing kanamycin.

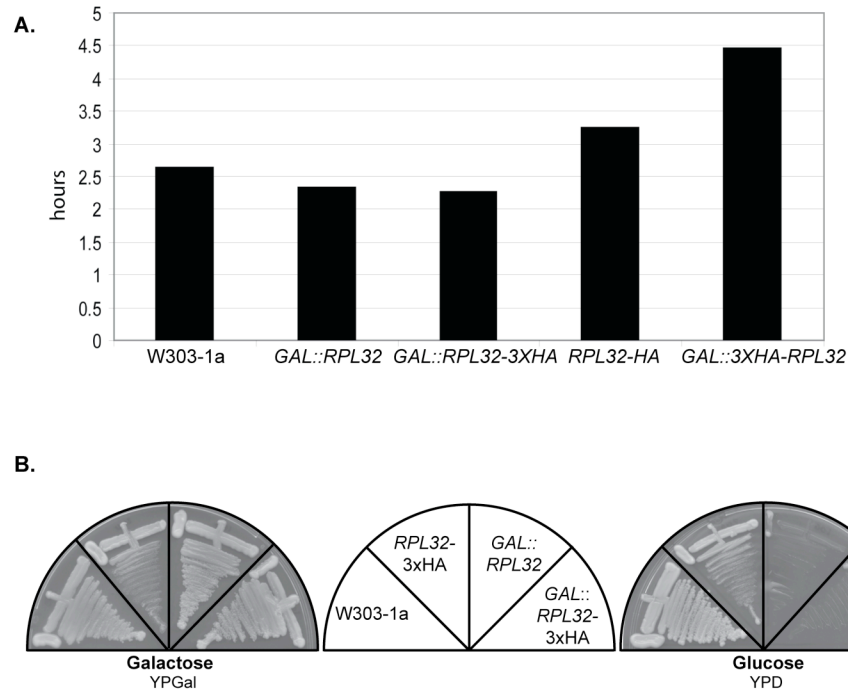


Figure 6. *RPL32* gene is essential for cell growth: (A) Population doubling times of *RPL32* tester strains: Yeast strains were grown in YPG medium and growth was recorded by measuring the OD₆₀₀ at regular intervals. When necessary, cultures were diluted to maintain exponential growth phase and population doubling time was calculated as $T(d) = (t_{final} - t_{initial}) \times \{\log(2)/[\log(OD_{final}/OD_{initial})]\}$. (B) The cellular phenotype for *GAL::RPL32* strains was tested on inductive (galactose) and repressive (glucose) medium for *RPL32* expression. Cells were grown for two days at 30°C. Strains containing *GAL::RPL32* as the only copy of *RPL32* in their genomes are dependent on galactose for growth and do not grow on glucose medium. The parent strain W303-1A was used as a control.

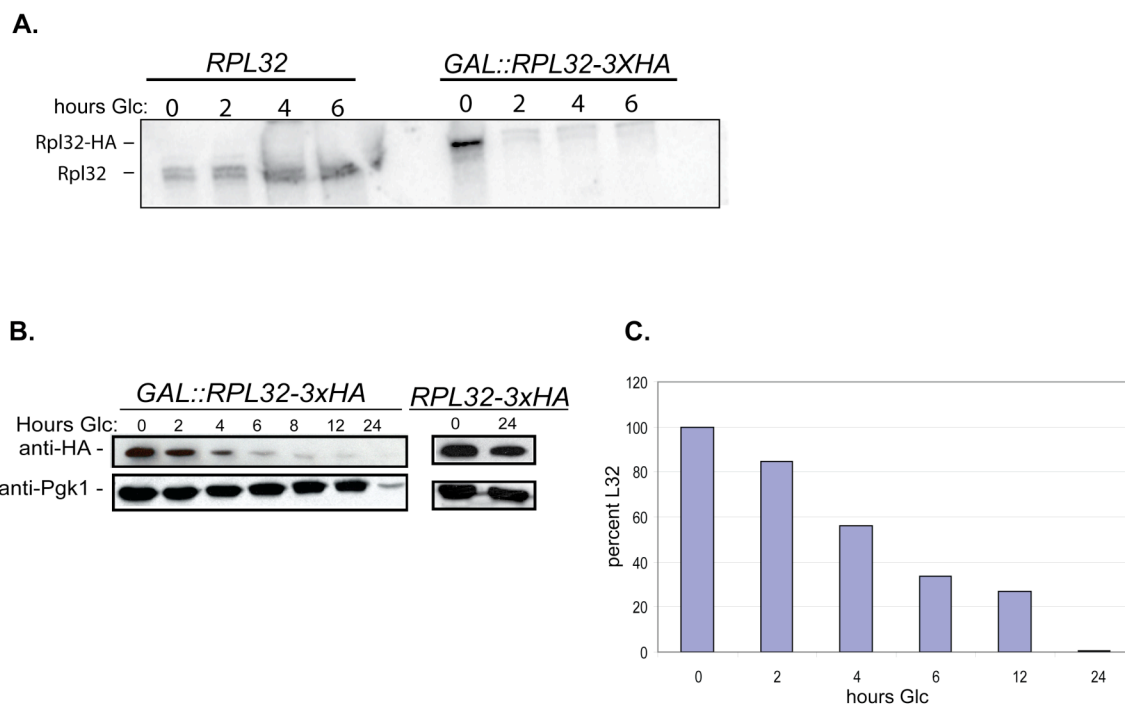


Figure 7. Efficient depletion of L32: Yeast cells were grown to exponential phase in galactose-based medium and then shifted to glucose (Glc)-based medium. Total RNA or total protein was extracted at the indicated time points and the expression of L32 was measured by Northern and Western blots. (A) Northern blot of L32 mRNA levels from 10 OD₆₀₀ equivalents of cells after shifting to glucose medium. (B) Western blot analysis of L32-HA tagged protein from 0.1 OD₆₀₀ equivalents. (C) Quantification of depletion signal in (B).

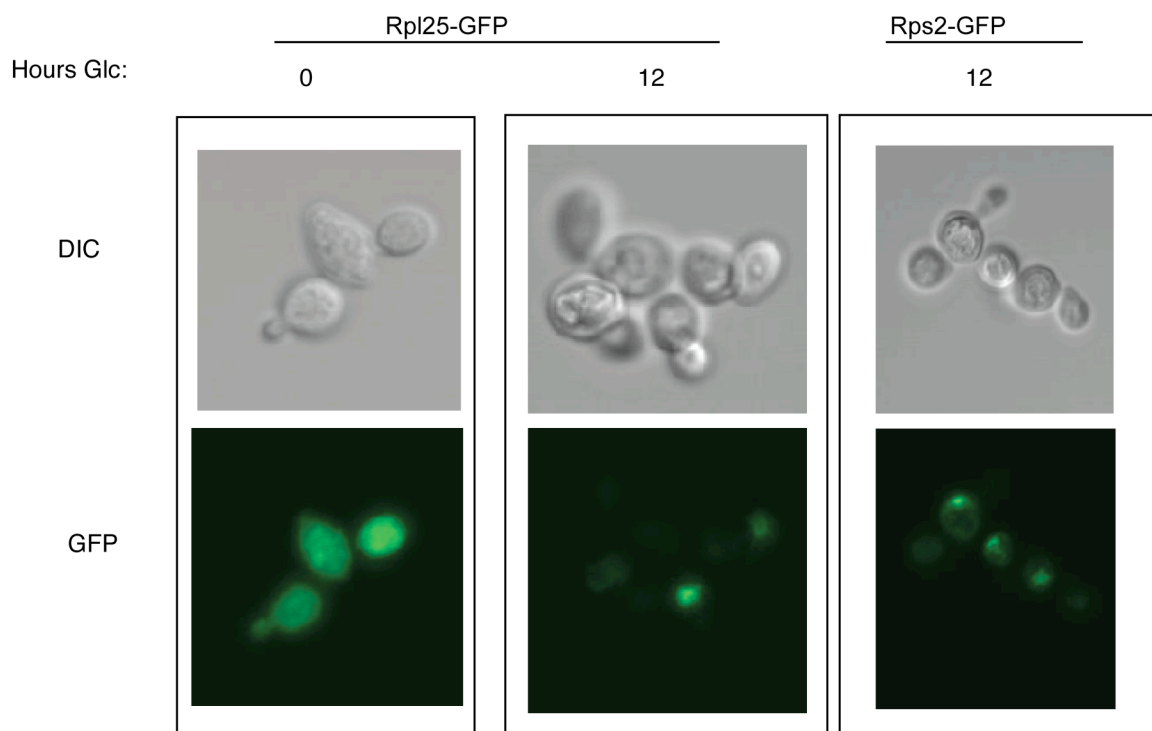


Figure 8. Nuclear accumulation of 40S and 60S subunits in Rpl32 depleted cells: The *GAL::RPL32* yeast strain was transformed with plasmids expressing Rpl25-eGFP or Rps2-GFP. Cells were pregrown in galactose medium (YPG) overnight at 30 °C and used to inoculate minimal galactose (SG-Ura) medium and cultured to exponential growth. Cells were then shifted to glucose medium (Glc, SD-Ura) and prepared for microscopy at 0 and 12 hours in glucose.

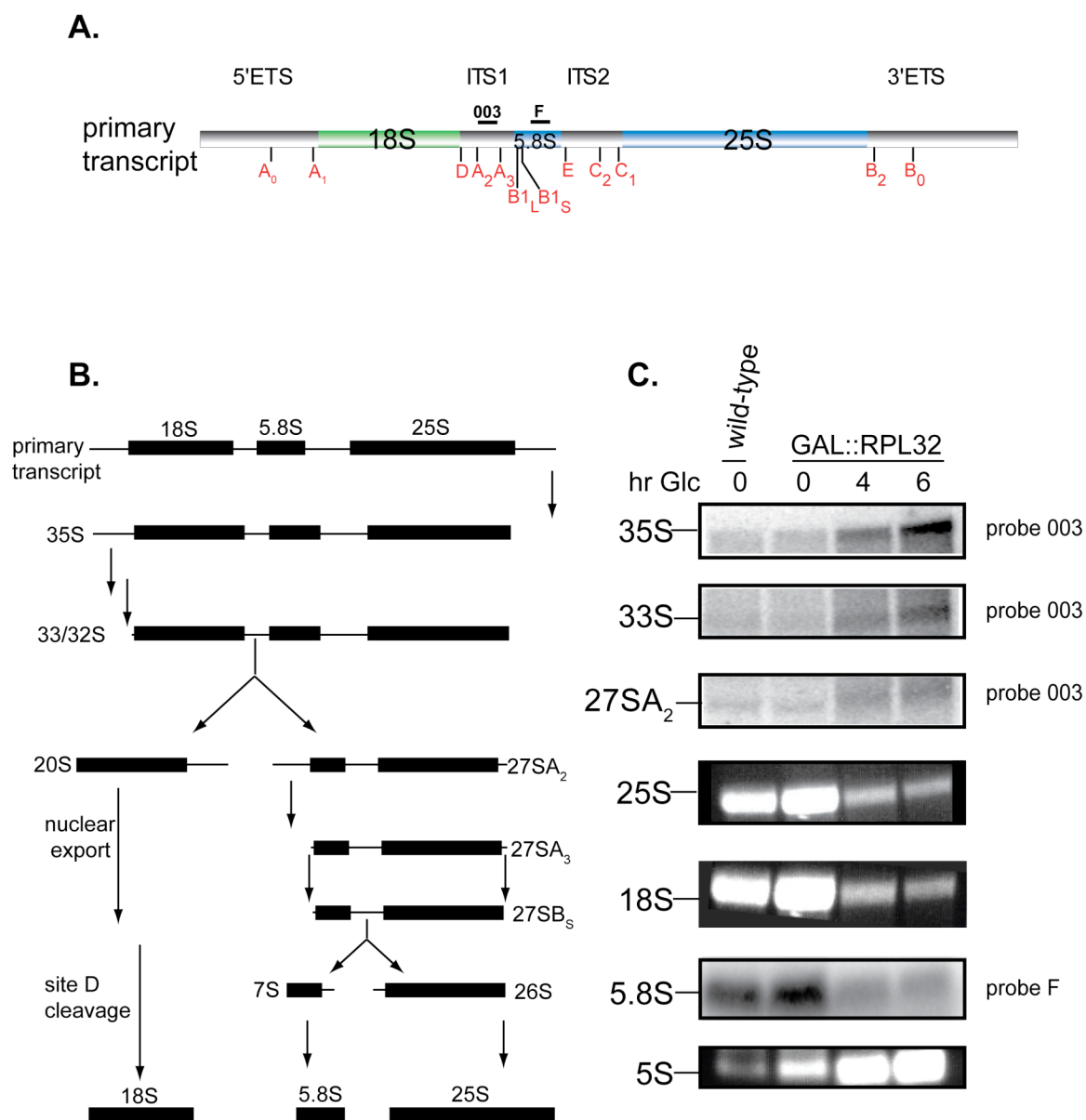


Figure 9. L32 is needed for 35S pre-rRNA processing: (A) The primary pre-rRNA transcript with oligonucleotide probes used in Northern analyses displayed above (F and 003) and the known processing sites marked in red below. (B) Schematic diagram of the major rRNA processing events in yeast. (C) *GAL::RPL32-HA* yeast cells were shifted from galactose based medium (YPG) to glucose based medium (YPD) and total RNA was isolated at 0, 4, and 6 hours. Northern analyses were carried out with 10 μ g of RNA using probes against 5.8S rRNA (probe F; van Beekvelt et al., 2001) or ITS1 (probe 003; Kos and Tollervey, 2005) to detect steady-state pre-rRNA processing intermediates compared to the wild-type strain W303-1a. Mature 25S, 18S, and 5S rRNA were detected by ethidium bromide staining prior to membrane capillary transfer.

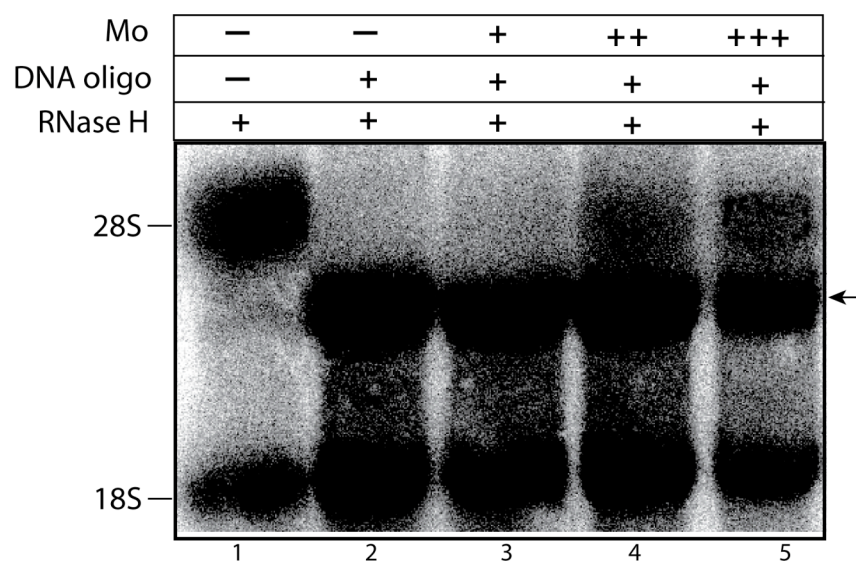


Figure 10. Morpholino oligo protects eCNE6 from RNase H cleavage: Stage V/VI *Xenopus* oocytes were injected with ^{32}P -UTP and total RNA isolated. Purified labeled RNA containing 28S and 18S rRNA was added to an RNase H reaction in the presence (lanes 2-5) or absence (lane 1) of 500 pmol of an antisense DNA oligo complementary to nt 966-998 which spans the eukaryotic conserved nucleotide element 1(eCNE6) in 28S rRNA. At time 0, a complementary morpholino oligo (Mo) to nt 966-990 was added (lanes 3-5). Competition assays contain an equal molar ratio of Mo:DNA oligo (lane 3) and increasing Mo in 10X (lane 4) and 50X (lane 5). The RNase H cleavage product is marked with arrow.

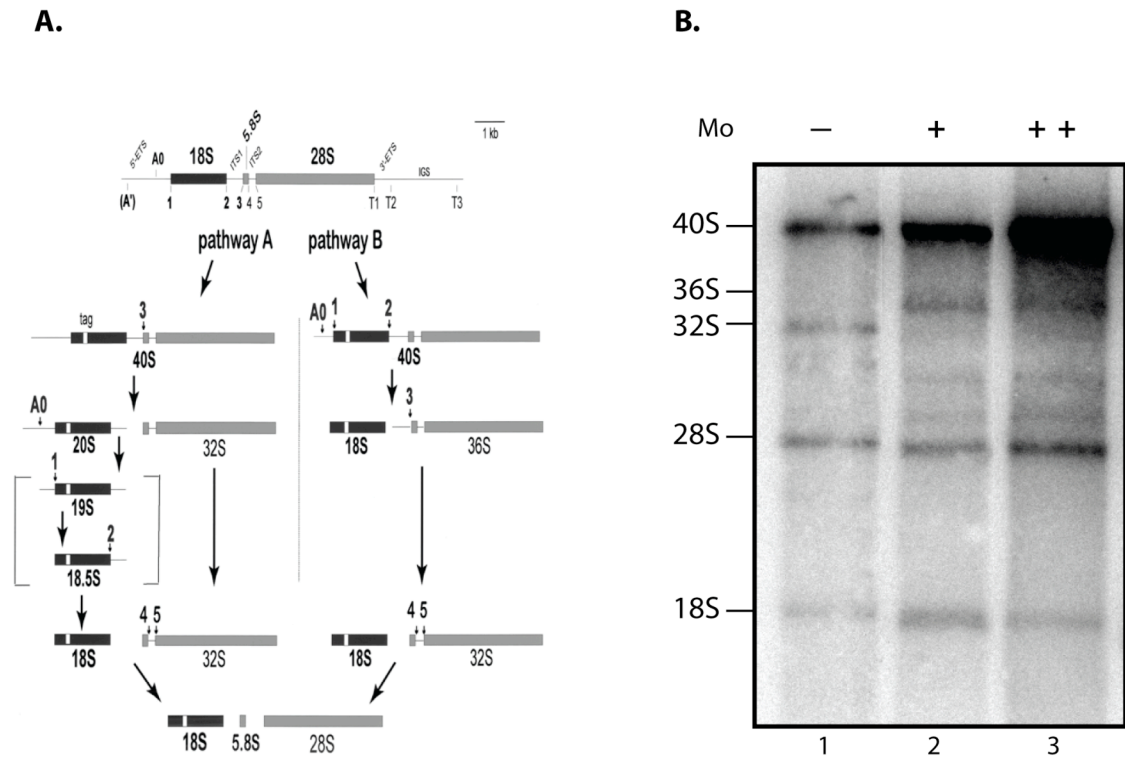


Figure 11. Morpholino oligo against eCNE6 blocks early rRNA processing: (A) rRNA processing scheme in *Xenopus* oocytes (Gerbi and Borovjagin, 2004) (B) Stage V/VI *Xenopus* oocytes were injected with ^{32}P -UTP alone (-) or coinjected with a morpholino oligo (Mo) complementary to the 28S rRNA eCNE6 (+900 fmol or ++9 pmol) into the nucleus. Five hours post-injection, nuclei were manually dissected and RNA was purified. Each lane represents total RNA isolated from ten nuclei and sizes of the nuclear rRNA indicated.

Chapter 4: Defects in Early Ribosome Biogenesis Stimulate Autophagy and Cell Cycle Arrest in *Saccharomyces Cerevisiae*

This project was done with the assistance of undergraduate student Elizabeth A. Schroeder.

ABSTRACT

Biogenesis of ribosomes is among the most energetically costly anabolic processes in the cell, and thus may serve to coordinate control of cell growth and proliferation. Previously we demonstrated that depletion of ribosomal protein L32 results in an immediate block in ribosomal RNA processing and an increase in the primary 35S precursor molecule. Here we show that in response to this processing defect cells rapidly arrest in G1 phase of the cell cycle and increase in cell volume. Unexpectedly, we detect a disproportional relationship between cell mass and volume due to degradation of mature ribosomal subunits. The accelerated degradation of ribosomes is dependent on the ubiquitin deconjugation enzyme Ubp3, an enzyme required for starvation-induced autophagy (ribophagy). Indeed, we detect autophagic bodies in cells with impaired ribosome biogenesis. These results imply that attenuating ribosome biogenesis at an early step mimics a starvation state, inhibits cell cycle progression, and promotes autophagic destruction of the ribosomal subunits.

INTRODUCTION

Biochemical reactions requiring large inputs of free energy are often subjected to tight regulation *in vivo*. The bioenergetics of building a ribosome is the most energy consuming process in the cell, and as such its regulation is central to maintaining metabolic balance and viability (Gourse et al., 1996; Warner, 1999). A growing yeast contains ~ 200,000 cytoplasmic ribosomes and during logarithmic proliferation the cells must synthesize 2,000 ribosomes per minute to maintain this concentration (Warner, 1999). This demanding biosynthetic pathway requires equimolar synthesis of four ribosomal RNAs (rRNA) and 79 ribosomal proteins (RP), utilizing all three major RNA polymerases. Eukaryotic cells have evolved a specialized RNA polymerase, RNA polymerase I, that is dedicated to the transcription of the polycistronic rDNA repeats encoding the 35S primary rRNA transcript (Nogi et al., 1991). The 35S pre-rRNA must be cleaved by nucleases to produce mature 18S, 5.8S, and 25S rRNA. RNA polymerase III produces 5S rRNA, which is cleaved and matured separately before assembling into a new ribosome. The expression of the 138 ribosomal protein genes (due to gene duplication) by RNA polymerase II accounts for 50% of the total mRNA pool and 90% of all splicing events (Warner, 1999). In addition to the native components of the ribosome, over 200 *trans*-factors are utilized in ribosomal RNA production (Wade et al., 2006).

Ribosome biogenesis and protein synthesis are massive consumers of metabolites, and the two processes must be coordinated with the nutritional state of the cell. The target of rapamycin (TOR) is a ser/thr protein kinase conserved in all eukaryotes that is part of a major signaling network for communicating nutrient availability within the cell. In yeast, TOR positively controls ribosome biogenesis at multiple steps including RNA Pol I activity (Claypool et al., 2004), 35S pre-rRNA processing (Powers and Walter, 1999), ribosome assembly and intranuclear transport, (Honma et al., 2006; Vanrobays et al., 2008), and RP gene expression (Martin et al., 2004). Additionally, TOR can negatively regulate factors that inhibit cell growth including autophagy and stress response pathways (De Virgilio and Loewith, 2006). Thus, TOR, and nutrient availability, broadly control ribosome biogenesis supporting the notion that ribosome biogenesis is tightly linked to growth control.

Cell growth and cell division are typically coupled processes that maintain cell size homeostasis. In budding yeast the commitment to divide is set at the end of G1-phase called Start, where a cell will commit to divide if a critical size is met and conditions are favorable (Hartwell et al., 1974). The precise features integrating cell size with cell division are unknown (Jorgensen and Tyers, 2004), but potentially involve several mechanisms that coordinate nucleolar assembly of ribosomes with genes controlling cell size (Jorgensen, 2002; Jorgensen, 2004). Indeed, the coordination of cell proliferation with ribosome biogenesis is a developing concept in eukaryotic cell biology (Dez and Tollervey, 2004). One of the first examples demonstrating a link between eukaryotic ribosome biogenesis and cell division came from a study of the mouse liver conditionally

knocked-out for the gene encoding ribosomal protein S6 (rpS6; Volarevic et al., 2000).

The mammalian liver is a well-suited model system to distinguish variables affecting cell growth versus cell proliferation. After a period of starvation the liver suffers a severe reduction in mass, and upon feeding will recover to its original mass; these responses induced by fasting and refeeding reflect only an change in cellular growth, since cell number and DNA content remain unchanged (Volarevic et al., 2000). This is in contrast to a partial liver hepatectomy where the remaining hepatocytes restore the organ mass through proliferation. Despite their inability to produce new 40S subunits, livers with a disrupted S6 gene were able to recover to their original mass after a period of starvation and refeeding, suggesting that ribosome biogenesis is not needed for cell growth. In contrast, the matched rpS6 knock-out livers however could not restore organ mass after partial hepatectomy showing that new ribosome biogenesis was needed for cells to enter the cell cycle (Volarevic, 2000). The inability to enter the cell cycle in rpS6 deficient livers could be due to a failure in cyclin E induction, which is required for entry into S-phase (Volarevic, 2000). In a subsequent study, rpS6 depletion in liver or siRNA mediated knockdown of rpS6, rpS23, or rpL7a in tissue culture cells induced expression of the G1 checkpoint protein p53, revealing a potential mechanism for the G1 arrest (Fumagalli et al., 2009). The induction of p53 was dependent on rpL11, which had previously been show to induce p53 upon disruption of rRNA synthesis and nucleolar integrity (Lohrum et al., 2003). The above findings result in the model that upon disruption of ribosome biogenesis, free rpL11 accumulates in the cell (Fumagalli et al., 2009). Free rpL11 can bind and inhibit Hdm2- a ubiquitin ligase involved in nuclear export and degradation of p53 (Di Ventura et al., 2008; Lohrum et al., 2003), thereby

causing a p53 dependent arrest in G1. The induction of p53 can also stimulate autophagy and apoptosis (Feng et al., 2005), but whether ribosome biogenesis is linked to these processes is not known.

In budding yeast, links between the biogenesis of ribosomes and cell cycle control have been recently reviewed (Dez and Tollervey, 2004). Genetic depletion of several small subunit processing factors impaired 18S processing and resulted in an arrest in G1 (Bernstein and Baserga, 2004; Bernstein et al., 2007). Importantly, this arrest occurred before any detectable changes in mature rRNA levels or global translation defects (Bernstein et al., 2007). The G1 arrest is likely sensed by Whi5p, as yeast cells depleted of the ribosome biogenesis factors Pwp2/Utp1 have a relative increase in nuclear Whi5p (Bernstein et al., 2007). Whi5p is the functional homologue of the mammalian retinoblastoma tumor suppressor protein (Rb), and functions as a transcriptional repressor of genes needed for the G1/S transition (Costanzo et al., 2004). Biogenesis defects and G1 arrest have also been documented for yeast cells depleted of ribosomal protein L3 (Rosado et al., 2007). The L3 protein has a defined role in protein synthesis needed for forming the peptidyl transferase center and A-site function (Meskauskas and Dinman, 2007). It is unknown if the G1 arrest caused from L3 depletion is a consequence of impaired translation or biogenesis or if the arrest depends on Whi5p. Although emerging data support a link between biogenesis of ribosomes and cellular proliferation, specific pathways that signal defective ribosome synthesis to the cell cycle machinery remain elusive.

In the abovementioned experiments, each gene studied specifically impaired the biogenesis of one ribosomal subunit and the defect was linked to a cell cycle arrest. Recently, we have shown that ribosomal protein L32 is needed for early steps in eukaryotic ribosome biogenesis. Depletion of L32 caused a rapid accumulation of the 35S transcript and inhibited the synthesis of both the large and small ribosomal subunits (Chapter 3). We reasoned that this severe attenuation in rRNA processing would impact cell physiology and prompted us to look at cell cycle progression in L32 depleted cells. Our results support previous findings that ribosome biogenesis is sensed at Start because cells depleted of L32 arrest in G1. In parallel to the G1 arrest, we observe a rapid decay of the mature small and large ribosomal subunits by autophagy (ribophagy). Taken together, our results reveal a novel link between early rRNA processing and the disassembly of cytoplasmic ribosomes and their concomitant degradation.

RESULTS

L32 genetically interacts with the Start machinery

Previously, we engineered a haploid yeast strain in which the only copy of the wild-type *RPL32* gene is under the control of the galactose inducible/glucose repressible *GAL1* promoter (Chapter 3). When galactose is the sole carbon source in the medium, the growth of the *GAL::RPL32* cells were indistinguishable from wild-type, but the cells were inviable on glucose medium showing that *RPL32* is essential (Chapter 3). To further examine this phenotype, an asynchronous culture of the *GAL::RPL32* strain was grown in liquid galactose-based medium, shifted to glucose medium and cell growth was monitored. After 5 hours of growth in glucose, the *GAL::RPL32* cells stop dividing in contrast to the wild-type strain (Figure 1A). Simultaneously, the two glucose-grown cell cultures were prepared for Fluorescence Activated Cell Sorting (FACS) to test if the growth arrest was at a specific stage in the cell cycle. Cells depleted for L32 begin to accumulate a single copy of their genome (1C signal, Figure 1B) by 3 hours, and by 5 hours the majority of the cells have a single copy of their DNA, strongly suggesting an arrest in the G1-phase of the cell cycle and an inability to proceed through Start.

For yeast cells to activate the G1/S-phase transcriptional network and initiate DNA replication, the transcription factor complexes SBF and MBF are required to induce transcription of the G1 cyclins Cln1/Cln2 (Cross, 1995). After 2 hours of L32 depletion, we observe a 60% down regulation in Cln1 transcript levels (Figure 2). Thus, L32 depletion causes a G1-phase arrest, which can be explained in part by the failure to

induce Cln1 expression. These data are largely in agreement with previously reported yeast strains deficient in ribosome biogenesis (Bernstein and Baserga, 2004; Bernstein et al., 2007; Rosado et al., 2007). Given that L32 depletion caused a rapid arrest in the cell cycle, we chose to investigate how L32 depletion impacts other known physiological requirements for yeast to reach Start including: rate of protein synthesis, reaching a critical cell mass, active ribosome biogenesis, and levels of cytoplasmic ribosomes (Rupes, 2002).

G1 arrest occurs before defective protein synthesis

Exposing yeast cells to low dosages of the general protein synthesis inhibitor cycloheximide, results in halfmer polyribosomes (Helser et al., 1981), slows protein synthesis (but not rRNA synthesis), and causes cells to arrest in G1 (Moore, 1988; Popolo et al., 1982). To test if protein synthesis is sensitive to depletion of L32, the *GAL::RPL32* and parental strains were grown to exponential growth phase in galactose-based medium, shifted to glucose, and translation efficiency was measured by incorporation of radio-labeled amino acids over a 24 hour time course. The L32 depleted cultures showed no significant difference in new protein synthesis during the first 6 hours of depletion compared to the wild-type strain (Figure 3). Twelve hours of depletion led to ~65% reduction in protein synthesis efficiency. This defect is several hours past the cell cycle arrest and eliminates global translation as a major determinant in the arrest from L32 depleted cells.

L32 depleted cells have an enlarged morphology

In budding yeast, cell size homeostasis is maintained through a requirement for each cell reaching a critical size prior to cell division (Johnston, 1977; Jorgensen, 2004).

Therefore, we used two optical techniques to assess the morphological properties of cells expressing L32 and compared with those depleted of L32. Yeast cells will scatter light proportional to their cellular volume (Beauvoit et al., 1993). We used Forward Light Scatter (FLS) analysis to understand the morphological changes of cells during an L32 depletion through the G1 arrest. At the onset of carbon source shift, the *GAL::RPL32* strains are indistinguishable from wild-type (Figure 4A). However, by 5 hours of depletion the majority of the population arrest in G1 (Figure 1B) and are larger in size (Figure 4A). Representative images of these two cell populations show that L32 depleted cells generally lack emerging bud tips and appear enlarged (Figure 4B and C), perhaps due to a vacuolar enlargement (Figure 4D). Together these data suggest that cells are achieving sufficient size to pass through Start.

Depletion of L32 stimulates cytoplasmic degradation of RNA and protein

To test if the morphological observation of enlarged L32 depleted cells is due to an increase in cell mass, we measured total RNA and protein levels during L32 depletion. Upon depleting L32 for 2 hours, total protein per cell is reduced ~30% and total RNA decreases ~40% (Figure 5). Gel analysis revealed a severe down regulation for all four mature rRNA species as early as 2 hours post L32 depletion (Figure 5B). The half-life of mature eukaryotic rRNA is on the order of several days (Kraft et al., 2008; Volarevic, 2000), suggesting cytoplasmic ribosomes are being degraded after L32 depletion. This

prompted us to examine if the stability of ribosomal proteins change in response to L32 depletion. Shifting the *GAL::RPL32-HA* strain into glucose-based medium causes an immediate repression of *RPL32* transcription, prevents new L32 synthesis, and the protein is subsequently depleted (Chapter 3). In order to understand if increased protein turnover, in addition to repression of gene expression, is involved in the down regulation of L32, we repeated the depletion in strains transformed with a plasmid encoding wild-type L32 or empty vector control. Once shifted to glucose medium, expression of the *GAL::RPL32-HA* chromosomally encoded L32 is shut off and protein stability is followed by immunoblotting for the unique HA tag. This analysis shows that the majority of L32 is depleted by 10 hours in minimal glucose medium (Figure 6A). In contrast, L32 is relatively unchanged compared to time zero in cells containing a plasmid born copy of *RPL32* that can support ribosome biogenesis and thus growth on glucose (Figure 6C). Because the L32-HA gene expression system is identical in both cell cultures, the difference in protein stability is likely due to a proteolysis mechanism that senses reduced ribosome biogenesis. Additionally, repeating the depletion experiment in a low pH medium also stabilizes L32, further supporting an enzyme mediated degradation event that was abrogated by low pH (Figure 6B). Therefore the increase in cell volume is not proportional to cell mass, suggesting that L32 depletion leads to osmotic swelling. A decrease in the ribosomal pool (Nakatogawa et al., 2009) and osmotic swelling (Nowikovsky et al., 2007) are both suggestive of macroautophagy.

L32 induces a novel form of autophagy

In eukaryotes, there are two major pathways directing protein degradation: ubiquitin-mediated proteolysis (Laney and Hochstrasser, 1999) and delivery of cytoplasmic components to the lysosome/vacuole compartment for degradation by autophagy (Nakatogawa et al., 2009). L32 deficient cells appear to have an enlarged vacuole (Figure 4D), as noted previously for strains deficient in ribosome biogenesis (Bernstein et al., 2007). The yeast vacuole contains various hydrolytic enzymes (e.g. RNases, proteases, glycosidases, and phosphatases) and responds to the nutritional needs of the cell, reaching maximum size and activity during starvation (Takeshige et al., 1992). During autophagy, spherical autophagic bodies form in the cytoplasm and deliver cytosolic components to the vacuole for degradation. These spherical bodies can be detected by light microscopy when cells are deficient in proteases, either by deleting *PEP4* or treating cells with the protease inhibitor PMSF (Takeshige et al., 1992). We explored if autophagy is being induced in response to L32 depletion. To observe autophagy, yeast cells were cultured under nitrogen deficient conditions (SG-N), whereby the resulting autophagic bodies can be visualized within the cell and vacuolar compartments after eight hours of starvation by DIC imaging (white arrowheads, Figure 7). Similar to starvation, autophagic spherical bodies were detected when cells are depleted of L32 for 2 hours (YPD medium) and increases by 8 hours of depletion (Figure 7, DIC arrowheads). These results are in contrast to those for cells not nitrogen starved nor depleted of L32 (YPG medium). As a second method to score autophagy, vacuolar membranes and autophagic bodies were visualized in live cells by fluorescence microscopy. In these experiments, yeast cell plasma membranes are labeled with a fluorescent dye FM 4-64 that accumulates in the

vacuole through normal endocytosis (Vida and Emr, 1995). After FM 4-64 labeling, starvation-induced autophagic body accumulation in the lumen of the vacuole is visualized (Figure 7, FM 4-64). An increase in vacuolar fluorescence is also observed when cells are depleted of L32 (Figure 7, YPD compared to YPG); however, the pattern of fluorescence is less robust compared to nitrogen starved cells suggesting a different or kinetically slowed autophagy mechanism.

Recently, genetic experiments in yeast identified a novel selective autophagic pathway termed ribophagy that targets ribosomes for destruction (Kraft et al., 2008). Ribophagy of the large subunit requires the deubiquitinating enzyme Ubp3p, which cleaves ubiquitin monoubiquitin but not polyubiquitin chains (Baker et al., 1992). To investigate if ribophagy mediates destruction of cytoplasmic ribosomes in L32 depleted cells, L32 was depleted in $\Delta ubp3$ cells and its stability was assayed by immunoblot (Figure 8). The kinetics of L32 depletion in the $\Delta ubp3$ are significantly slower than wild-type cells, implying that ribophagy is induced as a consequence of depletion of L32. This suggests that in response to ribosome biogenesis defects imposed by L32 depletion, cells activate ribophagy.

Ribosome biogenesis defects are epistatic to autophagy in cell cycle arrest

Both defective ribosome biogenesis and autophagy can bring about a G1-phase arrest in the cell cycle. Depletion of L32 results in defective ribosome biogenesis (Chapter 3), induces autophagy (ribophagy, Figure 7 and 8), and causes cells to accumulate in G1 (Figure 1). The fact that L32 induces two phenomena that lead to G1 arrest could explain

why L32 depletion leads to a more rapid G1 arrest (~ 3 hours, Figure 1B) than previously reported for other rRNA processing factors. For confirmation of this conclusion, we took advantage of the fact that L32 turnover is slowed when cells are cultured in acidic media. Using FACS analysis, we observed identical cell cycle arrest kinetics in acidic and neutral media (Figure 1B). Because acidic medium specifically alters L32 stability and not rRNA processing (data in progress), we conclude that rRNA processing defects are epistatic to autophagy in the L32 cell cycle arrest pathway.

DISCUSSION

In response to favorable growth stimuli, cells will up-regulate ribosome biogenesis. Conversely, during adverse growth conditions essential nutrients are supplied by autophagy for survival. In this report, we demonstrate that ongoing ribosome biogenesis is necessary to promote cell division and inhibit autophagy.

The majority of previous studies addressing the contribution of ribosome biogenesis to cell cycle progression used genetic manipulations that resulted in impaired synthesis for either the small or the large ribosomal subunit. From those data several connections have been established between ribosome biogenesis factors and specific cell cycle checkpoints (reviewed in (Dez and Tollervey, 2004). In some cases, the biogenesis defect resulted in an arrest in the G1 phase of the cell cycle (Bernstein and Baserga, 2004; Bernstein et al., 2007; Fumagalli et al., 2009; Rosado et al., 2007; Volarevic, 2000). A common theme in these different G1 arrest scenarios is the kinetic build up of the primary rRNA transcript, a common component to the small and large subunits. Previously, we showed that depletion of L32 in yeast causes a defect in pre-rRNA processing with a build up in the 35S precursor molecule and a block in the synthesis of both subunits (Chapter 3). In the current report we explored how this affects cell physiology. When a *GAL::RPL32-3XHA* strain is shifted from galactose to glucose, the cells stop dividing and accumulate in G1-phase of the cell cycle. The population doubling-time for our engineered *GAL::RPL32-3XHA* strain is ~2.5 hours in galactose-based medium, and when shifted to glucose a G1

arrest is seen as early as 3 hours. Thus the Start machinery immediately responds to L32 depletion within one round of cell division by arresting in G1. The kinetics of the G1 arrest imposed by L32 depletion is more rapid than the G1 arrest resulting from depletion of L3 (6-12 hr; Rosado et al., 2007) or Pwp2 depletion (8 hours; Bernstein et al., 2007). In the absence of Pwp2 and L3, a fraction of 35S pre-rRNA accumulates, but initially it still can be processed into mature 18S, 5.8S and 25S rRNA. This is in contrast to L32 depletion, which results in an immediate block in rRNA processing and a rapid accumulation in 35S precursor RNA. These observations suggest a model whereby the Start cell cycle machinery senses the concentration of the 35S precursor in the nucleolus. The sensors that link ribosome biogenesis to cell cycle machinery remain to be identified, but one such candidate is the transcriptional repressor protein Whi5p.

In mammalian cells, disruption of ribosome biogenesis of the small or large subunit leads to activation of p53 via rpL11 dependent inhibition of the ubiquitin ligase Hdm2 (MDM2 in mouse) and subsequent arrest in G1 (Fumagalli et al., 2009). The tumor suppressor retinoblastoma protein (Rb) can modulate the activity of p53 and form a trimeric complex with p53 and Hdm2 (Bates et al., 1998; Yap et al., 1999). Although a homologue of p53 has yet to be identified in yeast, recently a functional homologue of Rb, Whi5p, has been identified (Costanzo et al., 2004). The Whi5p associates with promoter elements acting as a transcriptional repressor of the G1/S gene expression program (e.g. Cln1 and Cln2) and is negatively regulated by high levels of cyclin-dependent kinase activity (Costanzo et al., 2004). Furthermore, in response to defects in ribosome biogenesis Whi5-GFP chimeras increase their nuclear residence, suggesting Whi5p as an effector protein of the ribosome

biogenesis/ cell cycle pathway (Bernstein et al., 2007). In this work we show that in response to L32 depletion Cln1, a target of Whi5, is rapidly down regulated (Figure 2). Reports have linked abnormal cell morphology with ribosome biogenesis mutants. One example is an enlarged vacuole and increased cell size (Bernstein et al., 2007). In response to L32 depletion, we also observe an increase in cell and vacuole size (Figure 4 and 7). However, in contrast to previous reports, this increase in cell size is not accompanied by an increase in cellular mass. Indeed, we detect a severe reduction in total RNA and protein during an L32 depletion experiment (Figure 5). Given the observation that both RNA and protein are being degraded in these cells, accompanied by vacuole expansion we reasoned that autophagy or perhaps ribophagy was being induced in response to L32 depletion. Several lines of evidence suggest that mature ribosomes are being degraded in response to L32 depletion. First, the total pool of mature rRNA is reduced by 50% after 6 hours of growth in glucose media (Figure 5B). This dramatic rate of down regulation is indicative of an active turnover mechanism since the normal half-life of mature rRNA is several days (Kraft et al., 2008; Volarevic, 2000). Next we tested if ribosomal proteins were also being turned over in this system. To this end, we measured the steady-state levels of tagged L32 after shutting off its transcription in cells transformed with wild-type *RPL32* (pRPL32) or empty vector control. Cells transformed with plasmid encoded L32 suppress the glucose growth defect of strain LSY102 and therefore restores normal ribosome biogenesis (Figure 6C). At 10 hours of growth in minimal glucose medium, *GAL::RPL32-HA* cultures have >90% of L32-HA depleted (Figure 6A). This is in contrast to cells transformed with wild-type *RPL32*, which show only a modest reduction in L32-HA (Figure 6A). It is unlikely that the increase in L32-

HA stability seen in cultures transformed with pL32 is due to overexpression of L32, since there are relatively equal amounts of L32-HA in the empty vector control cultures at the time of carbon source shift. Therefore, we conclude that pre-existing L32 is being degraded when new L32 synthesis is repressed. Interestingly, depletion of L32 is less efficient than in minimal medium (compare 6A to 6B) suggesting that depletion is sensitive to nutrient availability. Because L32 is required for early steps in ribosome biogenesis, these experiments reveal a genetic link between L32, nucleolar rRNA biogenesis, and the cytoplasmic degradation machinery. Although a direct effect of L32 on the execution of a cytoplasmic ribosome degradation signal cannot be excluded, this seems unlikely given the rapid response of L32 depletion on 35S accumulation.

Together these observations suggested that macroautophagy or selective ribophagy was activated upon L32 depletion. To test these possibilities, we examined the morphology of vacuoles and tested for the presence of autophagic bodies in L32 and starved cells. When yeast are grown in nutrient rich (YP) medium, their vacuoles are transparent and contain few if any autophagic bodies. During conditions of starvation, macroautophagy is stimulated whereby isolated membranes non-selectively engulf the cytoplasm and deliver their contents to the vacuole (analogous to the lysosome in metazoans) in the form of autophagic bodies (Nakatogawa et al., 2009). Autophagic bodies can be visualized using light microscopy when cells are protease deficient, which inhibits the proteolytic cascade and break down of the vesicles (Figure 7, SG-N DIC) (Takeshige et al., 1992). In our experiments, we detect autophagic bodies in response to L32 depletion that accumulate in number from 2 hours to 8 hours of growth in glucose. Using a more sensitive fluorescent

assay, we also detect autophagic bodies in the lumen of the vacuole in L32 depleted cells. Interestingly, the pattern of fluorescence from nitrogen starvation is more robust than L32 depletion. These patterns could be due to a difference in the rate of autophagic body accumulation or a reflect different mechanisms of autophagy. In addition to macroautophagy, cells also possess cargo-specific autophagy. This form of autophagy uses a similar membrane vesicle system to traffic cargo to the vacuole for degradation (Nakatogawa et al., 2009). A newly identified cargo-specific autophagic pathway has been recently described for ribosomes termed ribophagy (Kraft et al., 2008). The exact receptor(s) for ribophagy is unknown, but the authors showed that some components of the 60S subunit contain ubiquitin moieties that must be removed by the ubiquitin deconjugation enzyme Ubp3 for starvation-induced ribophagy to occur. In contrast to wild-type *GAL::RPL32-HA* strains, L32 is significantly stabilized in *UBP3* null yeast strains. From these experiments we conclude that rRNA processing and ribophagy are coordinated processes.

In the future, it will be exciting to dissect the pathways and effector molecules that integrate biogenesis of ribosomes, cell cycle, and autophagy. One obvious candidate is the TOR pathway, which responds to the nutritional state of the cell and positively regulates ribosome biogenesis and negatively regulates autophagy.

MATERIAL AND METHODS

Yeast Strains and Culturing

The *S. cerevisiae* strains used in this study were derived from strain W303-1a (*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*). Yeast strain LSY102 (*GALI::RPL32-3XHA*) has been previously described (Chapter 3). LSY502 is a derivative of LSY102 containing a deletion in *UBP3* using pAG61 as template in PCR for synthesizing a disruption cassette (Goldstein et al., 1999). Yeast transformations were performed as described by Elble (Elble, 1992). Cells were cultured in rich (YP) medium supplemented with 2% glucose (YPD) or 2% galactose (YPG) and 0.004% adenine (Sherman et al., 1986). Acidic medium was prepared by adding concentrated HCl to pH 3.9. For plasmid selection, cells were cultured in synthetic yeast nitrogen base medium (S) containing 2% carbon source as noted supplemented with 0.004% required amino acids and 0.004% adenine and uracil. Nitrogen starvation media (-N) was prepared from yeast nitrogen base lacking ammonium sulfate and containing the indicated carbon source and necessary supplements. For L32 depletion experiments cells were grown to mid-log phase in YPG at 30° C, washed in sterile water, and resuspended in YPD medium pre-warmed to 30° C. Cells were kept in constant logarithmic growth by monitoring OD₆₀₀ and diluting with pre-warmed medium.

Plasmid Construction

The wild-type *RPL32* gene was PCR amplified from yeast genomic DNA isolated from strain W303-1a using oligo primers that add a synthetic 5' *Bam*HI site TTTTTTTTggatccGACAATGCAGCCAGCGGAGGCATTGTATGG and 3' *Sac*II

AAAAAAAAAccgcggAGTTATATTTAAAGTGAGGACTAGTGCTCCC site. The 1600 bp PCR product contained 600 bp of genomic regulatory sequences flanking the *RPL32* open reading frame, and was digested with *Bam*HI and *Sac*I, ligated into pRS314, and sequenced.

Flow Cytometry

FACS analysis was performed essentially as described (Sherwood and Bennett, 2008). Yeast strains LSY102 and W303-1a were cultured in YPG and shifted to YPD. At periodic time points after the carbon source shift, cells were collected and fixed in ethanol at 4°C. Cells were treated with RNase A and pepsin, and stained with Sytox Green (Invitrogen). Prior to FACS analysis, cells were sonicated for 5 seconds at the lowest power setting. DNA content was analyzed on a FACS Aria (Brown University) or Lifespan FACS (Providence, RI). For cell volume analysis, cells were washed in 50 mM Tris-HCl pH 7.5, sonicated, and analyzed by forward light scatter on a FACS Aria.

RNA analysis

Yeast total RNA was isolated using the method of hot-acidic phenol extraction and was quantified using A₂₆₀ absorbance. RNA was analyzed on formaldehyde-containing 1.2% agarose MOPS gel and stained with ethidium bromide. For quantitative reverse-transcriptase PCR (QPCR), the TaqMan® Reverse Transcription Reagents kit (Applied Biosystems, Foster City) was used to prepare cDNA. QPCR was performed on the 7300 Real-Time PCR system (Applied Biosystems, Foster City) using oligo primer pairs for Cln1 (5'-CTTGGATACTCTGAACTGGGACG-3' and

5'-TGACTTTCTCTTACAGGACCATTCT-3') and Act1 (5'-TGGAATCTGCCGGTATTGACCAAAC-3' and 5'-CAAAGCGGTGATTCCTTTTGCATTC-3') with the SYBER Green PCR Master Mix Kit (Applied Biosystems, Foster City). All QPCR experiments were run in triplicate and repeated at least once. Data for Cln1 levels was normalized against Act1 mRNA levels and represented as a fold change relative to the amount of Cln1 mRNA present at the time of carbon source shift.

Protein Synthesis Assay

Yeast strains LSY102 and W303-1a were grown to mid-log phase in YPG at 30° C, washed in sterile water, and resuspended in YPD medium pre-warmed to 30° C. To determine protein synthesis efficiency, 1 ml aliquots of culture were pulsed with 11 µCi of ³⁵S-EasyTag Express Protein Labeling Mix (PerkinElmer) for 4 minutes at 30° C. Cells were then treated with trichloroacetic acid, incubated on ice, and then heated. Precipitated protein was adsorbed to a GF/C filter paper and labeling efficiency was measured in a scintillation counter.

Protein Analysis

Cultures of LSY102 and W303-1a were grown to mid-log and 6.25 OD₆₀₀ equivalents of cells were harvested, washed with cold water, and resuspended in 500 µl lysis buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 0.2% SDS, 1% Triton X-100, 2 mM PMSF). Cells were disintegrated with 300 µl of glass beads and Turbo-mixed for 7.5 minutes. After centrifugation, supernatant was removed with a Pasteur pipette and

centrifuged for 30 minutes at 14000 rpm. Protein concentration was determined using the Bradford assay (Bio-Rad) with BSA as a standard. Protein content was normalized to cell number using a hemacytometer.

For immunoblotting, 1 OD₆₀₀ equivalent of cells were harvested, washed, resuspended in 100 µl SDS-PAGE sample buffer and stored at -80°C. Samples were boiled and 0.1 OD₆₀₀ equivalents of cells were resolved on a 12% SDS-PAGE or a 4-12% gradient gel (Invitrogen). Protein was transferred to a nitrocellulose membrane using the bufferless Invitrogen iBlot system according to manufacturer's instructions or by wet transfer. Blots were subsequently probed with anti-HA antibodies to detect L32 (Roche, 3F10) or anti-Pgk1 antibodies (Molecular Probes, Eugene, OR) as a loading control.

Light and Fluorescence Microscopy

Imaging of yeast vacuoles and autophagic bodies was based on a previously described method (Journé et al., 2008) with some modifications. Yeast strain LSY102 was cultured in YPG to mid-log and 10 OD₆₀₀ equivalents were collected and treated with 0.8 µM FM 4-64 (Invitrogen). Cells were stained for 20 minutes at 30° C, harvested by centrifugation, washed with medium, and resuspended to their original OD₆₀₀ and the dye was chased into the vacuole for 30 minutes at 30° C. Cells were then washed three times in 50 mM HEPES buffer containing 1 mM PMSF, and resuspended in YPG containing 1 mM PMSF and incubated for 30 minutes at 30° C. For L32 depletion or starvation, cells were washed with sterile water and resuspended in YPD or SG-N respectively containing 1 mM PMSF. For slide preparation, cells were washed briefly in PBS and embedded in

1.5% low melting point agarose onto glass slides. Data were collected on a Zeiss LSM 510 confocal microscope at 63X magnification.

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The data in this chapter would not be possible without the contribution of Elizabeth Schroeder, who assisted this project. First, to the countless hours she spent optimizing flow cytometry experiments. Secondly, for establishing the protocol for flow cytometry in our lab originally obtained from Kim Sherwood. The findings in this chapter were dependent on the engineering of difficult yeast strains that required multiple integrations. I am indebted to Elizabeth for her cleverness with and persistence at overcoming the obstacles associated with engineering difficult yeast strains. I thank Katie Pratt and Elizabeth Schroeder for their assistance in Western blotting. A special thank you to Rich Frieman for providing the hemocytometer and to Susan Gerbi and Elizabeth Schroeder for standardizing the Bradford assays to cell number. Finally, I wish to thank Elizabeth Schroeder for her assistance in developing the FM 4-64 labeling protocol, for piloting the labeling conditions, and to Celina Juliano for assistance with confocal microscopy.

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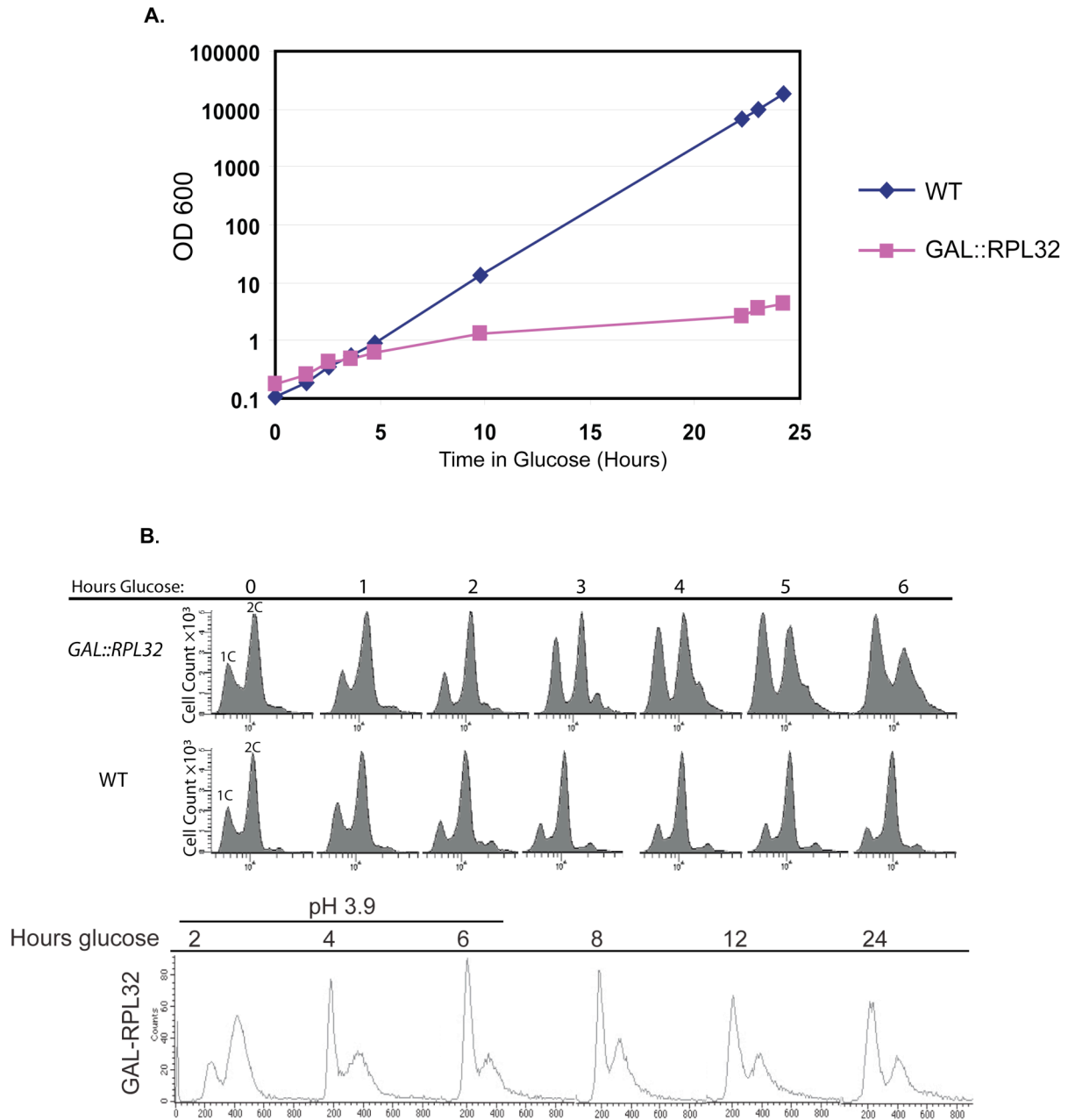


Figure 1. A genetic interaction between RPL32 and The G1 checkpoint: (A) L32 is needed for normal cell growth. Yeast strains were culture in rich galactose-based medium (YPG) to OD₆₀₀ = 0.6-0.8 and shifted to rich glucose-based medium (YPD) (time 0). Growth was measured by OD₆₀₀ at regular intervals. When necessary, cells were diluted with culture medium to maintain logarithmic growth. (B) L32 depleted cells arrest in G1. After shifting to neutral YPD or YPD pH 3.9, cells were stained with SYTOX Green at the indicated time points and sorted by FACS analysis. 2C peaks represent cells with replicated genomes and 1C peaks are cells with unreplicated genomes.

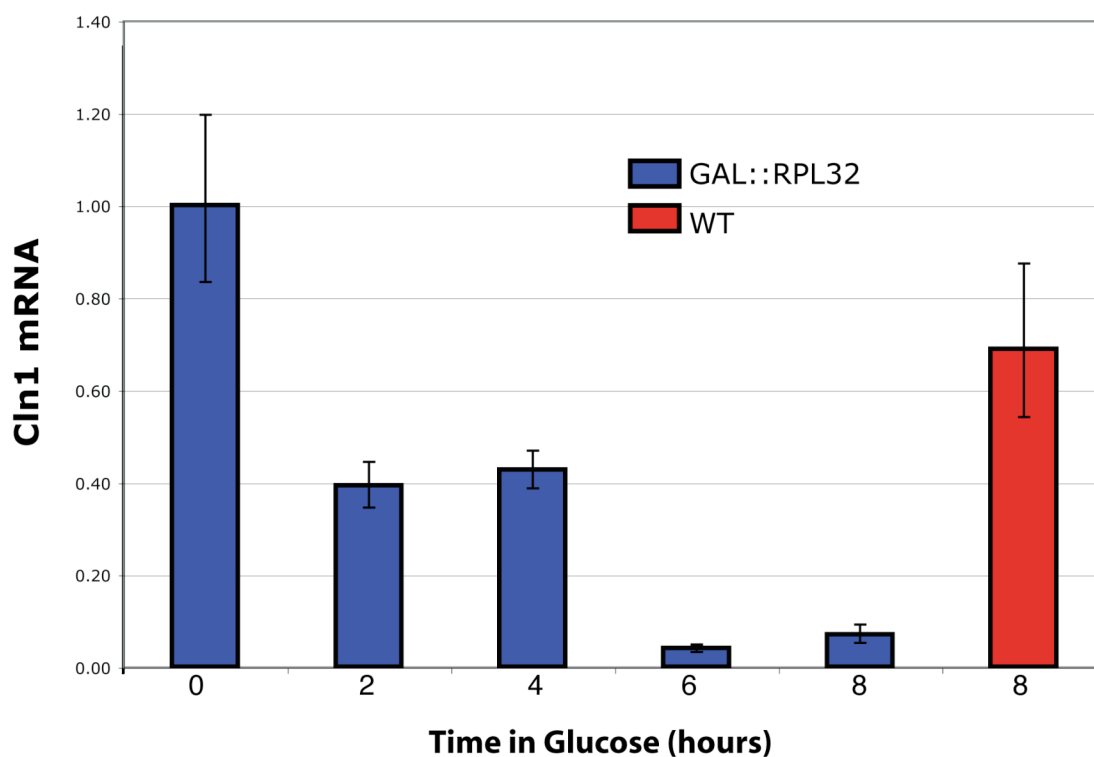


Figure 2. Negative Regulation of cyclin 1 (Cln1) expression by RPL32: Total RNA was isolated from yeast strains *GAL::RPL32* or W303-1a (WT) at the indicated times after shifting to YPD. Quantitative RT-PCR was used to measure the relative mRNA levels of Cln1 during an L32 depletion series. All values were normalized against Actin (Act1) mRNA and are represented as a fold change relative to the amount of RNA present at time zero.

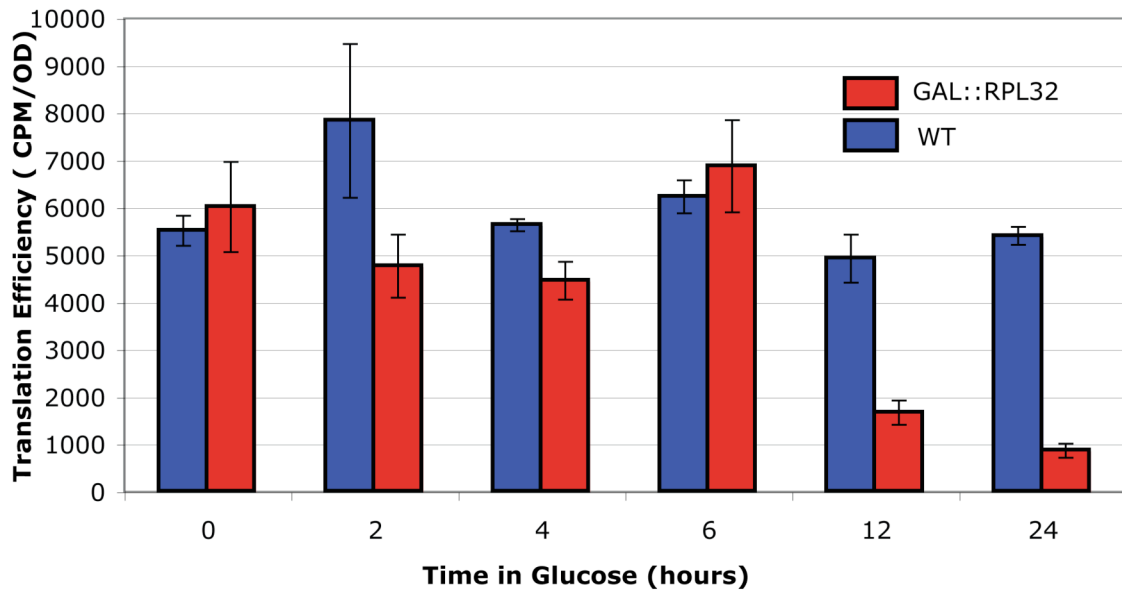


Figure 3. Effect of L32 depletion on protein synthesis: Exponentially growing wild-type (WT) and *GAL::RPL32* cultures were shifted from YPG to YPD. At the indicated times, 11 μCi of ^{35}S -Trans label were added to cells and labeled for 4 minutes at 30°C. The efficiency of translation was measured by trichloroacetic acid precipitation of radioactive peptides and represented as ^{35}S counts/min/ OD_{600} . Data represent the average of two independent cultures per strain with each time point assayed in triplicate.

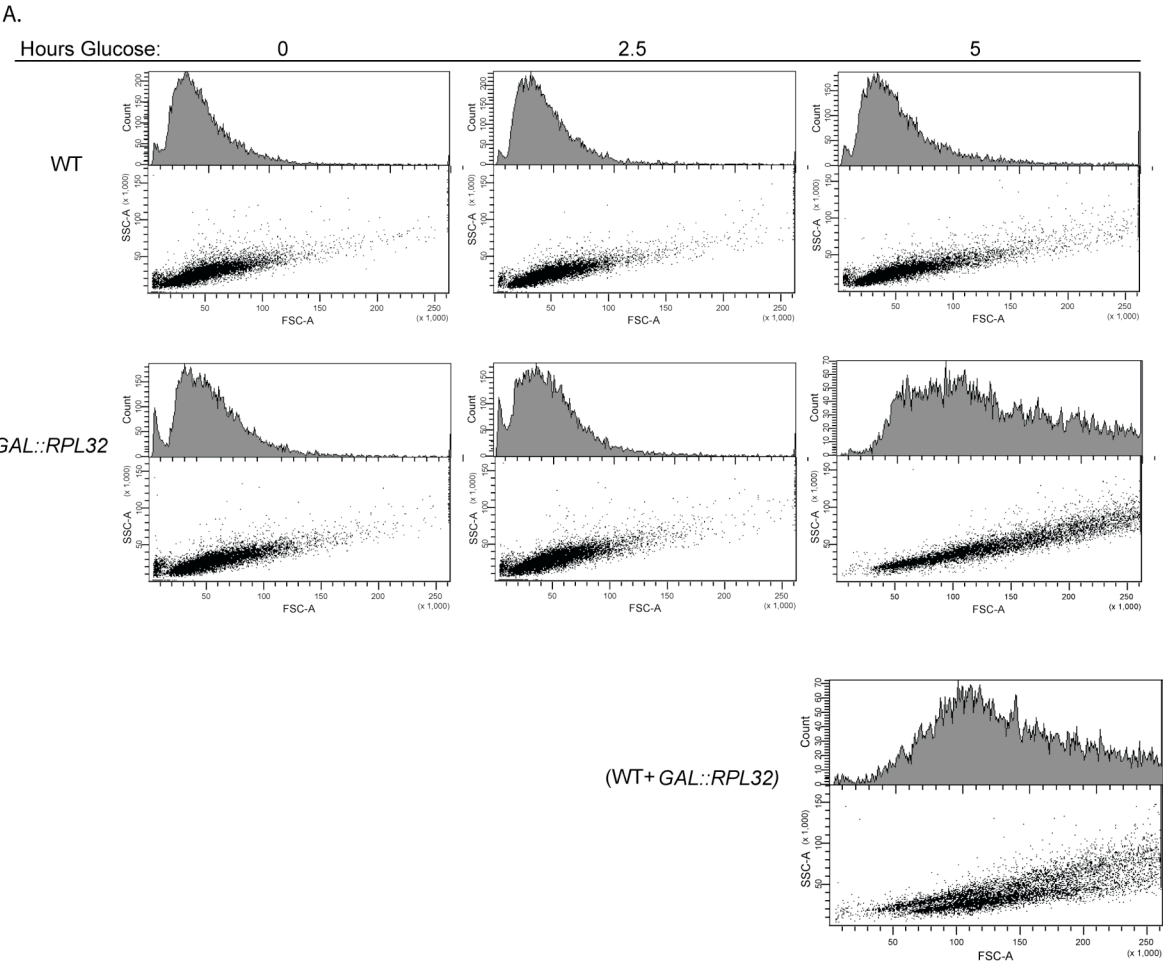


Figure 4. (legend opposite)

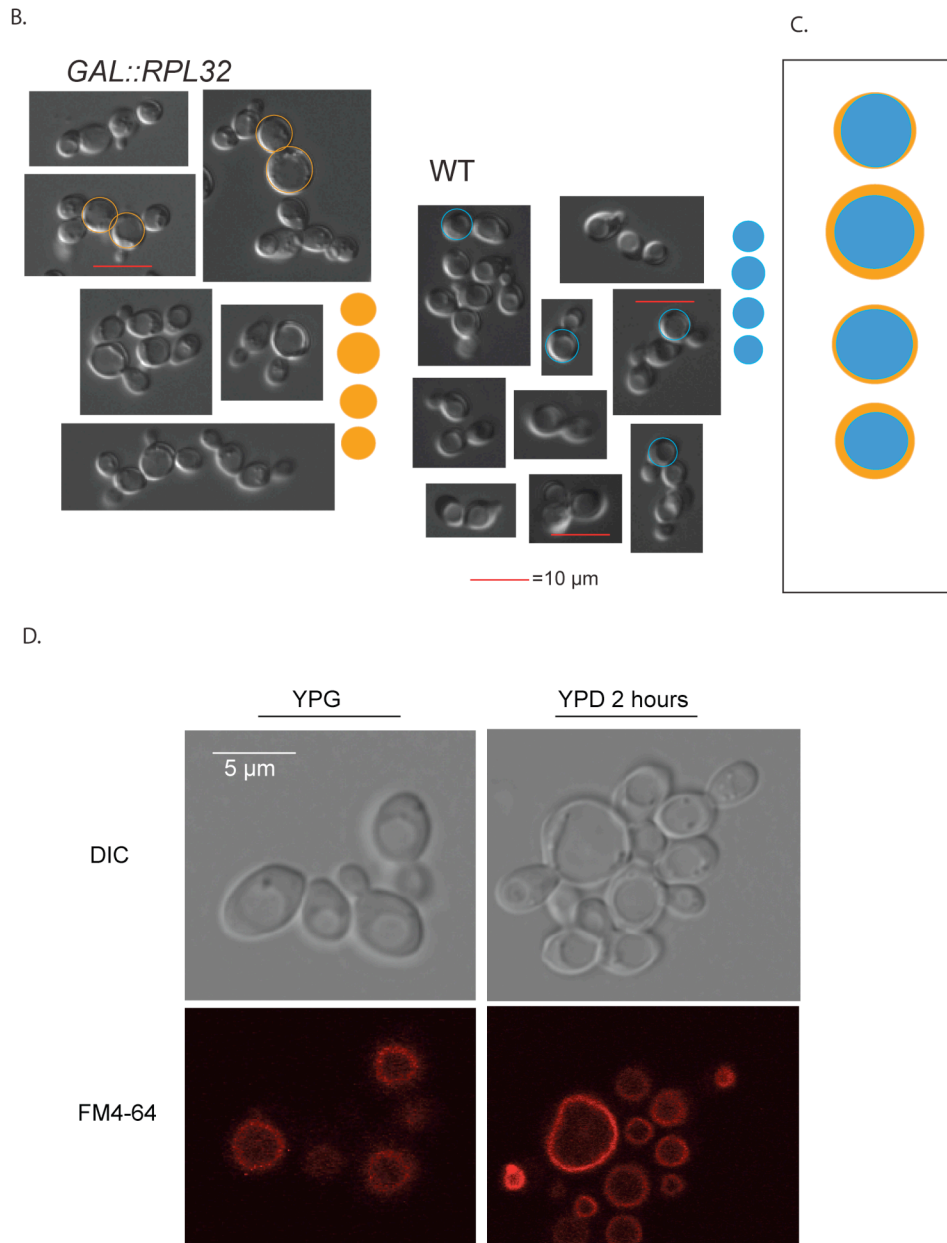


Figure 4. Morphological changes of enlarged L32 depleted cells: (A) Cell size was measured using Forward Light Scatter in a flow cytometer. Asynchronous cell cultures depleted of L32 show a shift in cell size towards larger size by 5 hours of glucose growth. (B) Morphometric analysis of wild-type versus L32 depleted cells. Phase contrast images of cells depleted of L32 are larger in size (orange circles) and than wild-type cells (blue circles). (C) Representation of cell size differences comparing wild-type (blue) to L32 depleted (orange). (D) Confocal microscopy of LSY102 (*GAL::RPL32-HA*) treated with the vital dye FM4-64 to label vacuoles in YPG or 2 hours YPD.

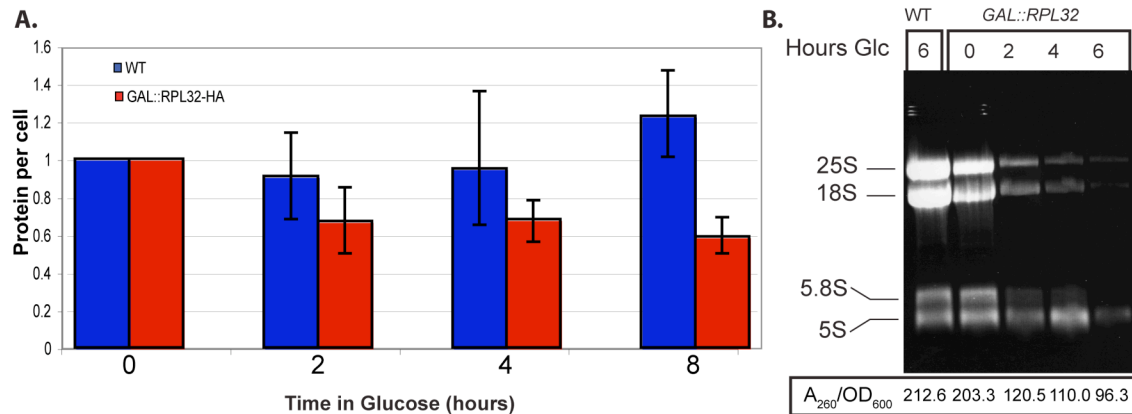


Figure 5. Decrease in total protein and RNA in L32 depleted cells: (A) Yeast cell extracts were made from glucose grown cells using glass bead disintegration. Protein concentration was measured using the Bradford assay and normalized to cell number as determined by hemocytometer counting. Protein concentration at time zero was set to 1.0 and subsequent measurements are expressed relative to time zero for each strain. Data represent the average of three independent measurements. (B) Total RNA was isolated using hot acidic phenol from 10 OD₆₀₀ equivalents at the indicated time points. 10 µg of RNA was electrophoresed on a 1.2% agarose MOPS gel containing formaldehyde stained with ethidium bromide. The ribosomal RNAs are labeled in the representative image. RNA was quantified using UV absorbance (A₂₆₀) and expressed relative to OD₆₀₀ (see ratio at bottom of gel image).

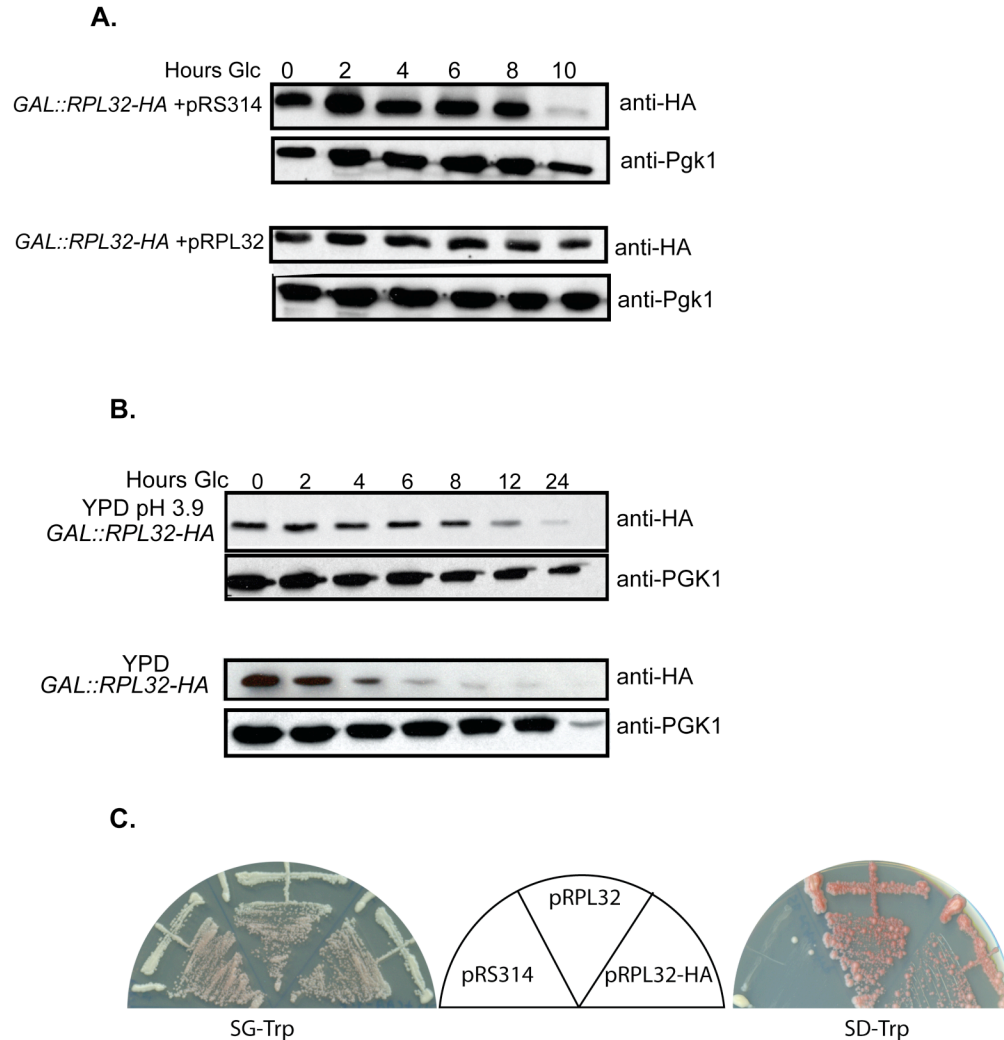


Figure 6. Depletion of L32 involves proteolysis: (A) Genetic suppression of L32 turnover. A *GAL::RPL32-HA* yeast strain was transformed with a plasmid containing *RPL32* (pRPL32) or empty vector control (pRS314). Plasmid containing cells were cultured in SG-Trp to exponential growth and at time zero shifted to SD-Trp. Yeast protein from 0.1 OD₆₀₀ equivalents were resolved on SDS-PAGE and subjected to immunoblot analysis with anti-HA and anti-Pgk1 antibodies. The L32-HA signal persists longer in cells transformed with pL32 than in the empty vector control. (B) Acidic medium slows L32 depletion. Cell were cultured in YPG and shifted to YPD or YPD pH 3.9 media. At the indicated times, total protein was analyzed as done in (A). (C) Strain *GAL::RPL32-HA* transformed with plasmid encoded *RPL32* restores normal growth on glucose.

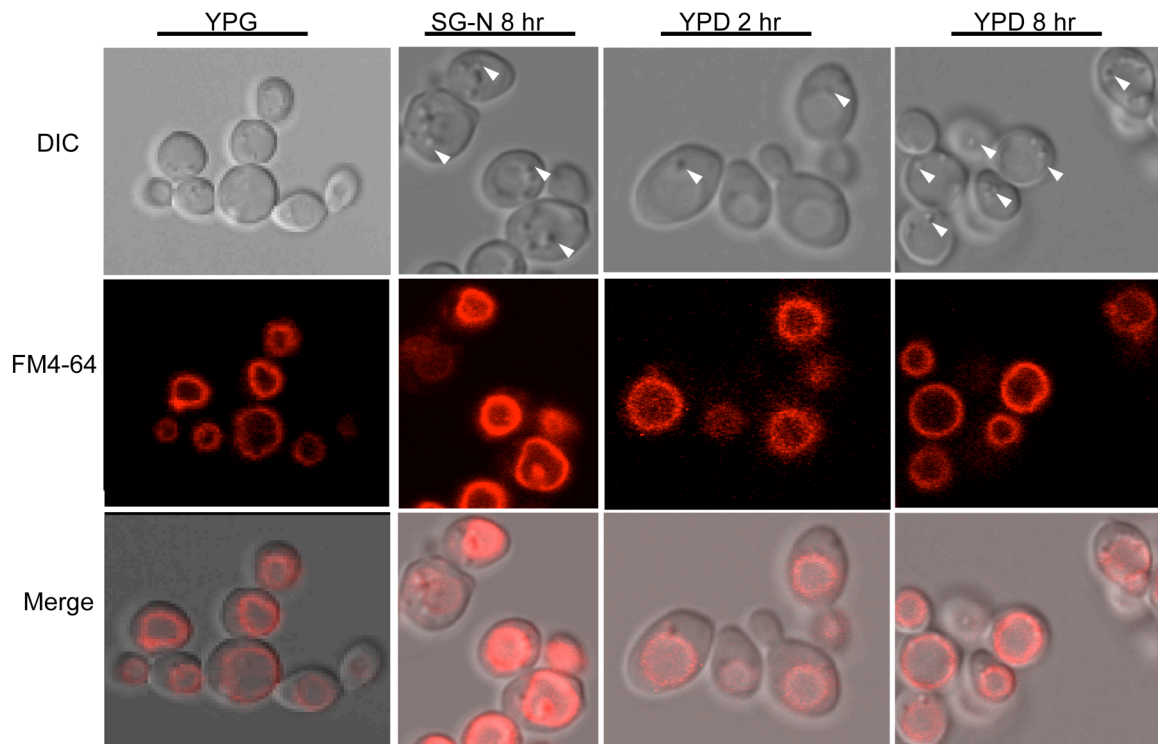


Figure 7. Detection of autophagic bodies: Yeast strain LSY102 (*GAL::RPL32-HA*) was cultured in YPG and treated with the vital dye FM 4-64 that stains the vacuolar membrane. The stained culture was washed with 50 mM HEPES buffer containing 1 mM PMSF and used to inoculate galactose-based medium (YPG), nitrogen starvation medium (SG-N) or glucose-based medium (YPD). Cells were photographed using confocal microscopy: DIC (differential interference contrast), FM 4-64 (Fluorescence imaging using a long-pass red filter), Merge (overlay of the two images). Autophagic bodies in the DIC images are marked with white arrowheads.

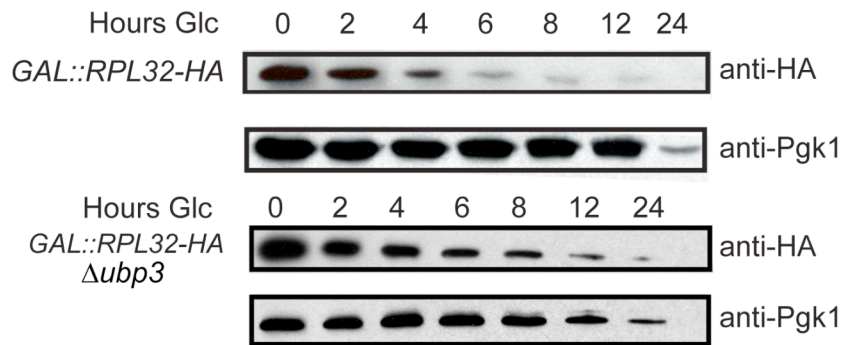


Figure 8. Effect of L32 depletion on Ubp3 dependent Ribophagy: *GAL::RPL32-HA* expression was analyzed in a wild-type and $\Delta ubp3$ yeast strains. Cells were cultured in YPG medium, shifted to YPD, and cell extracts were made at the indicated times. Protein levels from 0.1 OD₆₀₀ equivalents were resolved on SDS-PAGE and subjected to immunoblot analysis with anti-HA and anti-Pgk1 antibodies.

Chapter 5: Discussion

Ribosomes are the most complex ribonucleoprotein particles in the cell. These macromolecular machines are responsible for protein synthesis in all organisms. The two basic functions of the ribosome are decoding the genetic information in mRNA and catalyzing peptide bond formation between the growing peptidyl-tRNA and the selected aminoacyl-tRNA. The ribosome is a ribozyme as peptide bond formation occurs in an active site composed of only RNA (Beringer and Rodnina, 2007; Steitz, 2008), underscoring the importance of rRNA in the function of the ribosome. This investigation has highlighted the eukaryotic ribosome as a regulatory hub for metabolic health. Ribosome biogenesis balances two major cellular events: the synthesis and degradation of a cell.

Ribosomal RNA folding in ribosome assembly

A functional ribosome displays an exquisite architecture with an intricately molded RNA core and an outer coat of protein. Much of the knowledge about the stepwise assembly of the protein components of the ribosome come from *in vitro* reconstitution which produces functional bacterial ribosomes, showing that protein assembly is hierarchical, with obligatory primary binding proteins, and secondary binding proteins that require one or more of the primary proteins to be in place (Nierhaus, 1991). Assembly is also cooperative since protein addition stimulates conformational changes in the developing RNP creating new protein binding sites (Williamson, 2003). A question less understood in assembly is how the rRNA contributes to the hierarchical assembly landscape. Recently, the understanding of ribosomal RNA folding has been advanced using time resolved hydroxy radical footprinting of the entire 16S rRNA backbone during an *in vitro*

reconstitution assay (Adilakshmi et al., 2008). These experiments showed that the 16S ribosomal RNA could adopt native secondary structure and some native tertiary interactions in the absence of ribosomal proteins, providing evidence that rRNA intrinsically encodes part of the assembly pathway. From the RNA perspective of assembly, rRNA nucleates subunit assembly by self-folding and creating ribosomal protein binding sites. This self-folding event initiates a cascade of ribosomal protein assembly that ultimately stabilizes tertiary interactions in the subunit (Woodson, 2008). These detailed biochemical assembly maps are not available for eukaryotes, as *in vitro* ribosome reconstitution has not been achieved in a eukaryotic system (Dinman, 2009).

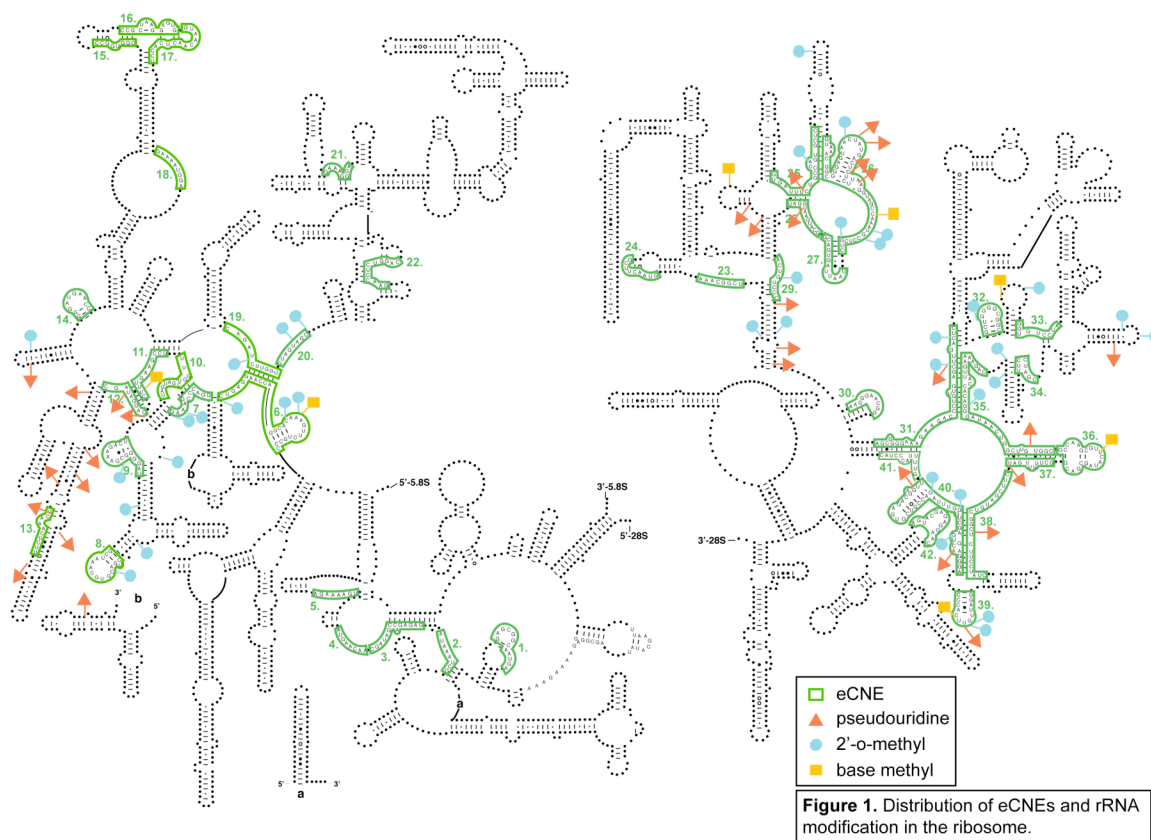
Eukaryotic rRNA is significantly larger than its bacterial counterpart owing to insertion elements that extend from the universal core structure termed expansion segments (Gerbi, 1996). Theoretically, larger multidomain RNAs such as eukaryotic 28S rRNA are more difficult to fold due to increased chances to mispair and consequently a tendency to become trapped in alternative stable structures (Herschlag, 1995). The increased size of the eukaryotic 28S rRNA as compared to bacterial 23S rRNA therefore presents a greater challenge to the cell to achieve the correct folded conformation. However, *in vivo* analysis of eukaryotic ribosomes shows that a very efficient assembly pathway is in place, as a yeast cell produces 2,000 ribosomes per minute (Warner, 1999). How does the cell accomplish this task? It is interesting to speculate that over eons of time, evolution has fine-tuned rRNA sequence to be maximally and efficiently self-folding.

The eukaryotic conserved nucleotide elements

Folding elements

We reasoned that if a specific rRNA sequence could acquire its native folded state using minimal energy and maximum kinetics, it would in theory be subjected to natural selection. To begin to test this theory, we took a computational phylogenetic approach to ask if rRNA motifs exist in a highly conserved format throughout eukaryotic evolution and tested their interdomain specificity. Historically, comparative phylogenetic studies of rRNA have examined the molecule at the single nucleotide level. Important as these results are, single nucleotides do not possess sufficient information to code for a single thermodynamically favored structure (Herschlag, 1995). Therefore, we set to test for the presence of motifs: contiguous stretches of highly invariant nucleotides. Using a new method that involves building a sample seed data set and employing cross-domain comparisons, we identified eukaryotic conserved nucleotide elements (CNEs). There are 42 conserved nucleotide elements in eukaryotic 28S rRNA, each showing high conservation in the entire eukaryotic domain (Chapter 2, Table II). Of the 42 elements, five show universal conservation and nine are highly degenerate in both Bacterial and Archaeal rRNA. Interestingly, these nine eCNEs are found within the universal core rRNA structure in the three main lineages and display a eukaryotic-specific conservation. We reason that eCNEs are plurifunctional, encoding (i) a maximally efficient RNA folding pathway, (ii) binding sites for biogenesis factors and/or ribosomal proteins, (iii) target sites for snoRNA mediated base modification, and/or (iv) a fine tuning mechanism for eukaryotic protein synthesis. Because eukaryotic assembly cannot be reconstituted *in vitro*, testing whether eCNEs drive the folding landscape cannot be assayed

biochemically. Almost all the post-transcriptional rRNA modifications in yeast are known (Decatur and Fournier, 2002). A summary map of eCNEs and rRNA modifications shows that the majority of modified nucleotides are in eCNEs. This remarkable superimposition of modifications and eCNEs highlights the functional importance of these rRNA structures.



A rapidly advancing approach in understanding the unique structure and function of eukaryotic ribosomes is cryo-electron microscopy (cryo-EM). It is anticipated that this method will soon achieve a 5 Å resolution of ribosomal subunit structures, which will permit individual RNA helix resolution (Dinman, 2009). Exciting directions for

elucidating eCNE function will be to engineer eCNE mutations in our RNA affinity tagged 60S subunits (Appendix II) and analyze mutant particles by mass spectrometry for protein assembly, coupling this with cryo-EM for structure-based analyses. An outstanding example of this type of collaborative approach to ribosome assembly comes from a study led by Ed Hurt and colleagues. Using a combination of biochemistry, genetics, and cryo-EM, these groups demonstrated a role for a protein kinase cascade in recruitment of ribosomal protein S3 and the concomitant formation of the 40S head domain (Schäfer et al., 2006). These multidisciplinary approaches represent the future frontier in unraveling the operations of the eukaryotic ribosome.

Ribosome biogenesis as a pivotal reporter on the metabolic state of the cell

L32, the eCNE interaction, and quality control

The large ribosomal subunit has six structural domains that make long-range interactions to form a functional RNA core. Ribosomal proteins stabilize the long-range interactions, giving the LSU its final shape. We took advantage of the high-resolution X-ray crystal structure from the archaeon *H. marismortui* (PDB No. 1S72) and modeled any r-protein that potentially contacts eCNEs. Attention was given to L32 because it draws together two eCNEs (eCNE6 and eCNE14) in domain II, and because L32 has no bacterial homologue. As a step towards understanding eCNE function *in vivo*, we disrupted the L32-eCNE interaction in yeast and frog cells. Interestingly, we observed an accumulation of the primary precursor rRNA in both cell types (Chapter 3), and a nuclear accumulation of both the small and large subunits (Chapter 3). This strongly suggests that

L32 is an early binding protein in the r-protein hierarchical assembly. Furthermore, L32 contains a large unstructured tail that penetrates into the RNA core (Chapter 3) and, for steric accessibility, would likely need to join early in the assembly pathway.

Given that genetic depletion of L32 inhibits rRNA processing of both subunits, it suggests the existence of a quality control mechanism for subunit assembly in the nucleolus. Such quality control mechanisms likely involve stepwise assembly factor recruitment that ensures the fidelity of assembly. In our model, the absence of L32 prevents the tertiary folding between eCNE helices in domain II and the defective pre-ribosome is not a substrate for early acting assembly factors- a structural proofreading model. The physiological consequences of activating this control pathway are discussed below.

Ribosome biogenesis coordinates the cell cycle and autophagy

At the very core of balancing cellular growth with proliferation lies a system for integrating nutritional information within the cell. Given the energetic cost to synthesize ribosomes, there is an obvious need to regulate ribosome biogenesis with the nutritional state of the cell. In response to favorable growth stimuli, cells will up-regulate macromolecular synthesis, namely ribosome biogenesis. Conversely, during adverse growth conditions, essential nutrients are supplied by autophagy for survival. In all eukaryotes, the TOR (target of rapamycin) pathway responds to the quality and quantity of metabolites in the cell, and controls multiple cellular events, directly or indirectly, including cell growth and negative regulation of autophagy (De Virgilio and Loewith,

2006). When the TOR pathway is inhibited, multiple steps in ribosomes biogenesis are negatively affected, including: formation of an active RNA polymerase I (Claypool et al., 2004), the processing of the 35S pre-rRNA (Powers and Walter, 1999), and the intranuclear transport of pre-ribosomes (Honma et al., 2006). These observations suggest that nutrient availability regulates early and late steps in the biogenesis pathway.

An increase in 35S rRNA in the nucleolus occurs during cellular stress such as limited nutrient availability. Depletion of L32 phenocopies this, with a severe rRNA processing defect, evident as a buildup of the 35S pre-rRNA in the nucleolus (Chapter 3, Figure 10). As a consequence of the processing defect, yeast cells will accumulate specifically in the G1-phase of the cell cycle within one cell division (Chapter 4, Figure 1). These data implicate ribosome synthesis as a key regulator of the G1/S-phase transition, since these arrested cells have adequate nutrients and apparently normal rates of protein biosynthesis. These data extend our quality control structural proofreading model beyond ribosome assembly to include a cell cycle checkpoint; in this scenario, the absence of L32 prevents proper ribosome assembly and the presence of defective pre-ribosomes in the nucleolus signals this adverse condition to the S-phase machinery. Although the molecular basis of the link is unknown, recent data have shown that many nucleolar proteins implicated in ribosome biogenesis are also required during cell cycle regulation (Dez and Tollervey, 2004). The Noc3 protein is essential for intranuclear transport of a nascent 60S ribosome from the nucleolus to the nucleoplasm (Milkereit et al., 2001), and Noc3 is required for formation of the pre-DNA replication complex onto origins of DNA synthesis (Zhang et al., 2002). Another biogenesis factor, Nop7, is required for maturation of 27S pre-rRNA

into 25S rRNA (Harnpicharnchai et al., 2001), and is needed for S-phase progression (Du and Stillman, 2002). Together these studies draw attention to the ribosome biogenesis pathway as a regulator of cellular proliferation.

When examining mature rRNA levels in the L32 depleted cells, we observed a dramatic decrease in mature rRNA and r-protein L32. This was highly unanticipated for two reasons: First, this has never been documented in a r-protein depletion study, and secondly, mature ribosomes are very stable with a half-life of days (Kraft et al., 2008; Volarevic, 2000). Morphological analysis of L32 depleted cells revealed an enlarged vacuole. Together these observations led us to test if autophagy was being stimulated in L32 depleted cells. However, in our immunoblot experiments, we observed that the abundant protein Pgk1p was not being turned over with the same efficiency as L32. We reasoned that if autophagy was occurring as a consequence of L32 depletion, it is likely through cargo selective autophagy and not bulk macrophagy (Nakatogawa et al., 2009). Recently, Kraft et al. (2008) demonstrated the selective autophagic breakdown of cytoplasmic ribosomal subunits can occur upon nitrogen deprivation and termed this process ribophagy. In a genetic screen, the authors identified the de-ubiquitinating enzyme Ubp3 as an essential factor in selective ribophagy, although the ubiquitin target in the ribosome remains unknown. We found that efficient depletion of L32 required Ubp3, supporting the idea that a selective type of autophagy, ribophagy, is induced in response to L32 depletion.

Why does L32 induce ribophagy?

We have postulated that the ribosomal protein L32 coordinates the folding of two eCNEs (eCNE6 and eCNE14). This interaction is required for ribosome biogenesis, as the absence of L32 attenuated all post-transcriptional pre-rRNA processing and stopped the production of both subunits. This is a novel molecular phenotype, as a typical r-protein depletion greatly inhibits the processing of the subunit in which it resides, and to a lesser extent the other subunit. We hypothesize that the severe increase in the 35S precursor rRNA caused by L32 depletion potentially mimics a starvation-like state in the cell and consequently stimulates ribophagy.

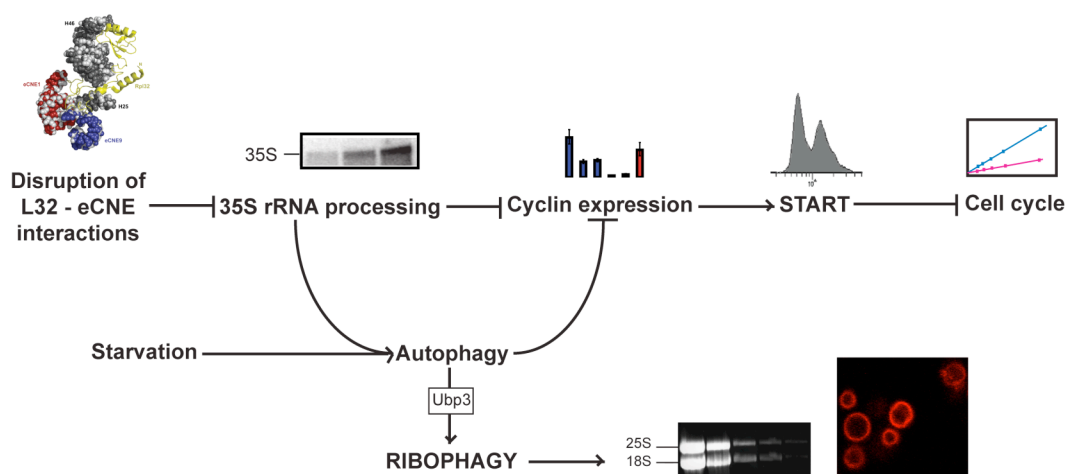


Figure 2. Model for the L32-eCNE interaction in cell cycle and autophagy

SUMMARY

The ribosome is an ancient macromolecular complex found ubiquitously in nature from subcellular organelles to the cytoplasm of human cells. Evolution has scrutinized this molecule over eons of time and reports on the functionally important regions of the

molecule. In this study we have focused on understanding the evolution of eukaryotic ribosomal RNA and have uncovered nucleotide elements that have remained unchanged in all domains of life as well as others that are specifically conserved in the eukaryotic domain. As a step towards understanding why these regions of rRNA have remained unchanged, we disrupted a three-way interaction between eCNE6, eCNE14, and ribosomal protein L32. We report here that this interaction is essential to the earliest steps in ribosome biogenesis. Furthermore, we learned that stopping ribosome biogenesis at this early step prevents the cells from entering S-phase and mimics a starvation phenotype (Figure 2). These data propose a model that ribosome biogenesis reports on the metabolic status of the cell, and thus promotes cell division and prevents autophagy.

In summary, the bioenergetics of the translation machinery makes it the most costly metabolic pathway in the cell. Logically, this pathway must be tightly coupled to the growth potential of the cell and its metabolic resources. In this study we verify this basic concept experimentally demonstrating that ribosome biogenesis controls cell growth by promoting cell proliferation and inhibiting its self-destruction by repressing autophagy. How this tightly controlled balance is achieved is an important direction for understanding a fundamental concept in cell biology

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**Appendix I: Does a Eukaryotic Ribosomal RNA Conserved Element Support
Nuclear Export of the Large Ribosomal Subunit?**

Injection of antisense oligos into *Xenopus* nuclei and gel analysis performed by Dr. Vassie Ware (Figure 4). All other experiments performed by SMD.

Summary

Eukaryotic ribosome biogenesis begins in the nucleolus where rRNA synthesis, processing, and subunit assembly initiates. Pre-ribosomal subunits mature in the nucleus and are subsequently exported to the cytoplasm where final steps in maturation occur. Ribosomal RNA sequences and folded RNA structures used in the precise regulation of export from the nucleus have not yet been identified. We describe a 29 nt element (CNE1, designated as eCNE6 in Chapters 2 and 3) that is perfectly conserved in 28S rRNA amongst eukaryotes but degenerate in all bacteria. This implicates the CNE1 sequence as a vital component in the eukaryotic ribosome. Indeed, the CNE1 unit is essential as yeast cells made dependent on CNE1 mutant 25S rRNA are inviable. Our preliminary data suggest that CNE1 is needed for export of the large 60S subunit in *Xenopus* oocytes and budding yeast *S. cerevisiae*. When an antisense DNA oligo against the 29 nt element is injected into the oocyte nucleus, 28S rRNA is retained in the nucleus. Chimeric transcripts containing CNE1 and the Box C/D stem motif of U14 snoRNA have sufficient information for nucleolar localization in oocytes and nuclear export in oocytes and yeast cells. This suggests the CNE1:U14 RNA chimera forms an export competent RNA-protein complex. We propose a role for CNE1 as a *cis* regulatory element in ribosome export that is conserved in all eukaryotes.

RESULTS AND DISCUSSION

Previously, our lab determined the complete rDNA sequence encoding *Xenopus laevis* 28S rRNA and from this analysis discovered eukaryotic rDNA expansion segments (Clark et al., 1984; Ware et al., 1983). Despite the limited number of eukaryotic rDNA sequences available at that time, a comparative analysis of the border of expansion segment 7 (numbered as per Gerbi, 1996) identified a small stretch of nucleotides (ca. 25 bases) highly conserved in sequenced eukaryotes but degenerate in bacteria (Ware et al., 1983). To test this preliminary result using modern eukaryotic rDNA databases, we found that this region contains a perfectly conserved nucleotide element (CNE1) of 29 core nucleotides flanked by a less conserved region (Figure 1A). The sequence maps to Domain II of the *S. cerevisiae* 25S rRNA molecule and forms helix 25.1 extending into half of helix 26, and ends at the top of helix 27 (Klein et al., 2004) (Figure 1B).

During biogenesis, rRNA undergoes nucleotide modification and the CNE1 nucleotides are a substrate for methylation including ribose and base methylation (Chapter 3 and 5). It is hypothesized that nucleotides targeted for modification occur in functionally important regions of the molecule and are often highly conserved in the three major domains of life. Most notable is the frequency of nucleotide modifications in rRNA that make up the A, P, and E sites on the ribosome (Decatur and Fournier, 2002). Four of the 29 CNE1 nucleotides are methylated including three 2'-O- methylations and one adenosine methylation (Figure 1B). These modifications are eukaryotic specific as the homologous bases are not modified in *E. coli* 23S rRNA (Decatur and Fournier, 2002).

Taken together, the sequence and modification data strongly suggest that the CNE1 must play a vital role to the eukaryotic ribosome.

The phylogenetic comparison data reveal that mutations in CNE1 are not tolerated in eukaryotic evolution from yeast to humans. To test this experimentally, we carried out sited directed mutagenesis substituting 32/33 nucleotides that comprise the core CNE1 and the conserved 3' flanking region. The CNE1 mutant retained the wild-type secondary structure: by using compensatory base changes we retained the short stem that forms helix 25.1 and by using G•U base pairs we retained the base-pair interaction that forms helices 26 and 27 (Figure 2A). This mutation permits the analysis of ribosomes that lack the CNE1 sequence without disrupting the core and higher order structure of the large subunit.

The phenotypic effects of CNE1 mutant ribosomes were analyzed using an rDNA expression system originally developed by Nomura and colleagues that makes use of a *S. cerevisiae* strain bearing a temperature sensitive RNA polymerase I (strain NOY504). The growth of this strain at the non-permissive temperature (37°C) can be rescued by introducing RNA polymerase II derived 35S rRNA from a plasmid (Liang and Fournier, 1997; Nogi et al., 1991). In the present experiments, a *GAL7* promoter was fused to the 35S rDNA operon that contains two hybridization tags- one in 18S and the other in the 25S rRNA coding regions to facilitate analysis of plasmid derived ribosomes (pSD264, Figure 2A). This unique *GAL7*-35S tagged system produces functional rRNA, as cells made dependent on this gene are viable (Figure 2B) and grew as well as cells expressing

plasmid encoded wild-type rRNA (data not shown). Next the CNE1 sequence mutation was introduced into the double-tagged *GAL7-35S* gene (pmutCNE-1). The CNE1 mutant rDNA gene cannot suppress the RNA polymerase I temperature sensitivity indicating that CNE1 is an essential component of the ribosome. To determine whether this lethality caused by the CNE1 mutation is due to an inhibition of ribosome biogenesis or ribosome function, total RNA was isolated from wild-type yeast cells transformed with *GAL7-35S* plasmids and Northern analysis was performed using the 25S hybridization tag to distinguish it from the chromosomally derived rRNA. The results demonstrate that the CNE1 mutant phenotype is due to defects in the amount of 25S rRNA synthesis and/or stability (Figure 3, lane 4).

There are several factors potentially responsible for the lethal CNE1 mutation and the down regulation of 25S rRNA. Since the nucleus is the defining feature of the eukaryote, a eukaryotic-specific nucleotide sequence may participate in the export of the large subunit from the nucleus to the cytoplasm. *Xenopus* oocytes have proven to be a superb model system for the study of RNA transport *in vivo* including ribosomal subunits (Trotta et al., 2003). Manual dissection of the *Xenopus* oocyte permits separation of nuclear and cytoplasmic compartments with little to no cross-contamination, and 28S and 18S rRNAs can be used as indicators of 60S and 40S subunits in the cytoplasm, respectively. Previously our lab has used the oocyte system to knockdown endogenous small nucleolar RNAs needed for rRNA processing in the nucleolus via injection of antisense DNA oligos (Savino and Gerbi, 1990). The formation of the DNA/RNA hybrid in the nucleus triggers the enzymatic cleavage of the RNA by endogenous RNase H. To test directly if

CNE1 is needed for nuclear export of nascent 60S subunits, an antisense oligo against that region (nucleotides 966-990 (Ajuh et al., 1991)) was injected into the nucleus of *Xenopus* oocytes. Injection of ^{32}P -GTP alone or with an antisense oligo against U1 snRNA (negative control) allows visualization of normal export of 18S and 28S rRNAs to the cytoplasm after 18 hours (Figure 4, lane 4). However, only 18S and not 28S rRNA is found exported to the cytoplasm if a DNA oligo complementary to 25 bases of the conserved element (c25mer) is injected (Figure 4, lane 5). Endogenous RNase H cleaves 28S rRNA at the position of hybridization with the c25mer oligo, thereby diminishing the amount of intact 28S rRNA. The resulting cleavage products that are slightly larger than 20S and slightly smaller than 18S are seen in nuclear but not cytoplasmic RNA (Figure 4, lane 2). From these experiments, we conclude that CNE1 is needed for the formation of an export competent large subunit.

Experiments that make use of mutant RNA to ask questions about trafficking patterns have to be interpreted with caution as the mutation could impair an upstream process such as the production of an export competent RNP. We tested a method for asking if an RNA sequence directs nuclear transport. This approach obviates concerns suggested in Figure 3 that mutations can cause destruction of RNA. With this optimized approach, a chimeric RNA was synthesized whereby a candidate export sequence is inserted into the Box C/D stem motif of a small nucleolar (sno)RNA. The C/D stem motif is utilized because the structure provides metabolic stability and as a nucleolar localization element (NoLe) to a snoRNA (Lange et al., 1998; Terns et al., 1995). In the tested chimeras, the C/D stem motif of U14 flanks the rRNA test sequence (C/D+CNE1, Figure 5A). To assay

if the C/D:CNE1 chimera localizes to nucleoli, the primary site of ribosome biogenesis, fluorescent transcripts were microinjected into the oocyte nucleus. The nuclei were then separated, the nuclear envelopes manually removed and the nucleoplasmic contents were spread on a glass slide by centrifugation for microscopy. The data indicate the fluorescent chimera localizes to nucleoli (Figure 5B). Next, nuclear export to the cytoplasm was tested using isotopically labeled wild-type U14 and chimeric snoRNA:rRNA clones. A visual comparison of the intracellular distributions of the co-injected U14 and chimeric RNA would imply that the chimera containing CNE1 from 28S is exported to the cytoplasm with greater efficiency than U14 (Figure 6A, lanes 2 and 3). However, quantification of the RNA levels indicates that the efficiency for the CNE1 sequence to override the nuclear retention signals of the C/D motif is not greater than the negative control adjacent 25 bases (nt 941-965) (Figure 6B). A possible explanation for the export efficiency of the negative control could be the action of the *Xenopus* Exportin-5 receptor, which preferentially exports small structured RNA (Jim Dahlberg, personal communication (Lund et al., 2004)).

To overcome the potential influence of Exportin-5 in the transport small RNA constructs, analogous experiments were conducted in *S. cerevisiae* that have a homologous ribosome export pathway, but lack microRNAs and thus an Exportin-5 receptor. Using a two plasmid-based system developed in the Singer lab (Bertrand et al., 1998), RNAs are visualized in yeast cells by virtue of a green fluorescent protein fused to an RNA binding domain (MS2cp:GFP, Figure 7B). The RNA of interest is tagged with the cognate MS2cp recognition site. A chimeric noncoding RNA gene encoding three domains was

constructed: 44 nt of yeast 25S containing the core CNE1 plus flanking rRNA, the C/D stem motif of yeast U14, and 2XMS2 containing two binding sites for the phage MS2 coat protein (Figure 7A). Importantly, the GFP-MS2cp fusion has an engineered nuclear localization sequence peptide (NLS, Figure 7B); therefore, unless the tagged RNA is exported the GFP signal remains nuclear (Figure 7B and 7C). Indeed when the CNE1:C/D chimera fused to the 2XMS2 RNA is introduced to the GFP expressing cells, the pattern of fluorescence changes from nuclear restricted to throughout the cell indicative of nuclear export (Figure 7B). As a second way to measure nuclear transport, the CNE1 construct was fused to the glucose repressible/galactose inducible *GAL7* promoter. When cultured in glucose, CNE1:C/D expression is repressed and the GFP signal remains nuclear (Figure 7C). After a 30 minute shift to galactose, the GFP signal is detected in the cytoplasm due to expression and export of the MS2 tagged CNE1:C/D. These experiments show that CNE1 in eukaryotic 25S/28S rRNA is dominant to the C/D Box and the NLS nuclear retention mechanism(s).

Results from yeast and *Xenopus* support a function for a conserved motif of LSU rRNA termed CNE1 as an export sequence. This suggests the CNE1 is capable of binding export proteins. CNE1 was tested for its ability to specifically bind oocyte factors by an RNA electrophoretic mobility shift assay (REMSA). Incubation of ³²P labeled CNE1 transcripts with an oocyte extract causes a shift in mobility on a non-denaturing gel (Figure 8, lane 2 RNP arrow). This complex is specific as judged by competition with unlabeled homologous CNE1 RNA (Figure 8, lane 3). To analyze and identify the proteins which complex with the CNE1, a modified transcript containing a 5' biotin and

3' ^{32}P -pCp was incubated in ^{35}S -labeled cell extract for streptavidin bead recovery.

Results in Table I show the biotinylated RNA is stable during binding and recovery as the 5' biotin and 3' ^{32}P co-migrate during chromatography, and furthermore, the method recovers 99% of the input RNA. Protein recovery analyzed by SDS-PAGE autoradiography shows an enrichment over mock for two polypeptides migrating at ~20 and 50 kDa (Figure 9 compare lanes 5 and 7). The polypeptide of 20 kDa is in the correct range to be a ribosomal protein or the export receptor protein Mtr2p- a 20.7 kDa protein. As a final way to identify CNE1 binding proteins, a yeast three-hybrid genetic screen was carried out using CNE1 as bait and a yeast cDNA library as prey. This screen did not reveal any CNE1 specific RNA binding proteins or proteins involved in nuclear export (Appendix III).

This study demonstrates that elements within rRNA can orchestrate important functions during ribosome biogenesis. While it remains unclear from the experimental data presented here what the exact function of CNE1 is, it is apparent from both antisense oligo injection and mutagenesis experiments that it is critical to the biogenesis and/or export of the eukaryotic ribosome. Together with our comprehensive CNE analysis (Chapter 2) and data from the yeast three-hybrid screen (Appendix III), we hope to further our understanding of the roles CNEs play in the eukaryotic ribosome biogenesis pathway.

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Hs (1287–1330)	acc	cg	acccgtctt	gaaacacggaccaaggagtct	aac	acgtgc
Xl (960–1003)	acc	cg	acccgtctt	gaaacacggaccaaggagtct	aac	gcgcgc
Dm (755–798)	tcgg	ga	cccggtctt	gaaacacggaccaaggagtct	aac	atatgt
At (1488–1531)	att	cg	acccgtctt	gaaacacggaccaaggagtct	gac	atgtgt
Pp (667–710)	tta	cc	acccgtctt	gaaacacggaccaaggagtct	aac	gtacat
Sc (630–673)	atg	ccg	cccgtctt	gaaacacggaccaaggagtct	aac	gtctat
Ec (552–595)	actg	cg	tac	ctttt	gtataat	gggtcagcgacttatattctgta

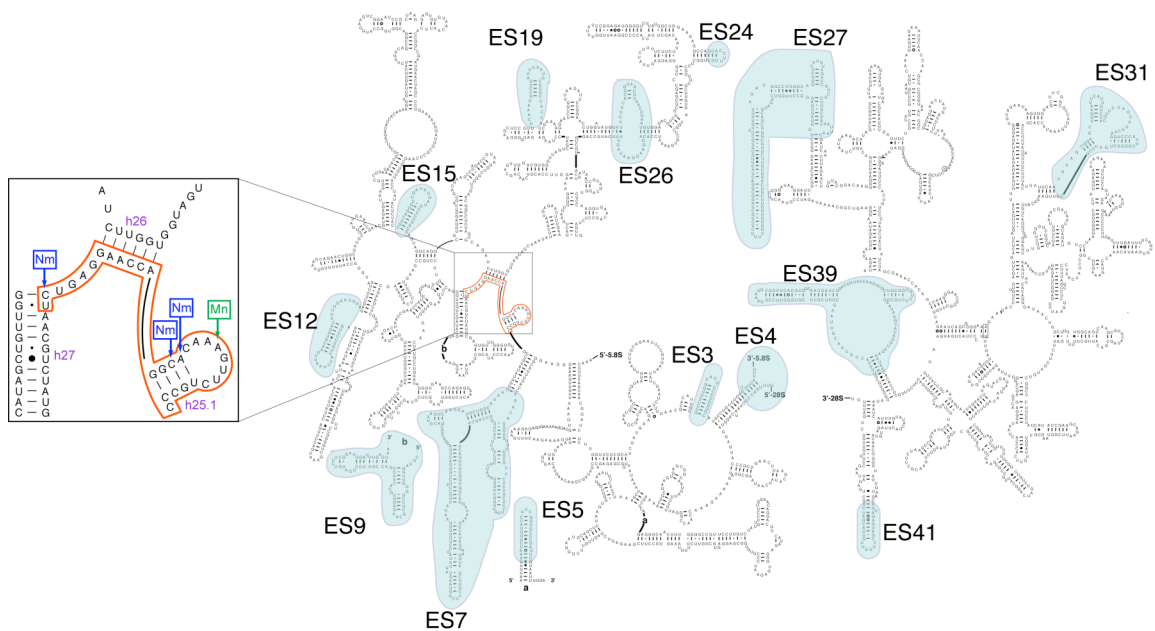


Figure 1. A perfectly conserved nucleotide element (CNE1) in eukaryotic rRNA. (A) Primary sequence alignment identifies a eukaryotic conserved nucleotide element (red- perfect conservation, blue- highly conserved, black- degenerate). Sc (*Saccharomyces cerevisiae*), Pp (*Polyspharum physarum*), At (*Arabidopsis thaliana*), Dm (*Drosophila melanogaster*), Xl (*Xenopus laevis*), Hs (*Homo sapiens*). (B) The yeast 25S Secondary structure with CNE1 mapped was adapted from (<http://www.rnaicmb.utexas.edu>). Helices (h) numbered as per Klein et al., 2004 and expansion segments (ES) as per Gerbi et al., 1996. The CNE1 structure is magnified in the left panel with the methylation of 2' hydroxyls (Nm) and the A645 methylation (Mn) indicated.

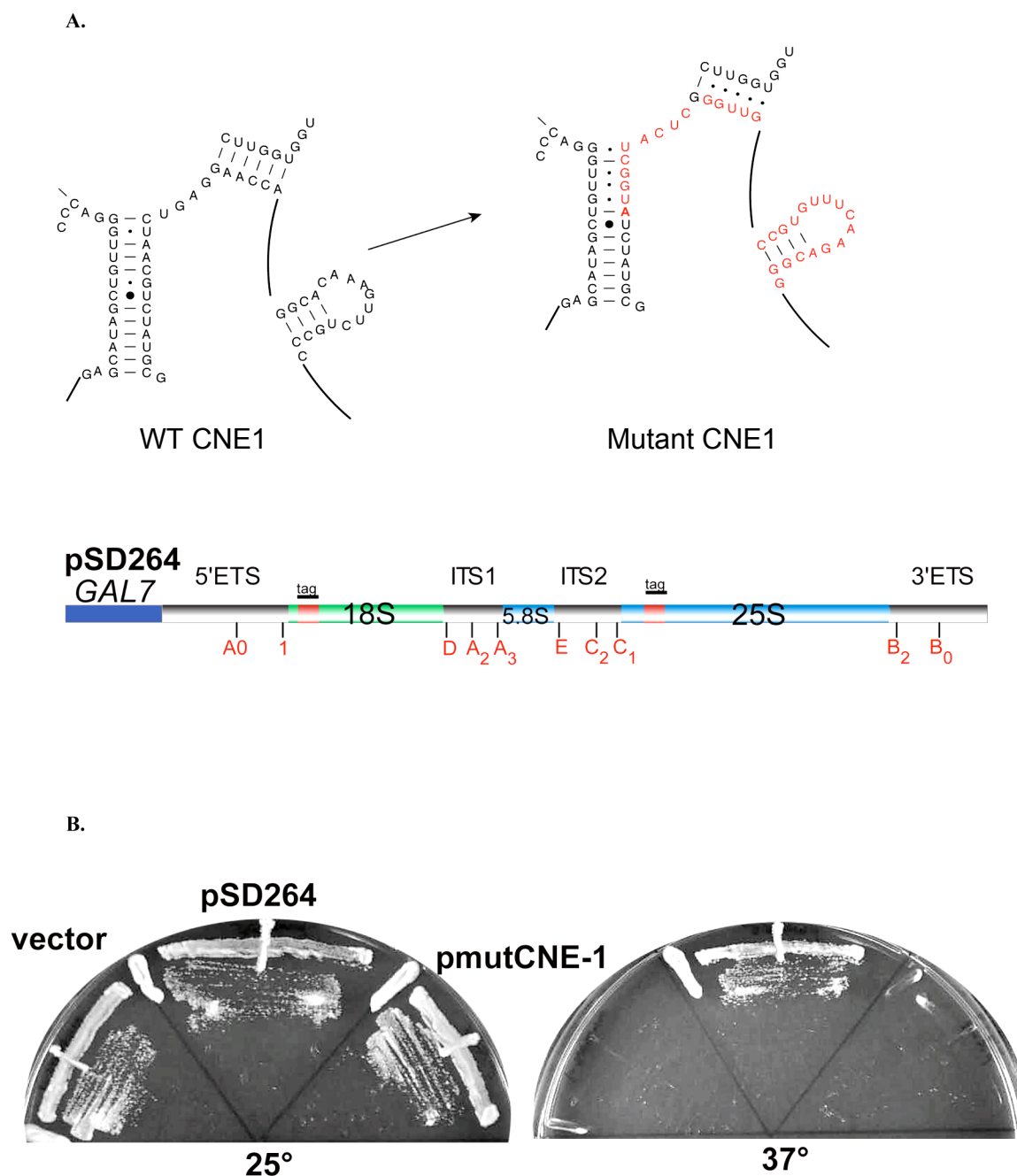


Figure 2. The conserved nucleotide element is essential. (A) The RNA secondary structures of wild-type and the CNE1 mutant that maintains base-pairing. The CNE1 mutation was introduced into a *GAL7*-35S clone containing two hybridization tags (pSD264) for expression in Pol I mutant yeast (NOY504). (B) Transformants were struck onto solid SG-Ura medium and incubated for 7 days at 25°C (left plate) or the nonpermissive 37°C (right plate). Cells made dependent on CNE1 mutant rDNA are inviable at the restricted 37°C temperature.

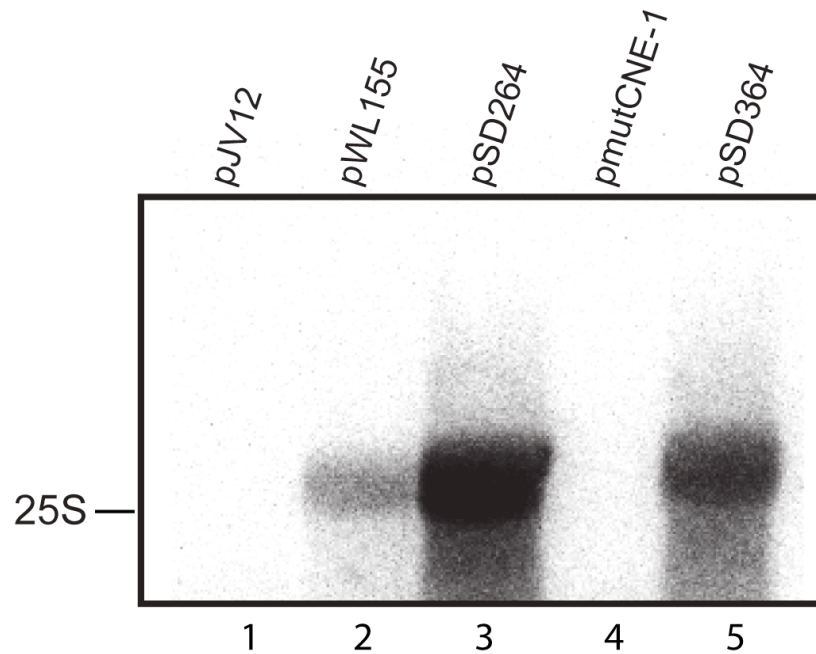


Figure 3. Northern analysis of CNE1 mutant 25S rRNA. A series of *GAL7-35S* rDNA clones were transformed into yeast strain W303-1a: CNE1 mutant rDNA (pmutCNE-1), wild-type 18S tag and 25S tag (pSD264), single-tagged 25S (pWL155), and triple-tagged (pSD364) rDNA. Negative control plasmid pJV12 contains different hybridization tags (Venema et al., 1995). Total RNA was extracted from logarithmically cultured cells in selective medium (SGal-Ura). RNA was analyzed by Northern hybridization with a probe directed against the engineered tag in 25S.

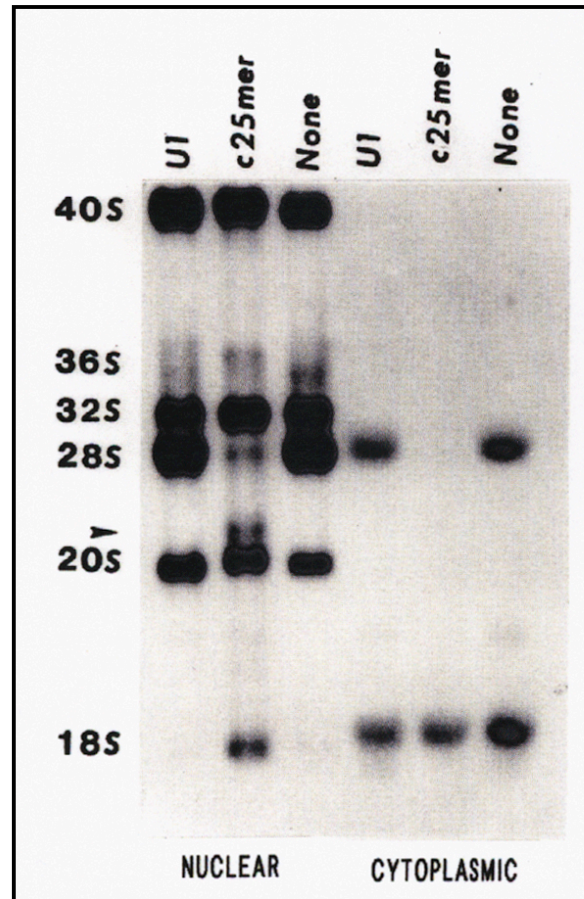


Figure 4. *In vivo* disruption of CNE1 in 28S rRNA in *Xenopus* oocytes. Nuclei of stage V/VI *Xenopus* oocytes were injected with 450 nCi of ^{32}P -GTP alone (None, lane 3), or coinjected with an antisense oligo against the 28S rRNA CNE1 (c25mer lanes 2) or against U1 (lane 1). Endogenous RNase H cleaves 28S rRNA CNE1 at the position of hybridization (arrow head lane 2). Nuclei were manually dissected and cytoplasmic (lanes 4-6) and nuclear (lanes 1-3) RNA was purified and electrophoresed on a 1.1% formaldehyde agarose gel. The cleaved 28S rRNA is not exported to the cytoplasm (lane 5).

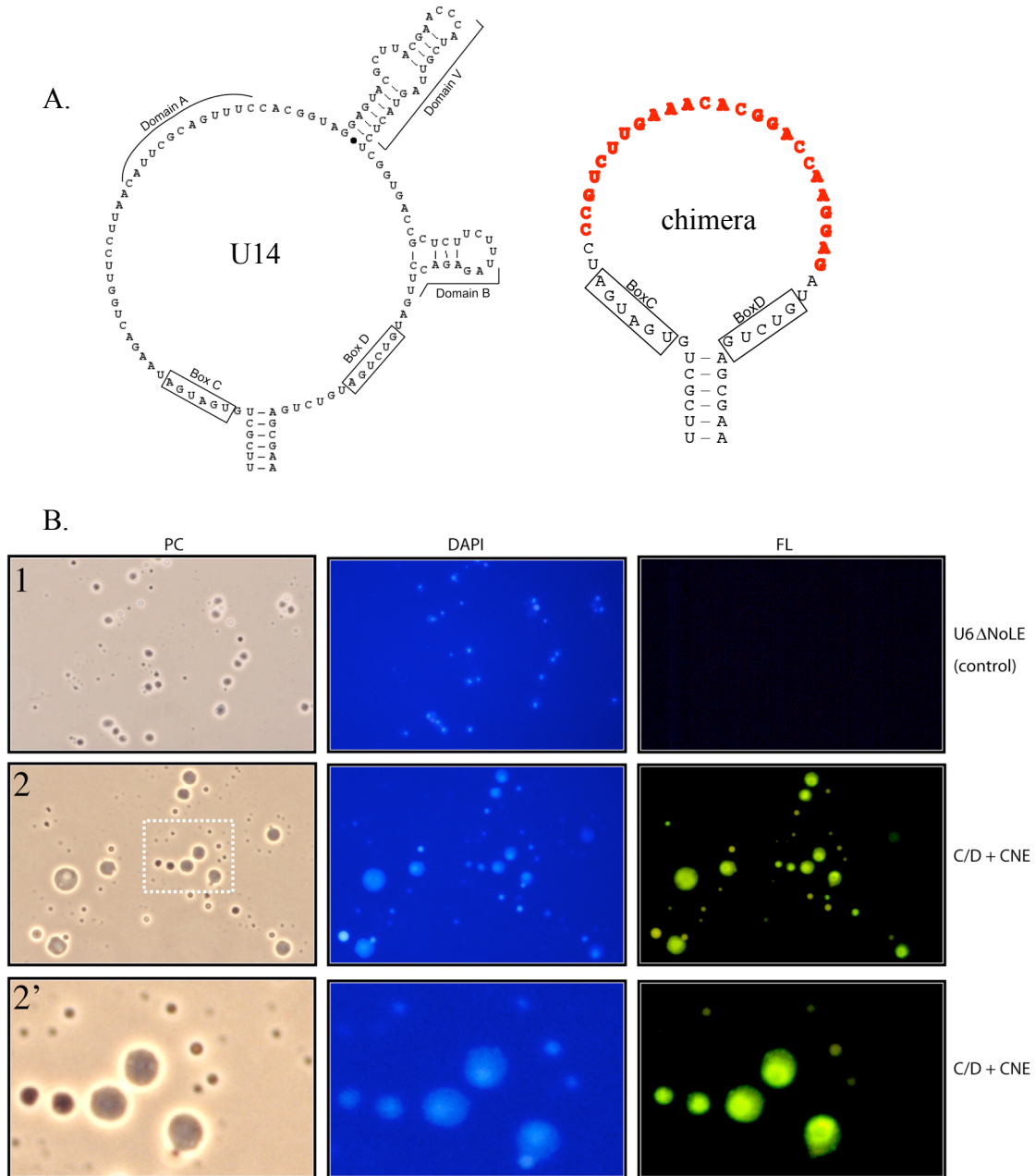


Figure 5. Nucleolar localization of the injected snoRNA:rRNA chimera (CNE1 + C/D). (A) Schematic of the yeast U14 snoRNA secondary structure (left) and the engineered chimeric C/D stem motif containing the CNE1. (B) Fluorescein-labeled U6ΔNoLE (control) or the CNE1+C/D chimera was injected into the nuclei of *Xenopus* oocytes. 30 min postinjection, nuclei were manually dissected, nuclear envelopes manually removed and nuclear contents spread on a glass slide and centrifuged. Nucleoli were analyzed by phase-contrast (PC) or fluorescence (FL) microscopy; the nucleolar rDNA is stained by DAPI. Panel 1- U6ΔNoLE. Panel 2- C/D + CNE1. Panel 2'- Magnification of boxed nucleoli in panel 2.

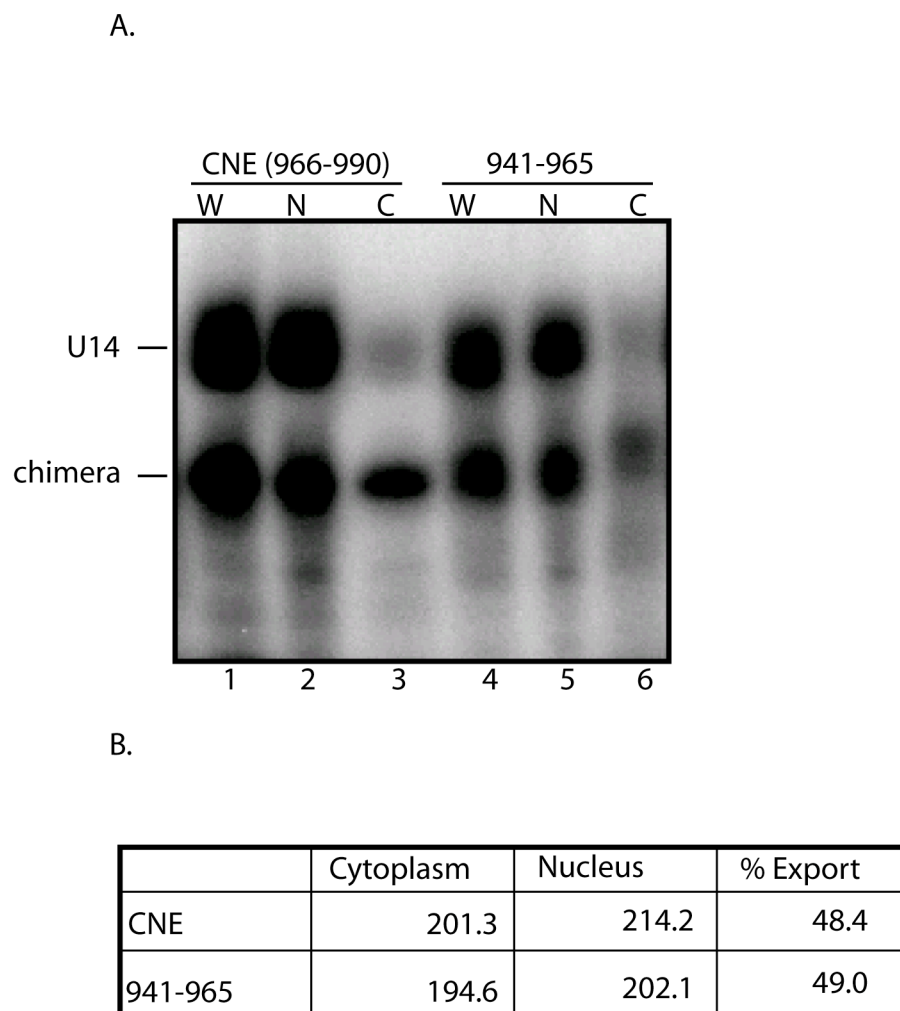


Figure 6. A Test for RNA export sufficiency of snoRNA:CNE1 chimera. (A) *In vitro* transcripts of wild-type *Xenopus* U14 and a rRNA:snoRNA chimera containing 25 nt from 28S rRNA (CNE1 nt 966-990 lanes 1-3 or negative control nt 941-965 lanes 4-6) were ^{32}P labeled and coinjected into *Xenopus* nuclei. Six hours post injection, the intracellular distributions of labeled RNAs were determined. (W) 5 whole oocytes (N) 5 nuclei (C) cytoplasm from 5 enucleated oocytes. **(B)** Quantification of RNA signals to measure export efficiency where percentage of Export is defined as, % Export = $\text{Cytoplasm} / (\text{Nucleus} + \text{Cytoplasm})$.

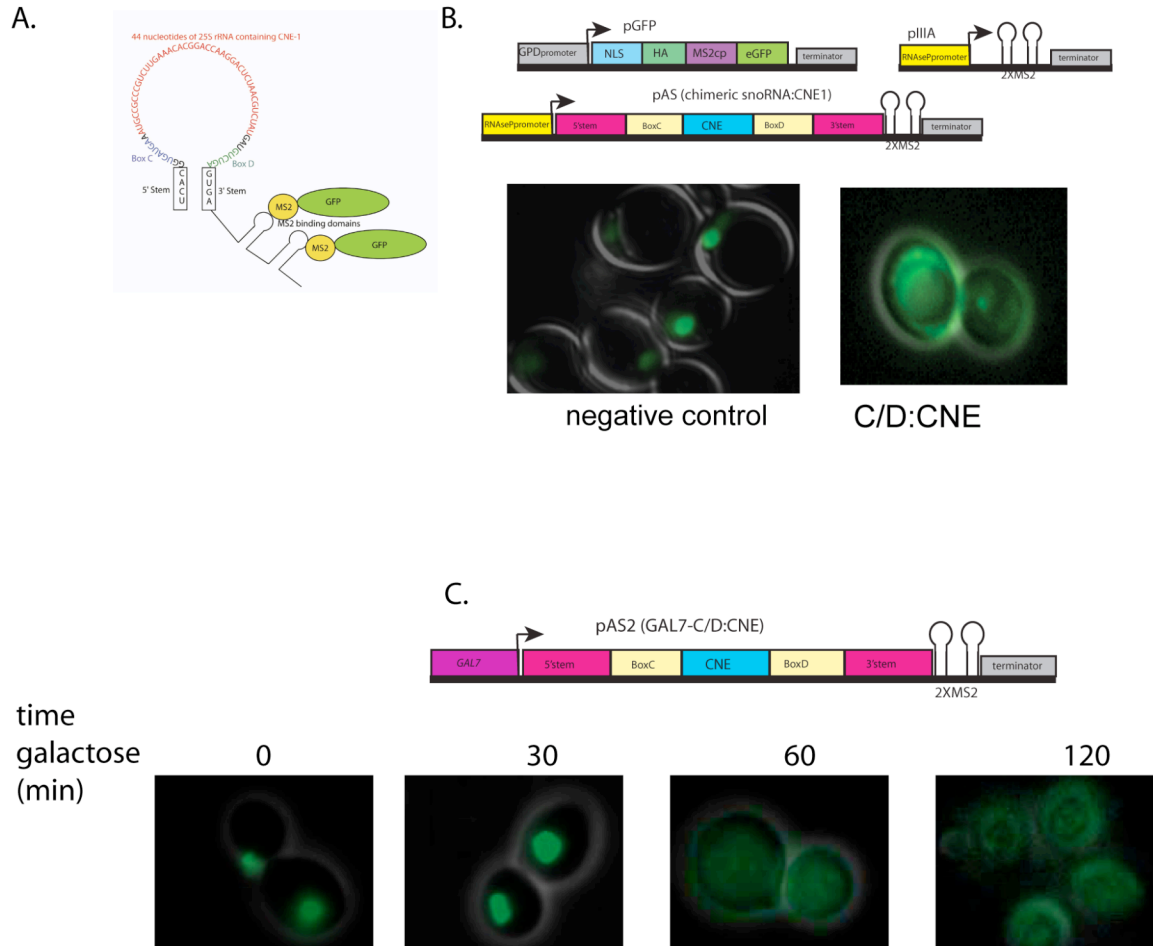


Figure 7. Visualization of RNA Export in Yeast: (A) Schematic of the C/D:CNE1 MS2 tagged RNA labeled with GFP. (B) The two-plasmid system used to fluorescently mark RNA *in vivo*. The GFP-MS2 coat protein (MS2cp) clone is expressed from the constitutive GDP promoter and the chimeric RNA tagged with two MS2 recognition sites is expressed from the RNase P Pol III promoter. The N-terminus of the GFP contains a nuclear localization sequence so that only GFP bound nuclear exported RNA is detected in the cytoplasm. (Bottom) Fluorescent microscopy of live yeast cells expressing the GFP-MS2 clone with either the MS2 RNA alone (negative control, right panel) or the chimeric C/D-CNE1-2XMS2 (right panel). (C) The C/D:CNE1:2XMS2 construct was expressed from the inducible *GAL7* promoter. In the absence of galactose ($t=0$) the GFP signal remains nuclear. After 30 min of induction, GFP is found in the cytoplasm indicative of nuclear export.

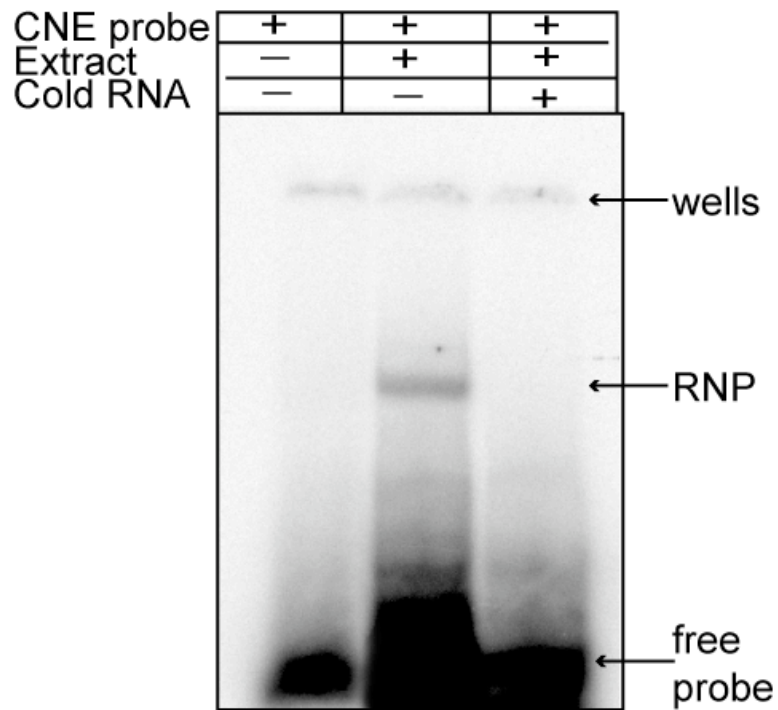


Figure 8. *In vitro* binding of oocyte factors to the CNE1 sequence: *In vitro* binding reactions contain ^{32}P -labeled CNE1 alone (lane 1) or with *Xenopus* oocyte extract from ten whole oocytes (lanes 2 and 3). The position of the free RNA or shifted complex is shown on the right. For specific competition, 40 molar excess homologous cold CNE1 RNA (lanes 3) was added to the binding reaction.

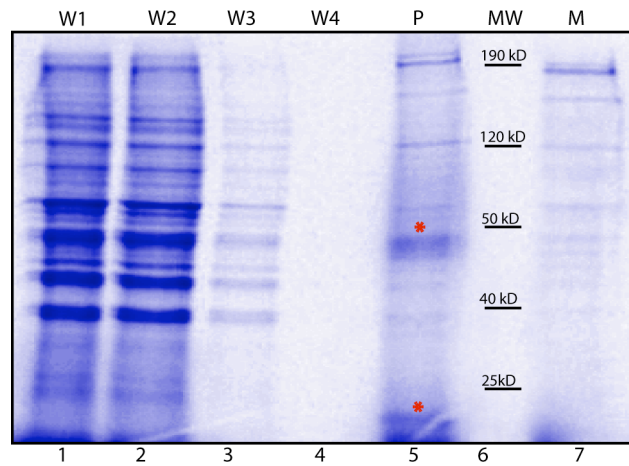


Figure 9. Streptavidin magnetic bead pull out of a biotinylated CNE1 and associated proteins: A 35 nt transcript containing the CNE1 was labeled at its 5' end with a single biotin and its 3' end with ^{32}P -pCp (b-CNE1-pCp). ^{35}S labeled yeast lysates were incubated with immobilized b-CNE1-pCp to streptavidin beads. The recovered RNA was washed four times with buffer (W, lanes 1-4). The pelleted protein components of the CNE1 RNP (P, lane 5 red asterisks) were identified as being specific by comparison to proteins pulled out from the beads alone (M, lane 7).

**Appendix II: A New System For The Analysis Of Ribosomal RNA Mutants in
*Saccharomyces Cerevisiae***

SUMMARY

Eukaryotes have a more complex ribosome biogenesis program relative to their bacterial and archaeal counterparts. Recent focus has been placed on cataloguing the multitude of *trans*-factors that transiently associate with the developing ribosomes from their synthesis in the nucleolus through export into the cytoplasm. In contrast, less attention has been focused on understanding the role of rRNA in the assembly and transport process. To facilitate the analysis of rRNA mutant populations of ribosomes, we introduced an RNA affinity tag (viral MS2 sequence) within the 25S rRNA coding region. We show that this tag is benign to ribosome function and that tagged ribosomes can selectively incorporate fusion proteins (e.g., GFP-MS2 or TAP-MS2) into the large subunit. This allows for the *in vivo* imaging and purification of ribosomes bearing mutations in 25S rRNA. Future experiments will test if eCNEs play a *cis*-regulatory role in assembly and nuclear transport of nascent ribosomes.

RESULTS AND DISCUSSION

We chose to apply the MS2 system to eukaryotic ribosomes, which has been used successfully to purify RNPs (Bardwell and Wickens, 1990), and for live cell imaging of mRNA localization in yeast (Bertrand et al., 1998). The MS2 RNA affinity tag (RAT tag) was introduced into the 25S coding region in a cloned yeast rDNA operon. The RNA tag itself comprises a small RNA hairpin of 19 nucleotides that is recognized by two copies of the viral MS2 coat protein (MS2cp; Lowary and Uhlenbeck, 1987). Fusions of this coat protein with protein affinity tags, such as the TAP tag, or with fluorescent proteins can be used to specifically purify MS2-tag-containing RNPs or image cellular localization respectively. In bacteria, insertion of a single MS2 hairpin was used to specifically purify ribosomes (Youngman and Green, 2005). This study showed that not only could this exogenous sequence be inserted into rRNA without having a negative effect on ribosome assembly and function, but that it could be effectively accessed and recognized by the MS2cp. It has been demonstrated that a markedly increased efficiency of purification, presumably through increased affinity of the RNA tag to its protein partner, is achieved when two hairpins are used (Bardwell and Wickens, 1990). We therefore chose to insert two MS2 sequences, separated by a 20 nucleotide spacer to ensure efficient binding of the MS2cp dimer, into 25S rRNA in a position comparable to that used in bacteria (Youngman and Green, 2005) (Figure 1A) in expansion segment 39. The tagged rRNA was introduced into cells as a plasmid-born 35S rDNA under the control of the inducible *GAL7* promoter (Figure 1B) in plasmid pSD364.

In order to test if the presence of the MS2 hairpins interferes with LSU function, we took advantage of the fact that endogenous 35S is transcribed by RNA polymerase I. In the RNA pol I temperature strain NOY1075, Pol I can be inactivated in a temperature sensitive manner (Nogi et al., 1991) resulting in an abrogation of cell growth. This defect can be rescued through the transformation of a plasmid containing the 35S rDNA unit under the control of a Pol II promoter such as *GAL7* (Figure 2A). At the non-permissive temperature, where cells rely entirely on plasmid-encoded 35S to grow, MS2-tagged rRNA was able to restore growth in a comparable manner to the wild-type sequence (Figure 2A), indicating that the MS2 sites do not interfere with LSU assembly or function. To verify these genetic data biochemically, yeast lysates were resolved on a sucrose gradient, and the resulting fractions analyzed by northern blotting (Figure 2B). Using a probe to MS2 insertion sequence, we observed that tagged ribosomes indeed fractionate in the heavy region of the gradient, suggesting that they indeed correctly incorporate into both the 60S and 80S ribosome and form polyribosomes. These genetic and biochemical tests showed that the small RNA affinity tag was not interfering with mature ribosome production or subsequent translation.

We next addressed whether the MS2cp in the context of the entire ribosome could recognize the MS2 hairpins. To do this we used a GFP-MS2cp fusion construct (Bertrand et al., 1998) in order to track cellular localization of tagged ribosomes by confocal microscopy. Cells were transformed with a plasmid encoding this fusion, in combination with either a control plasmid encoding the wild-type rDNA sequence or the MS2 tagged version of this same gene (Figure 3). In the control scenario (Figure 3, top panels) GFP

signal is observed solely in the nucleus as expected, given the presence of an NLS sequence in the GFP fusion protein. This is an inherent advantage of the MS2 imaging system since it restricts any background fluorescence to the nuclear compartment. However, upon expression of the MS2-tagged 25S rRNA, the GFP signal is observed throughout the cytoplasm, demonstrating that the GFP-MS2cp fusion is recognizing and binding its target structure in the mature 60S ribosome (Figure 3).

Ultimately, we would like to be able to use this system to specifically track and purify mutant ribosomes from the cell. As a pilot experiment, we asked if we could use a TAP-MS2 fusion protein to affinity purify tagged 25S rRNA (diagrammed in Figure 4A). The TAP protein affinity tag contains a Protein A moiety that is recognized efficiently by rabbit IgG. We therefore incubated lysates made from cells co-expressing TAP-MS2 and either wild-type or MS2-tagged 25S rRNA with IgG agarose beads to isolate rRNPs, and the RNA recovered from the pellet was used for RT-PCR. As shown in Figure 4B, rRNA was recovered in the pellet only if MS2 sequences were present in 25S rRNA. This effectively shows that MS2-tagged ribosomes can be purified away from endogenous ribosomes based on rRNA sequence.

The experiments detailed here show that the MS2 RNA tag can be used to great effect in the study of ribosome biogenesis and function in the widely used model system, *Saccharomyces cerevisiae*. We have shown that not only do the MS2 hairpins themselves have no influence over the biogenesis and function of the 80S ribosome, but that these

sites can be recognized by the MS2cp, thus providing an easily exploitable system for imaging and purification of the eukaryotic ribosome.

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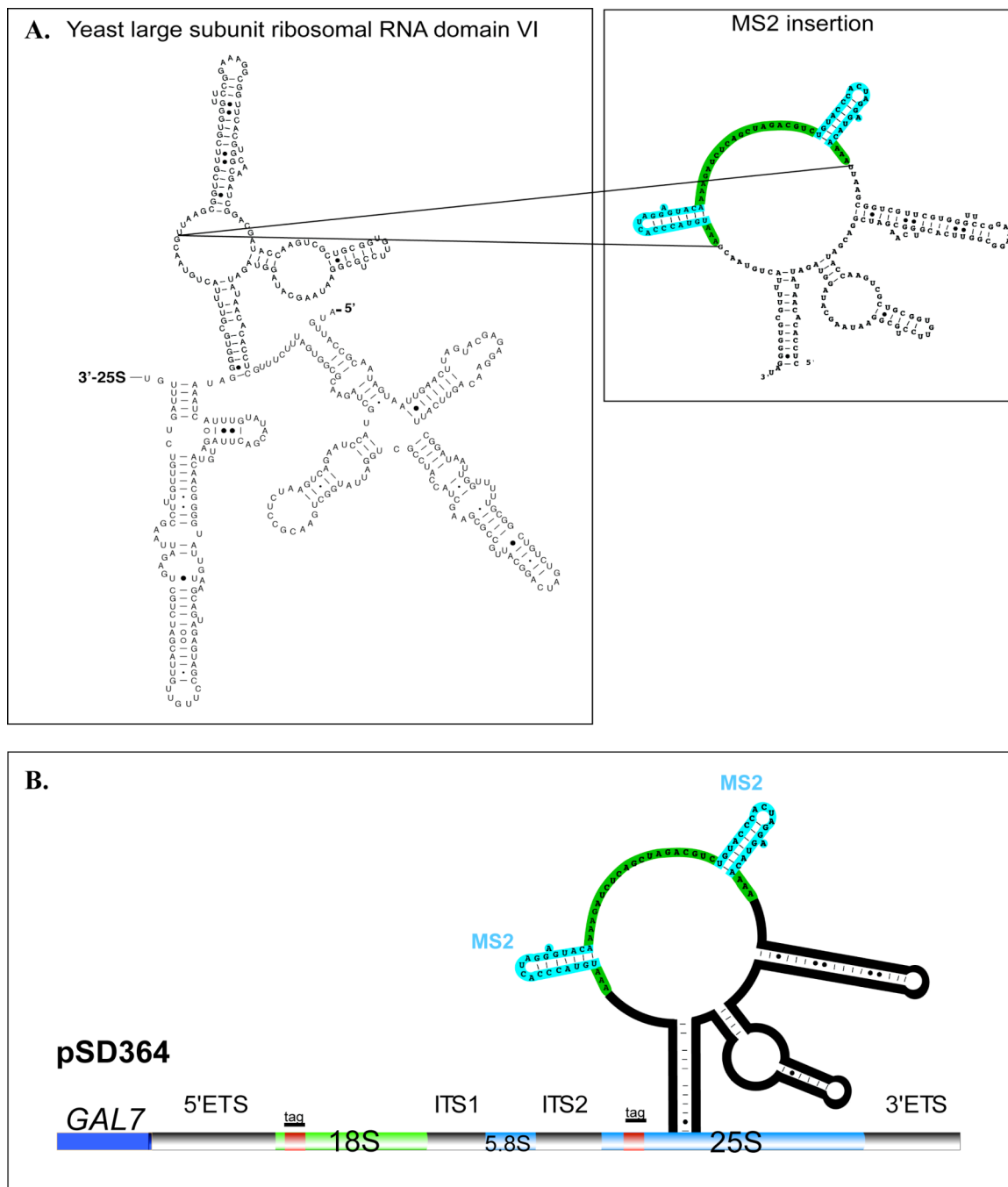


Figure 1. RNA affinity tagged 25S ribosomal RNA: (A) Two MS2 hairpins were inserted into expansion segment 39 in the positions diagrammed. (B) Tagged rRNA was transcribed from a plasmid-born full length 35S rDNA sequence under the control of the *GAL* promoter.

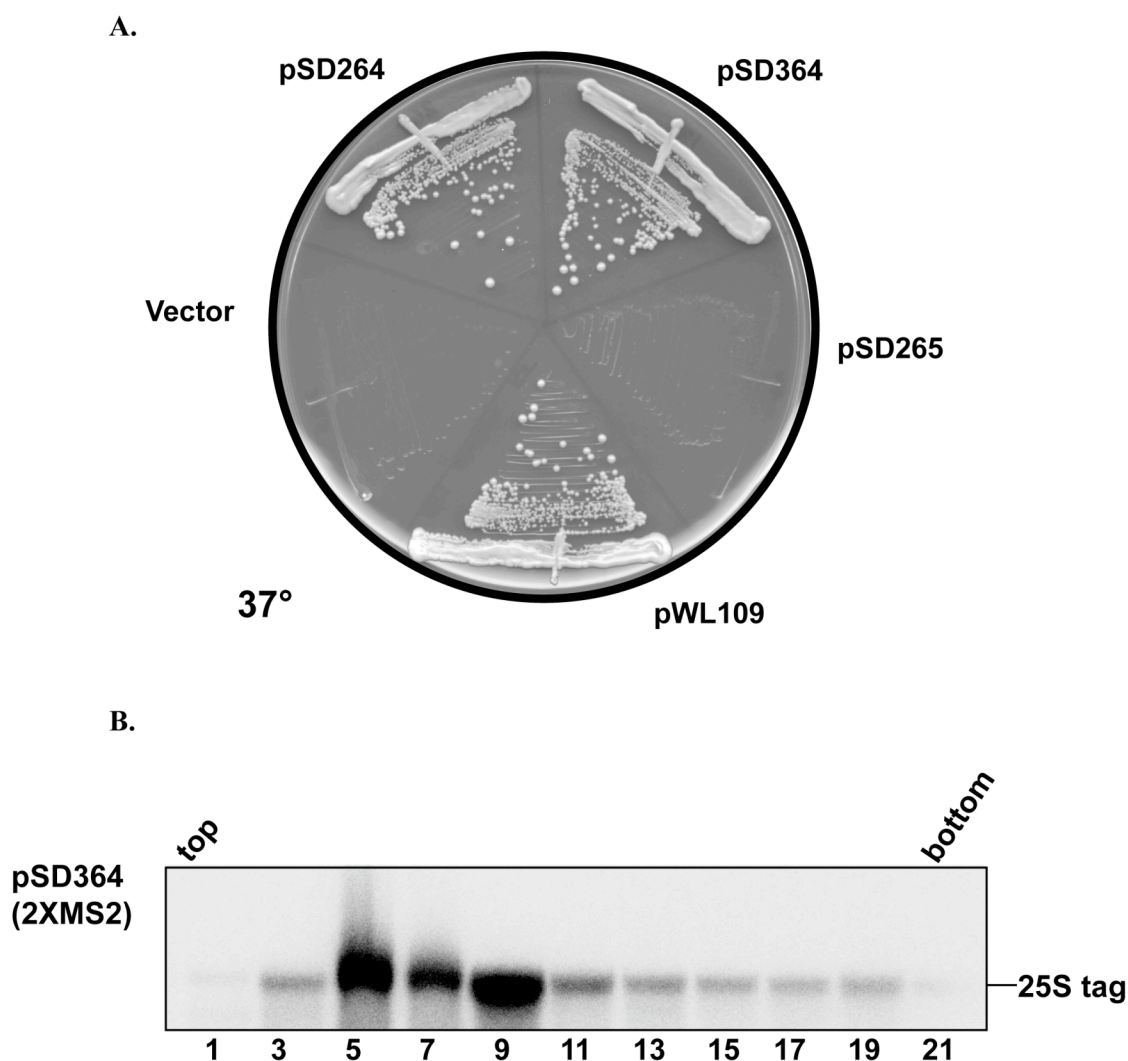


Figure 2. MS2 affinity tagged 25S rRNA produces functional large subunits. (A) The temperature sensitive RNA Pol I yeast strain NOY1075 was transformed with a series of GAL7-35S clones containing one hybridization tag (pWL109 Liang and Fournier, 1997), two hybridization tags (pSD264), mutation in CNE-1 (pSD265), two hybridization tags and the 2XMS2 affinity tag (pSD364) or empty vector. Cells made dependent on 2X MS2 tagged rDNA are viable at the non-permissive 37° temperature. (B) Sucrose gradient analysis of affinity tagged rRNA. Lysates from cells transformed with pSD364 were fractionated on a 15-50% sucrose gradient, and fractions were analyzed by Northern blot using a probe for the hybridization tag in 25S. The position of 25S is shown.

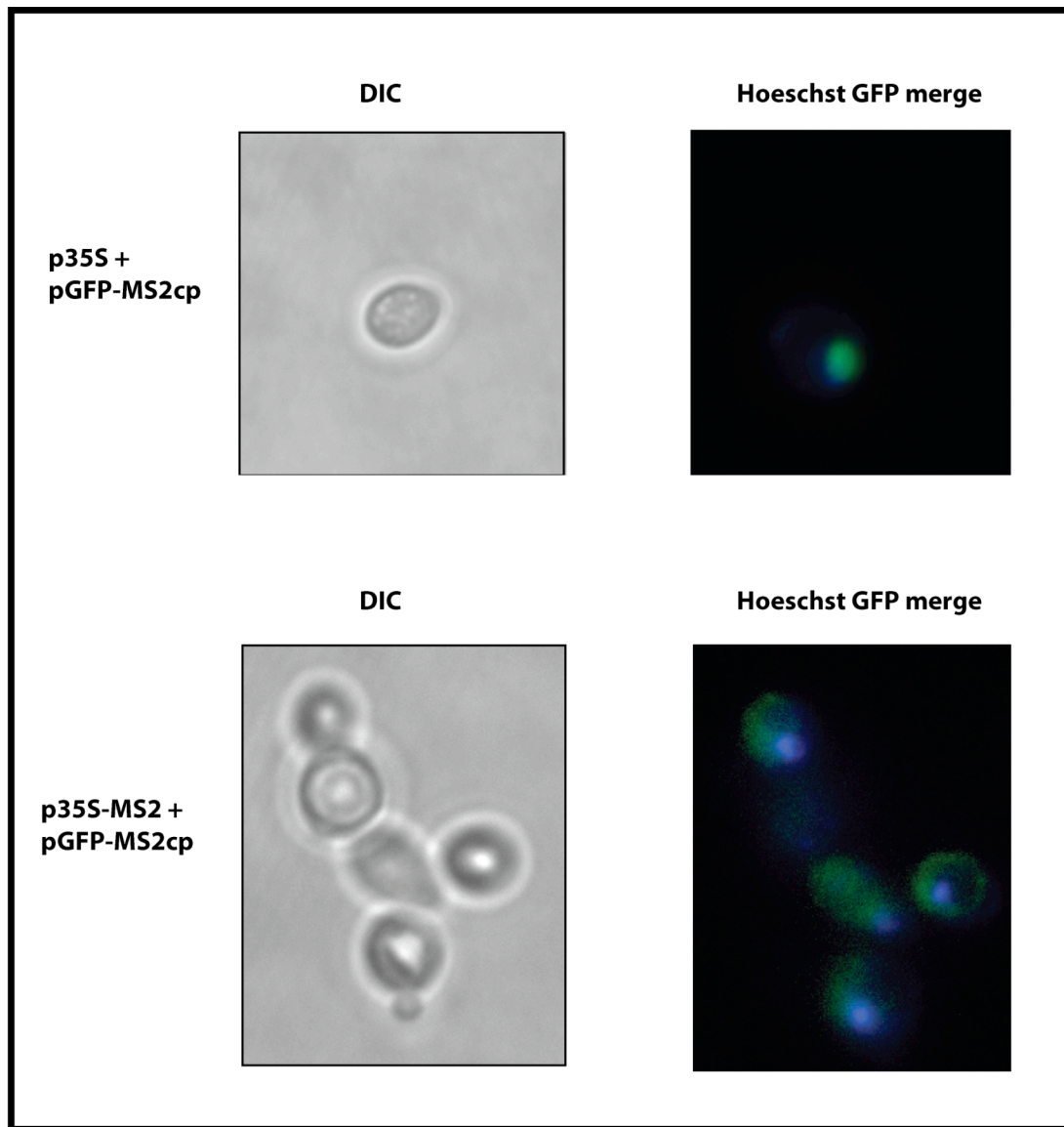


Figure 3. Visualization of MS2 tagged 25S rRNA in live cells. Yeast cells expressing the MS2 coat protein fused to GFP were transformed with either a control plasmid containing untagged 35S rDNA (top panels) or the affinity tagged construct (bottom panels). Cells were visualized using confocal microscopy after staining with Hoechst to stain DNA (blue, right panels). In the absence of MS2 tagged rRNA, GFP-MS2 is retained in the nucleus by virtue of an engineered NLS (top right). MS2 tagged ribosomes incorporate GFP-MS2, as shown by a diffuse GFP signal throughout the cytoplasm (bottom right).

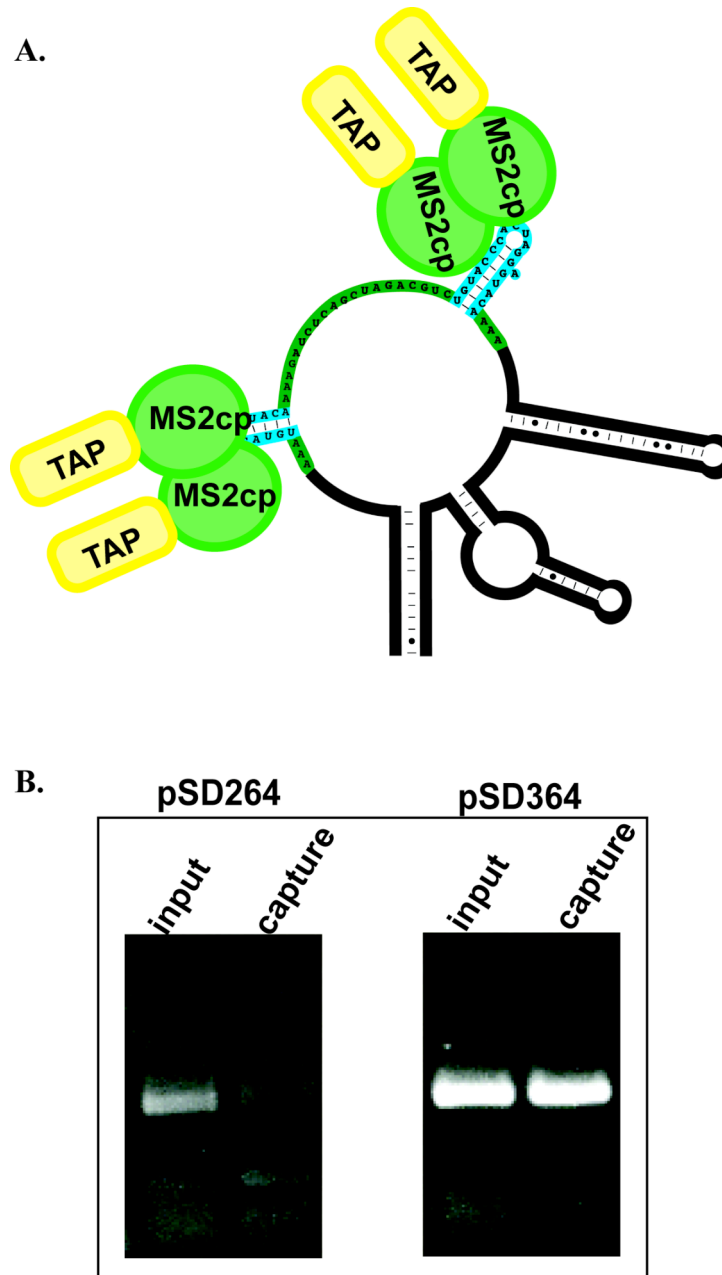


Figure 4. Affinity purification of MS2 tagged 25S rRNA. (A) Yeast cells expressing tagged 25S were transformed with a Tandem Affinity Protein fused to the MS2 coat protein (TAP-MS2cp, from D. Lafontaine) which binds to a single MS2 site as a dimer. The 25S expressed from pSD364 contains two MS2 sites and thus can bind 4 TAP:MS2cp fusions. (B) Cell extracts prepared from logarithmically growing yeast cells were reacted with IgG agarose beads, which have high affinity for the protein A molecule in the TAP tag. RT-PCR analysis on the input and IgG bead capture shows 25S rRNA is enriched in the presence of the RNA tag (right panel) compared to 25S rRNA lacking the MS2 tag (Left panel).

**Appendix III: The Yeast Three-Hybrid Screen as a Tool for Identification of
Eukaryotic Ribosomal RNA Binding Proteins**

SUMMARY

Eukaryotic ribosome biogenesis is a complex process that utilizes hundreds of *trans*-factors. Precise molecular information regarding the location and timing of accessory factor binding during ribosome biogenesis is largely unknown. We have used the yeast three-hybrid screen as a tool for identifying proteins that interact with nucleotide elements highly conserved in eukaryotic but not bacterial large subunit rRNA (CNEs). This screen uncovered several candidate CNE binding proteins, each one exhibiting an interaction with a given CNE molecule. One CNE sequence associates with *SEC65*, suggesting a role for this rRNA element in the protein secretory pathway. We also identified Cms1 as a potential rRNA binding protein. The *CMS1* gene has been implicated to facilitate the formation of the pre-replication complex at yeast origins of DNA replication (Wang and Wu, 2001). The CNEs studied here were identified using manual alignments of 28S rRNA prior to establishing the hierarchical CNE analysis and CORD as presented in Chapter 2.

RESULTS AND DISCUSSION

Eukaryotic ribosome biogenesis is a complex and energetically expensive process. Multiple tightly coordinated molecular events must occur before a mature 80S ribosome can assume its central role in translation. For example, the 35S rRNA is covalently modified and cleaved, both co- and post-transcriptionally, while concurrently associating with various ribosomal proteins to form the 90S pre-ribosome (Granneman and Baserga, 2005). These processes, and subsequent remodeling to form pre-40S and pre-60S subunits, are guided by hundreds of accessory factors, which is marked contrast to the bacterial system wherein the *in vitro* reconstitution of 70S ribosomes has been achieved using only rRNA and r-proteins (Nierhaus, 1991). In addition, the majority of eukaryotic biogenesis takes place within the nucleolus, a specialized compartment within the nucleus (Gerbi et al., 2003). Therefore in order for mature ribosomes to fulfill their function, they must be transported across the nuclear envelope (Tran and Wente, 2006). Due to the fact that in eukaryotes *in vitro* ribosome reconstitution experiments have been unsuccessful (Dinman, 2009), the location of accessory factor binding and the hierarchy of protein assembly onto rRNA are poorly understood.

To begin to address these eukaryotic specific problems in ribosome biogenesis, we first asked if there were conserved *nucleotide elements*, or CNEs, within eukaryotic 25S rRNA that are not present in bacteria or archaea. We rationalized that in order to serve as a potential protein-binding site, CNEs should be at least 10 nucleotides in length, and any element smaller than 10 nucleotides was discarded. Using ClustalW and comparison of

bacterial, archeal and eukaryal rRNA secondary structures, we manually identified six CNEs (Figure 1); CNE-1, CNE-6, CNE-8/9, CNE-12, CNE-16 and CNE-19. [NB: The CNE identification used for this work was achieved using an earlier method than that described in Chapter 2 of this thesis. Thus the nomenclature used in this appendix is different than that of Chapter 2. In order to facilitate a comparison between the data shown here and that of Chapter 2, Table I lists both sets of CNE nomenclature.]

Using homology modeling we mapped the six CNEs into LSU rRNA tertiary structure using the crystal structure of the archeal 50S subunit from *Haloarcula marismortui* (Ban et al., 2000; Klein et al., 2004). As shown in Figure 2, CNEs 1, 8/9, 12 and 19 cluster in the core of the subunit, whereas CNEs 6 and 16 are exposed at the surface of the rRNP. While this suggests that CNE-6 and CNE-16 are more accessible to accessory rRNA processing factors, given the complexity of ribosome biogenesis and the multiple conformations assumed by the rRNA and associated r-proteins during assembly, the core CNEs could potentially be exposed earlier in the biogenesis pathway. Alternatively the core CNEs, and the interactions between them, could contribute to the efficiency of rRNA folding and the stability of ribosomal precursor entities. This second scenario, though intriguing, was not further explored in this study.

The yeast three-hybrid system (Y3H) has been employed successfully in recent years to identify specific RNA-protein interactions in a number of systems (Bernstein et al., 2002). For example, the binding determinants for ribosomal protein S14 for helix 23 and a stem-loop in the body of its mRNA were mapped using this method (Antunez de

Mayolo and Woolford, 2003). The technique is based upon the yeast two-hybrid system, in which a DNA-binding protein domain is fused to a bait protein. Cells are then transformed with a cDNA prey library in which proteins are expressed as a fusion with a transcriptional activation protein domain. An interaction between bait and prey domains results in the expression of a reporter gene, thus previously unknown protein-protein interactions can be uncovered in a large-scale screen. In the Y3H method (diagrammed in Figure 3),

an RNA-protein interaction results in the reconstitution of a transcription activator complex thereby activating an essential reporter gene(s). We have obtained from Marvin Wickens the yeast strain YBZ1 which increases the number of sequence specific RNA-protein interactions and simultaneously reduces false-positives in the Y3H screen (Hook, 2005). This improved Y3H was achieved by expressing the MS2cp as a head-to-tail dimer fused to a LexA monomer. This minimizes nonspecific protein-protein interactions as the MS2cp now forms an intramolecular dimer. The method was further improved upon by engineering a point mutation in the MS2cp, N55K, which decreases the K_d of the RNA-protein interaction sevenfold (Lim and Peabody, 1994). These improved modifications to the Y3H screen coupled to the fact that we are screening for yeast proteins binding a small RNA (<50 nt) make the CNE an ideal candidate for Y3H screening (M. Wickens, personal communication).

We used the six CNEs as RNA bait (Figure 1), and as prey used a yeast cDNA:Activation Domain library (gift of S. Elledge). The yeast strain used for the screen contains two reporter genes controlled by the LexA operator; *HIS3* and *LacZ*. The *HIS3* gene allows

for initial selection based on growth phenotype on selective media containing the drug 3-AT (an inhibitor of His3p, therefore growth of each transformant depends on the levels of active His3p in the cell), whereas the LacZ gene allows for subsequent quantitative analysis of the bait-prey interaction. The activity of the LacZ reported is reflective of the amount of bait RNA in the cell. Northern blotting revealed an equivalent expression levels for each CNE bait (Figure 4A and B).

A summary of the results for each RNA bait is presented in Table II. A variety of concentrations of 3-AT were used for the initial selection of positive interactors in order to retrieve a manageable yet representative number of candidate CNE-binding proteins for each screen. Increasing 3-AT concentration increases the stringency of the screen, since a weak RNA-protein interaction will only weakly activate transcription from the LexA operator and thus lower levels of the His3p will be present in the cell. Each His⁺ clone was then assayed for β -galactosidase activity, precisely as done by Pinkham et al. (Pinkham et al., 1997) Clones that were both His⁺ and stained blue on medium containing X-gal were sequenced, and a summary of all the proteins identified is shown in Table III. Unfortunately, the Y3H using CNE-8/9 as bait did not react with any cDNA. This could be due to a variety of potential problems intrinsic to the system, such as the chimeric RNA forming an alternate structure through interactions between the MS2 sequence and the CNE-8/9 sequence.

Our screen yielded a vast number of candidate CNE-interacting proteins that function in a variety of cellular processes (Table III). One particularly strong candidate is *CMSI*, which interacts with CNE-6. CNE-6 is highly conserved in eukaryotes but degenerate in *E. coli* (Figure 5A). While CNE-6 failed to be identified as an eCNE in Chapter 2 (also see Table I), it should be noted that this CNE was highly conserved in the eukaryotic kingdom Opisthokonta, in which the yeast *Saccharomyces cerevisiae* is located, and furthermore *CMSI* has yet to be identified outside of the fungal subphylum. Little is known about *CMSI*. It was identified in a high copy suppressor screen using a temperature sensitive mutation in *mcm10* (Wang and Wu, 2001) hence its name, Complementation of *Mcm10* Suppressor. *Mcm10* is an essential DNA-replication factor involved in replication initiation. Since the overexpression of *CMSI* compensated for the loss of *mcm10* function (Wang and Wu, 2001), *CMSI* likely plays a role in the initiation of DNA replication and may therefore play a role in the control of the cell cycle. By β -galactosidase assay (Figure 5B), *CMSI* binds tightly and specifically to CNE-6. In this assay, the interaction between *CMSI* and CNE-6 was almost 10-fold tighter than with the other CNEs, and three-fold tighter than that of the characterized iron response protein (IRP) and its cognate recognition element (IRE). The fact that a factor involved in controlling the duplication of genetic information interacts with rRNA, in particular a highly conserved piece of eukaryotic rRNA, suggests that ribosome biogenesis and the cell cycle are directly linked. This is not entirely unexpected, given the many controls imposed upon a cell before it can divide, particularly those pertaining to cell size and DNA copy number. Upon cell division, cellular contents are divided between mother and daughter. For both to survive, they must be equipped with robust cellular machinery,

perhaps the most critical being the translational apparatus. Monitoring ribosome biogenesis in the nucleus and relaying the health of that process to the DNA replication machinery is a logical and efficient way for the cell to ensure that premature cell division is avoided.

A second gene identified in this screen captured our attention: *SEC65*. *SEC65* encodes a component of the yeast signal recognition particle (SRP), an RNP complex that is involved in the targeting of secreted proteins in the process of being translated to the endoplasmic reticulum (ER). *SEC65* showed a weak yet significant interaction with CNE-6 (and to a lesser extent CNE-19) in the Y3H (Figure 6). The secretory pathway in eukaryotes is vastly different than that of bacteria, involving the complex membrane bound structures of the ER and Golgi apparatus rather than membrane spanning transporter complexes. While SRP-like components exist in bacteria, none bear any homology to *SEC65* (Halic et al., 2004). CNE-6 is exposed at the surface of the 60S subunit, and is therefore poised to interact with extra-ribosomal particles in the cytoplasm (Figure 2). CNE-19 however, is in the 60S core, and therefore seems less likely to play a role in SRP binding. A detailed structure of the SRP-bound ribosome has yet to be captured, so we can only speculate as to the likelihood of CNE-19 being a *bone fide* *SEC65* binding partner. As mentioned earlier, the interaction between *SEC65* and the CNEs is weak, especially compared to that of *CMSI* with CNE-6 (Figure 5). This could be explained in one of two (not necessarily mutually exclusive) ways. The first is that the interaction between the ribosome and the SRP is transitory. Once the SRP has delivered the stalled elongation complex to the ER, it interacts with the SRP-receptor and upon

GTP hydrolysis is released. A tight interaction between the ribosome and the SRP could interfere with SRP recycling and/or translation of the nascent peptide into the ER lumen. An alternate explanation for the weak affinity of CNE-6 and 19 for *SEC65* is that multiple weak interactions are responsible for SRP recognition of the ribosome, and that these RNA elements account only for part of that recognition event.

This screen identified a vast array of proteins that interact with elements of the eukaryotic ribosome that are not only highly conserved, but also degenerate in the other two domains of life. Highlighted above are two examples of how this screen will become a valuable starting point for further study of extra-ribosomal accessory factors. Both ribosome biogenesis and translation consume vast amounts of energy, and the efficient and flawless production of these macromolecular machines is of utmost importance to the cell. From this screen, and the work that will come out of it, it is clear there is much that remains unknown about eukaryotic ribosome assembly, structure and function.

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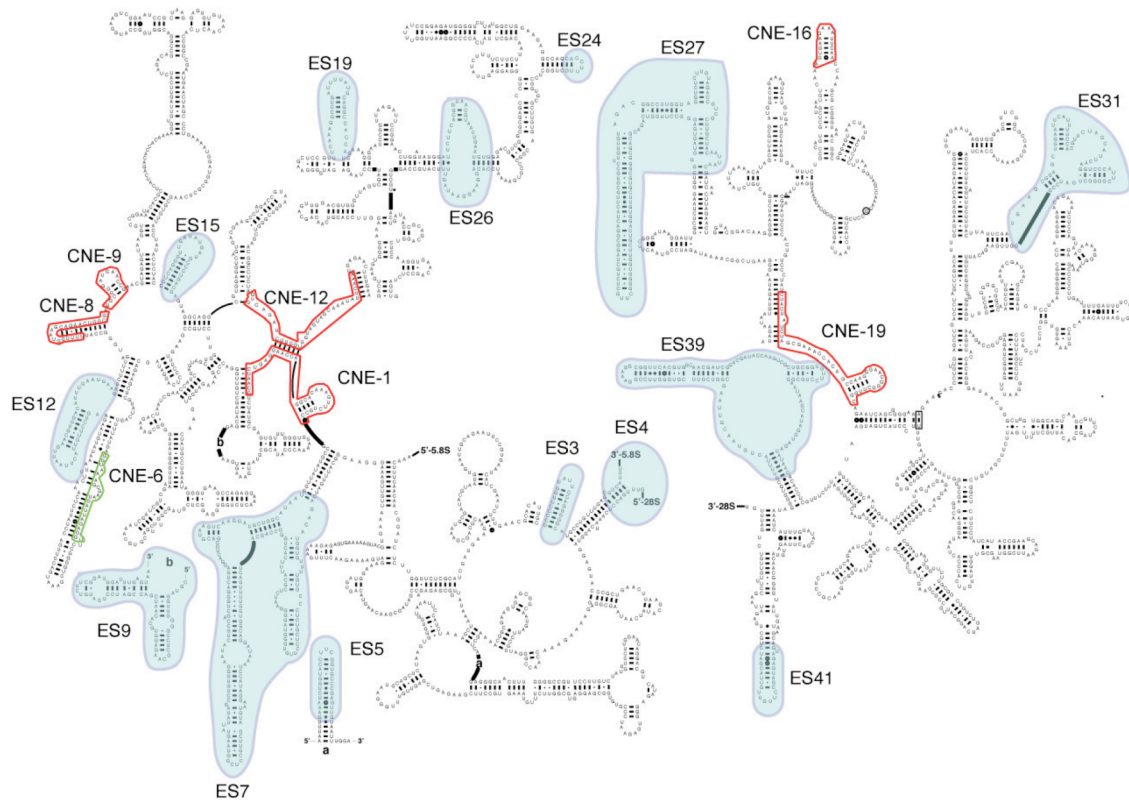


Figure 1. Secondary structure of CNE in 25S rRNA: The yeast 25S secondary structure with the six eukaryotic CNEs boxed (CNEs 1, 6, 8/9, 12, 16, 19) and expansion segments (ES) shaded in blue.

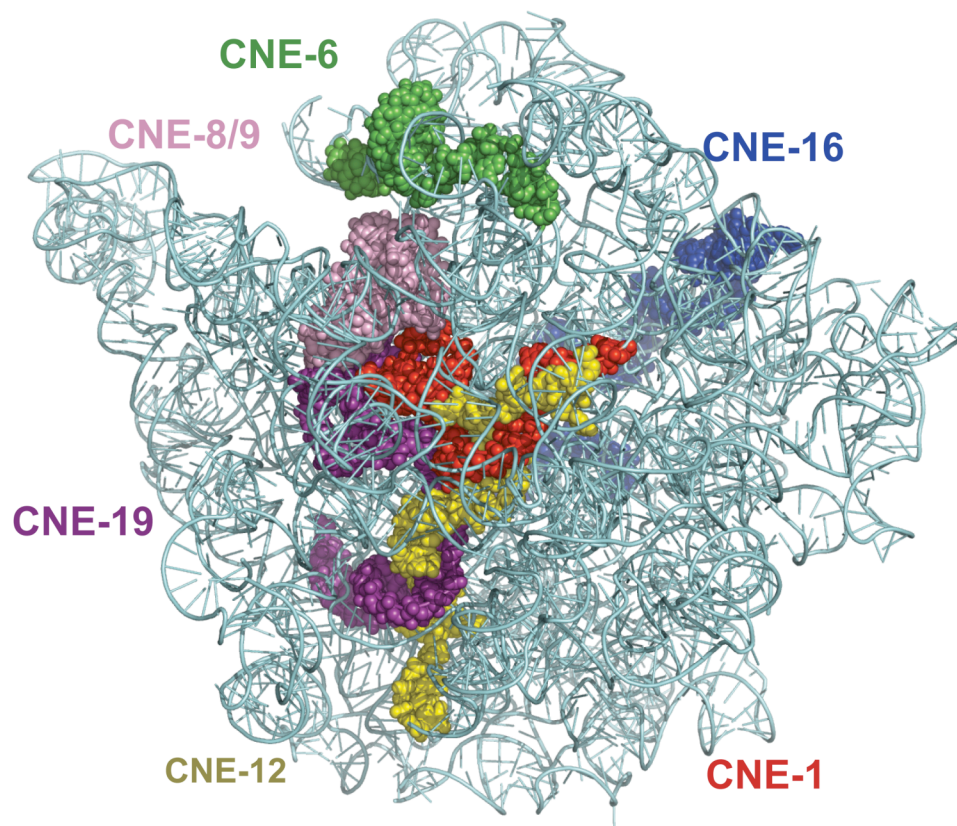


Figure 2. Homology modeling of CNE tertiary structure in the 50S subunit: Tertiary structure for the back view of the *H. marismortui* large subunit rRNA (1S72) with the CNE homologues shown as colored space filling atoms.

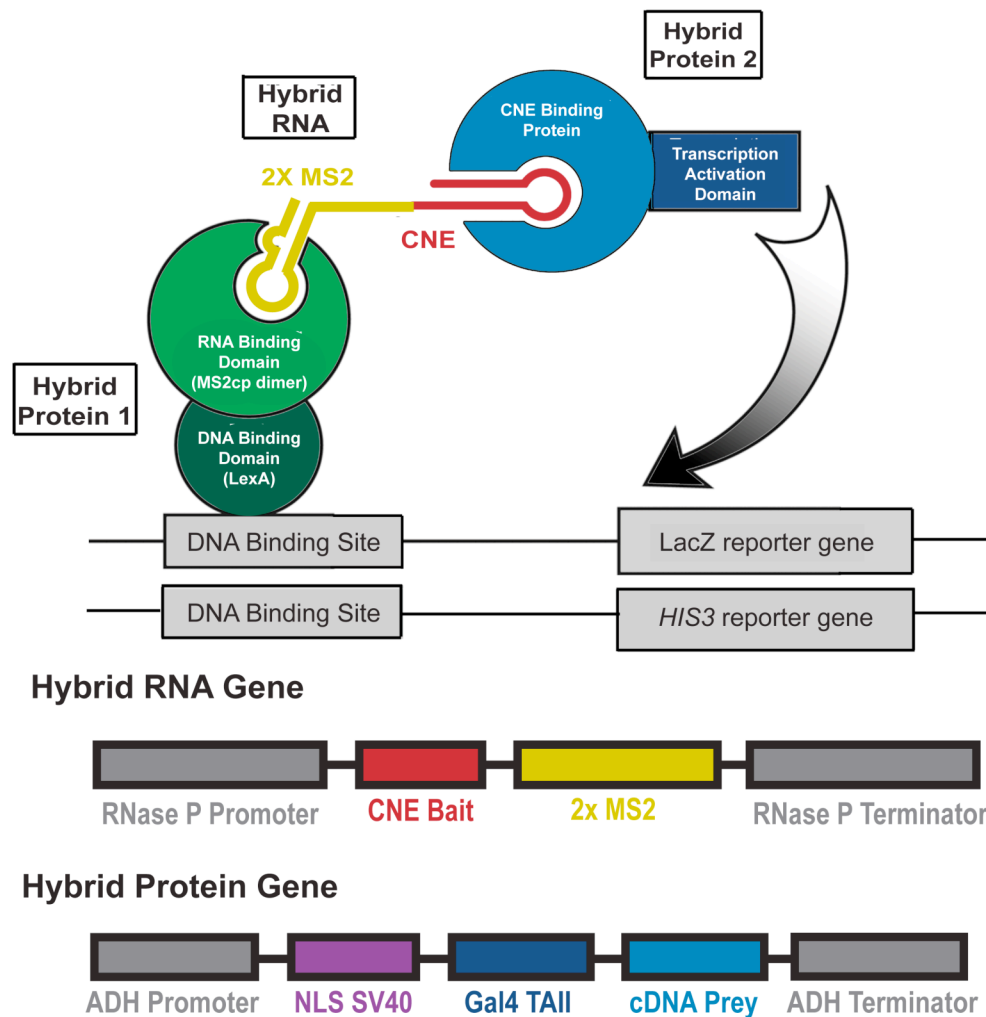


Figure 3. The Yeast Three-Hybrid Screen Identifies Proteins that Interact with an RNA Molecule of Interest: At the center of the yeast three-hybrid system is a hybrid RNA molecule containing the CNE of interest fused to two tandem MS2 sites. Hybrid Protein 1 has specific DNA and RNA binding activities (a fusion of LexA and the viral MS2 coat protein) that anchor the RNA to the promoter of two reporter genes: *LacZ* and *HIS3*. If the anchored CNE interacts with Hybrid Protein 2 (containing a candidate protein from the yeast genome fused to a transcription activation domain), reporter genes are transcribed. The hybrid RNA construct, containing the CNE fused to two tandem MS2 sequences, is transcribed from a plasmid-derived gene controlled by the RNase P promoter and terminator. Hybrid Protein 2 constructs, containing yeast cDNAs fused to a nuclear localization signal from SV40 (NLS SV40) and a Gal4 transcription activator II (Gal4 TAIL), are transcribed from plasmid-derived genes controlled by the ADH promoter and terminator (adapted from Marvin Wickens).

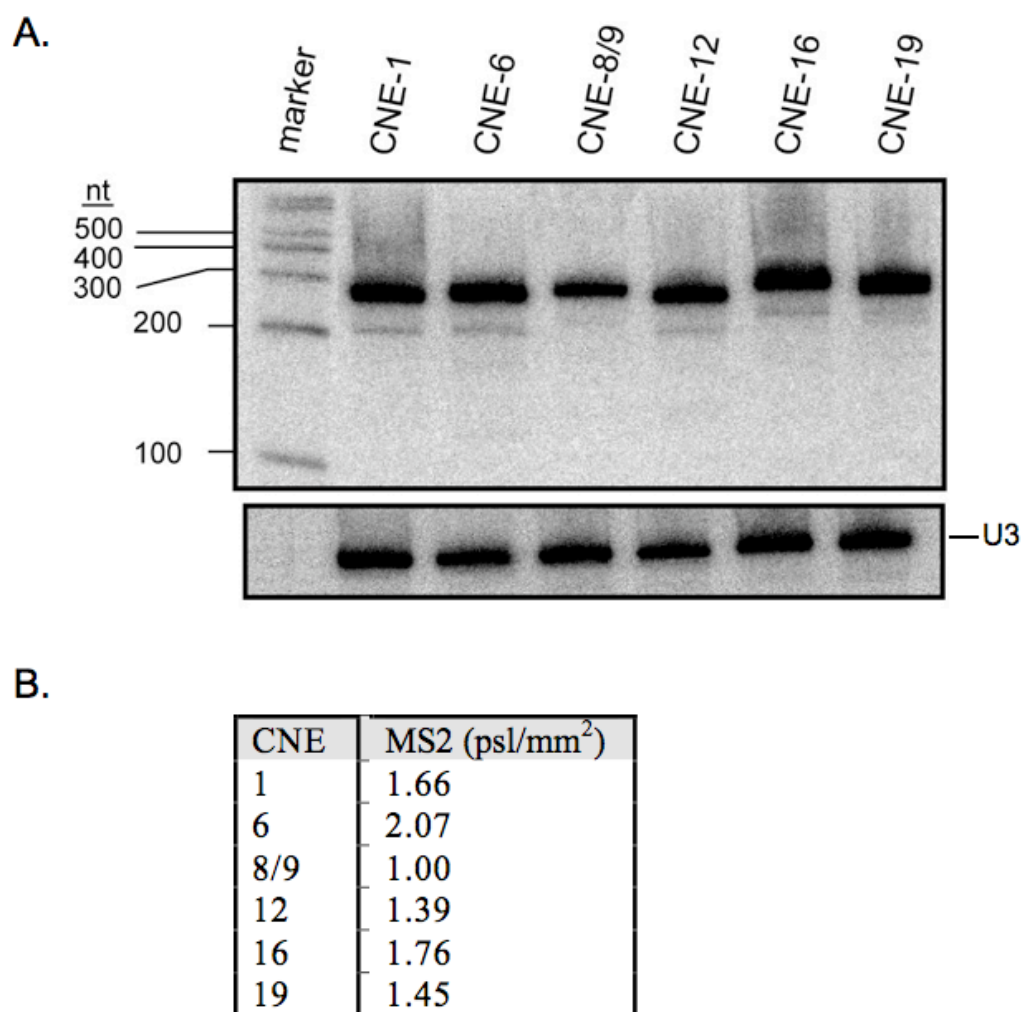


Figure 4. Expression of chimeric CNE:MS2 RNA baits: (A) A quantified Northern blot was conducted on cells expressing different RNA baits using a probe for the tandem MS2 sites. Results indicate that all six baits are being expressed within a two-fold range. The blot was then stripped and re-hybridized with a probe for U3 to confirm similar amounts of total RNA. 10 ug samples of RNA were run on an 8% PAGE 8M urea gel. (B) Hybridization of CNE probes was normalized relative to cell number and U3 signal.

A.

Prohibiting Gaps Between Nucleotides

H. sapien	9654	GGTAAAGCGAATGATTAGAGGT	9676
X. laevis	4909	GGTAAAGCGAATGATTAGAGGT	4930
D. melanogaster	4468	GGTAAAGCGAATGATTAGAGGC	4489
A. thaliana:	5201	GGTAAAGCCAATGATTAGAGGC	5222
S. cerevisiae:	4343	GGTAAAGCGAATGATTAGAGGT	4364
E. coli	4265	GGTAGAGC	4286
% Conservation of CNE-6: 9/22 = 41%			

Allowing Gaps Between Nucleotides

H. sapien	9654	GGTAAAGCGAATGATTAGAGGT	9676
X. laevis	4909	GGTAAAGCGAATGATTAGAGGT	4930
D. melanogaster	4468	GGTAAAGCGAATGATTAGAGGC	4489
A. thaliana:	5201	GGTAAAGCCAATGATTAGAGGC	5222
S. cerevisiae:	4343	GGTAAAGCGAATGATTAGAGGT	4364
E. coli	4265	GGTAGAGC- ACTGTTT --CGGC	4286
% Conservation of CNE-6: 14/22 = 64%			

B.

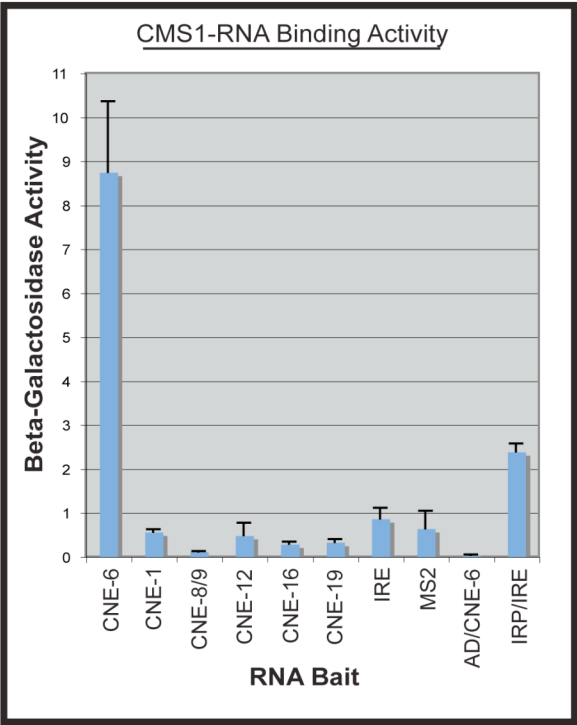


Figure 5. CMS1 (legend opposite)

Figure 5. CMS1 Interacts Specifically with CNE-6. (A) A nucleotide alignment of CNE-6 compares its universal conservation in eukaryotes to its partial conservation in the prokaryote *E. coli*. The degree of conservation of CNE-6 in *E. coli* is greater when gaps are allowed between nucleotides in the alignment. (B) A β -galactosidase assay of CMS1 paired with each CNE bait shows the levels of LacZ reporter gene expression. Negative controls pair CMS1 with both empty bait plasmid (MS2) and iron response element mRNA (IRE); CNE-6 was also paired with empty activation domain plasmid (AD/CNE-6). The positive control is the known interaction of IRE with iron response protein (IRP).

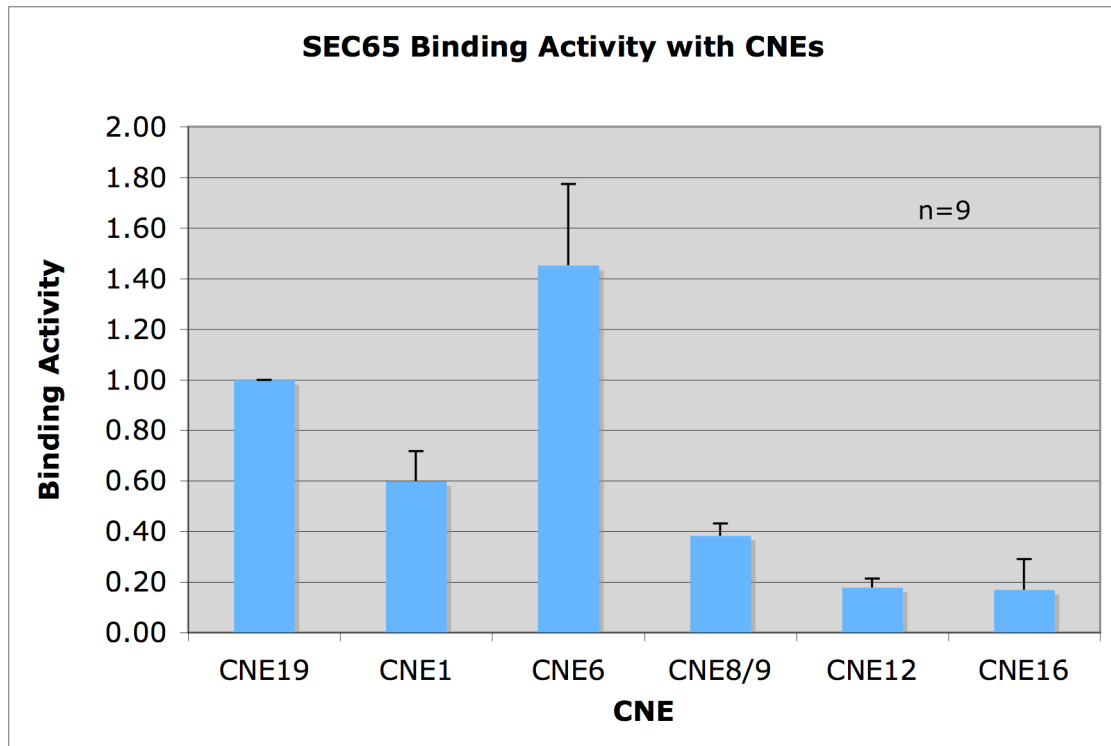


Figure 6. A β -galactosidase assay of SEC65 paired with each CNE bait shows the levels of LacZ reporter gene expression.

Table I. CNE nomenclature: manual identification vs. CORD analysis

Manual analysis	CORD analysis
CNE1	eCNE6
CNE6	-
CNE8/9	eCNE14
CNE12	eCNE19/20
CNE16	-
CNE19	eCNE30

Table II. Yeast three-hybrid screen conditions and results

	RNA Bait Length	3-AT Conc.	Total No. of Transformants	No. of His+ Transformants	No. of LacZ+ Transformants	No. Sequenced
CNE-1	52 nt	5 mM	9.6×10^4	19	18	16
CNE-6	48 nt	4 mM	8.6×10^5	92	81	30
CNE-8/9	63 nt	3 mM	1.1×10^5	0	-----	-----
CNE-12	52 nt	4 mM	4.9×10^5	174	166	54
CNE-16	75 nt	5 mM	1.9×10^6	67	64	64
CNE-19	54 nt	5 mM	4.6×10^4	320	300	57

Table III. Frequency and classification of proteins identified

	CNE1	CNE6	CNE8/9	CNE12	CNE16	CNE19
Ribosomal Proteins						
RPL9A	1	3	-	1	5	7
RPL13B	4	-	-	-	9	9
RPS9A	-	-	-	-	-	3
RPL13A	-	-	-	-	-	3
RPL41A	2	-	-	-	-	-
RPL11B	1	-	-	1	-	-
RPL41B	1	-	-	-	-	-
RPS1A	-	1	-	1	-	-
RPS20	-	1	-	-	-	-
RPS4A	-	-	-	1	-	-
RPL3	-	-	-	-	1	-
RPL21A	-	-	-	-	1	-
RPL28	-	-	-	-	1	-
RPS18A	-	-	-	1	1	-
RPL8B	-	-	-	-	-	1
RPS8B	-	-	-	-	-	1
RPS18B	-	-	-	-	-	1
Ribosome Biogenesis & rRNA Processing						
NOP6	4	6	-	5	21	12
LOC1	-	1	-	1	5	3
SAS10	1	-	-	-	1	1
CMS1	-	11	-	30	-	-
UTP23	-	-	-	-	3	-
IP13	-	-	-	-	1	-
NOP12	-	-	-	-	1	-
Protein Secretion/Degradation						
SEC65	-	1	-	8	-	6
KES1	-	1	-	-	-	-
PBA1	-	-	-	-	1	-
PRB1	-	-	-	-	-	1
SEC53	-	-	-	-	-	1
Bioenergetics						
ADH1	-	2	-	-	1	1
GPM1	-	-	-	-	1	1
TDH3	-	1	-	-	-	-
PNC1	-	-	-	1	-	-
HXK2	-	-	-	-	1	-
PGK1	-	-	-	-	-	1
Chaperones						
SSC1	-	1	-	-	-	-
SBA1	-	-	-	-	1	-
HSC82	-	-	-	-	-	1
SSB1	-	-	-	-	-	1
Other	-	-	-	-	-	-
RNY1 (Rnase)	1	-	-	-	5	-
IFH1 (polII transcription)	-	-	-	-	1	-
PRP40 (splicing)	-	-	-	-	1	-
MUD1 (splicing)	-	-	-	-	1	-
FPS1 (membrane transport)	-	-	-	-	-	1
FUN12 (translation initiation)	-	-	-	-	-	1
Unknown Function						
YNL050C	1	-	-	-	-	-
YMR315W	-	1	-	-	-	-
YBR096W	-	-	-	-	1	-
YOL053W	-	-	-	-	-	1