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THE DOMAINS OF THE CATALYTIC SUBUNIT OF THE EUKARYOTIC RNA DEGRADING EXOSOME, RRP44P, HAVE DISTINCT FUNCTIONS

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**THE DOMAINS OF THE CATALYTIC SUBUNIT OF THE EUKARYOTIC RNA
DEGRADING EXOSOME, RRP44P, HAVE DISTINCT FUNCTIONS**

by

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A

DISSERTATION

Presented to the Faculty of the University of Texas Health Science Center at Houston and
the University of Texas M.D. Anderson Cancer Center

Graduate School of Biomedical Sciences

In Partial Fulfillment of
the Requirements for
the Degree of

DOCTOR OF PHILOSOPHY

by

Daneen Lynn Schaeffer, B.S.

Houston, Texas

August 2010

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THE DOMAINS OF THE CATALYTIC SUBUNIT OF THE EUKARYOTIC RNA DEGRADING EXOSOME, RRP44P, HAVE DISTINCT FUNCTIONS

Publication No. _____

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The exosome is a 3' to 5' exoribonuclease complex that consists of ten essential subunits. In the cytoplasm, the exosome degrades mRNA in a general mRNA turnover pathway and in several mRNA surveillance pathways. In the nucleus, the exosome processes RNA precursors to form small, stable, mature RNA species, including rRNA, snRNA, and snoRNA. In addition to processing these RNAs, the nuclear exosome is also involved in degrading aberrantly processed forms of these RNAs, and others, including mRNA.

The 3' to 5' exoribonuclease activity of the exosome is contributed by the RNB domain of the only catalytically active subunit, Rrp44p, a member of the RNase II family of enzymes. In addition to the RNB domain, Rrp44p consists of three putative RNA binding domains and has an uncharacterized N-terminus, which includes a CR3 region and PIN domain. In an effort to characterize the cellular functions of the domains of Rrp44p, this study identified a second nuclease active site in the PIN domain. Specifically, the PIN domain exhibits endoribonuclease activity *in vitro* and is essential for exosome function. Further analysis of the nuclease activities of Rrp44p indicate a role for the exoribonuclease activity of Rrp44p in the cytoplasmic and nuclear exosome.

This work has also characterized the CR3 region of Rrp44p, a region that has not yet been characterized in any other protein. This region is needed for the majority, if not all, of the cytoplasmic exosome functions as well as for interaction with the exosome. The CR3 region, along with a histidine residue in the N-terminus of Rrp44p, may coordinate a zinc atom. Preliminary evidence supports a role for this coordination in exosome function. Further investigation, however, is needed to determine the molecular dependence of the exosome on the CR3 region of Rrp44p.

Despite its initial discovery thirteen years ago, the essential function of Rrp44p, and the exosome, is not yet known. The studies presented here, however, indicate that the essential function of Rrp44p and the exosome is in the nucleus and depends on the nuclease activities of Rrp44p.

TABLE OF CONTENTS

	Page No.
Approval Sheet	i
Title Page	ii
Acknowledgements	iii
Abstract	v
Table of Contents	vi
List of Figures	ix
List of Tables	xii
Chapter 1: Introduction	
INTRODUCTION AND BACKGROUND	1
Eukaryotic RNA metabolism and surveillance	2
The eukaryotic exosome	3
Eukaryotic exosome cofactors	8
Functions of the cytoplasmic exosome	10
Functions of the nuclear exosome	18
SIGNIFICANCE OF THE STUDY	23
Chapter 2: Materials and Methods	25
STRAINS AND PLASMIDS	26
YEAST GROWTH ASSAYS	32
Complementation growth assays	32
Synthetic lethality growth assays	33
<i>His3</i> -nonstop growth assays	33
SOLUBLE CELL LYSATE PREPARATION USING GLASS BEAD LYSIS	34
<i>IN VIVO</i> TANDEM AFFINITY PURIFICATION (TAP)	34
SDS-PAGE AND WESTERN BLOT ANALYSIS	34
PLASMID RESCUE FROM YEAST	34
OVEREXPRESSION AND PURIFICATION OF RECOMBINANT RRP44P	35
RNase ASSAYS	36
DETERMINING THE PROCESSING OF THE 5.8S rRNA AND THE	
DEGRADATION OF THE 5' ETS OF THE PRE-rRNA PRECURSOR	36
STABILITY OF THE <i>PGK1pG</i> -NONSTOP mRNA	36
RNA ISOLATION	36

NORTHERN BLOT ANALYSIS AND QUANTITATION OF GENE EXPRESSION	37
Chapter 3: The PIN domain of Rrp44p has endoribonuclease activity and is essential for exosome function	38
INTRODUCTION	39
RESULTS	41
DISCUSSION	55
Chapter 4: The exoribonuclease activity of Rrp44p is required for exosome- mediated mRNA decay	58
INTRODUCTION	59
RESULTS	61
DISCUSSION	80
Chapter 5: The CR3 region, a putative zinc binding site of Rrp44p, is important for exosome activity	82
INTRODUCTION	83
RESULTS	87
DISCUSSION	111
Chapter 6: The far N-terminus of Rrp44p is important in exosome function	115
INTRODUCTION	116
RESULTS	118
DISCUSSION	142
Chapter 7: Conclusions and Perspectives	145
SUMMARY AND FUTURE DIRECTIONS	146
The endoribonucleolytic PIN domain of Rrp44p is required for cell survival	146
The exoribonuclease activity of Rrp44p is needed for a functional cytoplasmic and nuclear exosome	146
Determining the RNA substrates of the endo- and exonuclease activities of Rrp44p	147
The CR3 region of Rrp44p may be a putative zinc-binding domain	154
The CR3 region of Rrp44p may be needed for 3' to 5' mRNA degradation because this region may interact with a protein involved in 3' to 5' mRNA decay	158
Nonstop mRNA degradation requires Rrp44p	159

The far N-terminus of Rrp44p is needed for viability and is only one of several regions of Rrp44p that is required for interaction with the exosome	162
Determining the function of the three conserved residues of the far N-terminus of Rrp44p	164
Determining the essential function of Rrp44p	165
CONCLUDING REMARKS	167
Appendix: The essential function of Rrp44p cannot be carried out by other 3' to 5'	
exoribonucleases	168
INTRODUCTION	169
RESULTS	171
DISCUSSION	180
Bibliography	183
Vita	195

LIST OF FIGURES

Page No.

Chapter 1: Introduction

1.1	Schematic representation of the eukaryotic exosome and domain organization of Rrp44p	5
1.2	Schematic representation of the two cytoplasmic mRNA degradation pathways in eukaryotes	11
1.3	Schematic representation of the cytoplasmic nonstop mRNA degradation pathway in eukaryotes	14
1.4	Schematic representation of the cytoplasmic no-go decay pathway in eukaryotes	17
1.5	Nuclear 35S pre-rRNA processing in <i>S. cerevisiae</i>	20

Chapter 3: The PIN domain of Rrp44p has endoribonuclease activity and is essential for exosome function.

3.1	The PIN domain of Rrp44p is needed for viability	43
3.2	At least one nuclease activity of Rrp44p is needed for viability	45
3.3	The exonuclease activity of Rrp44p is needed for both the RNA processing and RNA degradation activities of the nuclear exosome	50
3.4	The exonuclease activity of Rrp44p may have some overlapping functions with the nuclear exosome cofactor, Rrp6p	53

Chapter 4: The exoribonuclease activity of Rrp44p is required for exosome-mediated mRNA decay.

4.1	The exonuclease activity of Rrp44p is needed for exosome-mediated 3' to 5' mRNA degradation	63
4.2	The exonuclease activity of Rrp44p is not needed for nonstop mRNA degradation	66
4.3	Neither of the nuclease activities of Rrp44p are needed for nonstop mRNA degradation	68
4.4	Schematic representation of ribosome disassembly in nonstop mRNA degradation	71
4.5	The endonuclease activity of Rrp44p is not redundant with Ski7p in nonstop mRNA degradation	74

4.6	The Rrp44p endonuclease is not responsible for endonucleolytic cleavage in no-go decay	76
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Chapter 5: The CR3 region, a putative zinc binding region of Rrp44p, is important for exosome activity.

5.1	The three cysteines of the CR3 region and histidine 184 (H184) of Rrp44p have the proper geometry for the coordination of a zinc atom	84
5.2	Mutating the three conserved cysteine residues of Rrp44p results in a slow growth phenotype	88
5.3	The CR3 region of Rrp44p is needed for interaction with the exosome	90
5.4	The CR3 region of Rrp44p is needed for endonuclease activity <i>in vivo</i> and <i>in vitro</i>	93
5.5	The CR3 region of Rrp44p genetically interacts with the exonucleolytic RNB domain of Rrp44p	98
5.6	The CR3 region of Rrp44p genetically interacts with the nuclear exosome cofactor, Rrp6p	101
5.7	The CR3 region of Rrp44p is needed for nonstop mRNA degradation	104
5.8	The disulfide bond of the CR3 region of Rrp44p is not needed for optimal cell growth	107
5.9	The disulfide bond of the CR3 region is not needed for nonstop mRNA degradation	109

Chapter 6: The far N-terminus of Rrp44p is important in exosome function.

6.1	Residues 34 to 46 of Rrp44p are needed for viability	119
6.2	The N-terminus of Rrp44p is needed for interaction with the exosome	123
6.3	Several residues in the N-terminus of Rrp44p are needed for an essential function that also requires the C-terminus of Rrp44p	126
6.4	The first 33 residues of Rrp44p are not needed for exosome-mediated 3' to 5' mRNA degradation	130
6.5	The first 33 residues of Rrp44p do not genetically interact with the nuclear exosome cofactor, Rrp6p	132

6.6	The first 33 residues of Rrp44p are not needed for nonstop mRNA degradation	135
6.7	The first 33 residues of Rrp44p are not needed for the RNA processing or RNA degradation activities of the nuclear exosome	137
6.8	At least one putative nuclear localization signals (NLSs) of Rrp44p is needed for viability	140

Chapter 7: Conclusions and Perspectives

7.1	Schematic representation of the yeast killer assay	149
7.2	Schematic representation of the heterozygote diploid-based synthetic lethality analysis with microarrays (dSLAM) technique	155

Appendix: The essential function of Rrp44p cannot be carried out by other

3' to 5' exoribonucleases.

A.1	<i>A. thaliana</i> Rrp41p does not suppress a yeast <i>rrp44</i> deletion	172
A.2	<i>E. coli</i> RNase II or RNase R does not suppress a yeast <i>rrp44</i> deletion	175
A.2	<i>S. cerevisiae</i> Rrp6p does not suppress an <i>rrp44</i> deletion	178

LIST OF TABLES

	Page No.
Table 1.1 Eukaryotic exosome components and cofactors	4
Table 2.1 Yeast strains used	27
Table 2.2 Plasmids used	28
Table 2.3 Oligonucleotides used	30
Table 7.1 Strains and probes needed to test the role of the Rrp44p nucleases in nuclear RNA metabolism	152

Chapter 1: Introduction

INTRODUCTION AND BACKGROUND

Eukaryotic RNA metabolism and surveillance

RNA metabolism and function in eukaryotes requires a transition of RNAs between different cellular compartments and protein complexes. For example, mRNA metabolism involves transcription, nuclear processing, nuclear-cytoplasmic transport, translation, and ultimately cytoplasmic degradation. During these processes, mRNA associates with a variety of complexes that are needed for mRNA biogenesis, function, and degradation. In addition, these complexes promote the association and disassociation of cofactors that regulate these steps of mRNA metabolism and, thus, dictate the function and half-life of an mRNA (Dreyfuss et al., 2002; Moore, 2005).

Similarly, the small, stable RNAs of the nucleus, including small nuclear (sn)RNA, small nucleolar (sno)RNA, rRNA, and tRNA, are subject to the same mechanisms of metabolism. In the nucleus, however, the biogenesis of these RNAs, as well as the protein complexes they encounter, differ from that of mRNAs. These nuclear complexes are not only responsible for the synthesis of nuclear RNAs, but they also serve as a checkpoint to ensure RNAs are functional prior to their export into the cytoplasm (Nazar, 2004; Wolin and Matera, 1999).

Aberrant RNAs arise through mistakes in gene expression, and not surprisingly, there are many ways in which gene expression can be faulty. For example, aberrant mRNAs can arise from mutations in the gene, which can lead to transcripts that lack termination codons or contain premature termination codons. Typically these transcripts produce truncated proteins and/or proteins that have a dominant-negative effect. Aberrant RNAs can also arise due to a defect in ribonucleoprotein (RNP) assembly or nuclear pre-RNA processing, from the inherent error rate of transcription, or through transcription of intergenic regions. To prevent the harmful effects of these aberrant RNAs, the cell has adopted specialized RNA degradation pathways, termed RNA surveillance. Interestingly, the same cellular machinery can degrade normal and aberrant RNAs. Therefore, it is important to understand the regulation of RNA turnover, how normal and aberrant RNAs are differentiated, and what factors are involved.

One protein complex that is involved in both cytoplasmic and nuclear RNA metabolism is the eukaryotic exosome. This complex of proteins associates with a variety of cofactors to promote the processing and degradation of RNAs, and also acts as a quality control mechanism to rapidly degrade aberrant RNAs.

The eukaryotic exosome

The eukaryotic exosome consists of 10 essential subunits. These subunits were initially identified genetically in the budding yeast *Saccharomyces cerevisiae* in independent screens for mutants that were defective in rRNA maturation (Mitchell et al., 1996) and cytoplasmic mRNA transport, mutants that accumulated poly(A) RNA in the nucleus (Kadowaki et al., 1994), and for mutants that expressed viral toxins (Ridley et al., 1984). Although these studies identified the exosome subunits for their unique mutant phenotypes, further genetic and biochemical analysis was required to show that these subunits are a part of a large complex that was subsequently named the exosome (Mitchell et al., 1997). Further analysis indicated that the exosome is highly conserved in eukaryotes. Additionally, the 10 subunit exosome was found to be present in both the cytoplasm and the nucleus (Allmang et al., 1999b).

The composition and structure of the eukaryotic exosome

Sequence analysis of several exosome subunits revealed similarities to bacterial RNase PH, PNPase, RNase II, and RNase R. Specifically, six of the subunits (Rrp41p, Rrp42p, Rrp43p, Rrp45p, Rrp46p, and Mtr3p; Table 1.1) assemble into three distinct heterodimers to form a ring, or channel structure (Figure 1.1), that structurally resembles the PH ring in bacterial PNPase and the archaeal exosome (Buttner et al., 2005; Liu et al., 2006; Lorentzen and Conti, 2005). The subunits that constitute the PH ring were named for their homology to RNase PH (Zuo and Deutscher, 2001). RNase PH is a phosphorolytic 3' to 5' exoribonuclease, in that it uses an inorganic phosphate to cleave RNA from the 3'-end. Specifically, RNase PH processes, or trims, bacterial tRNA precursors to form mature tRNA species (Wen et al., 2005). Although these subunits are catalytically active in PNPase and the archaeal exosome (Buttner et al., 2005; Lorentzen and Conti, 2005; Navarro et al., 2008; Symmons et al., 2000), the six PH subunits of the yeast and human exosome are catalytically inactive due to mutations the RNase PH-like active sites (Dziembowski et al., 2007; Liu et al., 2006). This suggests that the ring of the exosome is not the catalytic center of the exosome, but rather acts as a protein scaffold that may be needed for the recruitment of exosome cofactors. This, however, is not the case in all eukaryotes. Interestingly, *Arabidopsis thaliana* Rrp41p has retained the phosphorolytic 3' to 5' exonuclease activity of RNase PH (Chekanova et al., 2000). It is not yet known how the enzymatic activity of this subunit contributes to the overall activity of the exosome in plants.

The PH ring of the eukaryotic exosome is not stable on its own *in vitro*. Specifically, formation of the ring requires three additional proteins (Rrp4p, Rrp40p, and Csl4p; Table 1.1) that bind to one side of the PH ring, forming a cap (Figure 1.1; Liu et al., 2006). These subunits

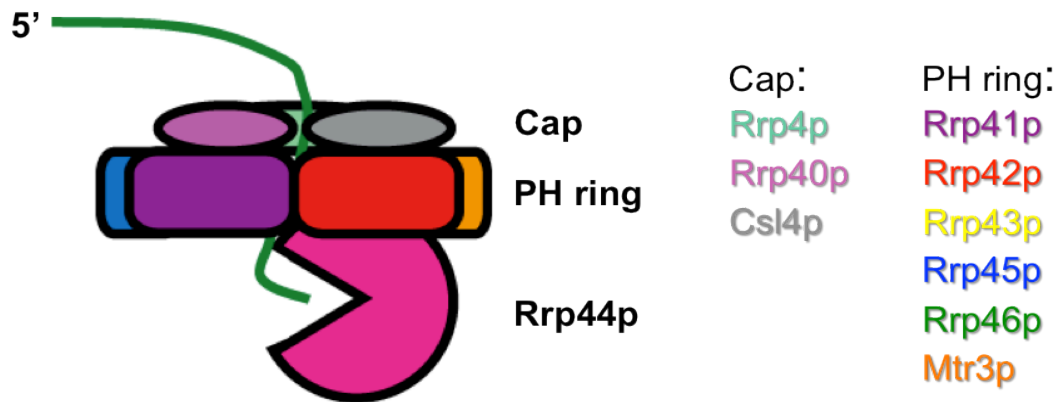
Table 1.1: Eukaryotic Exosome Components and Cofactors ¹						
Protein	Gene	Activity ²	Motifs/ Homologs	Deletion Phenotype	Human Homolog	Comments
Rrp4p	<i>YHR069c</i>		S1 and KH RBD	Inviable	hRrp4	
Csl4p/Ski4p	<i>YNL232w</i>		S1 RBD	Inviable	hCsl4	
Rrp40p	<i>YOL142w</i>		S1 and KH RBD	Inviable	hRrp40	
Rrp41p/Ski6p/Ecm20p	<i>YGR195w</i>		RNase PH	Inviable	hRrp41	
Rrp42p	<i>YDL111c</i>		RNase PH	Inviable	hRrp42	
Rrp43p	<i>YCR035c</i>		RNase PH	Inviable	Oip2	
Rrp44p/Dis3p	<i>YOL021c</i>	Hydrolytic, 3' to 5' exoribonuclease	RNase II/RNase R	Inviable	hDis3	
Rrp45p	<i>YDR280w</i>		RNase PH	Inviable	PM-Scl75	
Rrp46p	<i>YGR095c</i>		RNase PH	Inviable	hRrp46	
Mtr3p	<i>YGR158c</i>		RNase PH	Inviable		
Cytoplasmic Exosome Cofactors						
Ski2p	<i>YLR398c</i>	(ATP-dependent RNA helicase)	DEVH box	Viable	<i>SKIIV2L</i>	Ski complex
Ski3p/Ski5p	<i>YPR189w</i>		TPR repeat	Viable	<i>KIAA0372</i>	Ski complex
Ski7p	<i>YOR076c</i>	(GTPase)	GTP-binding	Viable	eRFS	Homologous to Hbs1p
Ski8p/Rec103p	<i>YGL213c</i>		WD repeat	Viable		Ski complex
Selected Nuclear Exosome Cofactors						
Rrp6p	<i>YOR001w</i>	Hydrolytic, 3' to 5' exoribonuclease	RNase D	<i>ts</i> lethal	PM-Scl100	
Rrp47p/Lrp1p	<i>YHR081w</i>			Viable	C1D	Synthetically lethal with deletion of <i>MPP6</i>
Mpp6p	<i>YNR024w</i>		Mphosph6	Viable	Mphosph6	Synthetically lethal with deletion of <i>RRP47</i>
<i>TRAMP Complex</i>						
Pap2p (Trf4p) or Trf5p	<i>YOL115w</i> or <i>YNL299w</i>	Poly(A) polymerase	Poly(A) polymerase	Viable	GLD-2	Synthetically lethal with deletion of <i>TRF5</i> or <i>TRF4</i>
Air1p or Air2p	<i>YIL079c</i> or <i>YDL175c</i>		Zinc knuckle domain	Viable	GLD-3	
Mtr4p/Dob1p	<i>YJL050w</i>	ATP-dependent RNA helicase	DEX/H box	Inviable	<i>SKIIV2L2</i>	

¹Activities shown in parentheses are predicted.

A.



B.



C.

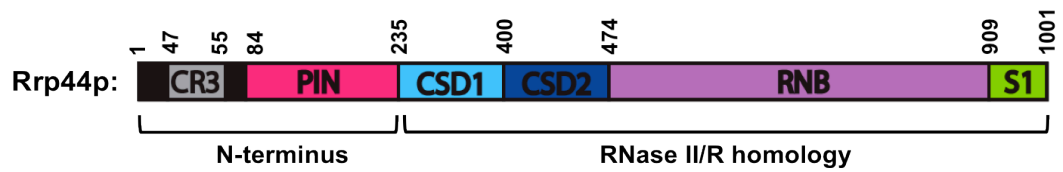


Figure 1.1: Schematic representation of the eukaryotic exosome and domain organization of Rrp44p.

Figure 1.1: Schematic representation of the eukaryotic exosome and domain organization of Rrp44p. A, A top view, looking down through the exosome. **B,** A side view of the exosome.

The exosome contains a PH ring of six proteins that have RNase PH domains. Assembly and/or stability of the PH ring require three additional proteins that form a cap on top of the ring.

Between the cap proteins, there are five putative RNA binding domains. The exoribonuclease activity of the exosome is provided by a tenth subunit, Rrp44p, that associates with the bottom of the PH ring. RNA substrates are thought to associate with the cap proteins, pass through the central channel, and are degraded by Rrp44p after they emerge from the bottom of the ring. **C,** Rrp44p consists of six putative regions/domains. In the N-terminus, is a CR3 region, which consists of at least three conserved cysteine residues, and a PIN domain, which is a putative nuclease domain. Rrp44p has homology to two bacterial proteins, RNase II and RNase R. In the RNase II/R homology region, are three putative RNA binding domains, including two consecutive Cold Shock Domains (CSD1 and CSD2) and an S1 domain. Separating the putative RNA binding domains is the 3' to 5' exoribonucleolytic RNB domain. This domain is solely responsible for the 3' to 5' exoribonuclease activity of the exosome.

are not homologous to any known nucleases, and instead contain RNA binding domains of either the KH (K-homology domain; Grishin, 2001) or S1 (ribosomal protein S1) type (Suryanarayana and Subramanian, 1984). Therefore, in addition to stabilizing the exosome, these cap proteins are thought to bind the RNA substrates of the exosome.

The catalytic subunit of the eukaryotic exosome, Rrp44p

The tenth subunit of the exosome, Rrp44p, is the only catalytically active subunit of the yeast (Dziembowski et al., 2007; Liu et al., 2006) and *Drosophila* exosome (Mamolen and Andrulis, 2009) with its 3' to 5' exoribonuclease activity contributed by the RNB domain (Dziembowski et al., 2007). This is also likely the case in humans, where the other nine exosome subunits are catalytically inactive (Liu et al., 2006). The RNB domain is also found in two bacterial enzymes that are homologous to Rrp44p, RNase II and RNase R (Table 1.1). These enzymes are hydrolytic 3' to 5' exoribonucleases, in that they use a water molecule to degrade mRNA from the 3'-end (Cannistraro and Kennell, 1994; Cheng and Deutscher, 2002). In addition to sharing the RNB domain, these proteins also share three putative RNA binding domains, two consecutive cold shock domains (CSDs) and a C-terminal S1 domain (Figure 1.1). In all three proteins, these domains are positioned on one side of the RNB domain, with ssRNA threaded through the putative RNA binding domains such that the 3'-end of the RNA rests in the catalytic site of the RNB domain (Bonneau et al., 2009; Frazao et al., 2006; Lorentzen et al., 2008; Zuo et al., 2006). Biochemically, Rrp44p is more similar to RNase R. Unlike RNase II, RNase R and Rrp44p are able to degrade structured RNAs and double stranded RNAs that contain a single stranded 3' overhang (Dziembowski et al., 2007; Vincent and Deutscher, 2006).

While the RNase II/R homologous region of Rrp44p constitutes the middle- and C-terminal portion of the protein, the N-terminus of Rrp44p is not well characterized. This is due, in part, to a lack of functional and structural data on this region. Despite the large number of recent studies on the catalytic subunit of the exosome, these reports have focused only on the nuclease activities of the Rrp44p (Dziembowski et al., 2007; Lorentzen et al., 2008; Mamolen and Andrulis, 2009; Schneider et al., 2007) or on the structure of the exosome (Bonneau et al., 2009; Liu et al., 2006; Lorentzen et al., 2008; Wang et al., 2007). Recently, crystal structures of Rrp44p were published (Bonneau et al., 2009; Lorentzen et al., 2008), however, both structures lack the first 33 residues (Bonneau et al., 2009). Sequence analysis shows that the far N-terminus of Rrp44p, which includes residues 1-46, contains a linker region of a conserved stretch of residues, residues 34-46. This region is then followed by a CR3 region, which consists of three conserved cysteine residues (C47, C52, and C55; Figure 1.1). Currently, no information exists on

the CR3 region in Rrp44p or in any other protein, and because the crystal structure of Rrp44p lacks the first 33 residues, it is not yet known whether the CR3 region consists of more than the three cysteine residues or if this region has a unique fold.

Sequence analysis of the N-terminus of Rrp44p also revealed the presence of a PIN domain (Figure 1.1). PIN domains, named for their homology to a bacterial pilus biosynthetic protein, are found in all three kingdoms of life. These domains are approximately 100 residues in length and are characterized by a motif of four conserved acidic residues (Clissold and Ponting, 2000; Makarova et al., 1999). Similar acidic residue conservation occurs in enzymes that ligate divalent cations, including nucleases. In support of this, several PIN domain-containing proteins exhibit nuclease activity *in vitro* and *in vivo* (Arcus et al., 2004; Bleichert et al., 2006; Daines et al., 2007; Fatica et al., 2004; Glavan et al., 2006; Levin et al., 2004). Because the PIN domain of Rrp44p has these four conserved acidic residues, this domain may also have nuclease activity. Despite the little information that is available on the N-terminus of Rrp44p, sequence analysis shows a possible link between the CR3 region and the PIN domain, as CR3 regions are present in most eukaryotic homologs of Rrp44p that have a PIN domain.

The association of Rrp44p with the exosome is controversial and seems to depend on the organism. For example, in yeast and *Drosophila*, Rrp44p only loosely associates with the exosome (Allmang et al., 1999b; Graham et al., 2009). In contrast, although homologs of Rrp44p are present in humans, *Arabidopsis*, and trypanosomes, Rrp44p does not associate with the exosome in these organisms (Allmang et al., 1999b; Chekanova et al., 2002; Estevez et al., 2001). This lack of interaction, however, could be an artifact of the biochemical assays used to detect exosome interaction. Regardless of its interaction with the exosome, Rrp44p is clearly important for the exosome in a variety of eukaryotes (Mamolen and Andrulis, 2009; Shiomi et al., 1998).

Eukaryotic exosome cofactors

The cellular localization of the exosome dictates its association with exosome cofactors. These cofactors play a dual role in regulating exosome activity. First, because the intrinsic exonucleolytic activity of the exosome is low, exosome cofactors are required to stimulate the catalytic activity of the exosome. Additionally, exosome cofactors are needed to direct the exosome to specific RNA substrates.

Cytoplasmic exosome cofactors

In the cytoplasm, the exosome is solely involved in mRNA degradation (see below). Here the exosome associates with, and is activated, by the Ski complex and Ski7p (Table 1.1). The Ski complex consists of Ski2p, Ski3p, and Ski8p. Ski2p is a putative DEVH-box RNA helicase (Widner and Wickner, 1993) that may function to remove mRNA-bound proteins or secondary structures from mRNAs. Ski7p is a small GTPase that is homologous to eEF1A and eRF3 (Benard et al., 1999) and interacts with both the Ski complex and the exosome (Araki et al., 2001). Because Ski7p can interact with both complexes, it may bridge the interactions between the Ski complex and the exosome. Additionally, Ski7p is thought to aid in the recognition of specific RNA substrates, causing recruitment of the exosome, and ultimately leading to the 3' to 5' decay of these mRNAs (Frischmeyer et al., 2002; Mitchell and Tollervey, 2003; van Hoof et al., 2002). Thus far, it is not yet known how the Ski complex and Ski7 activate the exosome.

Nuclear exosome cofactors

In contrast to the cytoplasmic exosome, the nuclear exosome is involved in RNA processing and RNA degradation. In RNA processing, an RNA precursor is trimmed to form a mature RNA, however, in RNA degradation, an RNA substrate is completely degraded to nucleotide monophosphates. The nuclear exosome processes and degrades “normal” RNAs, however, the major role of the nuclear exosome is its degradation of aberrantly processed RNAs (see below). The association of nuclear exosome cofactors determines whether the nuclear exosome processes or degrades a specific RNA substrate.

The nuclear exosome associates with at least three proteins, Rrp6p, Rrp47p, and Mpp6p, and at least one protein complex called TRAMP (Table 1.1). Rrp6p is the only other catalytically active nuclease subunit that associates with the exosome. Specifically, Rrp6p is a hydrolytic 3' to 5' exoribonuclease that is needed for most, if not all, of the nuclear exosome functions (Allmang et al., 1999a; Briggs et al., 1998; Butler, 2002; Fatica et al., 2000; van Hoof et al., 2000a). Rrp47p also associates with the nuclear exosome and is required for nuclear RNA processing, but not for nuclear RNA surveillance (Mitchell et al., 2003). Mpp6p is an RNA binding protein that, like Rrp47p, is only needed for specific nuclear exosome functions. Specifically, Mpp6p is needed for the recognition of specific RNA elements in nuclear RNA surveillance (Milligan et al., 2008; Schilders et al., 2005). Lastly, the nuclear exosome associates with, and is activated by, a complex of proteins called TRAMP (for Trf4/5-Air1/2-Mtr4). This complex constitutes a non-canonical poly(A) polymerase (Trf4p or Trf5), a zinc knuckle protein (Air1p or Air2p), and

a DEVH-box RNA helicase (Mtr4p). The TRAMP complex is solely involved in nuclear RNA surveillance, whereby Air1/2 is thought to bind an RNA substrate, which is then polyadenylated by Trf4/5, and subsequently degraded by the nuclear exosome (Davis and Ares, 2006; Eggecioglu et al., 2006; Houseley and Tollervey, 2006; LaCava et al., 2005; Vanacova et al., 2005; Wyers et al., 2005). Mtr4p is thought to unwind any secondary structures and also directly associates with the exosome (Milligan et al., 2008; Peng et al., 2003; Schilders et al., 2007), providing a link between the TRAMP complex and the exosome.

One similarity between the cytoplasmic and nuclear exosome is that both associate with a helicase. This association may facilitate the degradation of structured RNA substrates or protein-bound RNAs. Not surprisingly, a similar association is seen in the RNA degradation machine in *E. coli*, termed the degradosome. Here, the RhlB helicase is a key component of the at least four-member complex (Py et al., 1996). Therefore, the association of additional enzymatic activities with RNA degradation systems seems to be a common theme in nature.

Not surprisingly, exosome cofactors also impart differences between the cytoplasmic and nuclear exosome. Specifically, the cofactors influence how polyadenylation ultimately affects the fate of an RNA. In the cytoplasm, polyadenylation stabilizes transcripts, making them resistant to nucleolytic cleavage, and more amenable to translation. In contrast, polyadenylation in the nucleus is similar to that in bacteria, where polyadenylation triggers rapid degradation (Carpousis, 2007; Dreyfus and Regnier, 2002). This difference is one of many between the cytoplasmic and nuclear exosome. In fact, the cofactors exist in the cytoplasm and nucleus to direct the exosome to different RNA substrates and help tailor the catalytic activity of the exosome to these distinct RNAs.

Functions of the cytoplasmic exosome

General 3' to 5' mRNA degradation

Two general pathways of eukaryotic mRNA degradation have been identified using *S. cerevisiae* as a model system (Figure 1.2; Decker and Parker, 1993; Muhlrad and Parker, 1994). The initiating and rate-limiting step in both of the degradation pathways is the shortening of the poly(A) tail, in a process termed deadenylation. In yeast, the initial shortening of the poly(A) tail is catalyzed by the Pan2p/Pan3p complex, whereas the majority of deadenylation is performed by the Ccr4p/NOT complex (Muhlrad and Parker, 1992; Tucker et al., 2001). Removal of the poly(A) tail triggers cytoplasmic 5' to 3' decay in yeast. In this pathway, the 5' cap is removed by the decapping complex, which consists of Dcp1p and Dcp2p. This exposes the 5'-end of an mRNA to the 5' to 3' exoribonuclease, Xrn1p, which is responsible for degrading an mRNA

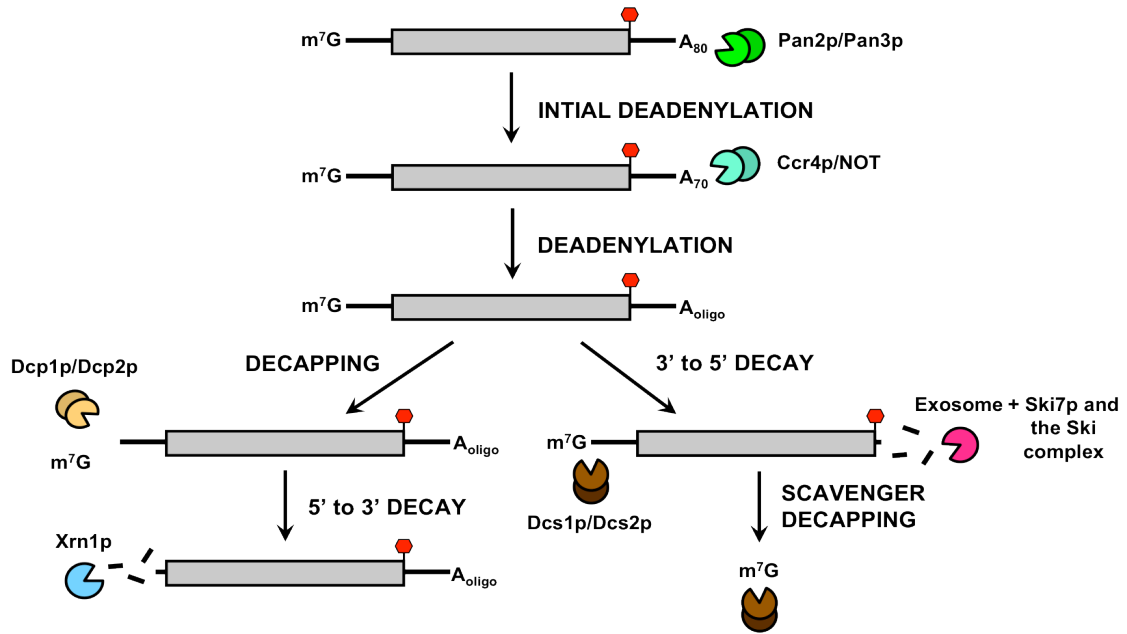


Figure 1.2: Schematic representation of the two cytoplasmic mRNA degradation pathways in eukaryotes.

Figure 1.2: Schematic representation of the two cytoplasmic mRNA degradation pathways in eukaryotes. Two general pathways exist in the cytoplasm to degrade eukaryotic transcripts. Both pathways require removal of the poly(A) tail (deadenylation) as the first step. In one pathway, deadenylation is followed by removal of the 5' cap (decapping) and degradation of the body of the mRNA by the 5' to 3' exoribonuclease, Xrn1p. In the second pathway, the body of the mRNA is degraded by the 3' to 5' exoribonucleolytic complex, the exosome.

from its 5'-end (Decker and Parker, 1993; Hsu and Stevens, 1993; Muhlrads et al., 1994). Deadenylation can also trigger the degradation of an mRNA from its 3'-end, a process catalyzed by the cytoplasmic exosome and the cytoplasmic exosome cofactors, Ski7p and the Ski complex (Anderson and Parker, 1998; Mukherjee et al., 2002; Rodgers et al., 2002). Following decay of the body of the transcript, the scavenger decapping complex, which consists of Dcs1p and Dcs2p, removes the 5' cap from the remaining oligonucleotide (Wang and Kiledjian, 2001). The enzymes involved in these decay pathways are conserved in other eukaryotes and inactivation of both mRNA decay pathways results in loss of viability (Anderson and Parker, 1998; Johnson and Kolodner, 1995). This conservation and the requirement for cellular mRNA decay underscore the importance of eukaryotic mRNA turnover.

Cytoplasmic degradation of aberrant transcripts

The cytoplasmic exosome is also involved in mRNA surveillance pathways, whereby exosome cofactors and adaptor proteins distinguish aberrant mRNAs from normal mRNAs, and ultimately direct the aberrant transcripts to the exosome for rapid degradation. Specifically, the cytoplasmic exosome degrades transcripts that lack termination codons (Frischmeyer et al., 2002; van Hoof et al., 2002) or poly(A) tails (Meaux and van Hoof, 2006), and transcripts that have premature termination codons (Mitchell and Tollervey, 2003; Takahashi et al., 2003) or cause stalls in translation elongation (Doma and Parker, 2006). Not surprisingly, the rate-limiting step of deadenylation is bypassed in these mRNA surveillance pathways to rapidly rid the cell of these potentially harmful transcripts.

Transcripts that lack termination codons, termed nonstop transcripts, can arise from mistakes in gene expression, including genetic mutations, defects in transcription, or premature polyadenylation, due to inaccurate 3'-end formation or through the use of a cryptic polyadenylation site (Bucheli et al., 2007). These transcripts are degraded in a process termed the nonstop mRNA degradation pathway (Figure 1.3; Frischmeyer et al., 2002; van Hoof et al., 2002). In the current model of nonstop decay, the translating ribosome reads through the poly(A) tail and stalls at the 3'-end. The cytoplasmic exosome cofactor, Ski7p, is then believed to recognize the ribosome with an empty A-site, and along with the Ski complex, recruits the exosome to rapidly degrade the transcript from the 3'-end (van Hoof et al., 2002).

Transcripts that cause stalls in translation elongation, perhaps due to secondary structure, are also degraded by the cytoplasmic exosome in a process termed no-go decay (Doma and Parker, 2006). In the current model of no-go decay, a ribosome stalls during translation elongation. Hbs1p and Dom34p, paralogs of eRF3 and eRF1, respectively, then recognize the

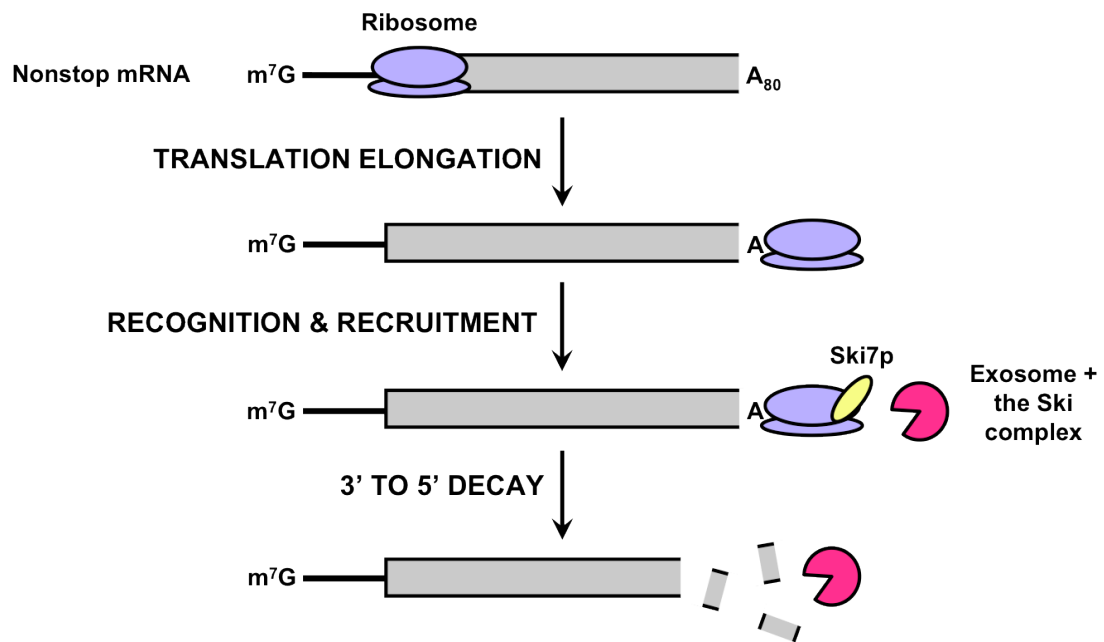


Figure 1.3: Schematic representation of the cytoplasmic nonstop mRNA degradation pathway in eukaryotes.

Figure 1.3: Schematic representation of the cytoplasmic nonstop mRNA degradation pathway in eukaryotes. In the current model of nonstop mRNA decay, a translating ribosome stalls at the 3'-end of an mRNA that lacks in-frame termination codons, termed a nonstop transcript. The stalled ribosome is then thought to be recognized by the cytoplasmic exosome cofactors, Ski7p and the Ski complex. Ski7p then recruits the exosome, which rapidly degrades the nonstop transcript from its 3'-end. This mRNA surveillance pathway differs from general 3' to 5' mRNA degradation in that prior removal of the poly(A) tail is not required.

stalled ribosome. The ribosome is then released by endonucleolytic cleavage, which generates a 5'- and 3'-degradation intermediate. The 5'-degradation fragment is degraded by the exosome, with help from the cytoplasmic exosome cofactors, Ski7p and the Ski complex. The 3'-degradation fragment is degraded by the 5' to 3' exoribonuclease, Xrn1p (Figure 1.4; Doma and Parker, 2006). The endoribonuclease responsible for the initial cleavage of the transcript has not yet been identified. Additionally, it is not yet known how Hbs1p and Dom34p recognize the stalled ribosome or recruit the endonuclease.

Several of the aberrant transcripts targeted by the cytoplasmic exosome have harmful and deleterious effects on the cell, and can even lead to disease (Ameri et al., 2007; Bulman et al., 1991; Chatr-Aryamontri et al., 2004; Kerem et al., 1990; Yasuda et al., 2003). The participation of the exosome in the cytoplasmic mRNA surveillance pathways used to degrade these aberrant mRNAs ensures that these transcripts are rapidly degraded and are, thus, unable to exert their negative effects on the cell.

Antiviral activity of the cytoplasmic exosome

Many yeast strains contain the L-A virus and the M satellite RNA. The L-A virus encodes an RNA-dependent RNA polymerase. The M satellite RNA encodes a protein toxin that is secreted from infected cells that ultimately kills uninfected cells. In a screen for “superkiller” mutants, or mutants that were able to kill uninfected cells more efficiently, several *SKI* genes were identified (Toh and Wickner, 1980). Many of these *SKI* genes encode exosome subunits and cytoplasmic exosome cofactors, including Ski7p and components of the Ski complex (Ski2p, Ski3, and Ski8p). The molecular mechanism of the exosome/Ski complex antiviral function is not completely understood, however, it seems that the cytoplasmic exosome limits killer toxin secretion by degrading unadenylated viral RNA (Brown et al., 2000; Meaux and van Hoof, 2006). Future studies are needed to determine whether the exoribonuclease activity of the exosome is needed for this degradation and, thus, for the antiviral function of the cytoplasmic exosome.

Functions of the nuclear exosome

General nuclear RNA processing and degradation

The nuclear exosome processes and degrades RNA. Specifically, the nuclear exosome processes RNA precursors, including snRNA, snoRNA and rRNA to form small, stable, mature RNA species. In yeast, ribosomal RNA processing begins with the 35S rRNA precursor, which contains the sequences for the mature 18S, 5.8S, and 25S rRNAs, which are separated by two

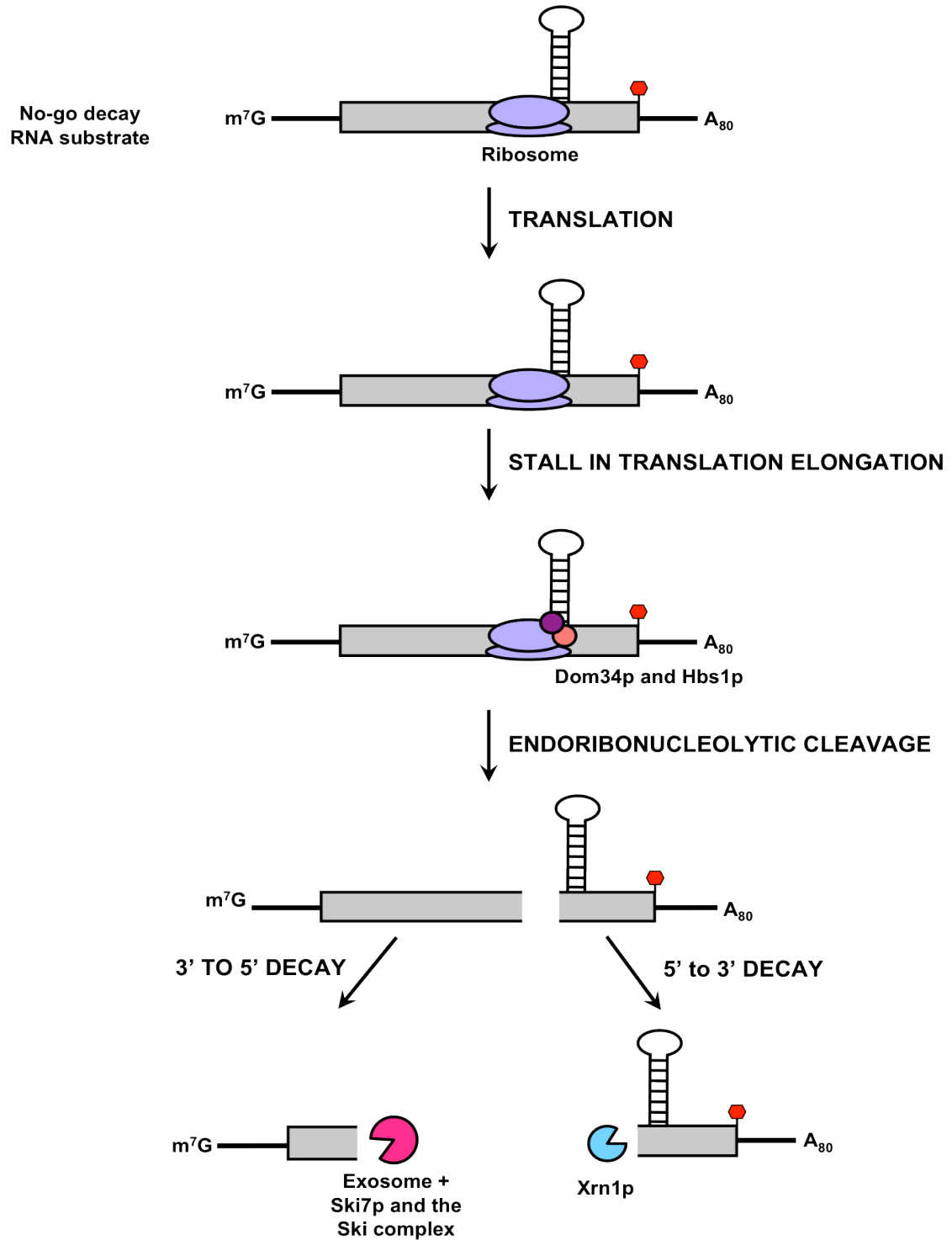


Figure 1.4: Schematic representation of the cytoplasmic no-go decay pathway in eukaryotes.

Figure 1.4: Schematic representation of the cytoplasmic no-go decay pathway in eukaryotes. In the current model of no-go decay, a translating ribosome stalls during translation elongation, possibly due to secondary structure in the transcript. The stalled ribosome is then thought to be recognized by the eRF3 and eRF1 paralogs, Hbs1p and Dom34p, respectively. An unidentified endoribonuclease then cleaves the transcript, releasing the stalled ribosome, and generating a 5' - and 3' -degradation fragment. The 5' - and 3' -degradation fragments are degraded by the cytoplasmic exosome and the 5' to 3' exoribonuclease, Xrn1p, respectively.

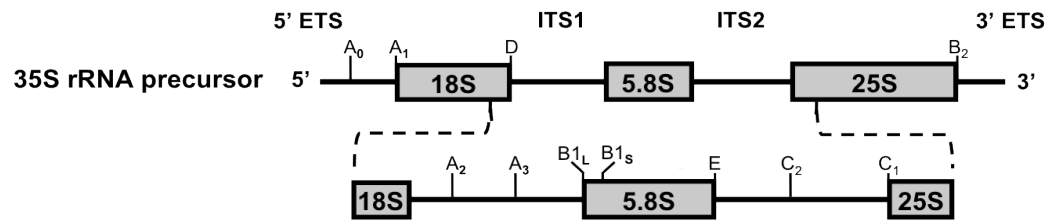
internal transcribed spacers, the ITS1 and the ITS2. On either ends of the 35S rRNA precursor, are two external transcribed spacers, the 5' ETS and the 3' ETS. Processing of 35S rRNA precursor liberates the mature rRNAs, which are subsequently packaged into the small and large ribosomal subunits. The nuclear exosome and the nuclear exosome cofactor, Rrp6p, are involved in two steps of 35S rRNA processing (Figure 1.5). In the first cleavage event, the 35S rRNA precursor is cleaved at site A₀, liberating the 5' ETS. The nuclear exosome and Rrp6p then degrade the 5' ETS to completion (de la Cruz et al., 1998).

The nuclear exosome and Rrp6p are also involved in processing the 7S rRNA precursor to form the mature 5.8S rRNA (Figure 1.5). Cleavage at the B1_L and B1_S sites generates the long and short forms of the 7S rRNA precursor (7S_L and 7S_S). The nuclear exosome then converts the 7S_{L/S} pre-rRNAs to 5.8S_{L/S}+30 [5.8S pre-rRNA species that are 3'-extended by approximately 30 nucleotides] (Allmang et al., 1999a). Rrp6p then processes the 5.8S_{L/S}+30 pre-rRNAs to 6S_{L/S} [5.8S pre-rRNA species that are 3'-extended by approximately five to eight nucleotides] (Briggs et al., 1998). The 6S_{L/S} pre-rRNAs are then exported to the cytoplasm where the final maturation step is completed to form the mature 5.8S_{L/S} rRNA (Thomson and Tollervey, 2010).

Nuclear RNA surveillance

Aberrant nuclear RNAs are typically generated due to defects in nuclear pre-RNA processing. There are three nuclear quality control systems to prevent the function of these defective RNAs, including export to the cytoplasm for cytoplasmic RNA decay, nuclear retention, and nuclear RNA degradation. The majority of transcripts that are targeted to the latter quality control system are degraded by the nuclear exosome, with the help of the nuclear exosome cofactors, Rrp6p and the TRAMP complex (Table 1.1). Specifically, the nuclear exosome degrades mRNAs that have defects in 3'-end processing (Libri et al., 2002; Torchet et al., 2002) or splicing (Bousquet-Antonelli et al., 2000). The nuclear exosome also degrades improperly modified tRNAs, including the hypomodified initiator tRNA_i^{Met} (Kadaba et al., 2004; Kadaba et al., 2006), as well as improperly processed rRNA, snRNA and snoRNA precursors (Egecioglu et al., 2006; LaCava et al., 2005). In addition to degrading RNAs that are improperly processed or modified, the nuclear exosome also degrades cryptic unstable transcripts (CUTs), which result from the transcription of intergenic regions. These transcripts ultimately result in small noncoding RNAs that are highly unstable in wild-type cells (Davis and Ares, 2006; Wyers et al., 2005).

A.



B.

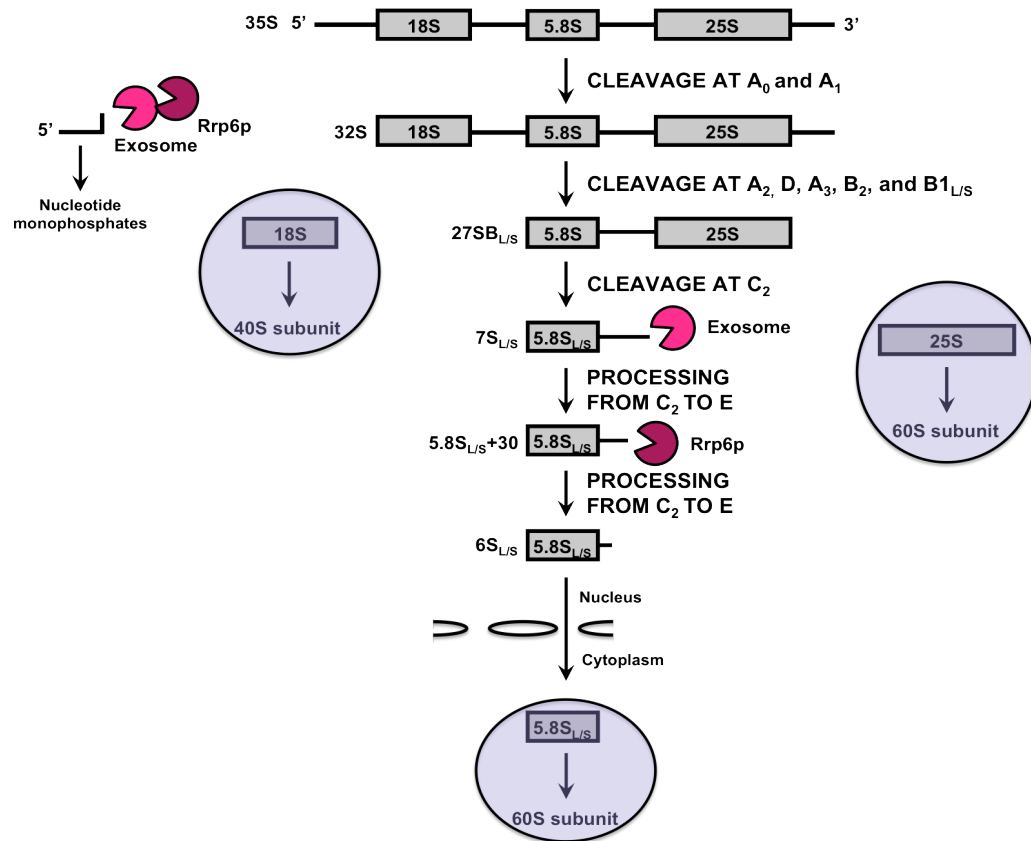


Figure 1.5: Nuclear 35S pre-rRNA processing in *S. cerevisiae*

Figure 1.5: Nuclear 35S pre-rRNA processing in *S. cerevisiae*. **A,** The 35S rRNA precursor contains the sequences for the mature 18S, 5.8S, and 25S rRNAs. These rRNA sequences are separated by two internal transcribed spacers, ITS1 and ITS2, and are flanked by two external transcribed spacers, the 5' ETS and the 3' ETS. There are 11 processing sites in the 35S rRNA precursor, which are used to generate the mature rRNAs. **B,** The 35S pre-rRNA is cleaved at sites A₀ and A₁, generating the 5' ETS fragment and the 32S pre-rRNA. This RNA is subsequently processed at sites A₂, D, A₃, B₂, and B1_{L/S}, resulting in the production of the long and short forms of the 27SB rRNA precursor (27SB_L and 27SB_S). The 27SB_{L/S} pre-rRNAs are cleaved at site C₂, generating the 7S_{L/S} pre-rRNAs. The nuclear exosome then processes these rRNA precursors to form the 5.8S_{L/S}+30 (5.8S pre-rRNA species that are 3' extended by approximately 30 nucleotides). The nuclear exosome cofactor, Rrp6p, then further processes the 5.8S_{L/S}+30 pre-rRNA to 6S_{L/S} (5.8S pre-rRNA species that are 3' extended by approximately five to eight nucleotides). The 6S_{L/S} pre-rRNAs are then exported to the cytoplasm where they undergo the final step of maturation, which generates the mature 5.8S rRNA.

The nuclear retention and degradation of these aberrant RNAs is an important checkpoint because cytoplasmic export of these defective RNAs could lead to deleterious effects on the cell if these RNAs escape the cytoplasmic RNA degradation machinery.

SIGNIFICANCE OF THE STUDY

Eukaryotic RNA metabolism is important in the post-transcriptional regulation of gene expression and is required to maintain the fidelity of gene expression. Not surprisingly, the key components involved in RNA metabolism are conserved in other eukaryotes and are required for optimal cell growth or viability. One key component of eukaryotic RNA metabolism is the exosome, a complex of ten essential proteins that is present in the nucleus and the cytoplasm.

When this study started, little was known about the catalytic subunit of the exosome, Rrp44p, and its involvement in regulating RNA metabolism. Specifically, it was not known which regions of the protein were needed for viability, exosome association, or exosome function. The data presented in these studies have uncovered new regions and activities of Rrp44p that influence the function of not only Rrp44p, but of the exosome.

Thirteen years after the discovery of the exosome, this study has demonstrated the presence of an additional nuclease active site in Rrp44p. Specifically, Chapter 3 describes the endoribonuclease active site of Rrp44p, the physiological importance of this nuclease activity, and how the endoribonucleolytic PIN domain interacts with the well-characterized exoribonucleolytic RNB domain of Rrp44p. This breakthrough finding will force the RNA metabolism field to re-evaluate how eukaryotic RNAs are recognized and degraded by the exosome.

Prior to this study, it was not known why Rrp44p was required for viability. Here, several Chapters describe the regions of Rrp44p that are needed for optimal cell growth and viability. Specifically, the N-terminus of Rrp44p, as well as the nuclease activities of the protein, are needed for cell survival. Most importantly, data in Chapter 6 suggest that the essential function of Rrp44p is likely nuclear.

Before this study was started, Rrp44p was known to loosely associate with the yeast exosome, however, it was not known which regions of the protein mediated this interaction. The data presented here show that not just one region of Rrp44p is needed for exosome interaction. Specifically, Chapters 5 and 6 show that two separate regions in the N-terminus of Rrp44p are needed for exosome association. These results, combined with several other biochemical and genetic analyses, demonstrate that multiple regions of Rrp44p are involved in exosome interaction, suggesting that these regions may be redundant for exosome association.

Lastly, this study uncovered a novel region of Rrp44p, the CR3 region, which has not yet been described in any other protein. The data in Chapter 5 suggest that CR3 region is needed for a large number of exosome functions, including the essential function. Additionally, recent structural data, coupled with *in vitro* biochemical analysis, suggest that this region is needed to

coordinate a zinc ion. The implications of this coordination may help to explain why mutating the CR3 region results in numerous exosome defects.

Taken together, the results of these studies show that the catalytic subunit of the eukaryotic RNA degrading exosome, Rrp44p, has novel nuclease activity and has a region that is important for the function of Rrp44p and the exosome. The studies presented in this dissertation contribute to a better understanding of eukaryotic RNA metabolism, both its role as a post-transcriptional regulator of gene expression and as a quality control mechanism to maintain the fidelity of gene expression. These studies also provide a foundation for further studies on the CR3 region, and nuclease activities, of Rrp44p and how they are involved in exosome function.

Chapter 2: Materials and Methods

Strains and Plasmids

The yeast strains used in these studies are described in Table 2.1. All of the *S. cerevisiae* strains were grown on rich yeast extract-peptone dextrose (YPD) [1% yeast extract, 2% peptone, 2% dextrose (or galactose, as indicated)] or synthetic complete (SC) media lacking the appropriate nutrient for plasmid selection (Sunrise Science Products). SC media was also supplemented with 5-fluoroorotic acid (5-FOA; 5.74 mM) during complementation growth assays. Unless otherwise noted, yeast strains were grown at 30°C. The heterozygous diploid *RRP44/rrp44* deletion has been previously described and was obtained from Open Biosystems (Giaever et al., 2002). This strain was transformed with a *URA3* plasmid that encoded wild-type *RRP44*.

The temperature-sensitive mRNA decapping mutant, *dcp1-2*, has been previously described (Anderson and Parker, 1998). To create a *dcp1-2, rrp44* deletion strain, the *dcp1-2* parent strain was crossed with an *rrp44* deletion strain that contained a *URA3* plasmid that encoded wild-type *RRP44*.

To create a *ski7, rrp44* deletion strain, a *ski7* deletion parent strain was crossed with an *rrp44* deletion strain that contained a *URA3* plasmid that encoded wild-type *RRP44*.

All diploid strains were sporulated, and haploid progeny spores were obtained by the hydrophobic spore isolation method as described (Rockmill et al., 1991). Isolated spores were germinated to generate a strain containing a genomic *rrp44* deletion, complemented with the *RRP44, URA3* plasmid, and where indicated, a second mutation.

The *GAL::rrp44* strains have been previously described and were obtained from Phil Mitchell and David Tollervey (Allmang et al., 1999b; Mitchell et al., 1997).

Rrp43p was C-terminally Myc-tagged using the one-step PCR system with pFA6a-13myc-His3MX6 as a template essentially as described (Longtine et al., 1998).

The plasmids used in these studies, and their creation, are described in Table 2.2. All plasmids were built using standard PCR and restriction enzyme cloning techniques, or were built using homologous recombination of PCR products or restriction digestion fragments of plasmids. All plasmids contained a yeast centromere, the endogenous promoter and 3' flanking region to control expression of Rrp44p and were verified by sequencing the insert.

For plasmids constructed using PCR and restriction enzyme cloning, the sites used are listed in Table 2.2 and the oligonucleotides used are listed in Table 2.3. To generate Rrp44p C-terminal truncations, 180 base pairs of the 3' flanking region was PCR amplified and cloned into pRS415 (Sikorski and Hieter, 1989), which contains a *LEU2* marker. 500 base pairs of the promoter and desired part of the coding region was PCR amplified and cloned into the plasmid

Table 2.1: Yeast strains used		
Strain Name	Genotype	Reference
BY4741	<i>MATa; his3ΔI; leu2Δ0; met15Δ0; ura3Δ</i>	Giaever et al., 2002
yAV862	<i>MATa; his3Δ200; leu2ΔI; trpI; ura3-52; gal108; GAL10::RRP44</i>	Mitchell et al., 1997
yAV1115	<i>MATa; his3ΔI; leu2Δ0; ura3Δ0; rrp44Δ::NEO [RRP44, URA3]</i>	This study
yAV1144	<i>MATa; his3ΔI; leu2Δ0; met15, ura3Δ; rrp6Δ::NEO</i>	This study
yAV1137	<i>MATa; his3ΔI; leu2Δ0; ura3Δ; rrp6Δ::NEO; rrp44Δ::HYG [RRP44, URA3]</i>	This study
yAV313	<i>MATa; leu2-3,112; lys2-20I; trpI; ura3-52; cup1::LEU2/PGK1pG/MFA2pG; dcp1-2::TRP1; ski7Δ::NEO</i>	van Hoof et al., 2000
yAV1143	<i>MATa; leu2Δ0; trpI; ura3Δ0; dcp1-2::TRP1; rrp44Δ::NEO [RRP44, URA3]</i>	This study
yAV856	<i>MATa; his3ΔI; leu2Δ0; met15Δ0; ura3Δ0; ski7Δ::NEO</i>	This study
yAV1166	<i>MATa; leu2Δ0; lys2Δ0; ura3Δ0; ski7Δ::HYG; rrp44Δ::NEO [RRP44, URA3]</i>	This study
yAV1179	<i>MATa; leu2Δ0; lys2Δ0; ura3Δ0; ski7Δ::HYG; rrp44Δ::NEO [RRP44, LEU2]</i>	This study
yAV1183	<i>MATa; leu2Δ0; lys2Δ0; ura3Δ0; ski7Δ::HYG; rrp44Δ::NEO [RRP44 (D171A), LEU2]</i>	This study
yAV1193	<i>MATa; leu2Δ0; ura3Δ0; xrm1Δ::NEO; rrp44Δ::HYG [RRP44, LEU2]</i>	This study
yAV1194	<i>MATa; leu2Δ0; ura3Δ0; xrm1Δ::NEO; rrp44Δ::HYG [RRP44 (D171A), LEU2]</i>	This study
yAV1117	<i>MATa; his3ΔI; leu2Δ0; met15Δ0; ura3Δ0; RRP43myc::HIS3</i>	This study

Table 2.2: Plasmids used (continued)				
Name	Description	Marker	Insert/ Origin of plasmid	Parent plasmid
pAV602	<i>RRP44</i> promoter, residues 1-1001 (I45A), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	Digest pAV593	pRS415
pAV603	<i>RRP44</i> promoter, residues 1-1001 (C47S), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	Digest pAV594	pRS415
pAV604	<i>RRP44</i> promoter, residues 1-1001 (C52S), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	Digest pAV595	pRS415
pAV605	<i>RRP44</i> promoter, residues 1-1001 (C55S), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	Digest pAV596	pRS415
pAV515	<i>RRP44</i> promoter, residues 1-1001 (D91A), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	Digest pAV506	pRS415
pAV502	<i>RRP44</i> promoter, residues 1-1001 (D91A), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	Digest pAV484	pRS415
pAV514	<i>RRP44</i> promoter, residues 1-1001 (E120A), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	Digest pAV505	pRS415
pAV503	<i>RRP44</i> promoter, residues 1-1001 (D171A), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	Digest pAV485	pRS415
pAV656	<i>RRP44</i> promoter, residues 1-1001 (D171A), <i>RRP44</i> 3' flanking region	<i>URA3</i>	Digest pAV503	pRS416
pAV504	<i>RRP44</i> promoter, residues 1-1001 (D198A), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	Digest pAV486	pRS415
pAV501	<i>RRP44</i> promoter, residues 1-1001 (D551N), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	Digest pAV482	pAV344
pAV657	<i>RRP44</i> promoter, residues 1-1001 (D551N), <i>RRP44</i> 3' flanking region	<i>URA3</i>	Digest pAV501	pAV361
pAV709	<i>RRP44</i> promoter, residues 1-1001 (Y40A, D551N), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	Digest pAV598	pAV501
pAV710	<i>RRP44</i> promoter, residues 1-1001 (L41A, D551N), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	Digest pAV599	pAV501
pAV711	<i>RRP44</i> promoter, residues 1-1001 (R42A, D551N), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	Digest pAV600	pAV501
pAV633	<i>RRP44</i> promoter, residues 1-1001 (C47S, C52S, C55S, D171A), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	Digest pAV629	pRS415
pAV536	<i>RRP44</i> promoter, residues 1-1001 (C47S, C52S, C55S, D551N), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	Digest pAV515	pAV501
pAV537	<i>RRP44</i> promoter, residues 1-1001 (D91A, D551N), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	Digest pAV502	pAV501
pAV538	<i>RRP44</i> promoter, residues 1-1001 (E120A, D551N), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	Digest pAV514	pAV501
pAV539	<i>RRP44</i> promoter, residues 1-1001 (D171A, D551N), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	Digest pAV503	pAV501
pAV740	<i>RRP44</i> promoter, residues 1-1001 (D171A, D551N), <i>RRP44</i> 3' flanking region	<i>URA3</i>	Digest pAV539	pRS416
pAV540	<i>RRP44</i> promoter, residues 1-1001 (D198A, D551N), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	Digest pAV504	pAV501
pAV528	<i>RRP44</i> promoter, residues 1-324 (D91A), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	oAV185 and oAV470 PCR	pRS415
pAV529	<i>RRP44</i> promoter, residues 1-324 (E120A), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	oAV185 and oAV470 PCR	pRS415
pAV530	<i>RRP44</i> promoter, residues 1-324 (D171A), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	oAV185 and oAV470 PCR	pRS415
pAV531	<i>RRP44</i> promoter, residues 1-324 (D198A), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	oAV185 and oAV470 PCR	pRS415
pAV606	<i>RRP44</i> promoter, residues 1-235 (E38A), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	oAV185 and oAV468 PCR	pRS415
pAV607	<i>RRP44</i> promoter, residues 1-235 (Y40A), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	oAV185 and oAV468 PCR	pAV454
pAV608	<i>RRP44</i> promoter, residues 1-235 (L41A), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	oAV185 and oAV468 PCR	pAV454
pAV609	<i>RRP44</i> promoter, residues 1-235 (R42A), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	oAV185 and oAV468 PCR	pAV454
pAV610	<i>RRP44</i> promoter, residues 1-235 (D44A), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	oAV185 and oAV468 PCR	pAV454
pAV611	<i>RRP44</i> promoter, residues 1-235 (I45A), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	oAV185 and oAV468 PCR	pAV454
pAV513	<i>RRP44</i> promoter, residues 1-235 (C47S, C52S, C55S), <i>RRP44</i> 3' flanking region	<i>URA3</i>	oAV185 and oAV468 PCR	pAV318
pAV615	<i>RRP44</i> promoter, residues 1-235 (C47S, C52S, C55S), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	Digest pAV513	pRS415
pAV714	<i>RRP44</i> promoter, residues 34-1001 (D171A), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	oAV186 and oAV678 PCR	pAV586
pAV715	<i>RRP44</i> promoter, residues 34-1001 (D551N), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	Digest pAV501	pAV586
pGEX-454	GST-RRP44 residues 1-235	-	Schaeffer et al., 2009	-
pAV575	GST-RRP44 residues 1-235 (C47S, C52S, C55S)	-	oAV647 and oAV648 PCR	pGEX4T1
pAV576	GST-RRP44 residues 84-235	-	oAV662 and oAV648 PCR	pGEX4T1
Reporter Plasmids				
pAV700	<i>his3</i> -nonstop	<i>LYS2</i>	Digest pAV188	pRS317
pAV188	<i>his3</i> -nonstop	<i>URA3</i>	van Hoof et al., 2002	-
pAV175	<i>GAL::pgkI</i> -nonstop	<i>URA3</i>	Frischmeyer et al., 2002	-
pAV701	<i>GAL::pgkI</i>	<i>URA3</i>	Doma and Parker, 2006	-
pAV702	<i>GAL::pgkI</i> -SL	<i>URA3</i>	Doma and Parker, 2006	-
Other plasmids				
pAV476	TAP	<i>LEU2</i>	oAV556 and oAV557 PCR	pRS415
pAV139	SK17 promoter, residues 1-748, SK17 3' flanking region	<i>URA3</i>	Benard et al., 1999	-
pAV179	SK17 promoter, residues 1-748 (P265*), SK17 3' flanking region	<i>URA3</i>	oRP1055 mutagenesis	pAV152
pAV402	GPD promoter, <i>A. thaliana</i> <i>RRP41</i> residues 1-241	<i>HIS3</i>	oAV464 and oAV465 PCR	p423GPD
pAV349	<i>E. coli</i> RNase II residues 1-644	-	Gift from Drs. Ana Barbas and Cecilia Arraiano	pET15b
pAV375	GPD promoter, <i>E. coli</i> RNase II residues 1-644	<i>HIS3</i>	Digest pAV349	p423GPD
pAV350	<i>E. coli</i> RNase R residues 1-813	-	Gift from Drs. Ana Barbas and Cecilia Arraiano	pET15b
pAV376	GPD promoter, <i>E. coli</i> RNase R residues 1-813	<i>HIS3</i>	Digest pAV350	p423GPD
pAV474	GPD promoter, <i>RRP6</i> residues 1-734	<i>HIS3</i>	oAV459 and oAV460 PCR	p423GPD

Table 2.3: Oligonucleotides used

PCR oligos	Sequence
oAV185	5' ATATAGAGCTCAAAAACGCCTACGTACCATTTAAC
oAV186	5' ATATACTCGAGCACCACCAAAATGTCAATTTTTTTG
oAV187	5' ATATAACTAGTAACAACATGTCCAATACCACCGTGATTC
oAV188	5' ATATAACTAGTTTTTGGCCTGTATGATGCAAG
oAV189	5' ATATAACTAGTTAGATGGAAGATGCTTCAGTATC
oAV190	5' ATATAACTAGTTTAACGCCTTGTCTGATTCTGAC
oAV271	5' ATATAACTAGTTTATTTCTCTATTCAAACGATCGTTGGTAAC
oAV282	5' ATATAACTAGTTTAGGAAGAGGACTCATTATTGTC
oAV354	5' ATATAACTAGTTTAATTCTCTCATTACTTGCCCCGAC
oAV356	5' ATATAACTAGTTTAAATTGTACCGAGGTCTCTCACAAG
oAV451	5' GTGTGTGCTACTGCAGTACTTCTTAGAG
oAV452	5' CGAGCGGCCGCGGTGTGTGGATCCCGAAGATCC
oAV459	5' ATATAACTAGTTGCGTTATGACTTCTGAAAATCCGGATG
oAV460	5' ATATACTCGAGTCACCTTTTAAATGACAGATTCTT
oAV464	5' ATATAACTAGTCCATGGAGTACGTAAACCCTGAAGGTC
oAV465	5' ATATACTCGAGTTATGCAGCTCGGCGATACTCTAGC
oAV468	5' ATATAACTAGTTTAAATAGAGTCTCTGATGTCGTCTGCA
oAV470	5' ATATAACTAGTTTATGATTGAGGTAGCAGTTCCACGATAAC
oAV472	5' ATATAACTAGTTTACCAGGATCTTCTTTGTATATATAAA
oAV482	5' GATCGGACATCCCAAGTCTTTCTAGAAGTAGTACCAAGAGTCCGCAAATTGTCTG
oAV484	5' TTGTCCCCCAGATTGTTCTAGATGCAGTGAGAAACAAGTC
oAV541	5' ATATAACTAGTATGGTAAGCATTACGTCTG
oAV542	5' GATCAAACGAGTTTATTTATCATACTTGCATCATACAGGCCAAAACAAC
oAV543	5' TTCTCTATTCAAACGATCGTTGGTAACAAGAACGACGTTTATGTCATAAG
oAV544	5' CTTATGACATAAACGTCGTTCTTGTACCAACGATCGTTTGAATAGAGAAGGTCGACGGATCCCCGGGTT
oAV545	5' TTTTAAACAATAATCTGCGCTTACGCTTGCTAGTAATTGGATCCATCACCG
oAV546	5' CGGTGATGGATCCAATTACTAGCAAGCGTAAGGCAGAATTATTGTTAAAAAGGTCGACGGATCCCCGGGTT
oAV547	5' AATAGAGTCTCTGATGTCGTCTGCATTGGTAGTAACCTCGATATACTGTA
oAV548	5' TACAGTATATCGAGTTACTACCAAATCGACACGACATCAGAGACTCTATTGGTCGACGGATCCCCGGGTT
oAV549	5' CACCACCAAAATGTCAATTTTTTGGCATTTTCTAAATAGTTTTTCTTCACTTATCGATGAATTCGAGCTCGTT
oAV565	5' CTCCAGGATGTGTTGATATCAACGATGCCCTACATGCG
oAV566	5' CGCATGTAGGGCATCGTTGATATCAACACATCCTGGAG
oAV569	5' CGACAATTTGCGGACTCTTGGTACTACTTCTAGAAAGACTTGGGATGTCCGATC
oAV570	5' GCATTACGTCGCTTGGCCACCAACGTTGGTGTAC
oAV571	5' GTAACACCACGTTGGTGGCCAAGACGACGTAATGC
oAV572	5' CGATTATGACAGAAACGCGCGCGCTATAAGGAAAACCTGTCAATGG
oAV573	5' CCATGACAGGTTTCTTATAGCGCGCGGTTTCTGTCATTAATCG
oAV574	5' CGTTCTTGTTACCAACGCTCGTTTGAATAGAGAAGC
oAV575	5' GCTTCTCTATTCAAACGAGCGTTGGTAACAAGAAGC
oAV578	5' GACTTGTCTCTACTGCATCTAGAACAATCTGGGGGACAA
oAV647	5' CGCAGAATTCATGTCAGTTCCCGCTATCG
oAV648	5' ATATACTCGAGTTAAATAGAGTCTCTGATGTCGTCTGCA
oAV662	5' CGCAGAATTCATGGGTAAGCATTACGTCTG
oAV663	5' ATATAACTAGTAACAACATGCTTGCAGATGGTTTAAGCG
oAV664	5' ATATAACTAGTAACAACATGTGTCTTTTCGAGAAGTTGTA
oAV678	5' ATATAACTAGTAACAACATGAAGATCGTAAGAGAACACTAT
oAV679	5' CAAGATCGTAAGAGCACACTACTTAAGATCGGACATC
oAV680	5' GATGTCCGATCTTAAGTAGTGTGCTCTTACGATCTTG
oAV681	5' CGTAAGAGAACACGCTTAAAGATCTGACATCCCATGTCTTTC
oAV682	5' GAAAGACATGGGATGTCAGATCTTAAAGCGTGTCTCTTACG
oAV683	5' GTAAGAGAACACTATGCAAGATCTGACATCCCATGTCTTTC
oAV684	5' GAAAGACATGGGATGTCAGATCTTGCATAGTGTCTCTTAC
oAV685	5' GAGAACTATTTAGCATCGGATATCCCATGTCTTTCG
oAV686	5' CGAAAGACATGGGATATCCGATGCTAAATAGTGTCTC
oAV687	5' CTATTTAAGATCGGCCATCCCATGTCTCTCGAGAAGTTGTACC
oAV688	5' GGTACAACCTTCTCGAGAGACATGGGATGGCCGATCTTAAATAG
oAV689	5' CTATTTAAGATCGGACGCCCATGTCTCTCGAGAAGTTGTAC
oAV690	5' GTACAACCTTCTCGAGAGACATGGGGCGTCCGATCTTAAATAG
oAV691	5' GATCGGACATCCCAAGTCTTTCTAGAAGTTGTACCAAG
oAV692	5' CTTGGTACAACCTCTAGAAAAGACTTGGGATGTCCGATC
oAV693	5' GATCGGACATCCCATGTCTTTCTAGAAGTAGTACCAAGTGCCG
oAV694	5' CGGACACTTGGTACTACTTCTAGAAAAGACATGGGATGTCCGATC
oAV695	5' CATCCCATGTCTTTCTAGAAGTTGTACCAAGAGTCCGCAAATTGTC
oAV696	5' GACAATTGCGGACTCTTGGTACAACCTCTAGAAAAGACATGGGATG
oAV720	5' ATATATCTAGAAACAACATGCTTGCAGATGGTTTAAGCG
oAV721	5' ATATAGGATCCTTAAATAGAGTCTCTGATGTCGTCTGCA
oAV722	5' ATATAGGATCTAGATGGAAGATGCTTCAAGTATC
oAV757	5' AATAGAGTCTCTGATGTCGTCTGCATTGGTAGTAACCTCGATATACTGTA
oAV758	5' TACAGTATATCGAGTTACTACCAAATCGACACGACATCAGAGACTCTATTGGTCGACGGATCCCCGGGTT
oRP1055	5' TGTCAAATTCAACTAGTGGGTGGCAATGAATG

Table 2.3: Oligonucleotides used (continued)	
Northern oligos	Sequence
oAV141	5' AATTGATCTATCGAGGAATTCC
oAV224	5' GTCTAGCCGCGAGGAAGG
oAV267	5' CTCTTGCTTCTCTGGAGAGATCGTCAGTC
oAV671	5' AAAATGGCGTGAGGTAGAGAGAAACCAGCG
oAV783	5' CGGATAAGAAAGCAACACCTGG
oAV784	5' GGTGGCAAAGCAGC
oJA003	5' TGAGAAGGAAATGACGCT
oRP993	5' CGAACGACAAGCCTACTCG

described above. N-terminal truncations were generated similarly, except that the promoter was PCR amplified and cloned first, with the truncated coding region and 3' flanking region cloned second.

Site-directed mutations of Rrp44p were created using the QuikChange II Kit (Stratagene) and the oligonucleotides listed in Table 2.3. Each oligonucleotide also changes a restriction site to aid in screening. Following restriction digestion, putative mutations were verified by sequencing. To combine point mutations in the CR3 region and PIN domain of Rrp44p with the D551N mutation in the RNB domain, plasmids encoding each single mutation were digested with *SacI* and *PshAI* and ligated together. *SacI* cuts at the beginning of the promoter, whereas *PshAI* cuts at a unique site within the CSD2 domain. To generate the point mutations of the CR3 region and PIN domain in the 1-324 truncation, the promoter and truncated coding region were PCR amplified from the full-length point mutations and cloned.

C-terminally TAP-tagged Rrp44 constructs were created using homologous recombination. To create a gapped plasmid, pAV473 was digested with *SpeI*, which cuts 3' of the Rrp44p promoter. A portion of the coding region of Rrp44p was PCR amplified from pAV344 so that the 5'-end of the resulting PCR product had at least 50 nucleotides of homology to the Rrp44p promoter. The TAP tag and *HIS3* marker were PCR amplified from a strain containing Rrp44p-TAP from the yeast TAP-tagged collection (Open Biosystems; Ghaemmaghami et al., 2003) so that the 5'-end of the resulting PCR product had at least 50 nucleotides of homology to the Rrp44p coding region and that the 3'-end of the resulting PCR product had at least 50 nucleotides of homology to the 3' UTR of Rrp44p. The gapped plasmid and both PCR products were transformed into yeast and subsequently rescued from yeast to verify the sequence of the plasmid.

Rrp44p GST fusion plasmids were generated by PCR amplifying the *RRP44* coding region from the plasmids described above, digesting the PCR product with *EcoRI* and *XhoI*, and ligating them into pGEX-4T-1 (GE Healthcare).

Yeast growth assays

Complementation growth assays

Complementation growth assays were performed using *rrp44* truncations and point mutations encoded on a *LEU2* plasmid. These plasmids were transformed into an *rrp44* deletion strain containing a *URA3* plasmid that encoded wild-type *RRP44*. Transformants were then grown on SC –LEU –URA + 2% dextrose overnight at 30°C. Cultures were then diluted in the same selection media to an optical density at 600 nm of 0.8. Next, cells were serially diluted in

96-well plates by a factor of 5 and spotted onto media containing 5-fluoroorotic acid (5-FOA). These plates were incubated at 30°C for 2-20 days. 5-FOA selects for cells that have lost the *URA3* plasmid. Therefore, cells that are viable on 5-FOA can lose the *URA3* plasmid encoding wild-type *RRP44*, which suggests that the *rrp44* truncation or point mutant can carry out the essential function of Rrp44p.

Synthetic lethality growth assays

Synthetic lethality growth assays were performed essentially as described (van Hoof et al., 2000b). Briefly, plasmids encoding *rrp44* truncations and point mutations were transformed into a *dcp1-2*, *rrp44* deletion strain containing a *URA3* plasmid encoding wild-type *RRP44*. Transformants were grown in SC –LEU –URA + 2% dextrose overnight at 23°C. Cultures were diluted in the same selection media to an optical density at 600 nm of 0.8. Next, cells were serially diluted in 96-well plates by a factor of 5 and spotted onto the same selection media. Plates were incubated at 23°C (permissive temperature), 30°C, and 37°C (non-permissive temperature) for 2-5 days. The basis of this assay is that one of the two general mRNA degradation pathways is required for viability. Cells with a functional 3' to 5' decay pathway are viable at all temperatures, whereas mutants in the 3' to 5' degradation pathway are synthetically lethal with *dcp1-2* at the non-permissive temperature.

His3-nonstop growth assays

The *his3*-nonstop growth assay was performed essentially as described (van Hoof et al., 2002). Briefly, *rrp44* deletion strains containing *LEU2* plasmids encoding *rrp44* truncations and point mutations were transformed with a *URA3* or *LYS2* plasmid encoding a *his3*-nonstop reporter. This reporter contains a nonstop mutation at the end of the *HIS3* open reading frame. Transformants were grown in SC –LEU –URA + 2% dextrose or SC –LEU –LYS + 2% dextrose overnight at 30°C. Cultures were diluted in the same selection media to an optical density at 600 nm of 0.8. Next, cells were serially diluted in 96-well plates and spotted onto media lacking histidine, SC –HIS –LEU –URA or SC –HIS –LEU –LYS. The plates were incubated at 30°C for 2-5 days. In cells with a functional nonstop mRNA degradation pathway, the nonstop mRNA degradation machinery rapidly degrades the *his3*-nonstop transcript. In contrast, in cells that are not able to degrade the *his3*-nonstop transcript, histidine is made, and cells are viable on media lacking histidine.

Soluble Cell Lysate Preparation Using Glass Bead Lysis

Cell cultures of optical density at 600 nm of 0.8 were pelleted and resuspended in 250 μ L of IP50 buffer [50mM NaCl, 50mM Tris-HCl (pH 7.5), 2mM MgCl₂, 0.1% Triton X, 0.00714% β -mercaptoethanol, 0.1M PMSF, with complete EDTA-free protease inhibitors (Roche)]. 50 μ L of glass beads were added to the cell suspension and agitated in a vortex mixer for 7 minutes at 4°C. Cell lysates were clarified by centrifugation at 4,500 \times g for 7 minutes at 4°C.

Tandem Affinity Purification (TAP)

Tandem affinity purifications were performed by adding 10 μ L of IgG Sepharose 6 Fast Flow beads (Amersham Biosciences) to 200 μ L of whole-cell lysates. After incubation for 1 hour at 4°C, the supernatant was discarded and the beads were washed two times with 200 μ L of IP50 buffer. The beads were washed two more times with 200 μ L of IP1000 buffer [1 M NaCl, 50 mM Tris-HCl (pH 7.5), 2 mM MgCl₂, 0.1% Triton X, 0.00714% β -mercaptoethanol, 0.1 M PMSF, with complete EDTA-free protease inhibitors (Roche)]. The beads were then resuspended in 100 μ L of IP50 buffer and 33.3 μ L of 6X protein sample buffer [350 mM Tris HCl (pH 6.8), 36% glycerol, 10% SDS, 5% β -mercaptoethanol, 0.012% bromophenol blue] was added. Bound proteins were eluted from beads by boiling for 3 minutes at 95°C and were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE and Western Blot analysis

Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad) for immunodetection essentially as described (Laemmli, 1970; Towbin et al., 1979). Antibodies were diluted in a 5% solution of non-fat milk in TBST (0.684 M NaCl, 0.1 M Tris base). Anti-Protein A monoclonal antibody was purchased from Sigma and used at a dilution of 1:1,000,000. Anti-Myc monoclonal antibody was used at a 1:1,000 dilution (kindly provided by E. Wagner, University of Texas Health Science Center at Houston, Houston, TX). Anti-Pgk1p monoclonal antibody was purchased from Invitrogen and used at a 1:6,000 dilution.

Plasmid Rescue from Yeast

The plasmid rescue from yeast protocol was adapted from instructions provided by the S. Fields Laboratory (University of Washington, Seattle, WA) and by using the QIAprep Spin Miniprep Kit (Qiagen). Briefly, yeast strains were grown in a 5 mL overnight culture of YPD. Cells were then pelleted and resuspended in 250 μ L of Qiagen buffer P1 and 250 μ L of acid-

washed glass beads. To disrupt the cell wall, the suspensions were agitated in a vortex mixer for 7 minutes. Next, 250 μ L of Qiagen buffer P2 was added and the suspensions were inverted 7 times. To obtain clarified cell lysates, the reactions were centrifuged at maximum speed for 10 minutes. The supernatant was transferred to a QIAprep spin column and spun at maximum speed for 1 minute. The column was washed once with 750 μ L of Qiagen buffer PE by centrifuging the column at maximum speed for 1 minute. The supernatant was discarded and the column removed to a clean microcentrifuge tube. Plasmid DNA was eluted by adding 50 μ L of sterile ddH₂O, followed by incubated at 37°C for 5 minutes.

Overexpression and Purification of Recombinant Rrp44p

Cell cultures of Rosetta (DE3) *E. coli* containing a plasmid encoding wild-type *RRP44* or *rrp44* mutations were grown at 30°C in 2 L of Terrific Broth (TB) [1.2% Tryptone, 2.4% Yeast Extract, 54.0 mM K₂HPO₄, 16.2 mM KH₂PO₄] supplemented with ampicillin (200 μ g/ml) and chloramphenicol (25 μ g/ml) to an optical density at 600 nm of 1.2. To induce expression of *rrp44*, 0.2 mM of isopropyl β -D-1-thiogalactopyranoside (IPTG) was added to the cultures, which were then switched to 18°C for overnight incubation. Cell cultures were then pelleted and resuspended in 30 mL phosphate buffered saline (PBS) [37 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, 10 mM DTT, and 1 mM phenylmethylsulfonyl fluoride (PMSF); pH 8]. Cell suspensions were then lysed using the French Press. Crude cell extracts were treated with 5 units of Benzonase (Sigma) for 30 minutes at 0°C and then clarified by a 30 minute centrifugation at 10,000 \times g. Clarified cell lysates were loaded onto a GSTrapTM FF 1 ml column (GE Healthcare) equilibrated in GST binding buffer [50 mM Tris-HCl (pH 8.5), 10 mM DTT]. Proteins were eluted with a 0-1610 mM glutathione gradient in GST Binding buffer and fractionated into 2 mL aliquots. The fractions containing purified protein were pooled and loaded onto a gel filtration column [Superdex 75 10/300 GL or a Superose 12 10/300 GL (GE Healthcare), depending on the protein size] equilibrated with gel filtration buffer [20 mM Tris-HCl, 150 mM NaCl, 5mM MgCl₂ and 10 mM DTT buffer (pH 8)]. The eluted proteins were loaded onto a Vivaspin 500 Centrifugal Concentrator column (Vivaspin) and concentrated by centrifugation at 4,500 \times g at 15°C until approximately 100 μ L of concentrated protein remained. Protein concentration was determined by spectrophotometry using a Nanodrop spectrophotometer. Glycerol [50% (v/v)] was added to the final fraction, which was stored at -20°C.

RNase assays

500 nM of Rrp44p GST-fusion recombinant proteins were incubated with 200 nM of ^{32}P 5'-end labeled A₃₀ oligonucleotide in a buffer consisting of 20 mM HEPES (pH 7.5), 150 mM NaCl, 3 mM MnCl₂, 1 mM DTT for the times indicated. Reactions were subject to denaturing urea-polyacrylamide gel electrophoresis for single nucleotide separation.

Determining the processing of the 5.8S rRNA and the degradation of the 5' ETS of the pre-rRNA precursor

Monitoring of 5.8S rRNA processing and 5' ETS degradation was carried out essentially as described (Allmang et al., 1999b). Briefly, *GAL::rrp44* strains containing *LEU2* plasmids encoding *rrp44* truncations and point mutations were grown in SC –LEU + 2% galactose overnight at 30°C. Cultures were diluted in the same selection media to an optical density at 600 nm of 0.2 and grown to an optical density at 600 nm of 0.8. The medium was replaced with SC –LEU + 4% glucose to inhibit expression of the endogenous *RRP44* allele. At the time points indicated, 2 mL cell aliquots were pelleted. RNA was isolated and subject to denaturing urea-polyacrylamide gel electrophoresis, followed by Northern blot analysis (see below).

Stability of *pgk1pG*-nonstop mRNA

To determine the half-life of the *pgk1pG*-nonstop reporter, yeast strains were grown in SC –URA + 2% galactose overnight at 30°C. Cultures were diluted in the same selection media to an optical density at 600 nm of 0.2 and grown to an optical density at 600 nm of 0.8. The medium was replaced with SC –URA + 4% glucose. At the time points indicated, 2 mL cell aliquots were pelleted. RNA was isolated and subjected to formaldehyde agarose gel electrophoresis, followed by Northern blot analysis (see below).

RNA Isolation

RNA isolations were performed essentially as described (Caponigro et al., 1993). Cell pellets were resuspended in 150 μL of LET [25 mM Tris (pH 8), 100 mM LiCl, 20 mM EDTA], 150 μL of phenol equilibrated with LET, and 250 μL of acid-washed glass beads in a 2 mL tube. To disrupt the cell wall, cell suspensions were agitated in a vortex mixer for 5 minutes. Next, 250 μL of chloroform and 250 μL of diethyl pyrocarbonate-treated (DEPC) water were added. To clarify the cell suspensions, the reactions were centrifuged at maximum speed for 2 minutes. The aqueous phase was transferred to a fresh 2 mL tube containing 400 μL of phenol-chloroform

equilibrated with LET. Samples were again centrifuged at maximum speed for 2 minutes. The aqueous phase was transferred to a fresh 2 mL tube containing 400 μ L of chloroform. Samples were again centrifuged at maximum speed for 2 minutes. The aqueous phase was transferred to a fresh 2 mL tube containing 1 mL 95% EtOH and 40 μ L of 3M NaAc. To precipitate the RNA, samples were incubated at -80°C for at least 30 minutes and then centrifuged at maximum speed for 30 minutes. RNA pellets were dried in a concentration/drying system and resuspended in 100 μ L of DEPC-treated water.

Northern Blot Analysis and Quantitation of Gene Expression

Northern blot analysis was performed essentially as described (Meaux and van Hoof, 2006). Blots were hybridized with 32 P 5'-end labeled oligonucleotides specific for the 5.8S rRNA (oJA003), 5' ETS (oRP993), *pgk1pG*-nonstop (oRP141), *GAL7* (oAV267), *ACT1* (oAV671), the 5'- and 3'-ends of *PGK1* and *pgk1*-SL (oAV784 and oAV783, respectively) and the RNA subunit of the signal recognition particle (oRP100). The oligonucleotide sequences are listed in Table 2.3. Signals were detected and quantitated using a STORM PhosphorImager (Amersham), and corrected for loading by quantitating the RNA subunit of the signal recognition particle, *SCR1*, or *ACT1*.

Chapter 3: The PIN domain of Rrp44p has endoribonuclease activity and is essential for exosome function.

NOTE: This Chapter is derived from work that was published in 2009: “The exosome contains domains with specific endoribonuclease, exoribonuclease and cytoplasmic mRNA decay activities.” *Nature Structural and Molecular Biology*. 16 (1): 56 – 62. I am the primary author on this paper and was involved in preparing the original manuscript. I performed all of the experiments described in this Chapter with the exception of the biochemical nuclease assays that were performed by our collaborators, Drs. Ana Barbas and Cecilia Arraiano (Instituto de Tecnologia Química e Biológica [ITQB]; Lisbon, Portugal). I have been given permission by the publisher of *Nature Structural and Molecular Biology*, the Nature Publishing Group, to reproduce any/all of my manuscript in print or electronically for the purposes of my dissertation.

INTRODUCTION

Rrp44p is the only catalytic subunit of the core exosome, with its 3' to 5' exonuclease activity contributed by the RNB domain. Besides the RNB domain, Rrp44p contains four other domains, three of which are putative RNA binding domains. The fourth domain, PIN, has not yet been characterized in eukaryotic Rrp44p.

PIN domains are named for their homology with the N-terminus of the bacterial type IV pilus biosynthetic protein, PilT (Wall and Kaiser, 1999), and are ubiquitous in all three kingdoms of life. These domains are approximately 100 residues in length and display a sequence conservation of four acidic residues (Clissold and Ponting, 2000; Makarova et al., 1999). The acidic residue conservation seen in PIN domains commonly occurs in enzymes that ligate divalent cations, such as nucleases (Clissold and Ponting, 2000). Although the conserved residues of the PIN domain are spread across the primary sequence of the protein, the active site residues come together and are thought to bind Mg^{2+} or Mn^{2+} . The central channel formed by the PIN domain is large enough to accommodate ssDNA or RNA, but not dsDNA (Daines et al., 2007; Fatica et al., 2004).

Bioinformatic analysis of PIN domains predicted a fold similar to the FLAP 5' to 3' exonuclease family of proteins, which includes T4 bacteriophage RNase H (Clissold and Ponting, 2000). Recent structural analysis of archaeal PIN domain-containing proteins, PAE2754 and AF0591, confirmed these predictions, showing a fold similar to the nuclease domain of T4 RNase H. In addition, PAE2754 its conserved acidic residues had a similar spatial arrangement, suggesting that these residues may also coordinate a metal ion for catalysis. *In vitro* experiments confirmed that these enzymes have Mg^{2+} -dependent exonuclease activity on ssDNA, similar to T4 RNase H (Arcus et al., 2004; Levin et al., 2004).

Importantly, several of the conserved acidic residues of PIN domains are present at the same structural position in eukaryotic PIN domain-containing proteins, which suggests that these proteins may also coordinate a divalent cation and exhibit nuclease activity. Recently three eukaryotic PIN domain-containing proteins, *S. cerevisiae* Nob1p and Utp24p and *H. sapiens* SMG6, were shown to have endoribonuclease activity on ssRNA, with Nob1p and SMG6 dependent on Mn^{2+} for activity. Importantly, mutations of the third conserved acidic residue in these proteins abolished PIN domain-mediated endonuclease activity *in vitro* and *in vivo* (Bleichert et al., 2006; Fatica et al., 2004; Glavan et al., 2006). Ribonuclease activity has also been demonstrated in bacterial PIN domain-containing proteins (Daines et al., 2007). Several of these proteins are found in toxin-antitoxin systems, where the RNase activity of the PIN domain

is responsible for degrading the labile antitoxin, resulting in a toxic effect (Anantharaman and Aravind, 2003; Gerdes et al., 2005).

PIN domain containing-proteins have nuclease activity in all three kingdoms of life, suggesting that this nuclease activity plays an important cellular role. To determine whether the PIN domain of *S. cerevisiae* Rrp44p also has nuclease activity, GST-fusions of Rrp44p were purified from *E. coli* and incubated with several RNA substrates. Here, the data indicate that the PIN domain of yeast Rrp44p has Mn^{2+} -dependent endoribonuclease activity that is not sequence specific. Additionally, the PIN domain is needed for viability. Data also suggest that while yeast cells require at least one of the nuclease activities of Rrp44p to survive, either activity is sufficient for viability. Lastly, the exoribonuclease, but not endoribonuclease, activity of Rrp44p is required for a functional nuclear exosome.

RESULTS

The PIN domain of Rrp44p has endoribonuclease activity.

Truncated Rrp44p, which includes the CR3 region and PIN domain (residues 1-235), was purified as a Glutathione S-transferase (GST) fusion from *E. coli*. Truncated Rrp44p was incubated individually with a ^{32}P 5'- and 3'-end labeled A_{30} oligonucleotide. Exonucleolytic degradation would yield labeled mononucleotides with only one of the substrates, whereas truncated Rrp44p showed a similar pattern of labeled oligonucleotides with both substrates (Schaeffer et al., 2009). This indicates that the PIN domain of Rrp44p acts as an endonuclease.

Truncated Rrp44p was also incubated individually with a ^{32}P 5'-end labeled U_{30} oligonucleotide and an *in vitro* transcribed, ^{32}P internally labeled 5' ETS substrate. Truncated Rrp44p was active on both, and on an A_{30} oligonucleotide, indicating that PIN domain endoribonuclease activity of Rrp44p is not sequence specific (Schaeffer et al., 2009).

Mutation of the third conserved acidic residue, Aspartate 171, abolished the endoribonuclease activity of Rrp44p (Lebreton et al., 2008; Schaeffer et al., 2009; Schneider et al., 2009), which has also been shown for other PIN domain-containing proteins (Bleichert et al., 2006; Fatica et al., 2004; Glavan et al., 2006).

The PIN domain of Rrp44p is needed for viability.

To determine whether the endoribonuclease activity of Rrp44p was needed for cell survival, C-terminal truncations of *RRP44* were generated. These mutants were transformed into an *rrp44* deletion strain that is complemented by full-length, wild-type *RRP44* on a plasmid with a *URA3* marker (*rrp44* Δ [*RRP44*, *URA3*]). Transformants were then spotted onto media containing 5-fluoroorotic acid (5-FOA) and grown for several days. Growth of the *rrp44* mutants was compared to two controls, empty vector and wild-type *RRP44* (Figure 3.1A). In cells that contain a wild-type *URA3* gene, the *URA3* gene product (orotidine-5'-monophosphate decarboxylase) converts 5-FOA to 5-fluorouracil, which is toxic to cells (Boeke et al., 1987). Since 5-FOA is used to select for cells that have lost the wild-type *RRP44*, *URA3* plasmid, only one mutant copy of *rrp44* remains in the cell. Therefore, growth on 5-FOA indicates that mutant Rrp44p can carry out the protein's essential function.

A C-terminal truncation that lacks the exoribonucleolytic RNB domain, but contains the CR3 region and PIN domain (residues 1-235), allowed for slow growth (Figure 3.1A)(Schaeffer et al., 2009). This result is not surprising given that it has been previously shown that a point mutant that inactivates the catalytic activity of the RNB domain, D551N, also has a slow growth phenotype (Amblar and Arraiano, 2005; Dziembowski et al., 2007). The slight growth observed

upon deletion of the RNB domain suggests that the D551N mutant is not viable only because of some low-level exonuclease activity *in vivo*. Instead, growth of the C-terminal truncation that lacks the RNB domain suggests that exonuclease activity is not crucial for the essential function of Rrp44p, or the exosome. A C-terminal truncation that lacks a portion of the PIN domain (residues 1-203) does not support viability (Figure 3.1A; Schaeffer et al., 2009). This lack of complementation is not due to a lack of expression as both C-terminal truncations are expressed (Figure 3.1B). This suggests that the PIN domain is required for viability, and thus, the essential function of Rrp44p.

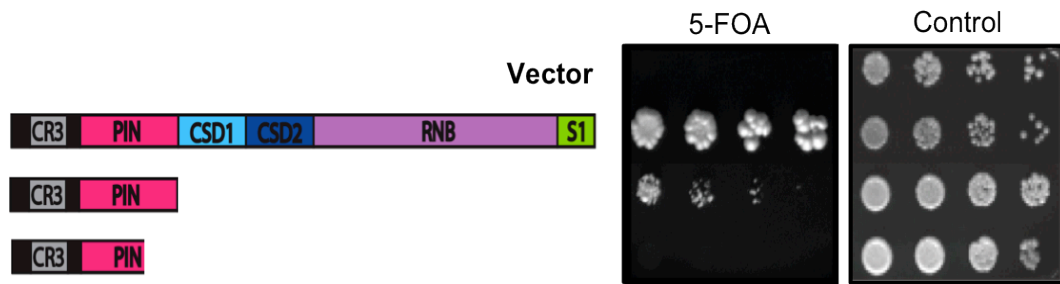
At least one nuclease activity of Rrp44p is needed for viability.

To determine whether the endonuclease activity of the PIN domain of Rrp44p is needed for viability, a catalytically inactive endonuclease point mutation (D171A) was generated and transformed into an *rrp44Δ* [*RRP44*, *URA3*] strain. Transformants were then spotted onto media containing 5-FOA and grown for several days. Growth of the D171A mutant was compared to empty vector, wild-type *RRP44* and the catalytically inactive exonuclease point mutant, D551N. The D171A mutant (Figure 3.2A), or mutants in the three other conserved acidic residues of the PIN domain (Figure 3.2B), had no obvious growth phenotype. In contrast, the D551N mutant had a slow growth phenotype (Figure 3.2A; Lebreton et al., 2008; Schaeffer et al., 2009; Schneider et al., 2009), which has been previously shown in mutants that inactivate Rrp44p exonuclease activity (Amblar and Arraiano, 2005; Dziembowski et al., 2007). These findings suggest that neither of the nuclease activities of Rrp44p is required for viability.

To determine whether cells require at least one nuclease activity of Rrp44p to survive, the double catalytically inactive point mutation (D171A, D551N) was generated and transformed into an *rrp44Δ* [*RRP44*, *URA3*] strain. Transformants were then spotted onto media containing 5-FOA and grown for several days. Growth of the D171A, D551N mutant was compared to empty vector, wild-type *RRP44*, the D171A mutant, and the D551N mutant. Combining the viable D171A mutant, or mutants in the other three conserved acidic residues of the PIN domain, with the D551N mutant resulted in no growth (Figure 3.2A and B; Lebreton et al., 2008; Schaeffer et al., 2009; Schneider et al., 2009). Similarly, combining the viable PIN mutations with a viable C-terminal truncation that lacks the exonucleolytic RNB domain, residues 1-324, also resulted in no growth (Figure 3.2C; Schaeffer et al., 2009). These results suggest that either nuclease active site is sufficient for viability, however, simultaneous inactivation of both of the catalytic sites results in a nonfunctional Rrp44p, and thus, no cell growth.

A.

rrp44 Δ [*RRP44*, *URA3*] +



B.

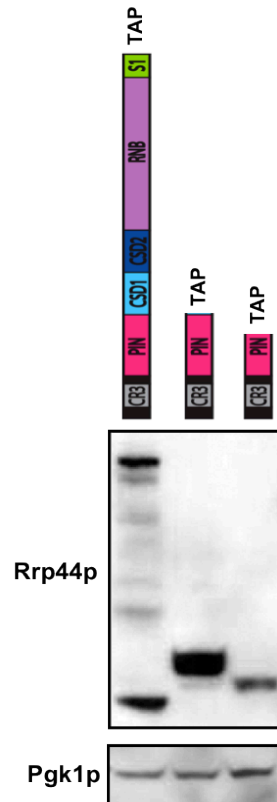
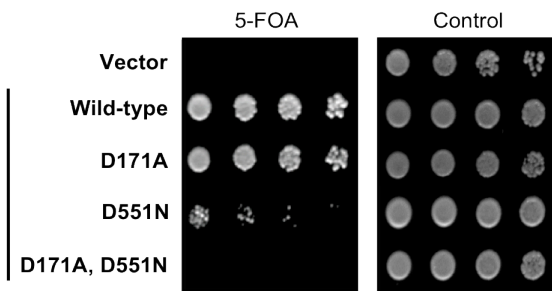


Figure 3.1: The PIN domain of Rrp44p is needed for viability.

Figure 3.1: The PIN domain of Rrp44p is needed for viability. **A,** An *rrp44* deletion strain complemented by full-length *RRP44* on a plasmid with a *URA3* marker was transformed with plasmids encoding each of the depicted truncations. Transformants were spotted onto media containing 5-fluoroorotic acid (5-FOA) and control media (SC –LEU–URA + 2% dextrose), and grown for several days. Growth of the *rrp44* mutants was compared to two control strains, empty vector and wild-type *RRP44*. Growth on 5-FOA indicates that truncated Rrp44p can carry out the essential function of Rrp44p. **B,** Whole-cell lysates were obtained from strains containing the C-terminally TAP-tagged Rrp44p truncations described in (A) and were analyzed by Western blot analysis with antibodies specific to Protein A to detect Rrp44p and to Pgk1p as a loading control. Expression of the Rrp44p truncated proteins was compared to wild-type Rrp44p.

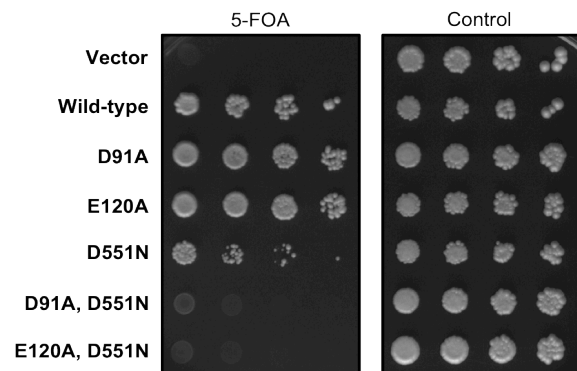
A.

rrp44 Δ [*RRP44*, *URA3*] +



B.

rrp44 Δ [*RRP44*, *URA3*] +



rrp44 Δ [*RRP44*, *URA3*] +

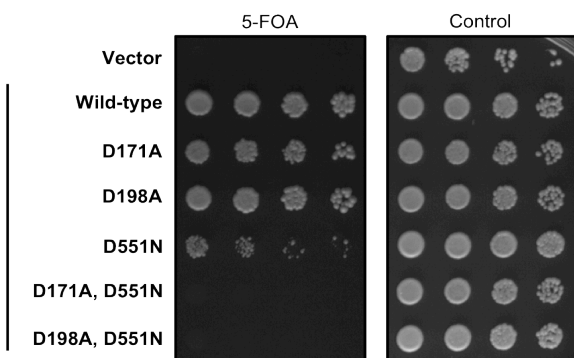


Figure 3.2: At least one nuclease activity of Rrp44p is needed for viability.

C.

*rrp44*Δ [*RRP44*, *URA3*] +

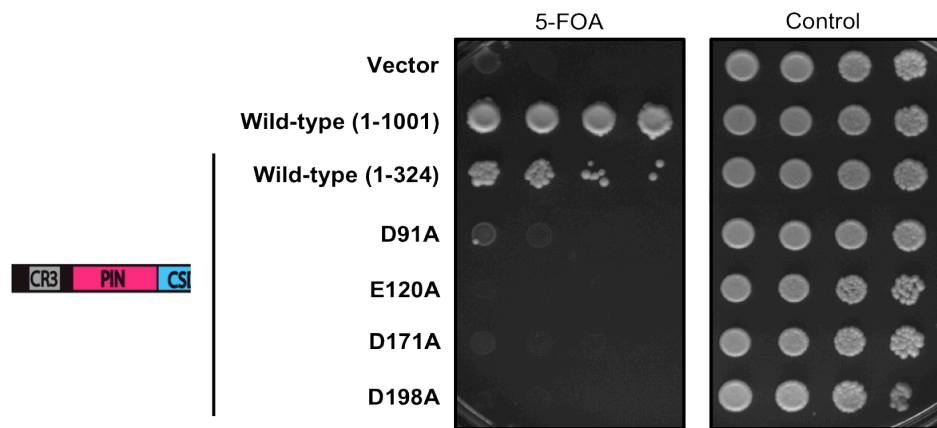


Figure 3.2: At least one nuclease activity of Rrp44p is needed for viability.

Figure 3.2: At least one nuclease activity of Rrp44p is needed for viability. An *rrp44* deletion strain complemented by full-length *RRP44* on a plasmid with a *URA3* marker was transformed with plasmids encoding the depicted mutations. Transformants were spotted onto media containing 5-FOA and control media (SC –LEU–URA + 2% dextrose), and grown for several days. Growth of the *rrp44* mutants was compared to two controls, empty vector and wild-type *RRP44*. Growth on 5-FOA indicates that mutated Rrp44p can carry out the essential function of the Rrp44p.

The exoribonuclease activity of Rrp44p is needed for both the RNA processing and RNA degradation activities of the nuclear exosome.

The nuclear exosome processes the 7S rRNA precursor to form the mature 5.8S rRNA and degrades the 5' external transcribed spacer (5' ETS) of the 35S rRNA precursor (Allmang et al., 1999a; Allmang et al., 1999b; Mitchell et al., 1997; Mitchell et al., 1996). In RNA processing, an RNA precursor is trimmed to form a mature, stable, small RNA. In RNA degradation, however, an RNA is cleaved many times to yield nucleotide monophosphates. This type of cleavage would require an efficient and processive enzyme that is able to degrade an RNA substrate to completion. Therefore, the endonuclease activity of the PIN domain of Rrp44p may be sufficient for nuclear exosome-mediated RNA processing, whereas the exonuclease activity of the RNB may be required for nuclear exosome-mediated RNA decay.

To test this hypothesis, several C-terminal truncations of *RRP44* were generated and transformed into a strain that contains endogenous *RRP44* under the control of the galactose promoter (*GAL::rrp44*). In the presence of galactose, endogenous *RRP44* is actively transcribed, however, transcription of *GAL::rrp44* is shut off in the presence of dextrose. Therefore, to inhibit expression of endogenous *RRP44*, these strains were grown in the presence of dextrose. RNA processing and degradation in the *rrp44* truncations was compared to two controls, a strain containing wild-type *RRP44* and an *rrp6* deletion strain. Rrp6p is a nuclear exosome cofactor and is required for nuclear RNA processing and degradation (Briggs et al., 1998). RNA was then isolated from these strains and subjected to denaturing urea-polyacrylamide gel electrophoresis. To determine if these strains were defective in RNA processing and/or RNA degradation, Northern blot analysis was performed using probes specific to the 7S rRNA precursor and to the 5' ETS, respectively. C-terminal truncations lacking the exonucleolytic RNB domain of Rrp44p caused the 7S precursor of the 5.8S rRNA to accumulate (Figure 3.3A, above) and caused a 20 to 24 fold increase in the accumulation of the 5' ETS (Figure 3.3A, middle; Lebreton et al., 2008; Schaeffer et al., 2009; Schneider et al., 2009). This suggests that the exonucleolytic RNB domain is needed for efficient nuclear RNA processing and degradation, but does not exclude the possibility that the endonuclease activity of Rrp44p is also required.

To test whether the endonuclease activity of the PIN domain is also needed for nuclear RNA processing and/or degradation, plasmids encoding the catalytically inactive endo- and exonuclease point mutations (D171A and D551N, respectively), as well as the double catalytically inactive point mutation (D171A, D551N) were transformed into *GAL::rrp44*. These strains were grown in the presence of galactose then shifted to media containing dextrose for the times indicated. Similar to the results obtained with the *rrp44* C-terminal truncations, 7S

processing and 5' ETS degradation intermediates accumulated in the D551N mutant and in the D171A, D551N mutant. There were no similar degradation or processing intermediates in the D171A mutant (Figure 3.B; Schaeffer et al., 2009; Schneider et al., 2009). Thus, both the RNA processing and degradation functions of the nuclear exosome require the exonuclease activity of the RNB domain of Rrp44p.

The exonuclease activity of Rrp44p may have some overlapping functions with the nuclear exosome cofactor, Rrp6p.

Rrp44p exonuclease activity and the nuclear exosome cofactor, Rrp6p, are involved in maintaining a functional nuclear exosome (Briggs et al., 1998; Dziembowski et al., 2007; Lebreton et al., 2008; Schaeffer et al., 2009; Schneider et al., 2009). To determine whether the endo- and/or exonuclease activities of Rrp44p were redundant with Rrp6p in the nucleus, the D171A and D551N point mutations were combined with an *rrp6* deletion. Briefly, plasmids encoding the D171A and D551N point mutations were transformed into an *rrp6* and *rrp44* deletion strain to generate the *rrp6*Δ D171A and *rrp6*Δ D551N strains, respectively. Because *RRP44* is essential, this strain is being complemented by wild-type *RRP44* on a plasmid with a *URA3* marker. Transformants were then spotted onto media containing 5-FOA and grown at three different temperatures (23°C, 30°C, 37°C) for several days. 5-FOA selects for cells that had lost the wild-type *RRP44*, *URA3* plasmid, therefore, the D171A and D551N point mutations were the only copies of *RRP44* in the cell. Although this particular *rrp6* deletion strain can grow at temperatures up to 37°C, its optimal growth temperature is at 25°C (Schneider et al., 2009). Additionally, other *rrp6* deletion strains are temperature-sensitive (Allmang et al., 1999b). Growth of the *rrp6*Δ D171A and *rrp6*Δ D551N strains at all three temperatures was compared to two controls, an *rrp6* and *rrp44* deletion strain (*rrp6*Δ *rrp44*Δ) and an *rrp6* deletion strain. The D551N mutant was synthetically sick with an *rrp6* deletion at all three temperatures, with the most severe growth defect at 37°C, whereas the *rrp6*Δ D171A strain had no observable growth defect (Figure 3.4). This suggests that the exonuclease activity of Rrp44p may have some overlapping functions with Rrp6p.

GAL::rrp44 +

Construct	Lane	Fold increase (7S pre-rRNA)	Fold increase (5' ETS)	Fold increase (SCR1)
CR3	1	1	1	1
PIN	2	8	11	-
CSD1	3	7	24	-
CSD2	4	8	23	-
RNB	5	6	20	-
S1	6	8	5	-
<i>rrp6Δ</i>	7	-	-	-

		GAL::rrp44 +															
		rrp6Δ				D171A				D171A D551N				D551N			
		WT Rrp44															
Time (h)		24	0	12	24	0	12	24	48	0	12	24	48	0	12	24	48
7S pre-rRNA																	
5.8S rRNA																	
5' ETS																	
SCR1																	

50

C.

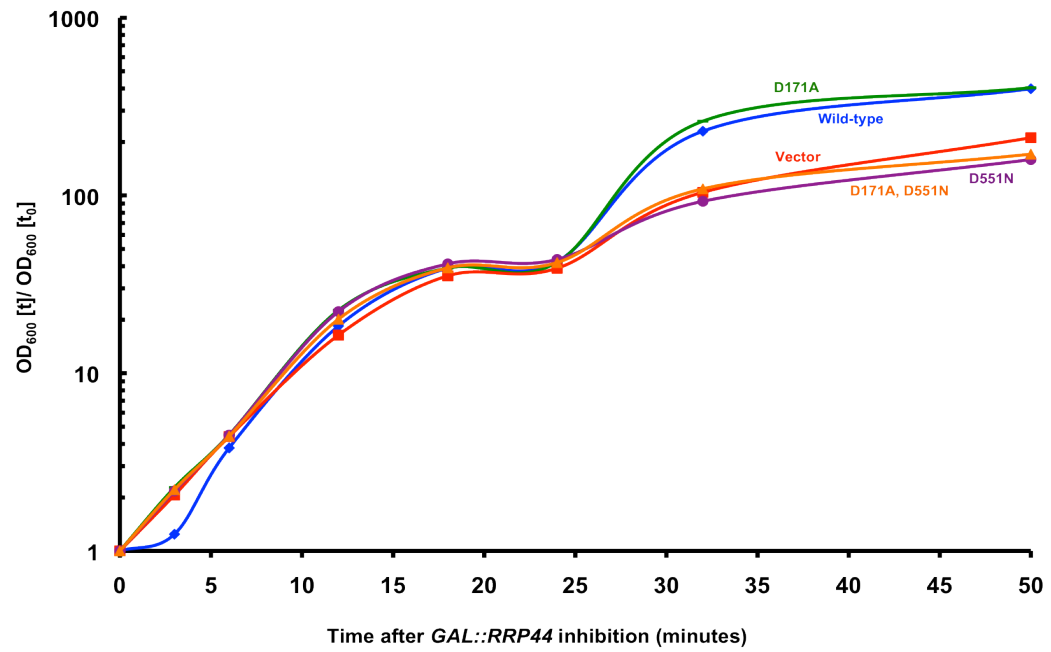


Figure 3.3: The exonuclease activity of Rrp44p is needed for both the RNA processing and RNA degradation activities of the nuclear exosome.

Figure 3.3: The exonuclease activity of Rrp44p is needed for both the RNA processing and RNA degradation activities of the nuclear exosome. **A**, The indicated Rrp44p truncations were expressed in a *GAL::rrp44* strain. RNA was isolated from cultures grown in dextrose (to inhibit expression of the *GAL::rrp44* gene) and analyzed by Northern blot analysis with probes that hybridize to the 7S precursor of 5.8S rRNA (above), the 5' ETS of the rRNA (middle) and the RNA subunit of the signal recognition particle (*SCR1*; below). The relative levels of the 7S pre-rRNA and the 5' ETS were normalized for loading using the *SCR1* signal and are indicated under the above and middle blots. **B**, *rrp44* alleles containing the D171A mutation, the D551N mutation or the D171A D551N double mutation was introduced into a *GAL::rrp44* strain. Cultures were first grown in media containing galactose and then incubated in media containing dextrose for the indicated time (in hours). RNA was isolated from these cultures and analyzed by Northern blot analysis with probes that hybridize to the 5.8S rRNA (above), the 5' ETS of the rRNA (middle) and *SCR1* (below). In both (**A**) and (**B**), RNA processing and RNA degradation in the *rrp44* mutants was compared to two control strains, wild-type *RRP44* and an *rrp6* deletion. **C**, A growth analysis of the strains described (**B**). The strains were grown in selective glucose media at 30°C to analyze growth.

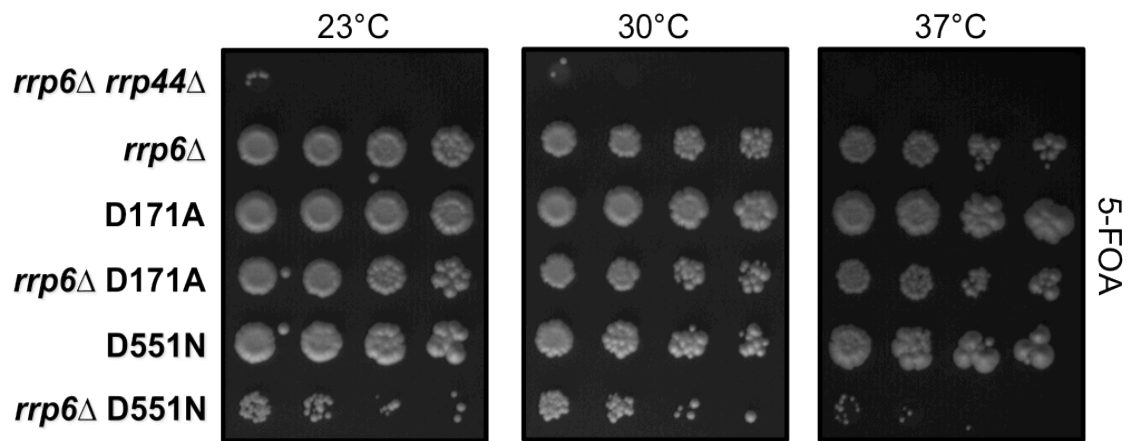


Figure 3.4: The exonuclease activity of Rrp44p may have some overlapping functions with the nuclear exosome cofactor, Rrp6p.

Figure 3.4: The exonuclease activity of Rrp44p may have some overlapping functions with the nuclear exosome cofactor, Rrp6p. An *rrp44* deletion strain or an *rrp6* and *rrp44* deletion strain was transformed with plasmids encoding each of the depicted mutations. Transformants were spotted onto media containing 5-FOA and grown for several days at the indicated temperatures. Growth of the *rrp6* and *rrp44* mutants was compared to two controls, an *rrp6* and *rrp44* deletion strain and an *rrp6* deletion strain. Growth at 37°C would suggest that the nuclease activities of Rrp44p are not redundant with Rrp6p.

DISCUSSION

Implications of two nuclease active sites in the catalytic subunit of the exosome

The data presented in this Chapter were published as the first report of a second nuclease active site in eukaryotic Rrp44p. Thirteen years after the discovery of the exosome (Mitchell et al., 1997), this breakthrough finding has revealed that it is possible for the exosome to cleave RNA *internally* as well as from the 3'-end.

It was widely accepted that Rrp44p had only 3' to 5' exoribonuclease activity. There are at least two reasons why the catalytically inactive exonuclease mutant, D551N, had no nuclease activity *in vitro*. First, the previously reported Rrp44p nuclease assays were performed using conditions that are favorable for 3' to 5' exonuclease activity (Tris buffer supplemented with Mg^{2+} ; Dziembowski et al., 2007; Liu et al., 2006; Lorentzen et al., 2008). When a C-terminal Rrp44p truncation that lacks the exonucleolytic RNB domain is tested in this buffer combination, there is no observable endonuclease activity (Schaeffer et al., 2009). Second, robust endonuclease activity was observed using truncated Rrp44p proteins, with much less activity seen with full-length Rrp44p (Schaeffer et al., 2009). This may suggest that the C-terminal domains of Rrp44p regulate the activity of the PIN domain. Nonetheless, the endonuclease activity of truncated Rrp44p, under conditions that are favorable for PIN domain activity (HEPES buffer supplemented with Mn^{2+}), is similar to the exonuclease activity of full-length Rrp44p under conditions that are amenable to RNB domain activity (Schaeffer et al., 2009). Therefore, although endonuclease activity had not been previously identified, it is robust and physiologically relevant under certain conditions and/or on RNA substrates that have not yet been determined.

The presence of two nuclease active sites in Rrp44p may indicate that the endo- and exonucleolytic domains act on different RNA substrates. Under this hypothesis, the endonucleolytic PIN domain may act on previously uncharacterized substrates, meaning that there may be more exosome substrates than previously thought. Additionally, the Rrp44p endonuclease may be responsible for an endonucleolytic cleavage event in an RNA processing and/or RNA degradation pathway where the endonuclease has not yet been identified. Alternatively, the presence of two catalytic sites may increase the overall efficiency of RNA processing and/or RNA degradation. Under this hypothesis, the endo- and exonuclease activities of Rrp44p do not have separate substrates, but rather can act together on most or all exosome substrates.

Combining RNases into a single RNA degradation machine is not unique in nature. In the *E. coli* degradosome, the RNase E endoribonuclease is combined with the 3' to 5'

exoribonuclease activity of PNPase (Carpousis et al., 1994; Py et al., 1994). In metazoan processing bodies (P-bodies), the Argonaute endoribonuclease is combined with several exoribonucleolytic deadenylation factors and the 5' to 3' exoribonuclease activity of Xrn1p (Andrei et al., 2005; Bashkirov et al., 1997; Cougot et al., 2004; Ingelfinger et al., 2002; Liu et al., 2005; Zheng et al., 2008). Because this combination has evolved multiple times independently, it likely offers a fundamental advantage to the cell.

The nuclease activities of Rrp44p may be functionally redundant.

The results shown in this Chapter also indicate that while cells require at least one nuclease activity of Rrp44p to survive, either is sufficient. This suggests that the Rrp44p endonuclease is active and is important *in vivo*. Additionally, this suggests that these nuclease activities are redundant for the essential function of Rrp44p. Such functional redundancy is not an unprecedented theme for RNases, and is similar to what is found in Rrp44p and Rrp6p in yeast (Dziembowski et al., 2007), the 5' to 3' exoribonuclease, Xrn1p, and the exosome in yeast (Anderson and Parker, 1998; Johnson and Kolodner, 1995), and several exoribonucleases in *E. coli* (Andrade et al., 2009; Deutscher and Li, 2001).

Although either nuclease activity is sufficient for viability, a C-terminal truncation lacking a portion of the endonucleolytic PIN domain cannot support growth. This result may indicate that the PIN domain, in addition to having nuclease activity, may also serve as a platform for crucial protein-protein interactions. The C-terminal region of the PIN domain (residues 203-235) may be needed for interaction with the core exosome and/or for interactions with a previously unidentified exosome cofactor. In support of this, it has been recently shown that the PIN domain of Rrp44p is important for exosome interaction (Schneider et al., 2009). Alternatively, this region may be needed to make critical contacts with an RNA substrate.

Rrp44p exoribonuclease activity and the nuclear exosome cofactor, Rrp6p, are needed for a functional nuclear exosome.

Lastly, the data presented here indicate that the exonuclease activity of Rrp44p is needed for a functional nuclear exosome, namely the RNA processing of the 7S precursor to form the mature 5.8S rRNA and the degradation of the 5' ETS. However, it was recently published that the endonuclease activity of Rrp44p is needed for 5' ETS degradation (Lebreton et al., 2008). This observation may be explained by the use of a different parental strain (W303) from what was used in this work (BY4741). Additionally, another study, in which the BY4741 strain was

used, confirmed the results presented here (Schneider et al., 2009). Therefore, the Rrp44p endonuclease may behave differently in different strain backgrounds.

The results shown here indicate that the exonuclease activities of Rrp44p and Rrp6p may have some overlapping functions. It is important to note that while the D551N mutant was synthetically sick with an *rrp6* deletion in this study, a previous study found that abolishing the exonuclease activity of Rrp44p in an *rrp6* deletion strain did not support viability. This observation can be explained by the use of a different parental strain (W303) from what was used in this work (BY4741). In support of the work presented here, another study, in which the BY4741 strain was used, confirmed the results shown here- the *rrp6* Δ D551N strain had a slow growth phenotype when compared to a wild-type strain, an *rrp6* deletion, and the D551N mutant (Schneider et al., 2009). Therefore, the Rrp44p and Rrp6p exonucleases seem to behave differently in different strain backgrounds. Accordingly, recent data suggest that the functional interactions between Rrp44p and Rrp6p are important for only certain Rrp6p substrates (Callahan and Butler, 2008).

The involvement of the Rrp44p endonuclease in other nuclear RNA processing and degradation reactions has not yet been tested. The possibility exists that the endonuclease activity of Rrp44p is needed for the processing of snRNA and/or snoRNA precursors. The Rrp44p endonuclease may also be involved in one of the multiple nuclear RNA surveillance pathways needed to degrade abnormally processed rRNA, tRNA, snRNA, snoRNA, and/or mRNA (see Chapter 7).

**Chapter 4: The exoribonuclease activity of Rrp44p is required for
exosome-mediated mRNA decay.**

INTRODUCTION

The eukaryotic exosome is a ten subunit complex that is involved in multiple RNA processing and RNA degradation reactions in both the nucleus and cytoplasm. The cytoplasmic exosome is strictly involved in mRNA degradation, where it degrades two types of transcripts. In one pathway, the exosome degrades normal transcripts to allow the cell to rapidly change mRNA levels, and ultimately protein levels, in response to a particular signal (Anderson and Parker, 1998). In mRNA surveillance, the exosome degrades aberrant transcripts in order to maintain the fidelity of gene expression. These types of aberrant mRNAs include host transcripts that lack termination codons (Frischmeyer et al., 2002; van Hoof et al., 2002) or poly(A) tails (Meaux and van Hoof, 2006), host transcripts that stall translation elongation (Doma and Parker, 2006) or contain premature termination codons (Gatfield and Izaurralde, 2004; Mitchell and Tollervey, 2003; Takahashi et al., 2003), and viral mRNAs (Guo et al., 2007).

In eukaryotes, normal transcripts are degraded by one of two pathways (Figure 1.2). In both cases, deadenylation, or removal of the poly(A) tail is the initiating and rate-limiting step (Muhlrad and Parker, 1992; Shyu et al., 1991). Degradation of the poly(A) tail triggers 5' to 3' mRNA decay in yeast. In this pathway, the 5' cap is removed by the decapping complex, Dcp1p and Dcp2, which then allows the 5' to 3' exoribonuclease, Xrn1p, to degrade the transcript from the 5'-end (Decker and Parker, 1993; Hsu and Stevens, 1993; Muhlrad et al., 1994). Following deadenylation, mRNA can also be degraded from the 3'-end, a process catalyzed by the exosome (Anderson and Parker, 1998; Mukherjee et al., 2002; Rodgers et al., 2002). Inactivation of both mRNA decay pathways results in loss of viability, underscoring the importance of mRNA turnover (Anderson and Parker, 1998).

In cytoplasmic mRNA surveillance pathways, the exosome degrades aberrant transcripts. One example of an mRNA surveillance pathway is the degradation of mRNAs that lack termination codons, termed nonstop transcripts, in the nonstop mRNA degradation pathway (Figure 1.3; Frischmeyer et al., 2002; van Hoof et al., 2002). Nonstop transcripts can arise from mistakes in gene expression, including premature polyadenylation, due to inaccurate 3'-end formation or through the use of a cryptic polyadenylation site (Bucheli et al., 2007). In the current nonstop decay model, the translating ribosome reads through the poly(A) tail and stalls at the 3'-end. The cytoplasmic exosome cofactor, Ski7p, is then believed to recognize the ribosome with an empty A-site and recruit the exosome to rapidly exonucleolytically degrade the transcript from the 3'-end (van Hoof et al., 2002).

Transcripts that stall translation elongation, perhaps due to secondary structure, are also degraded by the cytoplasmic exosome in an RNA surveillance pathway termed no-go decay

(Figure 1.4 and Figure 4.6). In the current model of no-go decay, a ribosome that stalls during translation elongation is recognized by two paralogs of translation release factors, Hbs1p and Dom34p. The transcript is then endonucleolytically cleaved near the stalled ribosome, releasing a 5'- and 3'-degradation fragment. The 5'-fragment is degraded by the cytoplasmic exosome, whereas the 3'-degradation fragment is degraded by the 5' to 3' exoribonuclease, Xrn1p, in the 5' to 3' mRNA degradation pathway (Doma and Parker, 2006). The endoribonuclease responsible for the initial cleavage event, and freeing of the ribosome, has not yet been identified.

The exosome subunit believed to be involved in these cytoplasmic mRNA degradation reactions is Rrp44p, as it is the only catalytically active subunit (Dziembowski et al., 2007). Specifically, the exonuclease activity of Rrp44p may be involved in general 3' to 5' mRNA degradation, nonstop mRNA decay, and in the degradation of the 5'-fragment in no-go decay. Additionally, the endonuclease activity of Rrp44p may be needed for the initial cleavage event in no-go decay. To determine whether the nuclease activities of Rrp44p are involved in these cytoplasmic mRNA degradation reactions, strains containing C-terminal truncations and catalytically inactive point mutations of Rrp44p were generated. The degradation of several representative reporter transcripts was then analyzed in these mutants. Here, the data indicate that the exonucleolytic RNB domain is required for exosome-mediated 3' to 5' mRNA decay. Interestingly, the data also suggest that neither the endo- or exonuclease activities of Rrp44p are needed for the degradation of nonstop transcripts and that the endonuclease activity of Rrp44p is not required for endonucleolytic cleavage in no-go decay. Additionally, the results suggest that the endonuclease activity of Rrp44p is not redundant with Ski7p in nonstop decay.

RESULTS

The exonuclease activity of Rrp44p is needed for exosome-mediated 3' to 5' mRNA decay.

To determine whether the endo- and/or exonuclease activities of Rrp44p were needed for general 3' to 5' decay, a synthetic lethality growth assay was performed (van Hoof et al., 2000b). The basis of this assay is that at least one of the two mRNA decay pathways is required for viability. Therefore, mutants in the 3' to 5' decay pathway are synthetically lethal with mutants in the 5' to 3' decay pathway (Anderson and Parker, 1998). Briefly, plasmids encoding the individual catalytically inactive Rrp44p nuclease point mutations, D171A and D551N, were transformed into an *rrp44* deletion strain that contains a temperature-sensitive allele of *dcp1* (*dcp1-2*), generating the *dcp1-2* D171A and *dcp1-2* D551N strains. Dcp1p is a subunit of the decapping complex needed for 5' to 3' mRNA decay (Coller and Parker, 2004). These strains also contain a plasmid encoding wild-type *RRP44* and *URA3* marker. Transformants were then spotted onto media containing 5-FOA to select for cells that had lost the wild-type *RRP44*, *URA3* plasmid. Therefore, the D171A and D551N point mutations were the only copies of *RRP44* in the cell. The *dcp1-2* D171A and *dcp1-2* D551N strains were then spotted onto selection media and grown at three different temperatures (23°C, 30°C, 37°C) for several days. Growth of these strains at the non-permissive temperature for *dcp1-2* (37°C) was compared to two control strains. The first control was a *ski7* deletion strain that also contained the *dcp1-2* allele (*dcp1-2 ski7Δ*). Ski7p is a cytoplasmic exosome cofactor that is required for general 3' to 5' decay (Araki et al., 2001; van Hoof et al., 2000b). The second control strain contained only the *dcp1-2* mutant. While the *dcp1-2* D171A strain had no detectable growth phenotype, the D551N mutant was synthetically lethal with *dcp1-2* at the non-permissive temperature (Figure 4.1A). In support of this finding, it has also been shown that deletion of Xrn1p, the 5' to 3' exoribonuclease, is also synthetically lethal with the D551N mutant (Schneider et al., 2009). Combined, these data suggest that the exonuclease activity, but not endonuclease activity, of Rrp44p is needed for exosome-mediated 3' to 5' mRNA decay.

To further test the importance of Rrp44p exonuclease activity in general 3' to 5' mRNA decay, the stability of an endogenous mRNA, *GAL7*, was measured. *GAL7* is a galactose metabolism gene and is expressed in media containing galactose. The degradation of the *GAL7* mRNA reporter was measured in the *dcp1-2* D171A and *dcp1-2* D551N strains. *GAL7* stability in these strains was compared to two control strains, *dcp1-2* and *dcp1-2 ski7Δ*. Briefly, these strains were grown in liquid media containing galactose at the permissive temperature for *dcp1-2* (23°C) and then shifted to the non-permissive temperature (37°C) for one hour. The strains were then grown in media containing dextrose to shut off transcription of *GAL7*. RNA was isolated

from these strains and subject to agarose formaldehyde gel electrophoresis. Northern blot analysis was then performed using probes specific to *GAL7*, and *SCR1* as a loading control. In the *dcp1-2* strain, *GAL7* was unstable since the 3' to 5' decay pathway was still functional. Conversely, in the *dcp1-2 ski7Δ* strain, *GAL7* was more than 4-times more stable, as neither the 5' to 3' or 3' to 5' mRNA decay pathway was functional. *GAL7* was unstable in the *dcp1-2* D171A strain, however, *GAL7* was 2-times more stable in the *dcp1-2* D551N strain as compared to the *dcp1-2* strain (Figure 4.1B and C). Although the exonuclease mutant stabilizes *GAL7*, there is some residual mRNA decay of the reporter in the *dcp1-2* D551N strain. This may be explained by incomplete inactivation of the *dcp1-2* allele, residual activity of the D551N mutant, or the presence of an exonuclease that is redundant with Rrp44p. Overall, these results suggest that, similar to the data obtained in the synthetic lethality growth assay, the exonuclease activity of Rrp44p is needed for the general 3' to 5' decay activity of the cytoplasmic exosome.

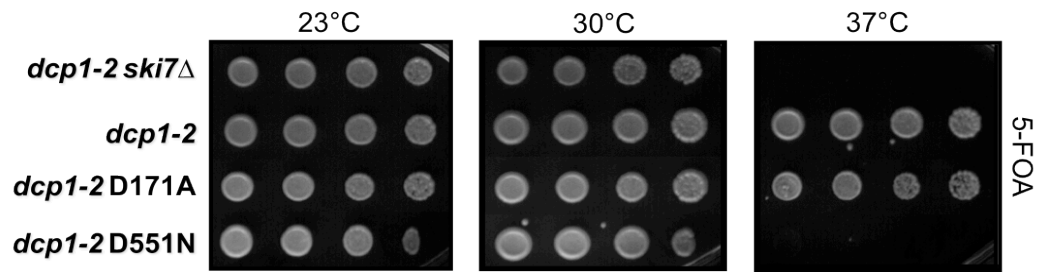
Neither of the nuclease activities of Rrp44p are required to degrade nonstop transcripts.

To determine whether the exonuclease activity of Rrp44p is required to degrade nonstop mRNAs, the stability of a representative nonstop transcript was measured in a strain that lacks the exonucleolytic RNB domain of Rrp44p. Briefly, the *GAL::pgk1pG*-nonstop reporter was transformed into an *rrp44* deletion strain that is complemented with a C-terminal truncation of Rrp44p that lacks the RNB domain.

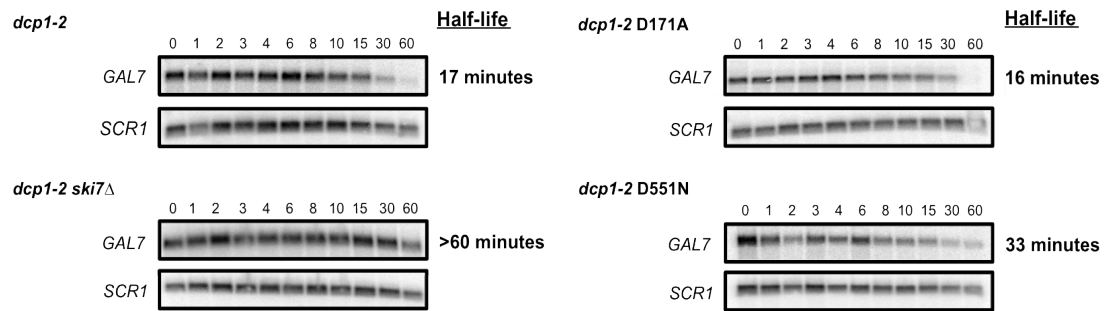
This reporter contains a nonstop mutation in the *PGK1* (3-phosphoglycerate kinase) open reading frame and is a known substrate of the nonstop decay pathway (van Hoof et al., 2002). Because this reporter is under the control of the galactose promoter, the *pgk1pG*-nonstop transcript is expressed in media containing galactose, however, transcription is rapidly shut off in media containing dextrose. This reporter also has a poly-guanine tract (pG) in order to differentiate between the stability of the reporter and the endogenous transcript.

The stability of the nonstop transcript in the *rrp44* deletion strain that lacks the RNB domain was compared to two control strains, a strain containing wild-type Rrp44p and a *ski7* deletion strain. These strains were grown in liquid media containing galactose and then shifted to media containing dextrose to shut off transcription of the *GAL::pgk1pG*-nonstop reporter. RNA was isolated from these strains and subject to agarose formaldehyde gel electrophoresis. Northern blot analysis was then performed using probes specific to *pgk1pG*, and to *SCR1* as a loading control. In the wild-type strain, *GAL::pgk1pG*-nonstop was unstable since the nonstop mRNA decay pathway was still functional. Conversely, when the nonstop decay pathway was inactivated in the *ski7* deletion strain, the nonstop transcript was 7-times more stable. In the

A.



B.



C.

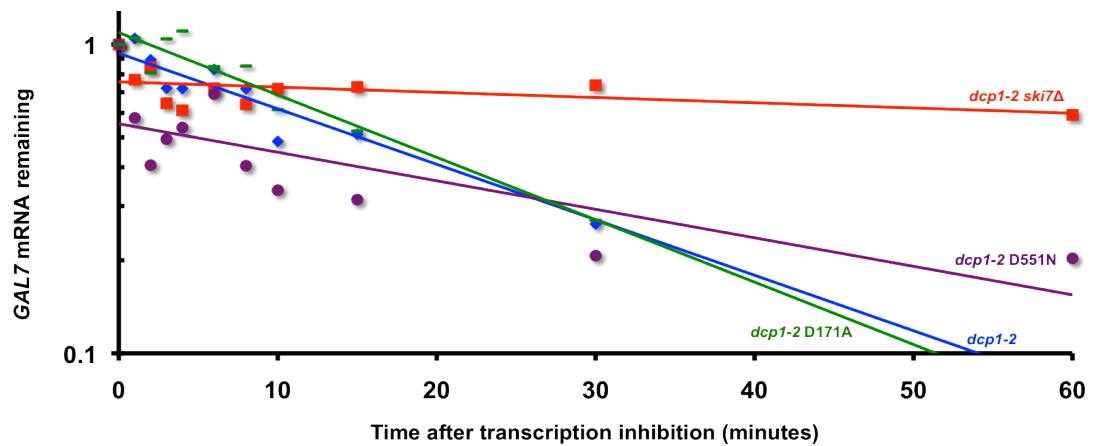


Figure 4.1: The exonuclease activity of Rrp44p is needed for exosome-mediated 3' to 5' mRNA degradation.

Figure 4.1: The exonuclease activity of Rrp44p is needed for exosome-mediated 3' to 5' mRNA degradation. **A,** An *rrp44* deletion strain containing a plasmid encoding wild-type *RRP44* and a *URA3* marker, as well as a temperature-sensitive allele of *dcp1* (*dcp1-2*), was transformed with plasmids encoding the depicted mutations. Transformants were spotted onto media containing 5-FOA and grown for several days at the indicated temperatures. Growth of the *dcp1-2 rrp44* mutants was compared to two control strains, a *ski7* deletion strain that also contains the *dcp1-2* allele (*dcp1-2 ski7Δ*) and a strain containing only the *dcp1-2* mutant. Growth on 5-FOA at 37°C, the non-permissive temperature of the *dcp1-2* allele, indicates that mutated Rrp44p can carry out exosome-mediated 3' to 5' mRNA degradation. **B,** RNA was isolated from each strain described in (A) and analyzed by Northern blot analysis with probes that hybridize to *GAL7* and *SCR1*. The half-life *GAL7* was normalized for loading using the *SCR1* signal and are as indicated (in minutes). **C,** A graphical representation of the half-life of the *GAL7* transcript.

strain that lacked the RNB domain of Rrp44p, *GAL::pgk1pG*-nonstop was as unstable as it was in the wild-type strain (Figure 4.2A and B), which suggests that exonucleolytic RNB domain of Rrp44p is not needed for the degradation of nonstop transcripts.

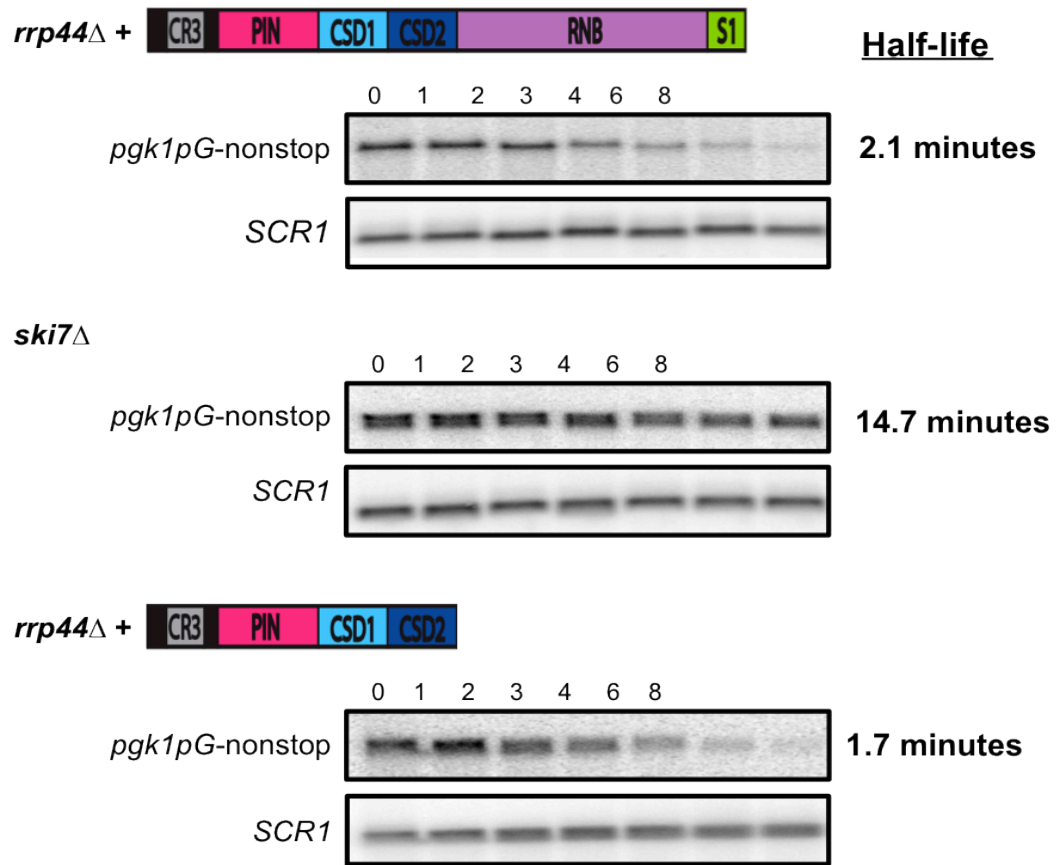
In order to determine whether the endonuclease activity of the PIN domain is needed for nonstop decay, the *his3*-nonstop growth assay was performed. Briefly, another representative nonstop reporter, *his3*-nonstop, was transformed into *rrp44* deletion strains that were individually complemented with the D171A and D551N mutants. The *his3*-nonstop growth assay is counter-intuitive in that in strains that have defects in nonstop decay, the *his3*-nonstop reporter is stable, which allows cells to grow on media lacking histidine. In contrast, cells that have a functional nonstop decay pathway rapidly degrade the *his3*-nonstop transcript, resulting in a lack of growth on media lacking histidine (van Hoof et al., 2002). The growth of the catalytically inactive point mutants on media lacking histidine was compared to two controls, a wild-type strain and a *ski7* deletion strain. Similar to the wild-type strain, both of the catalytically inactive point mutants were viable on media lacking histidine (Figure 4.3A). These results suggest that neither of the nuclease activities of Rrp44p are involved in degrading nonstop transcripts.

The *his3*-nonstop growth assay is an indirect measure of whether the nonstop decay pathway is functional. A more direct way to test for a functional nonstop decay pathway is to measure the stability of a representative nonstop mRNA. Therefore, the stability of the *GAL::pgk1pG*-nonstop transcript was measured in strains containing the catalytically inactive point mutants of Rrp44p. The stability of the nonstop transcript in the *rrp44* point mutants was compared to two controls, a strain containing wild-type Rrp44p and a *ski7* deletion strain. These strains were grown in liquid media containing galactose and then shifted to media containing dextrose to shut off transcription of the *GAL::pgk1pG*-nonstop reporter. RNA was isolated from these strains and subject to agarose formaldehyde gel electrophoresis. Northern blot analysis was then performed using probes specific to *pgk1pG*, and to *ACT1* as a loading control. Similar to what was observed in the *his3*-nonstop growth assay, the *GAL::pgk1pG*-nonstop transcript was as unstable in both of the catalytically inactive point mutants as it was in the wild-type strain (Figure 4.3B and C). Combined, these results indicate that neither of the nuclease activities of Rrp44p are needed for the degradation of nonstop transcripts.

The endonuclease activity of Rrp44p is not redundant with Ski7p in nonstop mRNA degradation.

In the current model of nonstop mRNA degradation, the C-terminus of Ski7p recognizes the ribosome with an empty A-site and recruits the exosome to rapidly degrade the nonstop

A.



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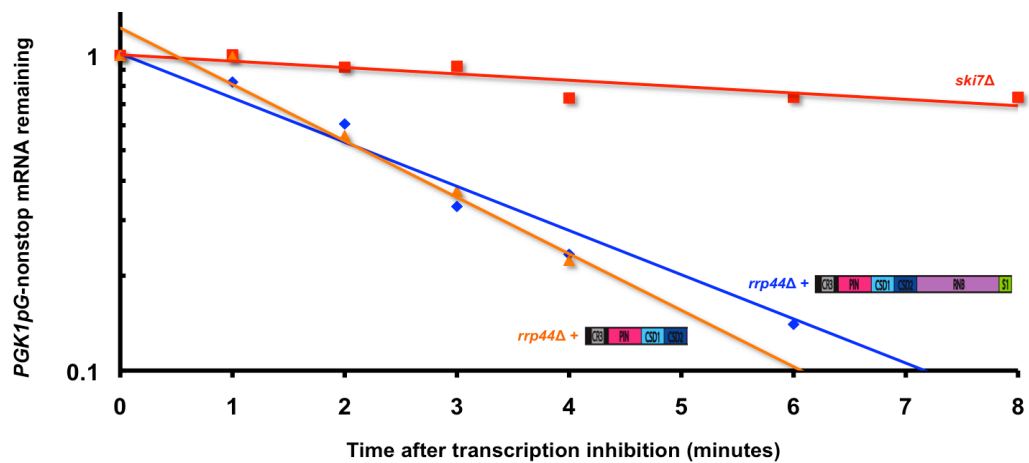
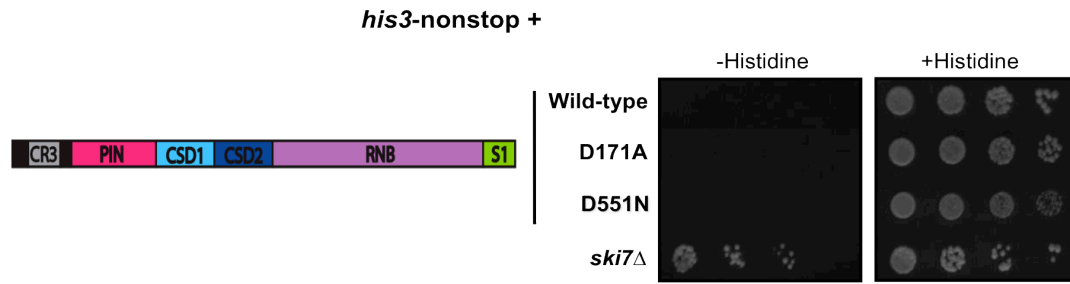


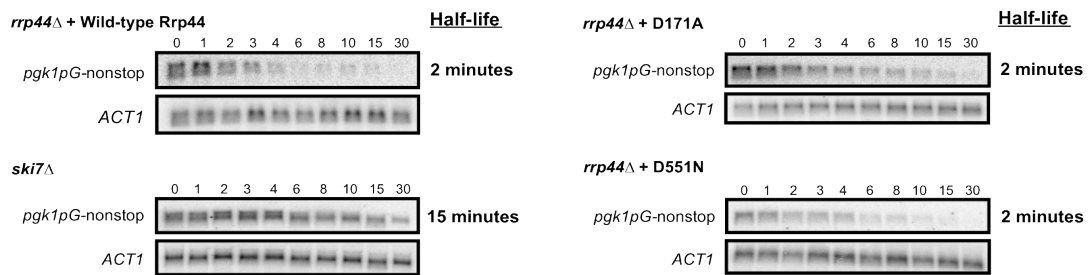
Figure 4.2: The exonuclease activity of Rrp44p is not needed for nonstop mRNA degradation.

Figure 4.2: The exonuclease activity of Rrp44p is not needed for nonstop mRNA degradation. **A,** An *rrp44* deletion strain complemented by a plasmid encoding a C-terminal truncation of Rrp44p that lacks the exonucleolytic RNB domain was transformed with the *GAL::pgk1pG*-nonstop reporter. The stability of the transcript in the *rrp44* mutant strain was compared to two control strains, wild-type *RRP44* and a *ski7* deletion. Cultures were first grown in media containing galactose and then incubated in media containing dextrose (to inhibit transcription of the *GAL::pgk1pG*-nonstop reporter) for the indicated time (in minutes). RNA was isolated from these strains and analyzed by Northern blot analysis with probes that hybridize to *PGK1* and *SCR1*. The half-life of the *GAL::pgk1pG*-nonstop transcript was normalized for loading using the *SCR1* signal and are as indicated (in minutes). **B,** A graphical representation of the half-life of the *GAL::pgk1pG*-nonstop transcript.

A.



B.



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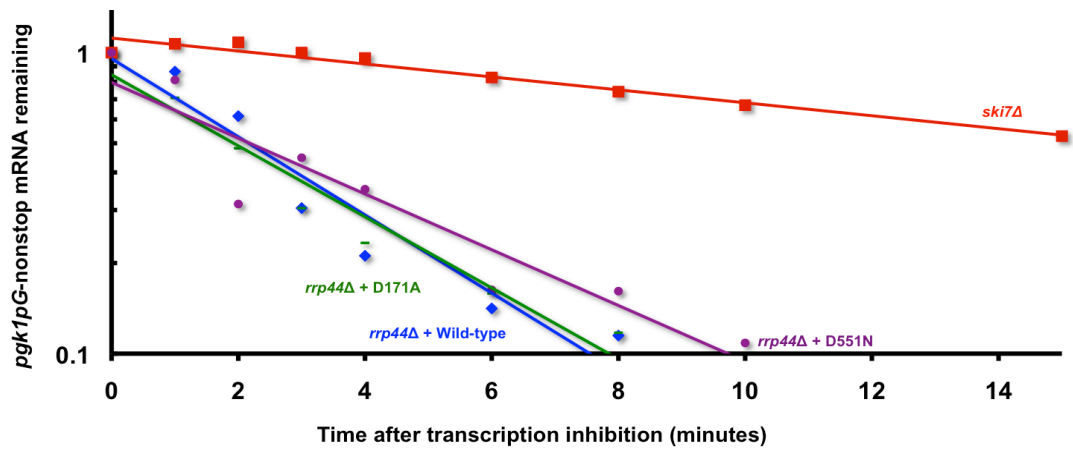


Figure 4.3: Neither of the nuclease activities of Rrp44p are needed to degrade nonstop transcripts.

Figure 4.3: Neither of the nuclease activities of Rrp44p are needed to degrade nonstop transcripts. **A**, An *rrp44* deletion strain containing the D171A mutant or the D551N mutant was transformed with a *his3*-nonstop reporter. Transformants were spotted onto media containing, or lacking, histidine and grown for several days. Growth of the *rrp44* mutants was compared to two control strains, wild-type *RRP44* and a *ski7* deletion. Growth on media lacking histidine indicates that the nonstop mRNA degradation machinery is not functional. **B**, An *rrp44* deletion strain complemented by a plasmid encoding the D171A mutation or the D551N mutation was transformed with the *GAL::pgk1pG*-nonstop reporter. The stability of the transcript in the *rrp44* mutant strains was compared to two control strains, wild-type *RRP44* and a *ski7* deletion. Cultures were first grown in media containing galactose and then incubated in media containing dextrose (to inhibit transcription of the *GAL::pgk1pG*-nonstop reporter) for the indicated time (in minutes). RNA was isolated from these strains and analyzed by Northern blot analysis with probes that hybridize to *PGK1* and *ACT1*. The half-life of the *GAL::pgk1pG*-nonstop transcript was normalized for loading using the *ACT1* signal and are as indicated. **C**, A graphical representation of the half-life of the *GAL::pgk1pG*-nonstop transcript.

transcript from the 3'-end (Figure 1.3; van Hoof et al., 2002). One unexpected issue with this model is that the 3'-end of the mRNA should be inaccessible to the exosome because it is presumably buried inside of the ribosome. One solution to this problem may be that Ski7p triggers ribosome disassembly. The C-terminus of Ski7p is similar to eRF3, and eRF1 and eRF3 are thought to trigger ribosome disassembly at termination codons. Therefore, the C-terminus of Ski7p may be needed to remove the stalled ribosome to allow the nonstop transcript to be degraded. Consistent with this idea, a C-terminal truncation of Ski7p that lacks the region homologous to eRF3 partially inhibits nonstop decay (van Hoof et al., 2002). This partial inhibition suggests that ribosome disassembly by Ski7p is one way, although not the only way, a stalled ribosome can be removed from a nonstop transcript. A second solution may be that the Rrp44p endonuclease cleaves the transcript near the ribosome, allowing for the 3'-end of the mRNA to be accessible. A third solution is a combination of the two, where either the C-terminus of Ski7p or the endonuclease activity of Rrp44p remove the ribosome (Figure 4.4). This third model predicts that a strain containing a C-terminal truncation of Ski7p and the catalytically inactive endonuclease point mutant of Rrp44p, D171A, would completely block nonstop decay.

To test whether the C-terminus of Ski7p and the endonuclease activity of Rrp44p were redundant in nonstop mRNA decay, a *his3*-nonstop growth assay was performed. In this assay, a representative nonstop reporter, *his3*-nonstop, was transformed into a strain containing a C-terminal truncation of Ski7p (as the only copy of Ski7p) and the D171A mutant of Rrp44p (as the only copy of Rrp44p). This reporter contains a nonstop mutation in the *HIS3* open reading frame and is a known substrate of the nonstop decay pathway. The basis for this assay is that in a strain that is defective in nonstop mRNA decay, the *his3*-nonstop reporter will be stable, thus allowing cells to synthesize histidine and grow on media lacking histidine. In contrast, in a wild-type strain, where the nonstop decay machinery is functional, the reporter will be rapidly degraded and cells will be inviable on media lacking histidine (van Hoof et al., 2002). Growth of the strain containing a C-terminal truncation of Ski7p and the D171A mutant of Rrp44p on media lacking histidine was compared to several controls, a wild-type strain, a *ski7* deletion strain, a strain lacking the C-terminus of Ski7p, and a strain containing only the D171A mutant of Rrp44p. Since the C-terminus of Ski7p is involved in nonstop decay, the strain lacking the C-terminus of Ski7p was able to grow on media lacking histidine. Because the N-terminus of Ski7p is needed for general 3' to 5' mRNA decay, the *ski7* deletion strain grew even better on media lacking histidine, indicating a stronger defect in nonstop mRNA decay. The strain containing a C-terminal truncation of Ski7p and the D171A mutant had the same growth phenotype on media

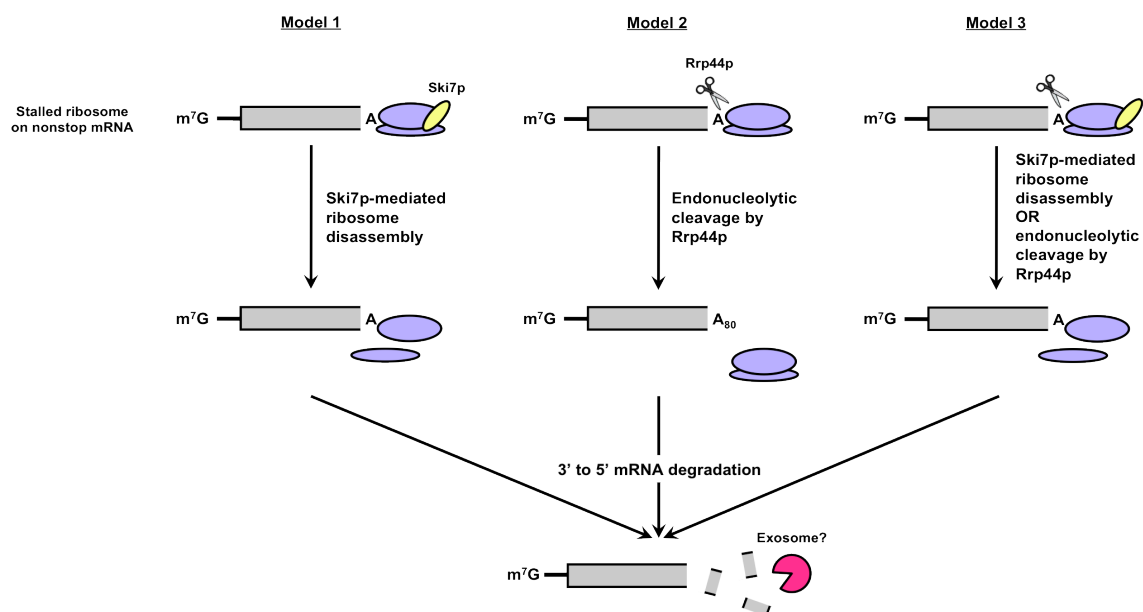


Figure 4.4: Schematic representation of ribosome disassembly in nonstop mRNA degradation.

Figure 4.4: Schematic representation of ribosome disassembly in nonstop mRNA

degradation. In the first model, the stalled ribosome is removed, and subsequently disassembled, by Ski7p. In the second model, endonucleolytic cleavage by Rrp44p removes the ribosome. In the third model, ribosome disassembly is mediated by both Ski7p and the endonuclease activity of Rrp44p, with either being sufficient.

lacking histidine as the strain containing a C-terminal truncation of Ski7p (Figure 4.5). This suggests that the endonuclease activity of Rrp44p is not redundant with the C-terminus of Ski7p for ribosome disassembly in nonstop decay.

The Rrp44p endonuclease is not responsible for endonucleolytic cleavage in no-go decay.

To determine whether the endonuclease activity of Rrp44p is involved in the no-go decay pathway, the steady-state level of a representative no-go decay transcript was measured in strains that lack Rrp44p endonuclease activity. Because the endonucleolytic 5'- and 3'-cleavage fragments can only be detected in strains that lack a functional cytoplasmic exosome and 5' to 3' mRNA degradation pathway, respectively, a plasmid encoding the catalytically inactive endonuclease point mutant, D171A, was transformed into two different strains, a *ski7* and *rrp44* deletion strain and an *xrn1* and *rrp44* deletion strain to generate the *ski7*Δ D171A and *xrn1*Δ D171A strains, respectively. These strains also contain a plasmid that encodes wild-type *RRP44* and a *URA3* marker. Transformants were then spotted onto media containing 5-FOA and grown for several days to obtain cells that could lose the wild-type *RRP44*, *URA3* plasmid. Thus, the *ski7*Δ D171A and *xrn1*Δ D171A strains contained only one copy of Rrp44p. These strains were then transformed with the *GAL::pgk1*-SL reporter, and as a control, a plasmid encoding wild-type *GAL::PGK1*. The *GAL::pgk1*-SL reporter contains a stem loop in the *pgk1* (3-phosphoglycerate kinase) open reading frame, which creates a stable secondary structure that is known to stall translation elongation (Figure 4.6A; Doma and Parker, 2006).

As a control, degradation of the *pgk1*-SL transcript was analyzed in three control strains, each complemented by a plasmid encoding wild-type *RRP44*: an *rrp44* deletion strain (Wild-type), a *ski7* and *rrp44* deletion strain (*ski7*Δ), and an *xrn1* and *rrp44* deletion strain (*xrn1*Δ). Cultures of these strains were grown in media containing galactose and then RNA was isolated and subjected to agarose formaldehyde gel electrophoresis. Northern blot analysis was then performed using probes specific to the 5'-end of the *pgk1* open reading frame, the 3'-end of the *pgk1* open reading frame just downstream of the stem loop, and to *SCR1* as a loading control. If the Rrp44p endonuclease is responsible for endonucleolytic cleavage of the *pgk1*-SL transcript, the 5'- and 3'-degradation fragments should not be detectable in the *ski7*Δ D171A and *xrn1*Δ D171A strains, respectively. The data presented here, however, show that the 5'-fragment is present at the same levels in the *ski7*Δ D171A strain as in the *ski7*Δ control strain (Figure 4.6B). Similarly, the 3'-fragment is present in both the *xrn1*Δ and *xrn1*Δ D171A strains (Figure 4.6C).

The 3'-degradation fragment is also present in the wild-type strain (Figure 4.6C). This may suggest that the stem-loop structure can block Xrn1p. It is not yet known whether this

***his3*-nonstop +**

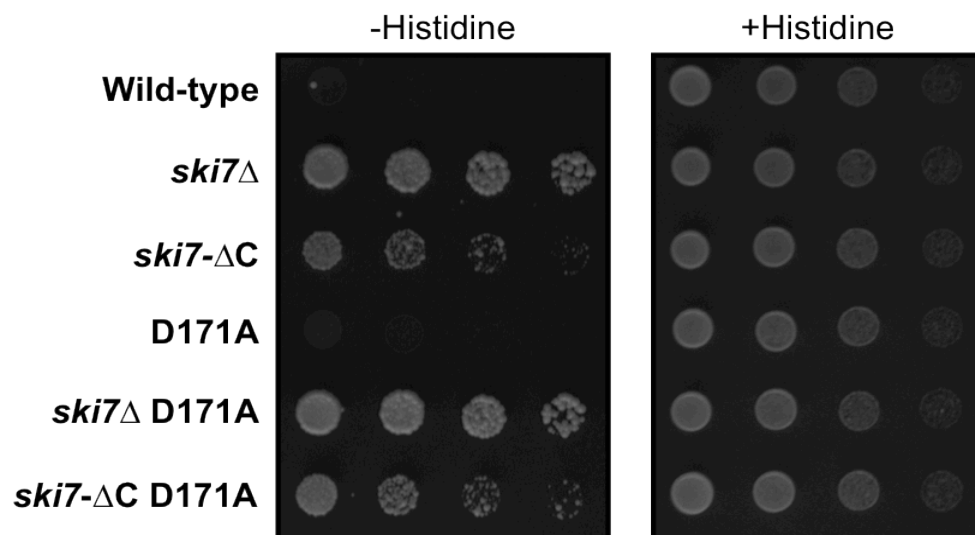


Figure 4.5: The endonuclease activity of Rrp44p is not redundant with Ski7p in nonstop mRNA degradation.

Figure 4.5: The endonuclease activity of Rrp44p is not redundant with Ski7p in nonstop mRNA degradation. A strain containing a C-terminal truncation of Ski7p and the D171A point mutant of Rrp44p was transformed with the *his3*-nonstop reporter. Transformants were spotted onto media containing, or lacking, histidine and grown for several days. Growth of the *ski7* and *rrp44* mutants was compared to several controls, a wild-type strain, a strain containing the D171A mutant, a strain lacking the C-terminus of Ski7p, and a *ski7* deletion strain. Growth on media lacking histidine indicates that the nonstop mRNA degradation machinery is not functional.

A.

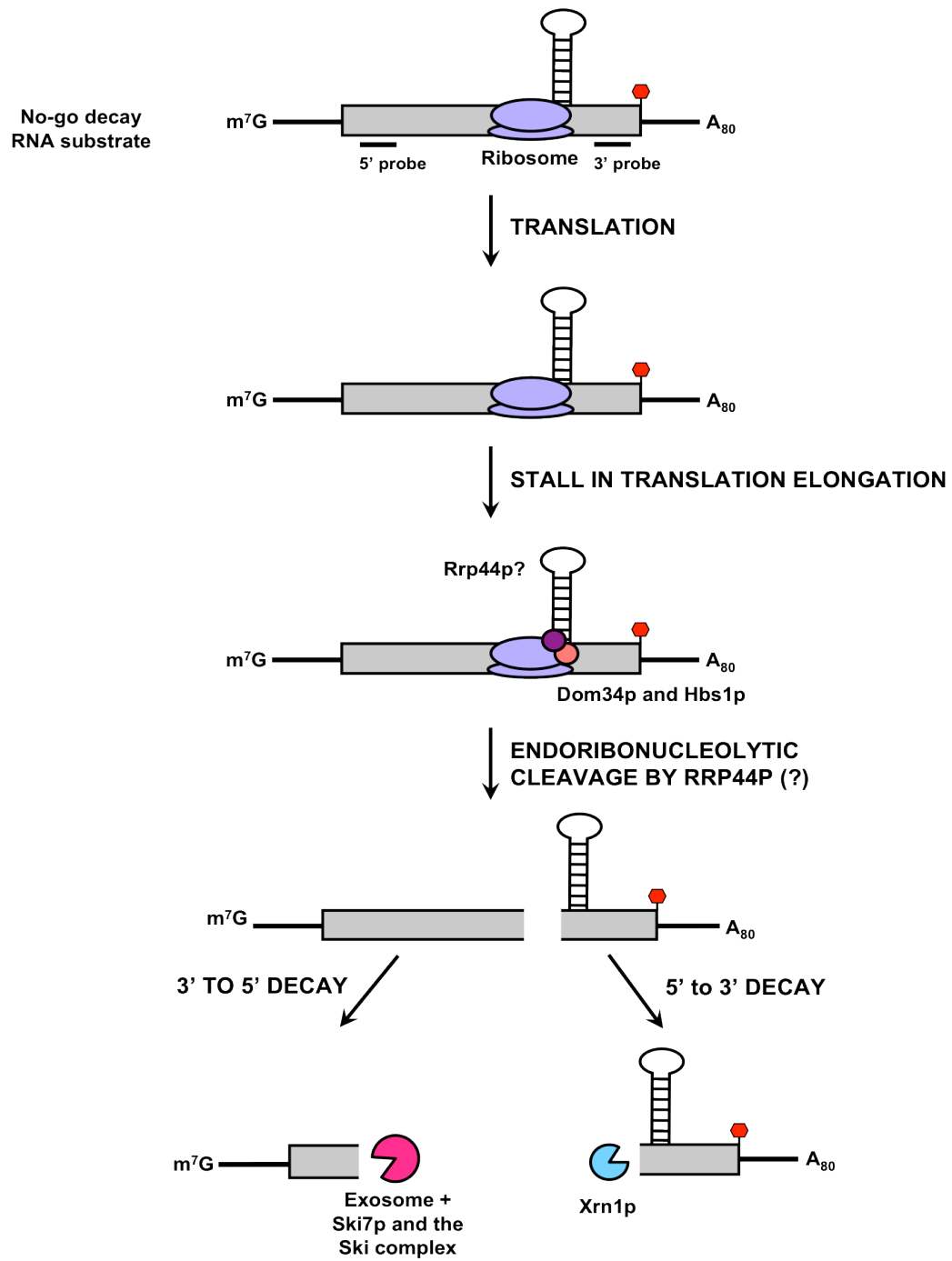
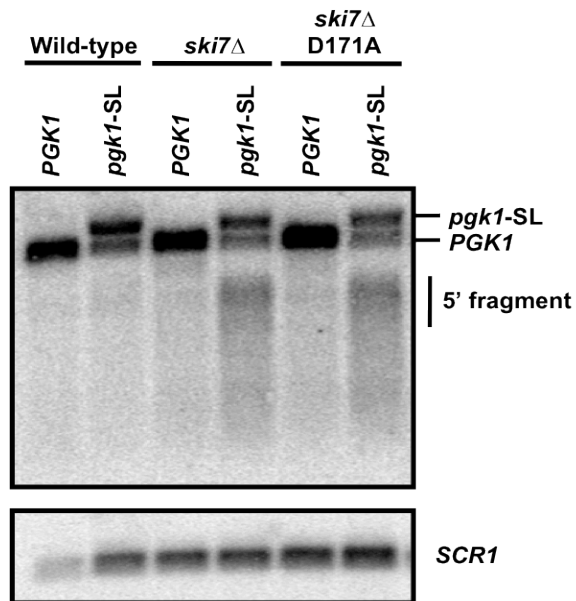


Figure 4.6: The Rrp44p endonuclease is not responsible for endonucleolytic cleavage in no-go decay.

B.



C.

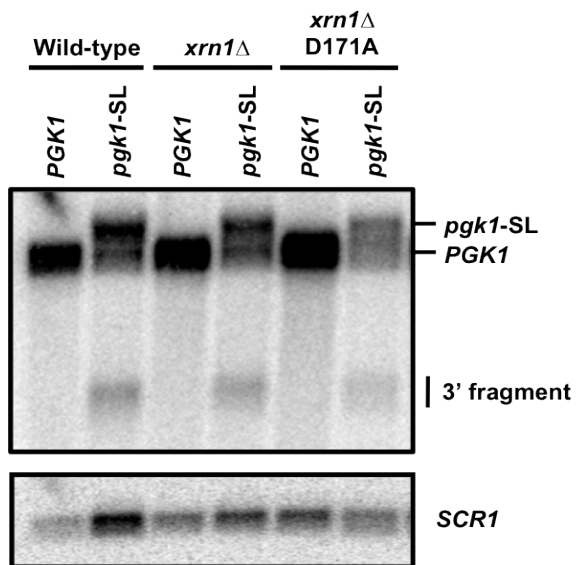


Figure 4.6: The Rrp44p endonuclease is not responsible for endonucleolytic cleavage in no-go decay.

Figure 4.6: The Rrp44p endonuclease is not responsible for endonucleolytic cleavage in no-go decay. **A**, In the current model of no-go decay, a translating ribosome stalls during translation elongation, possibly due to secondary structure in the transcript. The stalled ribosome is then thought to be recognized by the eRF3 and eRF1 paralogs, Hbs1p and Dom34p, respectively. The transcript is then endonucleolytically cleaved, possibly by the Rrp44p endonuclease, which releases the stalled ribosome and generates a 5'- and 3'-degradation fragment. The 5'- and 3'-degradation fragments are degraded by the cytoplasmic exosome and the 5' to 3' exoribonuclease, Xrn1p, respectively. **B** and **C**, A *ski7* Δ (**B**) and *xrn1* Δ (**C**) strain containing the *rrp44* D171A mutant were transformed with the *GAL::PGK1* and *GAL::pgk1*-SL reporters. The degradation of the *GAL::PGK1* and *GAL::pgk1*-SL transcripts in these strains were compared to three control strains, wild-type, a *ski7* Δ strain (**B**), and an *xrn1* Δ strain (**C**). RNA was isolated from cultures grown in galactose and analyzed by Northern blot analysis with probes that hybridize to the regions 5'- (**B**) or 3'- (**C**) of the stem-loop stall site, as illustrated (**A**).

degradation fragment arises by decapping followed by 5' to 3' degradation by Xrn1p, or by endonucleolytic cleavage. Despite the presence of this fragment in the wild-type strain, the 3' degradation fragment detected in the *xrn1* Δ strain must be produced by endonucleolytic cleavage, which suggests that at least some of the 3'-degradation fragment detected in the wild-type strain is produced by endonucleolytic cleavage.

The presence of the 5'- and 3'-degradation fragments in the *ski7* Δ D171A and *xrn1* Δ D171A strains suggest that the endonuclease activity of Rrp44p is not needed for the initial endonucleolytic cleavage event in no-go decay. These results, however, do not exclude the possibility that the exonuclease activity of Rrp44p is responsible for complete degradation of the 5'-degradation fragment.

DISCUSSION

The data presented in this Chapter analyze the roles of the nuclease activities of Rrp44p in the cytoplasmic exosome. Here, the exonuclease activity of Rrp44p is shown to be responsible for exosome-mediated 3' to 5' mRNA decay. This finding is supported by a previous study that showed that the D551N mutant of Rrp44p is synthetically lethal with a deletion of Xrn1p, the exoribonuclease involved in the 5' to 3' mRNA decay pathway (Schneider et al., 2009). Therefore, these data indicate that mRNA turnover is an essential process that is mediated by at least two key exoribonucleases, and that the endonuclease activity of Rrp44p contributes little to the degradation of normal transcripts.

The data presented here also show that the nuclease activities of Rrp44p are not needed for nonstop mRNA decay and that the endonuclease activity of Rrp44p is not needed for no-go decay.

Implications for nonstop mRNA degradation

The data shown here indicate that neither the endo- or exonuclease activities of Rrp44p are involved in degrading nonstop transcripts. This observation could be explained by one of three possibilities. First, the Rrp44p nucleases may be redundant in nonstop decay. This idea is difficult to test, however, because the double catalytically inactive point mutant (D171A, D551N) is not viable. Therefore, alternative means must be used to test whether nonstop transcripts are degraded when both the endo- and exonuclease activities of Rrp44p are abolished (see Chapter 7).

A second explanation is that nonstop decay is independent of the known catalytic activities of the cytoplasmic exosome. Under this hypothesis, a different nuclease would be responsible for the degradation of nonstop transcripts.

Lastly, a small fraction of the nuclear-localized exosome cofactor, Rrp6p, may associate with the cytoplasmic exosome to degrade nonstop transcripts. In this case, a small amount of Rrp6p activity may be enough for a functional nonstop mRNA decay pathway. In support of this, the human homolog of Rrp6p has been shown to localize to the nucleus and the cytoplasm in human cell lines (Lejeune et al., 2003). Additionally, Rrp6p has been shown to interact with the cytoplasmic exosome cofactor, Ski7p (Collins et al., 2007; Peng et al., 2003). In this explanation, however, the 3'-end of the nonstop transcript would still be buried in the ribosome. In this model, an unknown endonuclease may cleave the nonstop transcript near the ribosome, allowing the 3'-end to be accessible to Rrp6p.

The finding that the endonuclease activity of Rrp44p is not redundant with the C-terminus of Ski7p for ribosome disassembly suggests that either Ski7p functions alone or that another protein functions with Ski7p to free stalled ribosomes from a nonstop transcript. Because a C-terminal truncation of Ski7p only partially inhibits nonstop decay (van Hoof et al., 2002), it is more likely that another protein or endoribonuclease work with Ski7p in ribosome disassembly.

As will be discussed in the next Chapter, a different mutation in Rrp44p does stabilize nonstop reporter transcripts (see Figure 5.7). Thus, Rrp44p is required for nonstop mRNA degradation, and the most likely explanation is that the endo- and exonuclease activities of Rrp44p are redundant for nonstop decay (see Chapter 7).

Chapter 5: The CR3 region, a putative zinc binding site of Rrp44p, is important for exosome activity.

NOTE: I performed all of the experiments described in this Chapter with the exception of the biochemical nuclease assay that was performed by our collaborators, Filipa Reis and Dr. Cecilia Arraiano (Instituto de Tecnologia Química e Biológica [ITQB]; Lisbon, Portugal) – as denoted in the figure legend

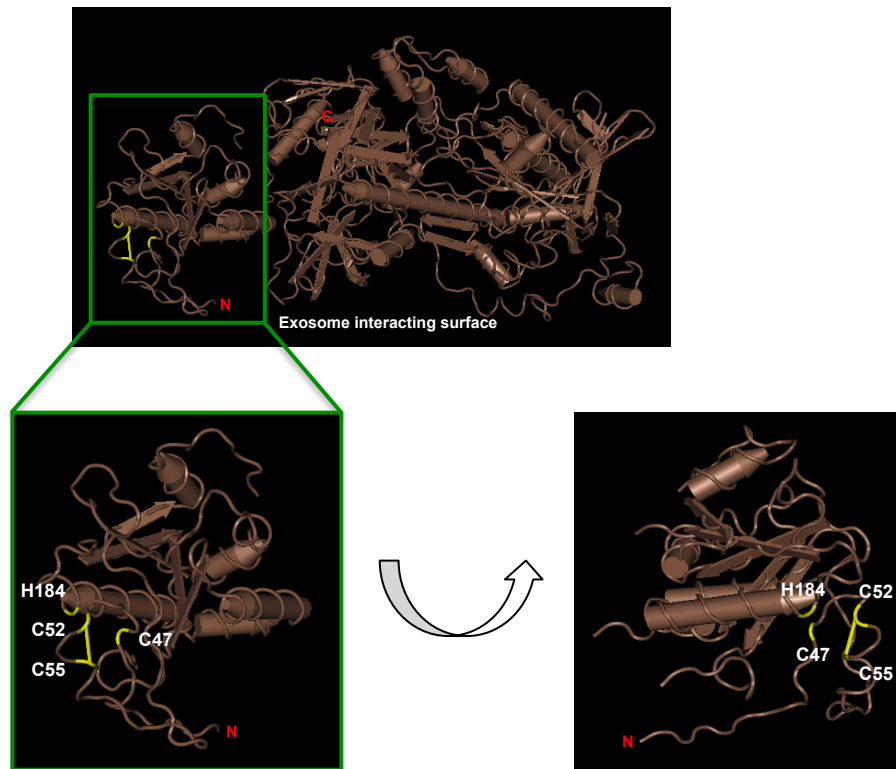
INTRODUCTION

Rrp44p is the catalytic subunit of the exosome and has two nuclease active sites. The PIN domain of Rrp44p has endonuclease activity and the RNB domain has 3' to 5' exonuclease activity. In addition to these two domains, a CR3 region is in the N-terminus and three putative RNA binding domains are spread throughout the protein. The CR3 region consists of three conserved cysteine residues (C47, C52, and C55) and is present in most eukaryotic homologs of Rrp44p that have a PIN domain. Currently, no information exists on the CR3 region in Rrp44p or in any other protein. Although it has been proposed that this region is important for protein-protein interactions (Ponting et al., 2000), no data exist to support this.

Recently the crystal structure of Rrp44p was published, however, the first 33 residues of the protein were not included (Bonneau et al., 2009). Therefore, it is not yet known whether the CR3 region consists of more than the three cysteine residues or if this region has a unique fold. For this reason, the three cysteine residues will be referred to as only the CR3 region. Further examination of the crystal structure of Rrp44p shows that two of the three cysteines are modeled to form a disulfide bond (C52 and C55; Figure 5.1A and B; Bonneau et al., 2009). However, because of the low resolution of the structure (3 Angstroms), the electron density is not defined enough to determine which residues, if any, form a disulfide bond. Another striking feature of the structure is that the three cysteines of the CR3 region are in close proximity to histidine 184 (H184; Figure 5.1A and B). This spatial organization is consistent with the coordination of a zinc ion, which may be needed, directly or indirectly, for protein stabilization, interaction with the exosome, and/or for Rrp44p nuclease activity. Indeed preliminary evidence suggests that Rrp44p endonuclease activity is stimulated by the addition of zinc (Filipa Reis and Dr. Cecilia Arraiano, personal communication). Interestingly, H184 is present in all eukaryotic homologs of Rrp44p that have a CR3 region.

Several studies have shown multiple interactions between Rrp44p and the exosome. In the first report, yeast two-hybrid data suggested an interaction between the CR3 region and the PIN domain of human Rrp44p with human Rrp43p, a subunit of the PH ring in the exosome (Lehner and Sanderson, 2004). In another study, a cryo-EM structure of the yeast exosome predicted that the CR3 region and the PIN domain of Rrp44p interact with Rrp41p, another subunit of the PH ring. This study also predicted major contacts between the cold shock domains (CSDs) of Rrp44p, and to a lesser extent the RNB domain, with Rrp45p, another PH ring subunit (Wang et al., 2007). Lastly, the most recent study suggests that the PIN domain of Rrp44p mediates interaction with the core exosome (Schneider et al., 2009). It is not yet known whether this region consists of only the PIN domain or includes both the CR3 region and PIN domain.

A.



B.

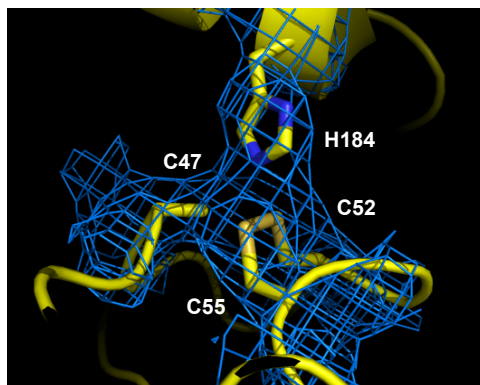


Figure 5.1: The three cysteines of the CR3 region and histidine 184 (H184) of Rrp44p have the proper geometry for the coordination of a zinc atom.

Figure 5.1: The three cysteines of the CR3 region and histidine 184 (H184) of Rrp44p have the proper geometry for the coordination of a zinc atom. **A,** Side view of the *S. cerevisiae* Rrp44p structure (Bonneau et al., 2009). The N- and C-termini are shown in red. The three cysteines of the CR3 region (C47, C52, C55) and H184 are shown in yellow. A disulfide bond is shown between C52 and C55 of the CR3 region. The zoomed-in view shows only the N-terminus of Rrp44p and better depicts the spatial organization between the three cysteines of the CR3 region and H184. **B,** The electron density of the N-terminus of Rrp44p is shown in blue mesh. The three cysteines of the CR3 region and H184 are shown in yellow. The disulfide bond between C52 and C55 is shown in orange and the nitrogen atoms of H184 are shown in blue (Bonneau et al., 2009). This figure was created by Sean Johnson (Utah State University, Utah).

Combined these studies suggest that the CR3 region and PIN domain of Rrp44p are important for exosome interaction. More importantly, these studies suggest that multiple regions of Rrp44p are needed for Rrp44p to interact with the exosome.

To determine the role(s) of the CR3 region of Rrp44p in the exosome, a triple CR3 mutant was generated, in which the three cysteine residues of the CR3 region were mutated. This triple CR3 mutant was then used to assay the interaction between Rrp44p and the exosome as well as exosome function. The data presented here are the first report of a CR3 region being needed for the formation of a complex *in vivo* and for enzymatic activity *in vitro*. Specifically, the data suggest that the CR3 region is needed for optimal cell growth and is needed for Rrp44p to interact with the exosome. The data presented here also show that the CR3 region is needed for Rrp44p endonuclease activity and for a functional cytoplasmic exosome. Importantly, these results also indicate that the modeled disulfide bond between the second and third cysteine residue of the CR3 region is not needed for exosome function.

RESULTS

Mutating the three conserved cysteine residues of Rrp44p results in a slow growth phenotype.

To determine whether the CR3 region was needed for cell survival, a triple CR3 mutant was generated, where the three cysteine residues were mutated to serine (C47S, C52S, C55S). The CR3 mutant was then transformed into an *rrp44Δ* [*RRP44*, *URA3*] strain. Transformants were spotted onto media containing 5-FOA and grown for several days. Growth of the CR3 mutant was compared to two controls, empty vector and wild-type *RRP44*. As compared to wild-type, the triple CR3 mutant allowed for only slow growth (Figure 5.2A). This suggests that the CR3 region is needed for optimal growth, however, this phenotype could be due to decreased expression of the triple CR3 mutant protein (Figure 5.3).

The CR3 region is necessary, but may not be sufficient, for Rrp44p to interact with the exosome.

To determine whether the CR3 region of Rrp44p mediated interaction with the exosome, wild-type Rrp44p and the triple CR3 mutant were C-terminally TAP-tagged. Wild-type and mutant Rrp44p were purified using IgG sepharose beads specific to the Protein A portion of the TAP tag. Proteins bound to Rrp44p were eluted and analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Whole cell lysates were obtained from the Rrp44p strains and were compared to the bound fraction. The interaction of wild-type Rrp44p and the CR3 mutant with the exosome was compared to empty vector. Unlike wild-type Rrp44p, the triple CR3 mutant was unable to interact with the exosome, suggesting that this region is necessary for exosome interaction (Figure 5.3). Because the CR3 region alone was not tested for exosome interaction, it is not yet known whether this region is sufficient for Rrp44p to associate with exosome. Additionally, previous studies indicate that other regions of Rrp44p are also needed for exosome interaction (Lehner and Sanderson, 2004; Schneider et al., 2009; Wang et al., 2007).

The CR3 region is needed for efficient Rrp44p endonuclease activity *in vivo* and *in vitro*.

To determine whether the CR3 region was needed for the endonuclease activity of Rrp44p, the triple CR3 mutant was tested in three different assays. First, the triple CR3 mutant combined with the catalytically inactive exonuclease point mutant, D551N, was tested for complementation of an *rrp44* deletion strain. Briefly, a plasmid containing the triple CR3 and D551N mutations was transformed into an *rrp44Δ* [*RRP44*, *URA3*] strain. Transformants were

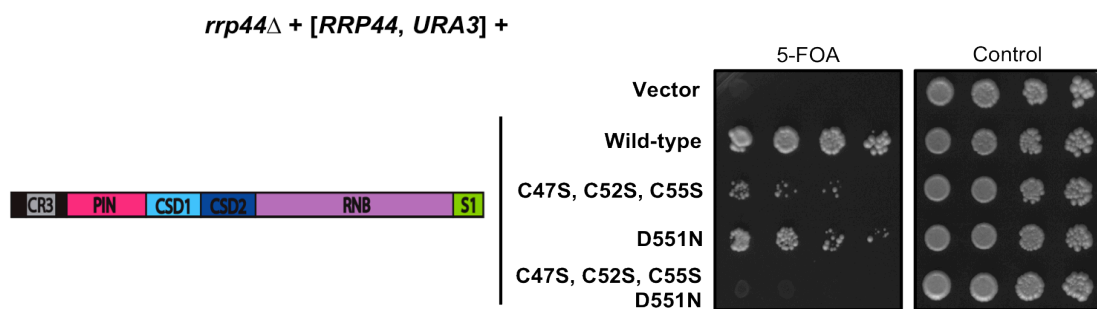


Figure 5.2: Mutating the three conserved cysteine residues of Rrp44p results in a slow growth phenotype.

Figure 5.2: Mutating the three conserved cysteine residues of Rrp44p results in a slow growth phenotype. An *rrp44* deletion strain complemented by full-length *RRP44* on a plasmid with a *URA3* marker was transformed with plasmid encoding the triple CR3 mutation. Transformants were spotted onto media containing 5-FOA and control media (SC –LEU–URA + 2% dextrose), and grown for several days. Growth of the triple CR3 mutant was compared to two controls, empty vector and wild-type *RRP44*. Growth on 5-FOA indicates that the CR3 region is not needed for the essential function of Rrp44p.

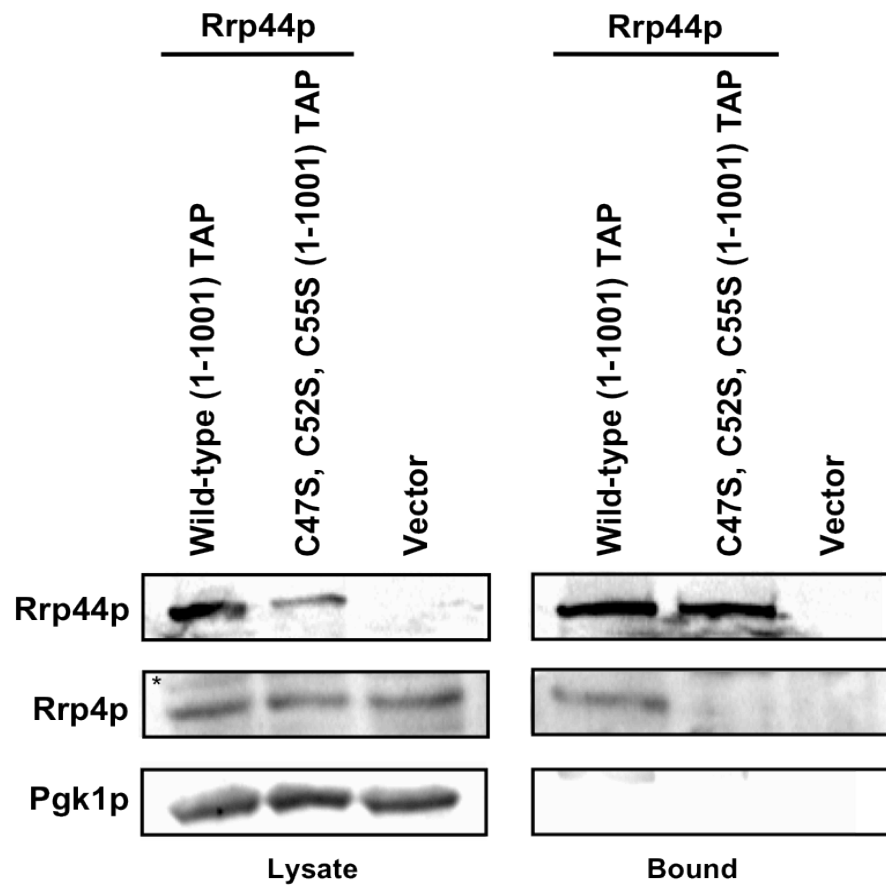


Figure 5.3: The CR3 region of Rrp44p is needed for interaction with the exosome.

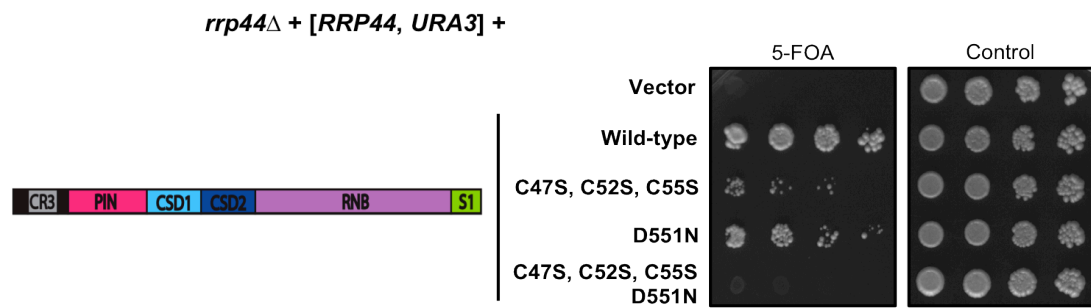
Figure 5.3: The CR3 region of Rrp44p is needed for interaction with the exosome. The C-terminal TAP-tagged triple CR3 mutant was purified using IgG sepharose beads. Prior to purification, whole-cell lysates were isolated (left). Purified proteins were eluted by boiling the beads (right). Interaction of the triple CR3 mutant with the exosome was compared to two controls, wild-type Rrp44p and empty vector. Whole-cell lysates and purified proteins were analyzed by Western blot analysis with antibodies specific to Protein A (above) to detect Rrp44p, Rrp4p (middle) to detect interaction with the exosome, and Pgk1p (below) as a loading control. The asterisk in the middle blot indicates a nonspecific protein.

spotted onto media containing 5-FOA and grown for several days. Growth of the CR3, D551N strain was compared to two controls, empty vector and wild-type *RRP44*. If the CR3 region was needed for Rrp44p endonuclease activity, the CR3 mutant should be synthetically lethal with D551N because cells lacking both the endo- and exonuclease activities of Rrp44p are not viable (Lebreton et al., 2008; Schaeffer et al., 2009; Schneider et al., 2009). Indeed, the CR3, D551N mutant did not support viability (Figure 5.4A). This suggests that the CR3 region may be needed for the endonuclease activity of Rrp44p.

To further test the genetic interaction between the CR3 region and the endonucleolytic PIN domain of Rrp44p, the triple CR3 mutation was generated in a C-terminal truncation of Rrp44p that lacks the exonucleolytic RNB domain. This truncation was also tested for complementation of an *rrp44Δ* [*RRP44*, *URA3*] strain. Again, if the CR3 region was needed for Rrp44p endonuclease activity, the CR3 mutant should be synthetically lethal with a truncation lacking the exoribonucleolytic domain because cells lacking both the endo- and exonuclease activities of Rrp44p are not viable (Lebreton et al., 2008; Schaeffer et al., 2009; Schneider et al., 2009). Similar to the results obtained with the CR3, D551N mutant, the CR3 truncation lacking exonuclease activity did not support viability (Figure 5.4B). This further suggests that the CR3 region is needed for Rrp44p endonuclease activity.

To more directly test the importance of the CR3 region in PIN domain nuclease activity, two mutant, truncated Rrp44p proteins were tested for endonuclease activity *in vitro*. In the first mutant, the CR3 mutation was again combined with a C-terminal truncation of Rrp44p that lacked the exonucleolytic RNB domain (residues 1-235). In the second truncation, both the CR3 region and the RNB domain were removed, leaving only the PIN domain (residues 84-235). Both of the Rrp44p truncated proteins were purified as GST fusions from *E. coli* and incubated individually with a ³²P 5'-end labeled A₃₀ oligonucleotide. Degradation of the RNA substrate in the Rrp44p mutants was compared to two controls, a reaction that did not contain any protein (control) and a C-terminal truncated Rrp44p that lacked the RNB domain but had an intact CR3 region and PIN domain (residues 1-235; Wild-type). The efficiency of endonucleolytic cleavage was reduced in both of the Rrp44p mutants, with a more severe defect seen in the Rrp44p truncation that lacked the CR3 region and RNB domain (Figure 5.4C). Combined, these results indicate that the CR3 region of Rrp44p is needed for efficient endonuclease activity.

A.



B.

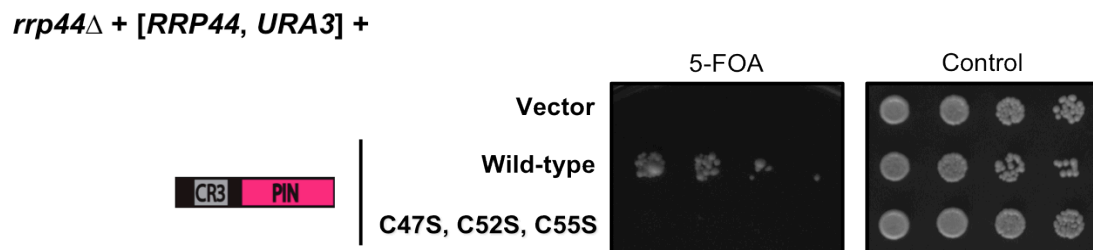


Figure 5.4: The CR3 region of Rrp44p is needed for endonuclease activity *in vivo* and *in vitro*.

C.

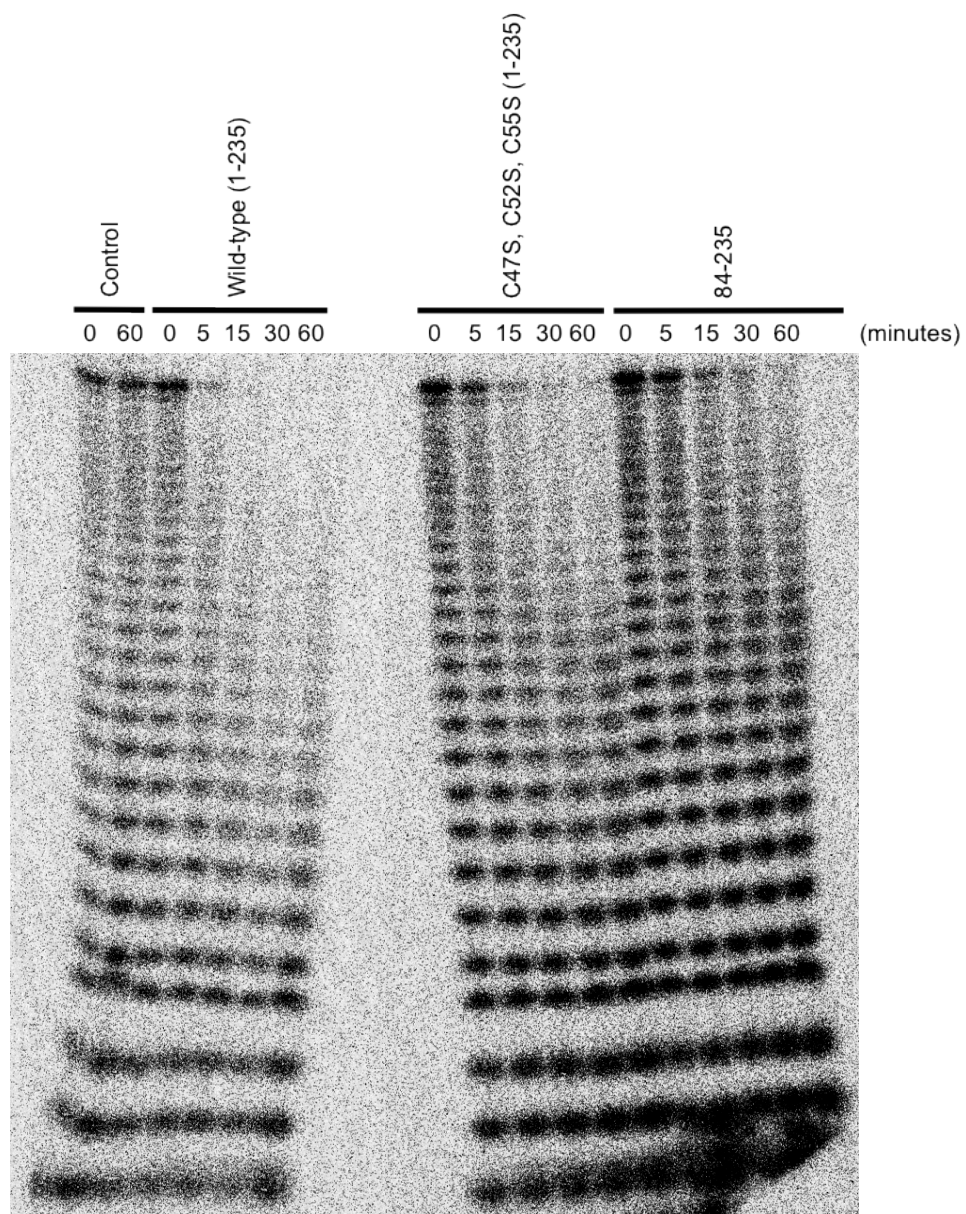


Figure 5.4: The CR3 region of Rrp44p is needed for endonuclease activity *in vivo* and *in vitro*.

Figure 5.4: The CR3 region of Rrp44p is needed for endonuclease activity *in vivo* and *in vitro*. **A and B**, An *rrp44* deletion strain complemented by full-length *RRP44* on a plasmid with a *URA3* marker was transformed with plasmids encoding each of the depicted mutations or truncations. Transformants were spotted onto media containing 5-FOA and control media (SC – LEU–URA + 2% dextrose), and grown for several days. Growth of the *rrp44* mutants was compared to two controls, empty vector and wild-type Rrp44p. Growth on 5-FOA would suggest that the CR3 region is not needed for Rrp44p endonuclease activity. **C**, The depicted Rrp44p mutants were purified as GST-fusion recombinant proteins from *E. coli* and incubated with a 5'-end labeled A₃₀ oligonucleotide for the time indicated (in minutes). Reactions were subjected to denaturing urea-polyacrylamide gel electrophoresis for single nucleotide separation. This experiment was performed by F. Reis in the laboratory of Dr. Cecilia Arraiano.

The CR3 region of Rrp44p genetically interacts with the nuclease activities of the exosome.

The CR3 region genetically interacts with the exonucleolytic RNB domain of Rrp44p.

To determine whether the CR3 region was needed for Rrp44p exonuclease activity, the triple CR3 mutant was tested in three different assays. First, a plasmid containing the triple CR3 mutation and the catalytically inactive endonuclease point mutation, D171A, was tested for complementation of an *rrp44Δ* [*RRP44*, *URA3*] strain. Growth of the CR3, D171A mutant strain was compared to two controls, empty vector and wild-type *RRP44*. If the CR3 region was needed for Rrp44p exonuclease activity, the triple CR3 mutant should be synthetically lethal with D171A because cells lacking both the endo- and exonuclease activities of Rrp44p are not viable (Lebreton et al., 2008; Schaeffer et al., 2009; Schneider et al., 2009). Indeed, the CR3, D171A mutant did not support viability (Figure 5.5A). This suggests that the CR3 region genetically interacts with the exonucleolytic RNB domain of Rrp44p, and that the CR3 region may also be needed for exonuclease activity.

To further test the functional interaction between the CR3 region and the RNB domain, a synthetic lethality growth assay was performed using the triple CR3 mutant. Briefly, a plasmid encoding the triple CR3 point mutation was transformed into an *rrp44* deletion strain that contains a temperature-sensitive allele of *dcp1* (*dcp1-2*) and a plasmid encoding wild-type *RRP44* and a *URA3* marker, generating the *dcp1-2* CR3 mutant strain. Dcp1p is a subunit of the decapping complex needed for 5' to 3' mRNA decay (Coller and Parker, 2004). The basis of this assay is that at least one of the two mRNA decay pathways is required for viability (Anderson and Parker, 1998). Therefore, if the CR3 region of Rrp44p is needed for 3' to 5' decay pathway, the triple CR3 mutant should be synthetically lethal with *dcp1-2*. The *dcp1-2* CR3 strain was then spotted onto media containing 5-FOA and grown at three different temperatures (23°C, 30°C, 37°C) for several days. Growth of this strain at the non-permissive temperature for *dcp1-2* (37°C) was compared to two control strains. The first control was a *ski7* deletion strain that also contained the *dcp1-2* allele (*dcp1-2 ski7Δ*). Ski7p is a cytoplasmic exosome cofactor that is required for general 3' to 5' decay (Araki et al., 2001; van Hoof et al., 2000b). The second control strain contained only the *dcp1-2* mutant. The triple CR3 point mutant was synthetically lethal with *dcp1-2* at the non-permissive temperature (Figure 5.5B), again indicating a genetic interaction between the CR3 region and RNB domain of Rrp44p.

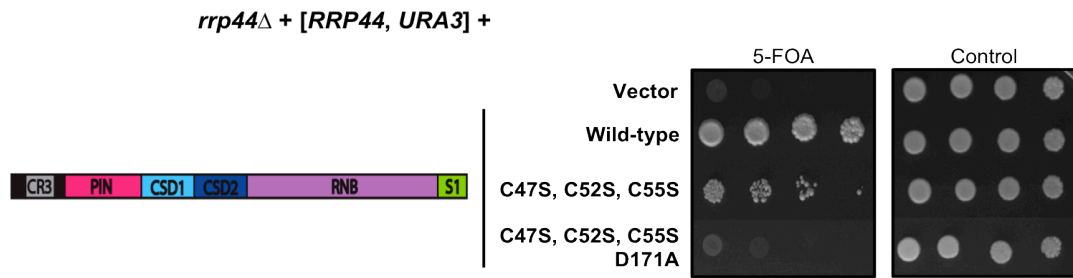
Because the synthetic lethality growth assay used above is only an indirect measure of 3' to 5' mRNA degradation, the stability of an endogenous mRNA, *GAL7*, was measured in the *dcp1-2* CR3 mutant strain. *GAL7* is a galactose metabolism gene and is expressed in media

containing galactose. *GAL7* stability in the *dcp1-2* CR3 mutant strain was compared to two control strains, a strain containing the *dcp1-2* mutant and the *dcp1-2 ski7Δ* strain. Briefly, these strains were grown in liquid media containing galactose at the permissive temperature for *dcp1-2* (23°C) and then shifted to the non-permissive temperature (37°C) for one hour. The strains were then grown in media containing dextrose to shut off transcription of *GAL7*. RNA was isolated from these strains and subject to agarose formaldehyde gel electrophoresis. Northern blot analysis was then performed using probes specific to *GAL7*, and *SCR1* as a loading control. In the *dcp1-2* strain, *GAL7* was unstable since the 3' to 5' decay pathway was still functional. Conversely, in the *dcp1-2 ski7Δ* strain, *GAL7* was more than 4-times more stable, as neither the 5' to 3' or 3' to 5' mRNA decay pathway was functional. Similar to the *dcp1-2 ski7Δ* control strain, *GAL7* was more than 4-times more stable in the *dcp1-2* CR3 strain than the *dcp1-2* strain alone. (Figure 5.5C and D). Since the exonuclease activity of Rrp44p is known to degrade mRNAs, these results suggest that the CR3 region of Rrp44p may be needed for the exonuclease activity of Rrp44p.

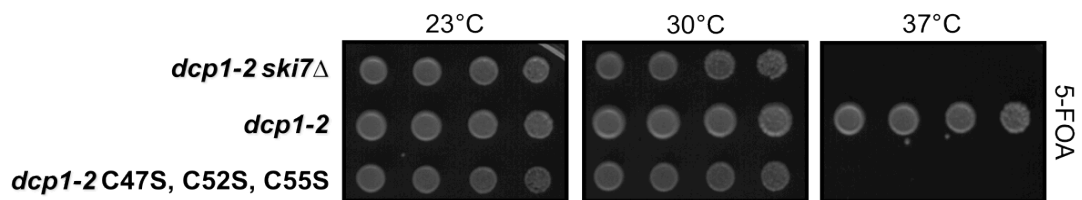
The CR3 region genetically interacts with the nuclear exosome cofactor, Rrp6p.

The catalytically inactive mutant of Rrp44p, D551N, is synthetically sick upon deletion of Rrp6p, a nuclear exosome cofactor. This indicates that the RNB domain of Rrp44p genetically interacts with Rrp6p. To determine whether the CR3 region of Rrp44p also genetically interacts with Rrp6p, a plasmid encoding the triple CR3 mutation was transformed into an *rrp44* and *rrp6* deletion strain, generating the *rrp6Δ* CR3 mutant. This strain also contains a plasmid encoding wild-type *RRP44* and a *URA3* marker. Transformants were then spotted onto media containing 5-FOA and grown at three different temperatures (23°C, 30°C, 37°C) for several days. Although this particular *rrp6* deletion strain can grow at temperatures up to 37°C, its optimal growth temperature is at 25°C (Schneider et al., 2009). Additionally, other *rrp6* deletion strains are temperature-sensitive (Allmang et al., 1999b). Growth of the *rrp6Δ* CR3 strain at all three temperatures was compared to two controls, an *rrp6* and *rrp44* deletion strain and an *rrp6* deletion strain. If the CR3 region genetically interacts with Rrp6p, the triple CR3 mutant should be synthetically sick or synthetically lethal with an *rrp6* deletion. Indeed, the triple CR3 mutant was synthetically sick with an *rrp6* deletion at lower temperatures, and synthetically lethal with an *rrp6* deletion at 37°C (Figure 5.6). Combined, these results indicate that the CR3 region genetically interacts with the exonucleolytic RNB domain of Rrp44p and with Rrp6p.

A.



B.



C.

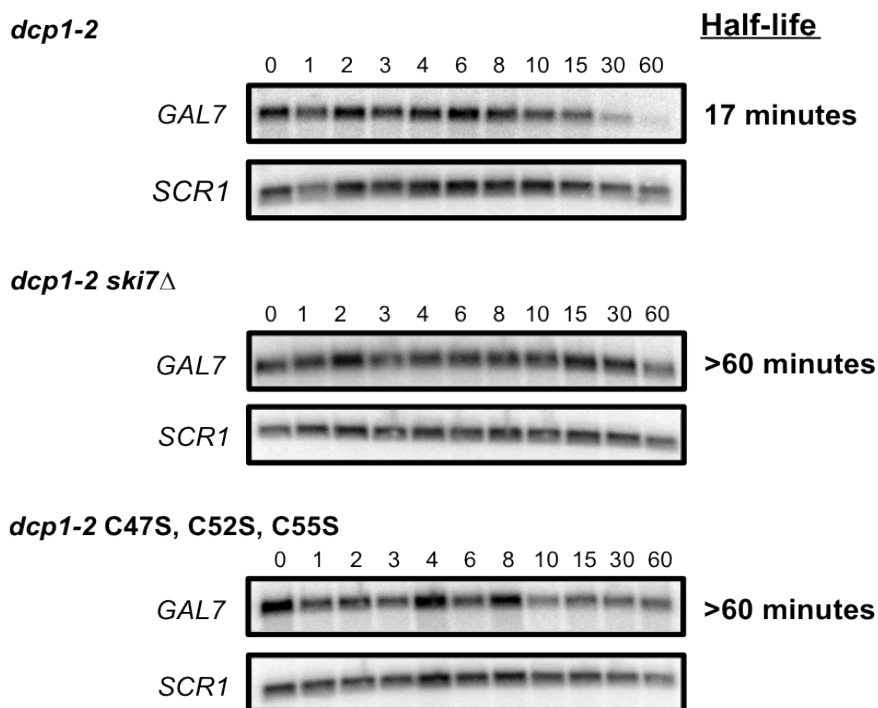


Figure 5.5: The CR3 region of Rrp44p genetically interacts with the exonucleolytic RNB domain of Rrp44p.

D.

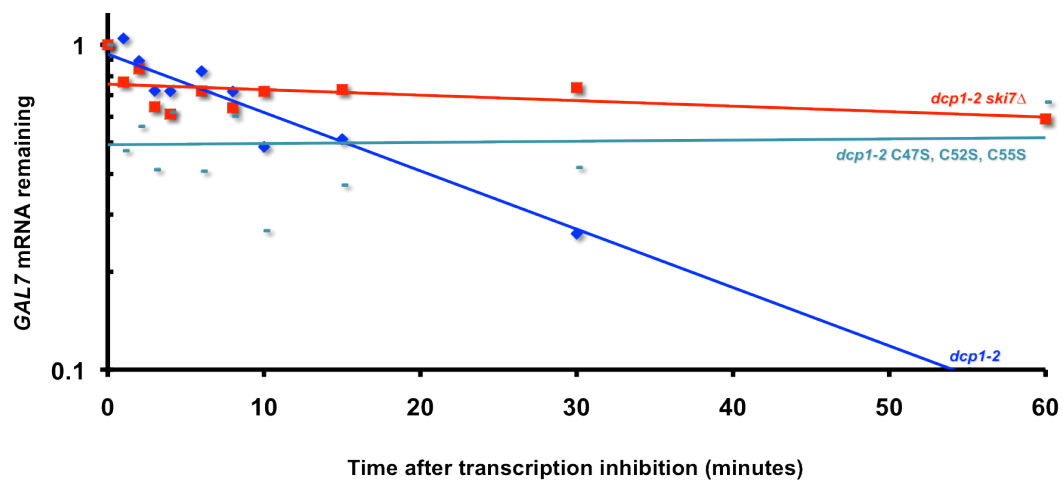


Figure 5.5: The CR3 region of Rrp44p genetically interacts with the exonucleolytic RNB domain of Rrp44p.

Figure 5.5: The CR3 region of Rrp44p genetically interacts with the exonucleolytic RNB domain of Rrp44p. **A**, An *rrp44* deletion strain complemented by full-length *RRP44* on a plasmid with a *URA3* marker was transformed with plasmids encoding each of the depicted mutations. Transformants were spotted onto media containing 5-FOA and control media (SC – LEU–URA + 2% dextrose), and grown for several days. Growth of the *rrp44* mutants was compared to two controls, empty vector and wild-type *RRP44*. Growth on 5-FOA would suggest that the CR3 region is not needed for Rrp44p exonuclease activity. **B**, An *rrp44* deletion strain containing a plasmid encoding wild-type *RRP44* and a *URA3* marker, as well as a temperature-sensitive allele of *dcp1* (*dcp1-2*), was transformed with a plasmid encoding the triple CR3 mutation. Transformants were spotted onto media containing 5-FOA and grown for several days at the indicated temperatures. Growth of the *dcp1-2 rrp44* triple CR3 mutant was compared to two control strains, a *ski7* deletion strain that also contains the *dcp1-2* allele (*dcp1-2 ski7Δ*) and a strain containing only the *dcp1-2* mutant. Growth on 5-FOA at 37°C, the non-permissive temperature of the *dcp1-2* allele, would suggest that the CR3 region of Rrp44p does not genetically interact with the exonucleolytic RNB domain.

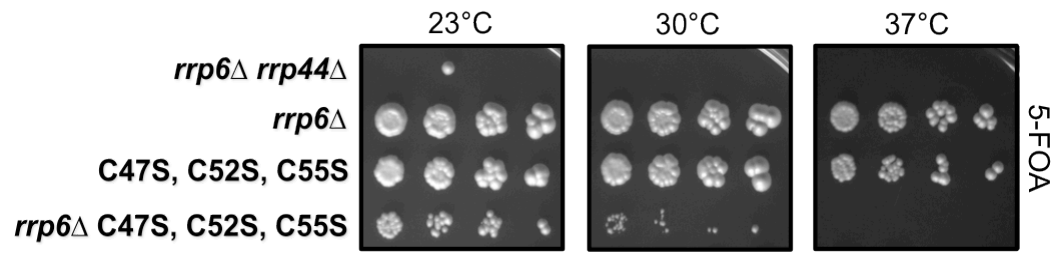


Figure 5.6: The CR3 region of Rrp44p genetically interacts with the nuclear exosome cofactor, Rrp6p.

Figure 5.6: The CR3 region of Rrp44p genetically interacts with the nuclear exosome cofactor, Rrp6p. An *rrp44* deletion strain or an *rrp6* and *rrp44* deletion strain was transformed with a plasmid encoding the triple CR3 mutation. Transformants were spotted onto media containing 5-FOA and grown for several days at the indicated temperatures. Growth of the *rrp6* and *rrp44* mutants was compared to two controls, an *rrp6* and *rrp44* deletion strain and an *rrp44* deletion strain. Growth at 37°C would suggest that the CR3 region of Rrp44p does not genetically interact with Rrp6p.

The CR3 region of Rrp44p is important in nonstop mRNA degradation.

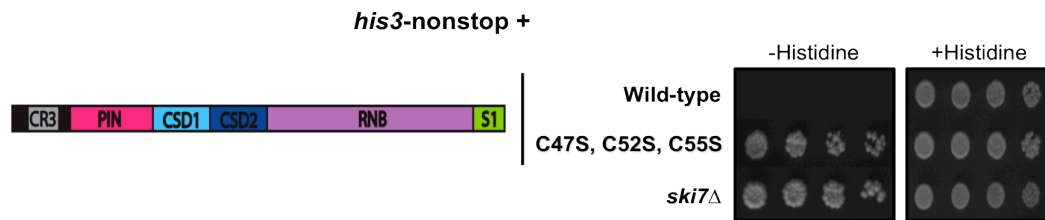
The data presented in Chapter 4 indicate that neither the endo- or exonuclease activities of Rrp44p are needed for a functional nonstop mRNA degradation pathway. The CR3 region has already been shown to be needed for both of the Rrp44p nuclease activities. Therefore, if these nucleases are redundant in nonstop decay, then the triple CR3 mutant should be unable to degrade nonstop transcripts. In order to test the effects of the triple CR3 mutant in nonstop decay, a *his3*-nonstop growth assay was performed. In this assay, the *his3*-nonstop reporter was transformed into an *rrp44Δ* strain that was complemented by the triple CR3 mutant. The basis for this assay is that the *his3*-nonstop reporter is stable in a strain that is defective in nonstop mRNA decay, thus allowing cells to make histidine and grow on media lacking histidine (van Hoof et al., 2002). The growth of the triple CR3 mutant on media lacking histidine was compared to two controls, a wild-type strain and a *ski7* deletion strain. In addition to being required for general 3' to 5' mRNA decay, Ski7p is needed for the degradation of nonstop transcripts (Frischmeyer et al., 2002). Similar to the *ski7* deletion strain, the triple CR3 mutant did not support growth on media lacking histidine (Figure 5.7A). These results suggest that the CR3 region is needed for a functional nonstop mRNA decay pathway.

Because the *his3*-nonstop growth assay is an indirect measure of whether the nonstop decay pathway is functional, a more direct assay was used. Specifically, the stability of the *GAL::pgk1pG*-nonstop transcript was measured in a strain containing the triple CR3 mutant of Rrp44p. The stability of the nonstop transcript in this strain was compared to two controls, a strain containing wild-type Rrp44p and a *ski7* deletion strain. These strains were grown in liquid media containing galactose and then shifted to media containing dextrose to shut off transcription of the *GAL::pgk1pG*-nonstop reporter. RNA was isolated from these strains and subject to agarose formaldehyde gel electrophoresis. Northern blot analysis was then performed using probes specific to *pgk1pG*, and to *ACT1* as a loading control. The *GAL::pgk1pG*-nonstop transcript was as stable in the triple CR3 mutant as it was in the *ski7* deletion strain (Figure 5.7B and C). Combined, these results indicate that the CR3 region of Rrp44p is needed for the degradation of nonstop transcripts.

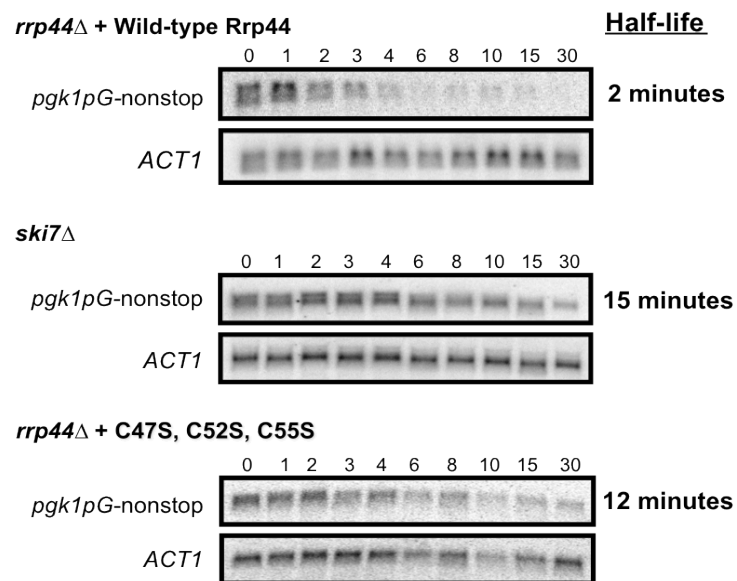
The disulfide bond of the CR3 region is not needed for optimal cell growth.

A disulfide bond is modeled to form between the second and third residues of the CR3 region (Bonneau et al., 2009). To determine if this disulfide bond is needed for optimal cell growth, the three individual cysteine residues of the CR3 region were mutated to serine. The individual cysteine mutants were then transformed into an *rrp44Δ* [*RRP44*, *URA3*] strain.

A.



B.



C.

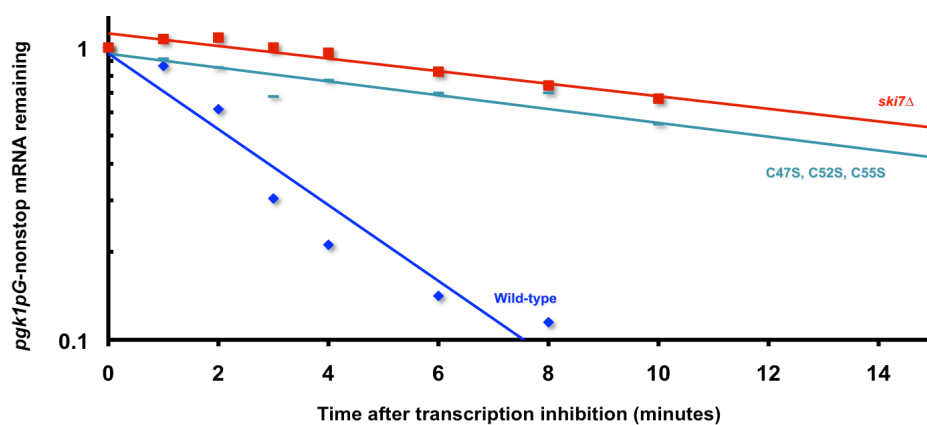


Figure 5.7: The CR3 region of Rrp44p is needed for nonstop mRNA degradation.

Figure 5.7: The CR3 region of Rrp44p is needed for nonstop mRNA degradation. **A**, An *rrp44* deletion strain complemented by a plasmid encoding the triple CR3 mutant was transformed with the *his3*-nonstop reporter. Transformants were spotted onto media containing, or lacking, histidine and grown for several days. Growth of the triple CR3 mutant was compared to two control strains, wild-type *RRP44* and a *ski7* deletion. Growth on media lacking histidine indicates that the nonstop mRNA degradation machinery is not functional. **B**, An *rrp44* deletion strain complemented by a plasmid encoding the triple CR3 mutant was transformed with the *GAL::pgk1pG*-nonstop reporter. The stability of the transcript in the triple CR3 mutant was compared to two control strains, wild-type *RRP44* and a *ski7* deletion. Cultures were first grown in media containing galactose and then incubated in media containing dextrose (to inhibit transcription of the *GAL::pgk1pG*-nonstop reporter) for the indicated time (in minutes). RNA was isolated from these strains and analyzed by Northern blot analysis with probes that hybridize to *PGK1* and *ACT1*. The half-life of the *GAL::pgk1pG*-nonstop transcript was normalized for loading using the *ACT1* signal and are as indicated (in minutes). **C**, A graphical representation of the half-life of the *GAL::pgk1pG*-nonstop transcript.

Transformants were spotted onto media containing 5-FOA and grown for several days. Growth of the individual cysteine mutants was compared to three controls, empty vector, wild-type *RRP44*, and the triple CR3 mutant. As compared to wild-type and the triple CR3 mutant, the individual cysteine mutants had an intermediate growth phenotype- their growth was not as robust as wild-type but was not as slow as the triple CR3 mutant. In particular, the C55S mutant had a growth phenotype similar to wild-type Rrp44p (Figure 5.8). This suggests that the disulfide bond modeled between C52 and C55 of the CR3 region is not needed for optimal growth. These results do not exclude the possibility that the disulfide bond exists between C47 and C52 of the CR3 region.

The disulfide bond of the CR3 region is not important in nonstop mRNA degradation.

Because the CR3 region is needed for nonstop mRNA decay, the role of the disulfide bond between the second and third cysteine residues of the CR3 region was tested in this mRNA surveillance pathway. Briefly, the *his3*-nonstop reporter was transformed into *rrp44* deletion strains that were complemented by the individual cysteine mutants. In this assay, cells with a non-functional nonstop mRNA degradation pathway are viable on media lacking histidine, whereas wild-type cells are inviable on media lacking histidine because they are able to rapidly degrade the *his3*-nonstop transcript. The growth of the individual cysteine mutants on media lacking histidine was compared to three controls, a wild-type strain, a *ski7* deletion strain, and the triple CR3 mutant. In contrast to the triple CR3 mutant, the individual cysteine mutants were viable on media lacking histidine (Figure 5.9). This suggests that the nonstop decay defect of the triple CR3 mutant is not due to the lack of a disulfide bond between the second and third cysteine residues of the CR3 region and that nonstop decay does not require a disulfide bond in the CR3 region of Rrp44p.

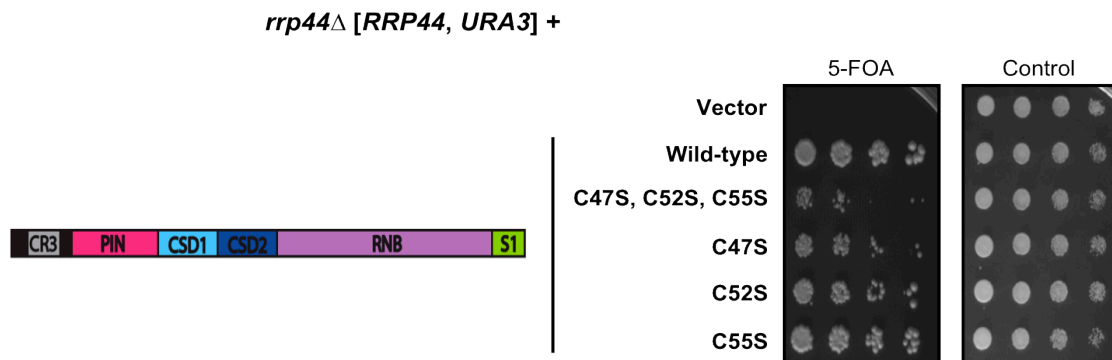


Figure 5.8: The disulfide bond of the CR3 region is not needed for optimal cell growth.

Figure 5.8: The disulfide bond of the CR3 region is not needed for optimal cell growth. An *rrp44* deletion strain complemented by full-length *RRP44* on a plasmid with a *URA3* marker was transformed with plasmids encoding each of the depicted mutations. Transformants were spotted onto media containing 5-FOA and control media (SC –LEU–URA + 2% dextrose), and grown for several days. Growth of the *rrp44* mutants was compared to two controls, empty vector and wild-type *RRP44*. Growth on 5-FOA indicates that mutated Rrp44p can carry out the essential function of Rrp44p.

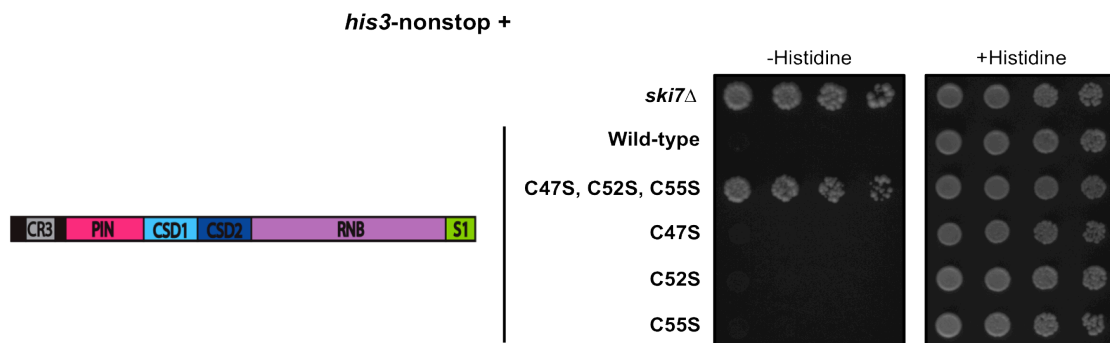


Figure 5.9: The disulfide bond of CR3 region is not needed for nonstop mRNA degradation.

Figure 5.9: The disulfide bond of the CR3 region is not needed for nonstop mRNA degradation. *rrp44* deletions strain complemented by plasmids encoding the depicted mutations were transformed with the *his3*-nonstop reporter. Transformants were spotted onto media containing, or lacking, histidine and grown for several days. Growth of the *rrp44* mutants was compared to three control strains, wild-type *RRP44*, a *ski7* deletion, and the triple CR3 mutant. Growth on media lacking histidine indicates that the nonstop mRNA degradation machinery is not functional.

DISCUSSION

The CR3 region may be a putative zinc binding domain.

The spatial organization of the three cysteines of the CR3 region and histidine 184 (H184; Figure 5.1A and B) suggests that these residues may coordinate a zinc ion. Therefore, the defects seen in the triple CR3 mutant may be explained by the lack of this coordination. The presence of a zinc ion may be directly or indirectly required for Rrp44p protein expression/stabilization, Rrp44p nuclease activity, and/or may be needed for Rrp44p to interact with the exosome. Specifically, there are several ways in which this coordination may impact the interaction between Rrp44p and the exosome and/or exosome function, none of which are mutually exclusive.

One possibility is that the lack of coordination may decrease the expression or stabilization of the Rrp44p protein, which in turn reduces cell growth and may decrease the interaction between Rrp44p and the exosome. Specifically, the coordination of a zinc ion may be needed for the correct folding of the CR3 region, and thus Rrp44p. Under this hypothesis, the lack of a functional exosome may be a secondary consequence of Rrp44p protein misfolding. Another possibility is that the lack of a zinc ion may interfere with Rrp44p endo- and/or exonuclease activity. If the coordination of zinc is important for both of these nuclease activities, this may explain why the triple CR3 mutant has a slow growth phenotype. Additionally, if these nuclease activities are redundant in nonstop mRNA decay, this could also explain the nonstop decay defect observed in the triple CR3 mutant. In support of this, preliminary evidence suggests that Rrp44p endonuclease activity is stimulated by the addition of zinc (Filipa Reis and Dr. Cecilia Arraiano, personal communication). For a more detailed explanation as to how the coordination of zinc may influence the CR3 region of Rrp44p, and thus the exosome, see Chapter 7.

Interestingly, the data presented here also show that the disulfide bond modeled between the second and third residues of the CR3 region is not required for optimal cell growth or for nonstop mRNA degradation. Because of the low resolution of the latest crystal structure of Rrp44p, the electron density is not defined enough to determine which residues, if any, of the CR3 region form a disulfide bond. Therefore, the results shown here, combined with the low resolution of the crystal structure, indicate that the disulfide bond of the CR3 region is not biologically important and suggests that it may not be present in the N-terminus of Rrp44p. In addition, a stable disulfide bond is unlikely to form in the reducing environment of the nucleus or cytoplasm.

The CR3 region is one of many regions of Rrp44p that is needed for exosome interaction.

The observation that the CR3 region mediates interaction between Rrp44p and the exosome is not surprising given that this interaction was predicted in human Rrp44p (Lehner and Sanderson, 2004) and by a cryo-EM structure of the yeast exosome (Wang et al., 2007). In the most recent study to show an interaction between Rrp44p and the core exosome, the authors concluded that the PIN domain of Rrp44p is needed for exosome interaction. In the study, however, it is not clear whether the region of Rrp44p that interacts with the exosome consists of only the PIN domain or the CR3 region and PIN domain. Additionally, the interaction detected when just the PIN domain was used (residues 86-203) was much weaker than in full-length Rrp44p (Schneider et al., 2009), which suggests that more than just the PIN domain is needed for Rrp44p to interact with the exosome. Additionally, the lack of interaction seen when a deletion of the PIN domain was used (a deletion of residues 86-203), could be explained by a transient interaction of mutant Rrp44p with the exosome or it may be an artifact of the immunoprecipitation conditions that were used. This latter possibility is likely since the interaction between Rrp44p and the exosome is salt-sensitive (Allmang et al., 1999b).

Since Rrp44p only loosely associates with the exosome (Allmang et al., 1999b), interaction of the CR3 mutant with the core exosome was tested under low and high salt conditions (50 mM and 1 M NaCl, respectively). The inability of the CR3 mutant to interact with the exosome, even under low salt conditions, further supports the importance of this region in mediating interaction with the exosome.

The data presented here, combined with the three previous studies on Rrp44p-exosome interaction, suggest that the CR3 region of Rrp44p is just one region of the protein that is needed to interact with the exosome. Specifically, five of the six domains of Rrp44p have been implicated in exosome interaction (Lehner and Sanderson, 2004; Schneider et al., 2009; Wang et al., 2007).

The functional interaction between the CR3 region and the PIN domain of Rrp44p

The observation that the triple CR3 mutant was synthetically lethal with the D551N mutant and with a C-terminal truncation of Rrp44p that lacked the RNB domain, suggests that the CR3 region is needed for the endonuclease activity of Rrp44p. One possible reason for this is that the CR3 region may be needed for the 5'-end dependency exhibited by the PIN domain. It has been previously shown that the Rrp44p endonuclease more efficiently degrades an RNA substrate that contains a 5' monophosphate than a 5' hydroxyl (Schaeffer et al., 2009). Indeed

preliminary evidence suggests that the CR3 region is important in differentiating between the 5'-ends of RNA substrates (Filipa Reis and Dr. Cecilia Arraiano, personal communication).

Another possibility is that the CR3 region is indirectly needed for Rrp44p endonuclease activity. Specifically, the coordination of a zinc ion between the CR3 region and H184 may be needed for a functional endonuclease active site. In the triple CR3 mutant, the CR3 region may be folded in such a way that precludes coordination of zinc, which may, therefore, decrease the endonuclease activity of Rrp44p. In support of this, H184 is on the same alpha-helix as D171, the active site residue of the endonucleolytic PIN domain.

The finding that the triple CR3 mutant is synthetically lethal with the D551N mutant and with a C-terminal truncation of Rrp44p may also be explained by a lack of protein expression, since the CR3 mutant is expressed at lower levels than wild-type Rrp44p. To determine whether the lack of growth is an artifact of expression, the CR3, D551N mutant and the triple CR3 mutant combined with a C-terminal truncation that lacks the RNB domain will be C-terminally TAP-tagged and expression of the mutant proteins will be analyzed. Regardless of whether these CR3 mutant proteins are expressed, the CR3 region is needed for efficient endonucleolytic cleavage *in vitro*. Additionally, the genetic interaction between the CR3 region and PIN domain is supported by the close proximity of the three cysteine residues and the active site of the PIN domain in the crystal structure (Bonneau et al., 2009).

The functional interaction between the CR3 region and the RNB domain of Rrp44p

The observation that the triple CR3 mutant was synthetically lethal with the D171A mutant suggests that the CR3 region is needed for exonuclease activity of Rrp44p. Although the CR3 region and the active site of the RNB domain are not in close proximity in the crystal structure, the CR3 region may be needed to interact with cytoplasmic exosome cofactors that are required for 3' to 5' mRNA degradation (see Chapter 7). Another possible explanation is that the CR3, D171A mutant is not expressed. A potential decrease in expression, coupled with a loss of exosome interaction, may explain the lack of complementation by the CR3, D171A mutant.

The CR3 region has a novel role in nonstop mRNA degradation.

The defect in nonstop decay seen in the triple CR3 mutant can be explained by several possibilities. In the first two explanations, it is assumed that the endo- and exonuclease activities of Rrp44p are redundant in nonstop decay. If this were the case, the nonstop decay defect is not surprising given that the CR3 region seems to be important for both of the nuclease activities of Rrp44p. Therefore, if the Rrp44p nuclease active sites have decreased nuclease activities in a

triple CR3 mutant, nonstop transcripts would be stabilized. Alternatively, it may be that the reduced expression of the triple CR3 mutant causes a reduction in the nuclease activities of Rrp44p, again resulting in the inability of Rrp44p to degrade nonstop transcripts (see Chapter 7).

If the nuclease activities of Rrp44p are not redundant in nonstop mRNA decay another unidentified nuclease is responsible for the degradation of nonstop transcripts. It may be that the CR3 region of Rrp44p is needed to recruit this nuclease. Under this hypothesis, if the triple CR3 mutant cannot recruit the nonstop decay nuclease, nonstop transcripts would be stabilized.

One more possibility, that does not require that the nuclease activities of Rrp44p be redundant in nonstop decay, is that the CR3 region of Rrp44p may interact with the nonstop mRNA decay machinery. In this model, abolishing this interaction would inactivate the nonstop decay pathway prior to mRNA degradation (see Chapter 7).

Chapter 6: The far N-terminus of Rrp44p is important for exosome function.

INTRODUCTION

The eukaryotic exosome is involved in the 3'-end processing and 3'-end degradation of various RNAs in the nucleus and the cytoplasm. The nuclear exosome processes RNA precursors to form small, stable, mature RNAs, including the 5.8S rRNA (Mitchell et al., 1997; Mitchell et al., 1996). The nuclear exosome also degrades RNA, specifically the 5' external transcribed spacer (5' ETS) of the 35S rRNA precursor (Allmang et al., 1999a). In contrast to the nuclear exosome, the cytoplasmic exosome is solely involved in mRNA degradation. Here, the exosome degrades normal and aberrant transcripts as a means of regulating and maintaining the fidelity of gene expression, respectively. Specifically, the cytoplasmic exosome is involved in degrading nonstop transcripts, or mRNAs that lack termination codons (Frischmeyer et al., 2002; van Hoof et al., 2002). Several studies suggest that the essential function of Rrp44p, and the exosome, is in the nucleus because none of the known cytoplasmic exosome functions are required for viability.

Although the exosome contains 10 subunits, only one subunit, Rrp44p, is catalytically active (Dziembowski et al., 2007; Liu et al., 2006; Mamolen and Andrulis, 2009). Rrp44p consists of six putative domains, two of which have nuclease activity. The N-terminal PIN domain has endoribonuclease activity (Lebreton et al., 2008; Schaeffer et al., 2009; Schneider et al., 2009) and the C-terminal RNB domain has 3' to 5' exoribonuclease activity (Dziembowski et al., 2007). Three of the six domains are putative RNA binding domains and are spread throughout the protein. Lastly, the CR3 region, which may not fit the classical definition of a domain, lies in the N-terminus of Rrp44p and seems to be important for most, if not all, of the known functions of the exosome (see Chapter 5). Previous studies on Rrp44p have focused only on the protein's endo- and exonuclease activities. Therefore, the far N-terminus of Rrp44p, specifically residues 1-46, has not yet been characterized. Recently, the crystal structure of Rrp44p was published, however, the structure lacked the first 33 residues (Bonneau et al., 2009). Bioinformatic analysis suggests that the far N-terminus of Rrp44p contains a linker region that consists of residues 34 to 46, with six of these residues highly conserved in eukaryotic Rrp44p homologs (Figure 6.1A). Importantly, four of these highly conserved residues have been implicated in exosome interaction (Bonneau et al., 2009).

Because the exosome is present in both the nucleus and the cytoplasm, at least one subunit requires a nuclear localization signal (NLS). Of the ten subunits, only Rrp44p has a putative NLS, in fact, it has four putative NLSs spread throughout the protein. It is not yet known which, if any, of these NLSs are required for Rrp44p, or any of the other exosome subunits, to localize to the nucleus. In support of the importance of the NLSs in Rrp44p,

Drosophila Rrp44p requires a C-terminal NLS for nuclear localization (Graham et al., 2009). It may be that the four putative NLSs of yeast Rrp44p are redundant for localization.

To determine the function of the far N-terminus of Rrp44p, specifically residues 1-46, and whether this region is needed for the essential function of Rrp44p, several N-terminal truncations and point mutations were generated and assayed for exosome function *in vivo*. The data here show that the far N-terminus of Rrp44p, specifically a linker region consisting of conserved residues, including residues 34 to 46, is needed for viability. In addition, at least the first 33 residues of the far N-terminus are needed for Rrp44p to interact with the exosome. Combining N-terminal mutants with catalytically inactive nuclease mutants of Rrp44p suggests that the N-terminus is not needed for the endo- or exonuclease activities of Rrp44p. Lastly, the data presented here suggest at least one of the putative NLSs of Rrp44p is required for viability.

RESULTS

A conserved stretch of residues in the far N-terminus of Rrp44p is needed for viability.

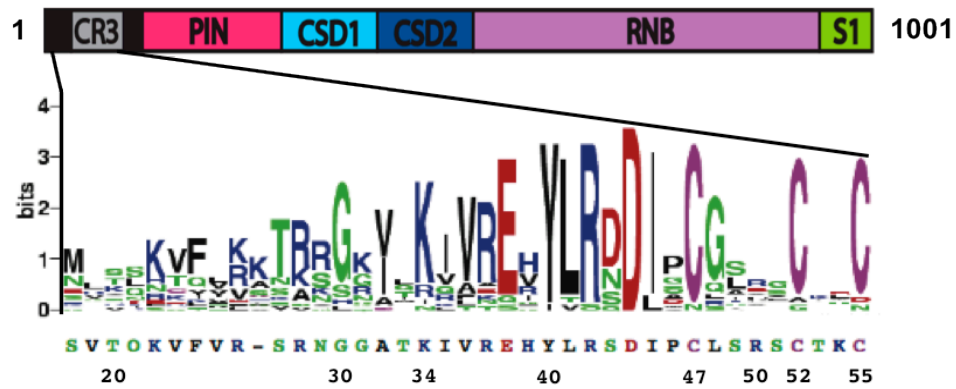
To determine whether the far N-terminus of Rrp44p, specifically residues 1-46, is needed for cell survival, plasmids encoding N-terminal truncations were generated and transformed into an *rrp44Δ* [*RRP44*, *URA3*] strain. Transformants were then spotted onto media containing 5-FOA and grown for several days. Growth of the *rrp44* truncations was compared to two controls, empty vector and wild-type *RRP44*. N-terminal truncations that lack up to the first 33 residues of Rrp44p had no obvious growth phenotype, with the smallest complementing truncation encoding residues 34-1001. In contrast, N-terminal truncations that lack the first 46 residues (47-1001), or more, did not support viability (Figure 6.1B). This lack of complementation is not due to a lack of protein expression, as both the 34-1001 and 47-1001 truncations are expressed at levels similar to wild-type (Figure 6.2). This suggests that the linker region, or residues 34 to 46, of Rrp44p is required for cell survival.

To determine whether the lack of complementation seen in 47-1001 truncation can be attributed to the lack of one of the conserved residues of the linker region, plasmids encoding point mutations in six of the most conserved residues were generated and tested for complementation of an *rrp44Δ* [*RRP44*, *URA3*] strain. Transformants were then spotted onto media containing 5-FOA and grown for several days. Growth of the Rrp44p point mutants was compared to two controls, empty vector and wild-type *RRP44*. Similar to wild-type, the point mutants had no obvious growth defects (Figure 6.1C). Therefore, although residues 34 to 46 of the linker region are needed for viability, point mutants in the most conserved residues of this region have no effect on cell growth.

The N-terminus of Rrp44p is necessary, but may not be sufficient, for Rrp44p to interact with the exosome.

One explanation for why the linker region, or residues 34 to 46, is needed for viability is that this region is needed for Rrp44p to interact with the core exosome. Indeed, four of the most highly conserved residues of this region have been implicated in exosome interaction (Bonneau et al., 2009). To test whether this region mediates interaction with the exosome *in vivo*, wild-type Rrp44p and the N-terminal truncations of Rrp44p, 34-1001 and 47-1001, were C-terminally TAP-tagged. Wild-type and the truncated Rrp44p proteins were purified using IgG sepharose beads specific to the protein A portion of the TAP tag. Proteins bound to the Rrp44p proteins were eluted and analyzed using SDS-PAGE. Whole cell lysates from the wild-type and truncated Rrp44p strains were compared to the bound fractions from these strains. The interaction of the

A.



B.

*rrp44*Δ [*RRP44*, *URA3*] +

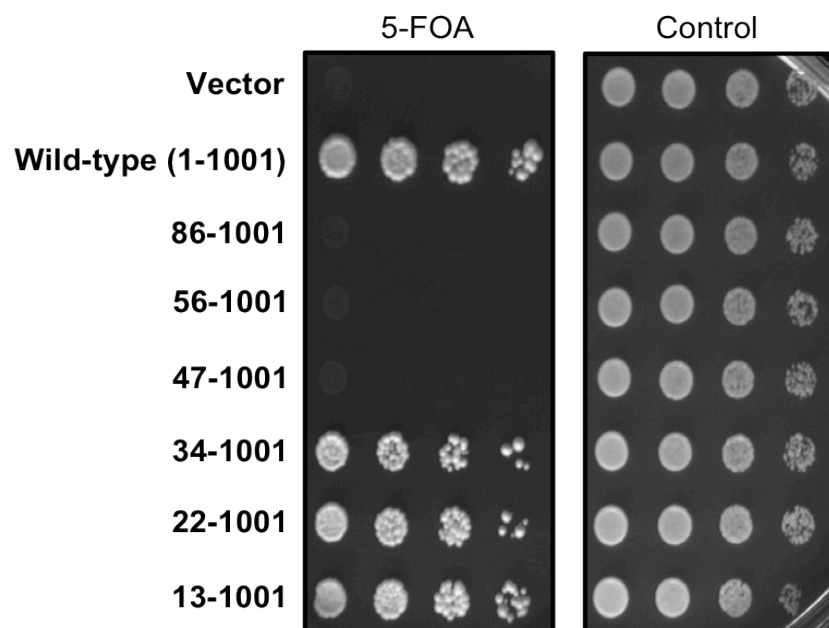


Figure 6.1: Residues 34 to 46 of Rrp44p are needed for viability.

C.

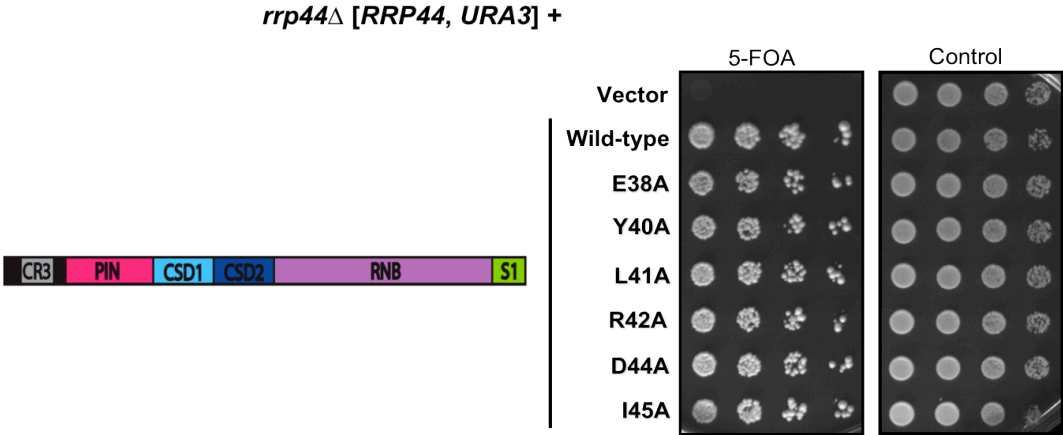


Figure 6.1: Residues 34 to 46 of Rrp44p are needed for viability.

Figure 6.1: Residues 34 to 46 of Rrp44p are needed for viability. **A,** A schematic representation of the N-terminus (residues 18 to 55) of Rrp44p. Conservation is indicated by the height of each amino acid. **B,** An *rrp44* deletion strain complemented by full-length *RRP44* on a plasmid with a *URA3* marker was transformed with plasmids encoding each of the depicted truncations. Transformants were spotted onto media containing 5-FOA and control media (SC – LEU–URA + 2% dextrose), and grown for several days. Growth of the *rrp44* truncations was compared to two controls, empty vector and wild-type *RRP44*. Growth on 5-FOA indicates that truncated Rrp44p can carry out the essential function of Rrp44p. **C,** An *rrp44* deletion strain complemented by full-length *RRP44* on a plasmid with a *URA3* marker was transformed with plasmids encoding each of the depicted mutations. Transformants were spotted onto media containing 5-FOA and control media (SC – LEU–URA + 2% dextrose), and grown for several days. Growth of the *rrp44* mutants was compared to two controls, empty vector and wild-type *RRP44*. Growth on 5-FOA indicates that mutated Rrp44p can carry out the essential function of Rrp44p.

wild-type and truncated Rrp44p proteins with the exosome was compared to empty vector. In contrast to wild-type, both of the N-terminal truncated Rrp44p proteins were unable to interact with the exosome suggesting that at least the first 33 residues of Rrp44p residues are needed for exosome interaction (Figure 6.2). Because this region alone was not tested for exosome interaction, it is not yet known whether this region is sufficient for Rrp44p to interact with the exosome. Additionally, previous studies indicate that other regions of Rrp44p are also needed for exosome interaction (Lehner and Sanderson, 2004; Schneider et al., 2009; Wang et al., 2007). The results presented so far indicate that deletion of the first 33 residues disrupts exosome interaction, but not exosome function.

Several residues in the N-terminus of Rrp44p are needed for an essential function that also requires the C-terminus of Rrp44p.

To determine whether the N-terminus of Rrp44p is needed for the protein's endonuclease activity, an N-terminal truncation and N-terminal point mutants were tested for whether they were synthetically lethal with mutants of Rrp44p that lack exonuclease activity. If the N-terminus of Rrp44p is needed for endonuclease activity, the combination of N-terminal truncations/point mutants with a mutant lacking exonuclease activity should not complement an *rrp44Δ* [*RRP44*, *URA3*] strain because cells lacking both the endo- and exonuclease activities of Rrp44p are not viable (Lebreton et al., 2008; Schaeffer et al., 2009; Schneider et al., 2009).

To determine whether the first 33 residues of the far N-terminus of Rrp44p are needed for the protein's endonuclease activity, the catalytically inactive exonuclease point mutant, D551N, was generated in the 34-1001 truncation (D551N 34-1001) and tested for complementation of an *rrp44Δ* [*RRP44*, *URA3*] strain. Growth of the D551N 34-1001 mutant strain was compared to four controls, empty vector, wild-type *RRP44*, the 34-1001 truncation, and the D551N mutant. The D551N 34-1001 mutant did have a slight growth defect when compared to a strain containing the 34-1001 truncation and a strain containing the D551N mutant, however, deletion of the first 33 residues was not synthetically lethal with the D551N mutant (Figure 6.3A). This suggests that the first 33 residues of Rrp44p are not needed for the protein's endonuclease activity.

To determine whether any of the six conserved residues of the linker region in the far N-terminus of Rrp44p (E38, Y40, L41, R42, D44, and I45), are needed for endonuclease activity, point mutations in these residues were generated in a C-terminal Rrp44p truncation that lacks exonuclease activity. These truncations were then tested for complementation of an *rrp44Δ* [*RRP44*, *URA3*] strain. Growth of the *rrp44Δ* mutant truncations was compared to two controls,

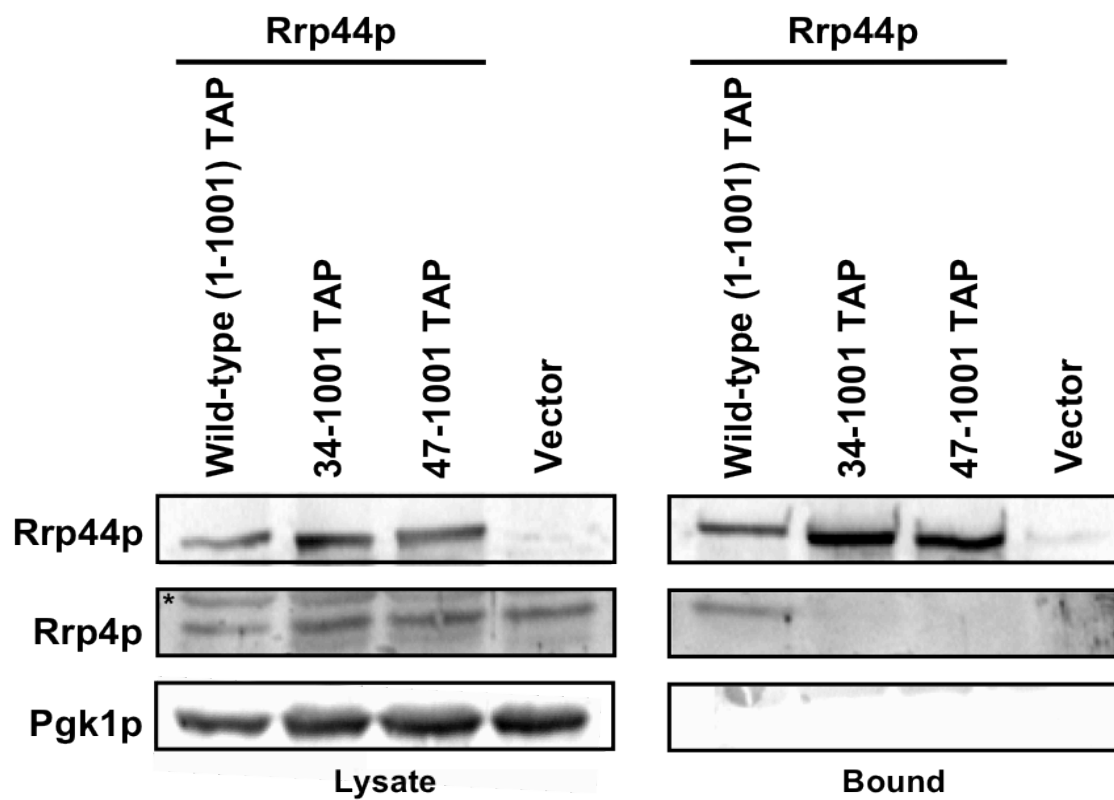


Figure 6.2: The N-terminus of Rrp44p is needed for interaction with the exosome.

Figure 6.2: The N-terminus of Rrp44p is needed for interaction with the exosome. C-terminal TAP-tagged Rrp44p mutants were purified using IgG sepharose beads. Prior to purification, whole-cell lysates were isolated (left). Purified proteins were eluted by boiling the beads (right). Interaction of the Rrp44p truncated proteins with the exosome was compared to two controls, wild-type Rrp44p and empty vector. Whole-cell lysates and purified proteins were analyzed by Western blot analysis with antibodies specific to Protein A (above) to detect Rrp44p, Rrp4p (middle) to detect interaction with the exosome, and Pgk1p (below) as a loading control. The asterisk in the middle blot indicates a nonspecific protein.

empty vector and wild-type *RRP44*. Three of the six point mutants (Y40A, L41A, and R42A) were synthetically lethal with a truncation lacking exonuclease activity, whereas the other three point mutants exhibited no growth defect when combined with a C-terminal truncation of Rrp44p (Figure 6.3B and C). This suggests that some of the six conserved residues of the linker region in the far N-terminus of Rrp44p may be needed for PIN domain endonuclease activity.

To further test the genetic interaction between these three conserved residues and the endonucleolytic PIN domain, the Y40A, L41A, and R42A mutants were combined, individually, with the D551N mutant and tested for complementation of an *rrp44Δ* [*RRP44*, *URA3*] strain. In contrast to the results obtained when these point mutants were generated in a truncation lacking the exonucleolytic RNB domain, the Y40A, L41A, and R42A mutants were not synthetically lethal with the D551N mutant (Figure 6.3C). These results suggest that the three conserved residues of the linker region in the far N-terminus of Rrp44p are likely not required for the endonuclease activity of the protein but rather for an essential function of Rrp44p that also requires the C-terminus of the protein (see Discussion)

The first 33 residues of Rrp44p do not genetically interact with the exonuclease activities of the exosome.

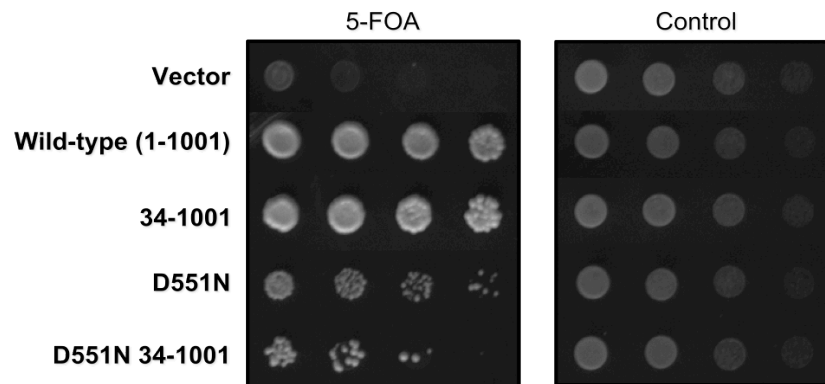
The first 33 residues are not needed for exosome-mediated 3' to 5' mRNA degradation.

To determine whether the first 33 residues of Rrp44p are needed for the protein's exonuclease activity, the N-terminal truncation, 34-1001, was tested in two different assays. First, the catalytically inactive endonuclease point mutant, D171A, was generated in the 34-1001 truncation (D171A 34-1001) and tested for complementation of an *rrp44Δ* [*RRP44*, *URA3*] strain. Growth of the D171A 34-1001 mutant was compared to two controls, empty vector and wild-type *RRP44*. If the first 33 residues are needed for the exonuclease activity of Rrp44p, a truncation lacking these residues should be synthetically lethal with D171A because cells lacking both the endo- and exonuclease activities of Rrp44p are not viable (Lebreton et al., 2008; Schaeffer et al., 2009; Schneider et al., 2009). Similar to wild-type Rrp44p, the D171A 34-1001 mutant had no obvious growth defect (Figure 6.4A), suggesting that the first 33 residues of Rrp44p are not needed for the exonuclease activity of the protein's RNB domain.

To further test the functional interaction between the first 33 residues of Rrp44p and the RNB domain, a synthetic lethality growth assay was performed. Briefly, a plasmid encoding residues 34-1001 was transformed into an *rrp44* deletion strain that also contained a temperature-sensitive allele of *dcp1* (*dcp1-2*) and a plasmid encoding wild-type *RRP44* and a *URA3* marker, generating the *dcp1-2* 34-1001 strain. Dcp1p is a subunit of the decapping

A.

rrp44 Δ + [*RRP44*, *URA3*] +



B.

rrp44 Δ + [*RRP44*, *URA3*] +

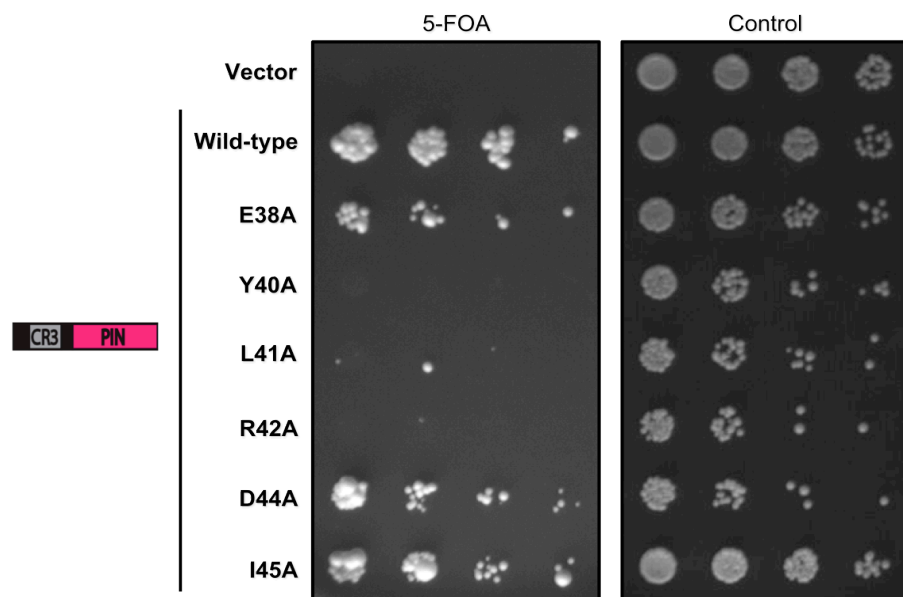


Figure 6.3: Several residues in the N-terminus of Rrp44p are needed for an essential function that also requires the C-terminus of Rrp44p.

C.

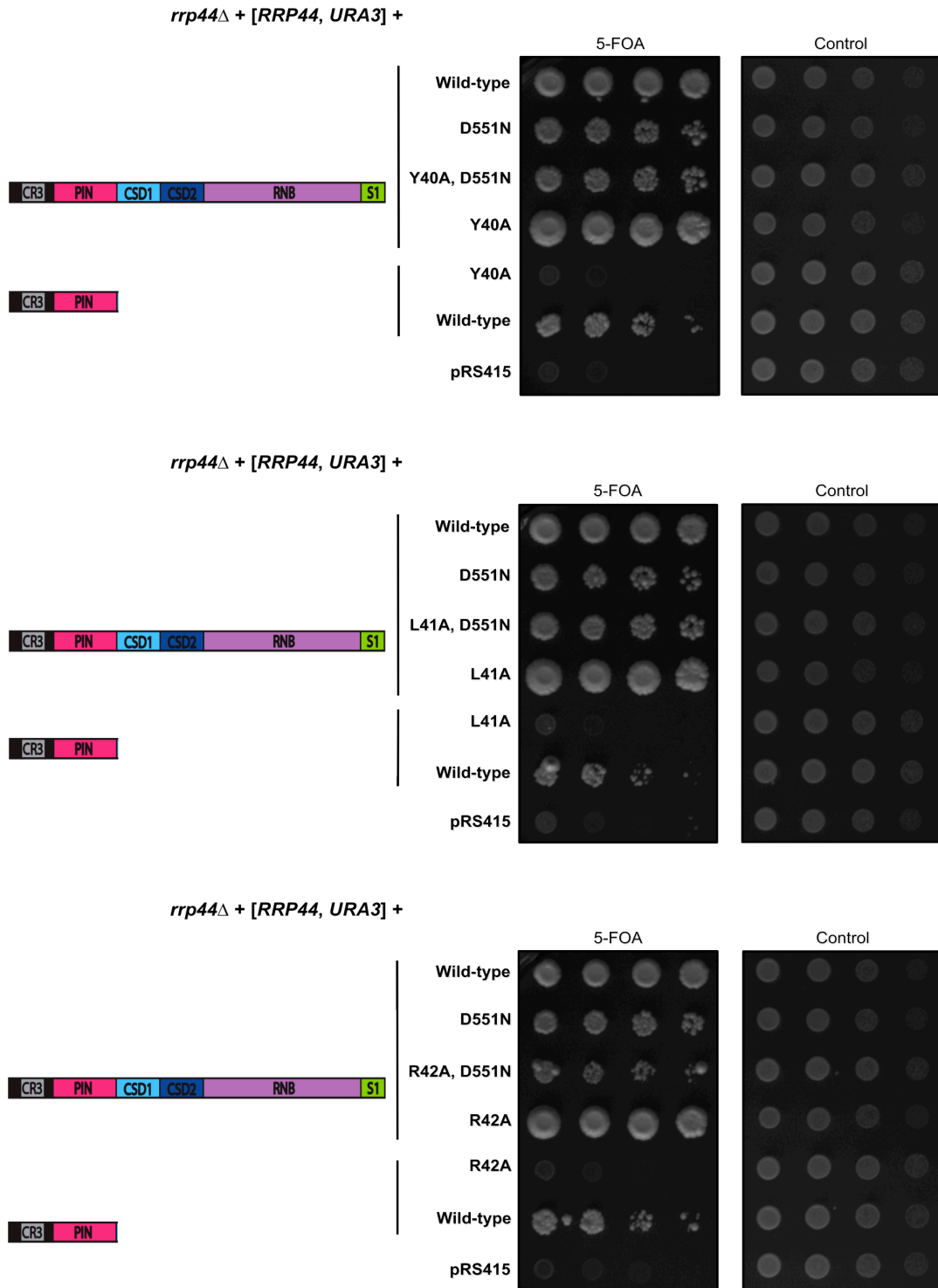


Figure 6.3: Several residues in the N-terminus of Rrp44p are needed for an essential function that also requires the C-terminus of Rrp44p.

Figure 6.3: Several residues in the N-terminus of Rrp44p are needed for an essential function that also requires the C-terminus of Rrp44p. An *rrp44* deletion strain complemented by full-length *RRP44* on a plasmid with a *URA3* marker was transformed with plasmids encoding each of the depicted mutations. Transformants were spotted onto media containing 5-FOA and control media (SC –LEU–URA + 2% dextrose), and grown for several days. Growth of the *rrp44* mutants was compared to empty vector and wild-type *RRP44*. Growth on 5-FOA would suggest that mutated Rrp44p can carry out the essential function of Rrp44p.

complex needed for 5' to 3' mRNA decay (Coller and Parker, 2004). The basis of this assay is that at least one of the two mRNA decay pathways is required for viability (Anderson and Parker, 1998). Therefore, if the first 33 residues of Rrp44p are needed for a functional 3' to 5' decay pathway, a truncation lacking these residues should be synthetically lethal with the *dcp1-2* mutant. The *dcp1-2* 34-1001 strain was then spotted onto media containing 5-FOA and grown at three different temperatures (23°C, 30°C, 37°C) for several days. Growth of the strain at the non-permissive temperature for *dcp1-2* (37°C) was compared to two control strains. The first control was a *ski7* deletion strain that also contained the *dcp1-2* allele. Ski7p is a cytoplasmic exosome cofactor that is required for general 3' to 5' decay (Araki et al., 2001; van Hoof et al., 2000b). The second control strain contained only the *dcp1-2* mutant. The N-terminal truncation was not synthetically lethal with *dcp1-2* at the non-permissive temperature (Figure 6.4B), again indicating that these residues are not needed for Rrp44p exonuclease activity.

The first 33 residues of Rrp44p do not genetically interact with the nuclear exosome cofactor, Rrp6p.

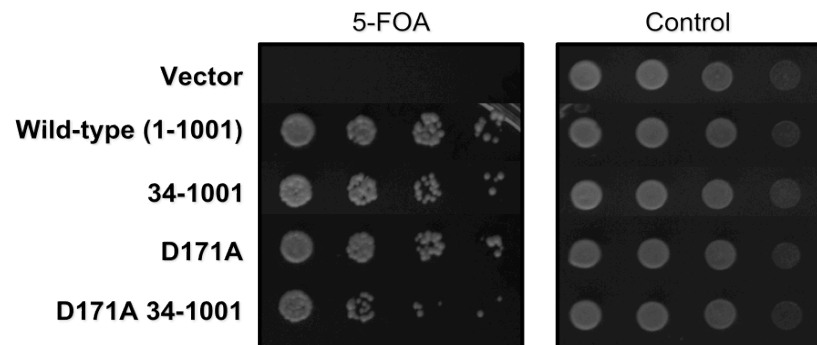
The catalytically inactive exonuclease mutant of Rrp44p, D551N, is synthetically sick upon deletion of Rrp6p, a nuclear exosome cofactor. This indicates that the RNB domain of Rrp44p genetically interacts with Rrp6p. To determine whether the first 33 residues of Rrp44p also genetically interact with Rrp6p, a plasmid encoding residues 34-1001 was transformed into an *rrp44* and *rrp6* deletion strain, generating the *rrp6Δ* 34-1001 mutant. This strain also contains a plasmid encoding wild-type *RRP44* and a *URA3* marker. Transformants were then spotted onto media containing 5-FOA and grown at three different temperatures (23°C, 30°C, 37°C) for several days. Although this particular *rrp6* deletion strain can grow at temperatures up to 37°C, its optimal growth temperature is at 25°C (Schneider et al., 2009). Additionally, other *rrp6* deletion strains are temperature-sensitive (Allmang et al., 1999b). Growth of the *rrp6Δ* 34-1001 strain at all three temperatures was compared to two controls, an *rrp6* and *rrp44* deletion strain and *rrp6* deletion strain. The 34-1001 truncation was not synthetically sick or lethal with an *rrp6* deletion at any of the three temperatures (Figure 6.5). These results suggest that the first 33 residues of Rrp44p do not genetically interact with Rrp6p.

The first 33 residues of Rrp44p are not needed for nonstop mRNA degradation.

Thus far, the data presented here indicate that neither the endo- or exonuclease activities of Rrp44p are needed for a functional nonstop mRNA degradation pathway, however, the CR3 region is needed for the degradation of nonstop transcripts. To determine whether the first 33

A.

*rrp44*Δ + [*RRP44*, *URA3*] +



B.

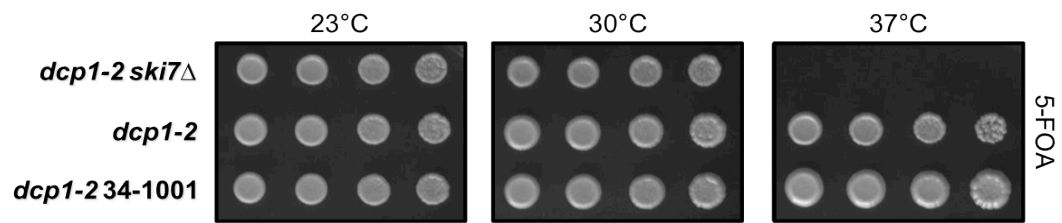


Figure 6.4: The first 33 residues of Rrp44p are not needed for exosome-mediated 3' to 5' mRNA degradation.

Figure 6.4: The first 33 residues of Rrp44p are not needed for exosome-mediated 3' to 5' mRNA degradation. **A**, An *rrp44* deletion strain complemented by full-length *RRP44* on a plasmid with a *URA3* marker was transformed with plasmids encoding each of the depicted mutations. Transformants were spotted onto media containing 5-FOA and control media (SC – LEU–URA + 2% dextrose), and grown for several days. Growth of the *rrp44* mutants was compared to two controls, empty vector and wild-type *RRP44*. Growth on 5-FOA indicates that mutated Rrp44p can carry exosome-mediated 3' to 5' mRNA degradation. **B**, An *rrp44* deletion strain containing a plasmid encoding wild-type *RRP44* and a *URA3* marker, as well as a temperature-sensitive allele of *dcp1* (*dcp1-2*), was transformed with a plasmid encoding residues 34 to 1001. Transformants were spotted onto media containing 5-FOA and grown for several days at the indicated temperatures. Growth of the *dcp1-2 rrp44* 34-1001 mutant was compared to two control strains, a *ski7* deletion strain that also contains the *dcp1-2* allele (*dcp1-2 ski7Δ*) and a strain containing only the *dcp1-2* mutant. Growth on 5-FOA at 37°C, the non-permissive temperature of the *dcp1-2* allele, indicates that the first 33 residues of Rrp44p are not needed for exosome-mediated 3' to 5' mRNA degradation.

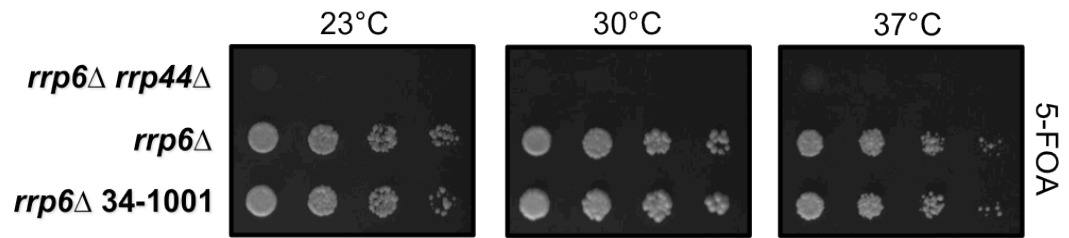


Figure 6.5: The first 33 residues of Rrp44p do not genetically interact with the nuclear exosome cofactor, Rrp6p.

Figure 6.5: The first 33 residues of Rrp44p do not genetically interact with the nuclear exosome cofactor, Rrp6p. An *rrp44* deletion strain or an *rrp6* and *rrp44* deletion strain was transformed with a plasmid encoding residues 34 to 1001. Transformants were spotted onto media containing 5-FOA and grown for several days at the indicated temperatures. Growth of the *rrp6* 34-1001 mutant was compared to two controls, an *rrp6* and *rrp44* deletion strain and an *rrp44* deletion strain. Growth at 37°C indicates that the first 33 residues of Rrp44p do not genetically interact with Rrp6p.

residues of Rrp44p are also needed for nonstop decay, a *his3*-nonstop growth assay was performed. In this assay, the *his3*-nonstop reporter was transformed into an *rrp44* deletion strain that was complemented by the 34-1001 truncation. The basis for this assay is that the *his3*-nonstop reporter is stable in a strain that is defective in nonstop mRNA decay, thus allowing cells to make histidine and grow on media lacking histidine (van Hoof et al., 2002). The growth of the 34-1001 truncation on media lacking histidine was compared to two controls, a wild-type strain and a *ski7* deletion strain. Similar to the wild-type strain, the 34-1001 truncation did not support viability on media lacking histidine (Figure 6.6). These results suggest that the first 33 residues of Rrp44p are not needed for a functional nonstop mRNA decay pathway.

The first 33 residues of Rrp44p are not required for the RNA processing or RNA degradation activities of the nuclear exosome.

The nuclear exosome processes the 7S rRNA precursor to form the mature 5.8S rRNA and degrades the 5' external transcribed spacer (5' ETS) of the 35S rRNA precursor (Allmang et al., 1999a; Allmang et al., 1999b; Mitchell et al., 1997; Mitchell et al., 1996). To test whether the first 33 residues of Rrp44p are needed for these nuclear RNA processing and/or degradation reactions, RNA was isolated from an *rrp44* deletion strain that was complemented with the 34-1001 truncation. RNA was also isolated from three controls, a strain containing an *rrp6* deletion, a strain containing wild-type *RRP44*, and a strain containing the D551N mutant. Rrp6p, a nuclear exosome cofactor, is required for nuclear RNA processing and degradation (Briggs et al., 1998). The RNA from these strains was then subject to denaturing urea-polyacrylamide gel electrophoresis. To determine if these strains were defective in RNA processing and/or degradation, Northern blot analysis was performed using probes specific to the 7S rRNA precursor and to the 5' ETS, respectively. Intermediates of 5.8S rRNA processing and 5' ETS degradation accumulate in an *rrp6* deletion strain and in the D551N mutant, since Rrp6p and the exonuclease activity of Rrp44p have been shown to be required for a functional nuclear exosome. In contrast to these strains, the 5.8S rRNA and the 5' ETS were efficiently processed and degraded, respectively, in the strain containing the 34-1001 truncation (Figure 6.7). This suggests that the first 33 residues of Rrp44p are not needed for the RNA processing and degradation functions of the nuclear exosome.

***his3-nonstop* +**

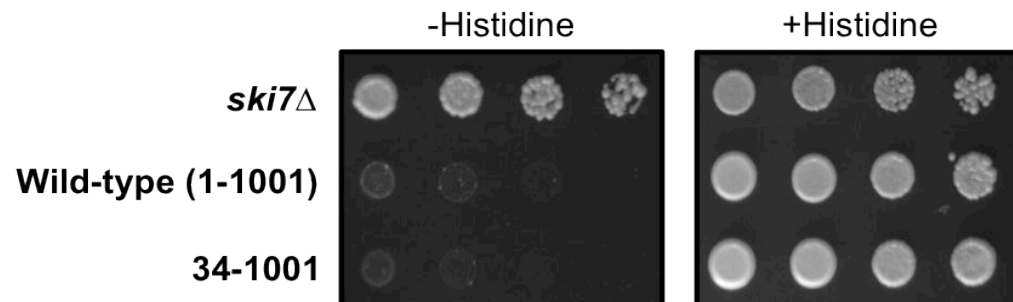


Figure 6.6: The first 33 residues of Rrp44p are not needed for nonstop mRNA degradation.

Figure 6.6: The first 33 residues of Rrp44p are not needed for nonstop mRNA degradation.

An *rrp44* deletion strain complemented by a plasmid encoding residues 34 to 1001 was transformed with the *his3*-nonstop reporter. Transformants were spotted onto media containing, or lacking, histidine and grown for several days. Growth of *rrp44* truncation was compared to two controls strains, a *ski7* deletion and wild-type *RRP44*. Growth on media lacking histidine indicates that the nonstop mRNA degradation machinery is not functional.

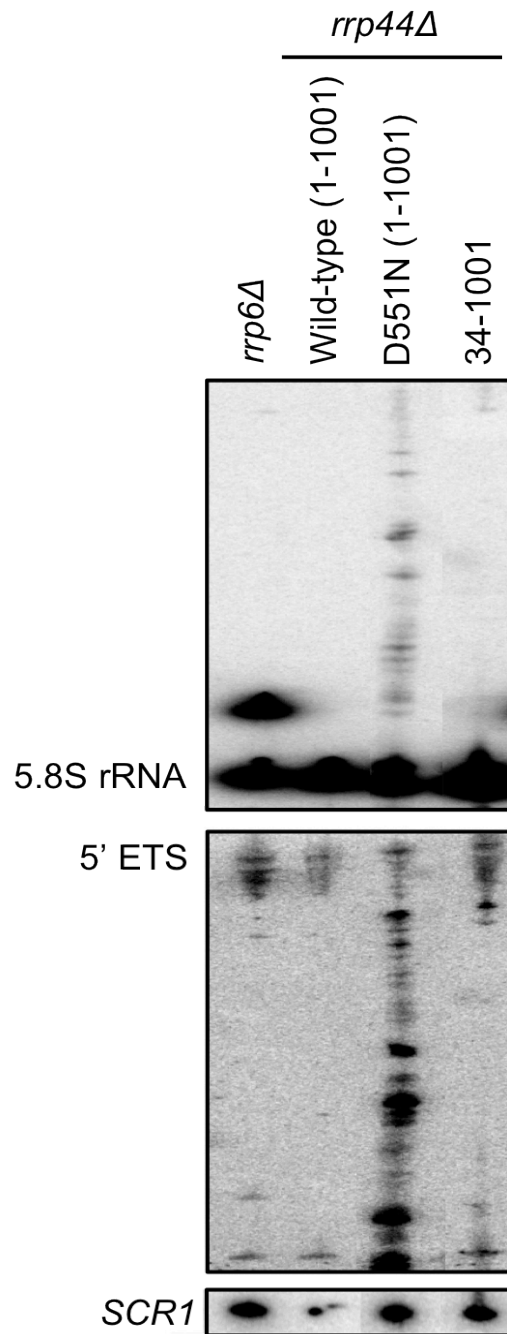


Figure 6.7: first 33 residues of Rrp44p are not needed for the RNA processing or RNA degradation activities of the nuclear exosome.

Figure 6.7: The first 33 residues of Rrp44p are not needed for the RNA processing or RNA degradation activities of the nuclear exosome. The indicated *rrp44* mutants were expressed in an *rrp44* deletion strain. RNA was isolated and analyzed by Northern blot analysis with probes that hybridize to the 7S precursor of 5.8S rRNA (above), the 5' ETS of the rRNA (middle) and the RNA subunit of the signal recognition particle (*SCR1*; below). RNA processing and RNA degradation in the *rrp44* mutants was compared to two control strains, an *rrp6* deletion and wild-type *RRP44*. The relative levels of the 7S pre-rRNA and the 5' ETS were normalized for loading using the *SCR1* signal.

At least one of the putative nuclear localization signals (NLSs) of Rrp44p is needed for viability.

The exosomes' presence in the nucleus suggests that at least one exosome subunit has an NLS. Indeed, Rrp44p has four putative NLSs spread throughout the protein. The first NLS is in the far N-terminus of Rrp44p, while the second and third are in the cold shock domains (CSD1 and CSD2, respectively). The fourth putative NLS is in the C-terminal S1 domain, one of the three putative RNA binding domains (Figure 6.8A). To determine whether these NLSs are required for viability, an N- and C-terminal truncation of Rrp44p were generated. Specifically, the N-terminal truncation, 13-1001, lacks the first putative NLS and the C-terminal truncation, 1-235, lacks the second, third, and fourth putative NLS. These truncations were transformed into an *rrp44Δ* [*RRP44*, *URA3*] strain. Transformants were then spotted onto media containing 5-FOA and grown for several days. Growth of the *rrp44* truncations was compared to two controls, empty vector and wild-type *RRP44*. The 13-1001 truncation had no obvious growth defect, whereas the 1-235 truncation had a slow growth phenotype (Figure 6.8B). The slow growth of the 1-235 truncation has been previously reported (see Chapter 3; Schaeffer et al., 2009) and is likely due to a lack of Rrp44p exonuclease activity. These results suggest that none of the putative NLSs of Rrp44p are required for viability, but does not exclude the possibility that these NLSs are redundant.

To determine whether cells require at least one putative NLS to survive, an N- and C-terminal truncation, 13-235, was generated and tested for complementation of an *rrp44Δ* [*RRP44*, *URA3*] strain. Growth of the 13-235 truncation was compared to four controls, empty vector, wild-type *RRP44*, the 13-1001 truncation, and the 1-235 truncation. The 13-235 truncation resulted in no growth (Figure 6.8B), however, this lack of complementation was not due to a lack of protein expression, as the 13-235 truncation was expressed at levels higher than wild-type Rrp44 (Figure 6.8C). These results suggest that any of the putative NLSs of Rrp44p are sufficient for viability, however, the absence of all putative NLSs results in a nonfunctional Rrp44p, and thus, no cell growth.

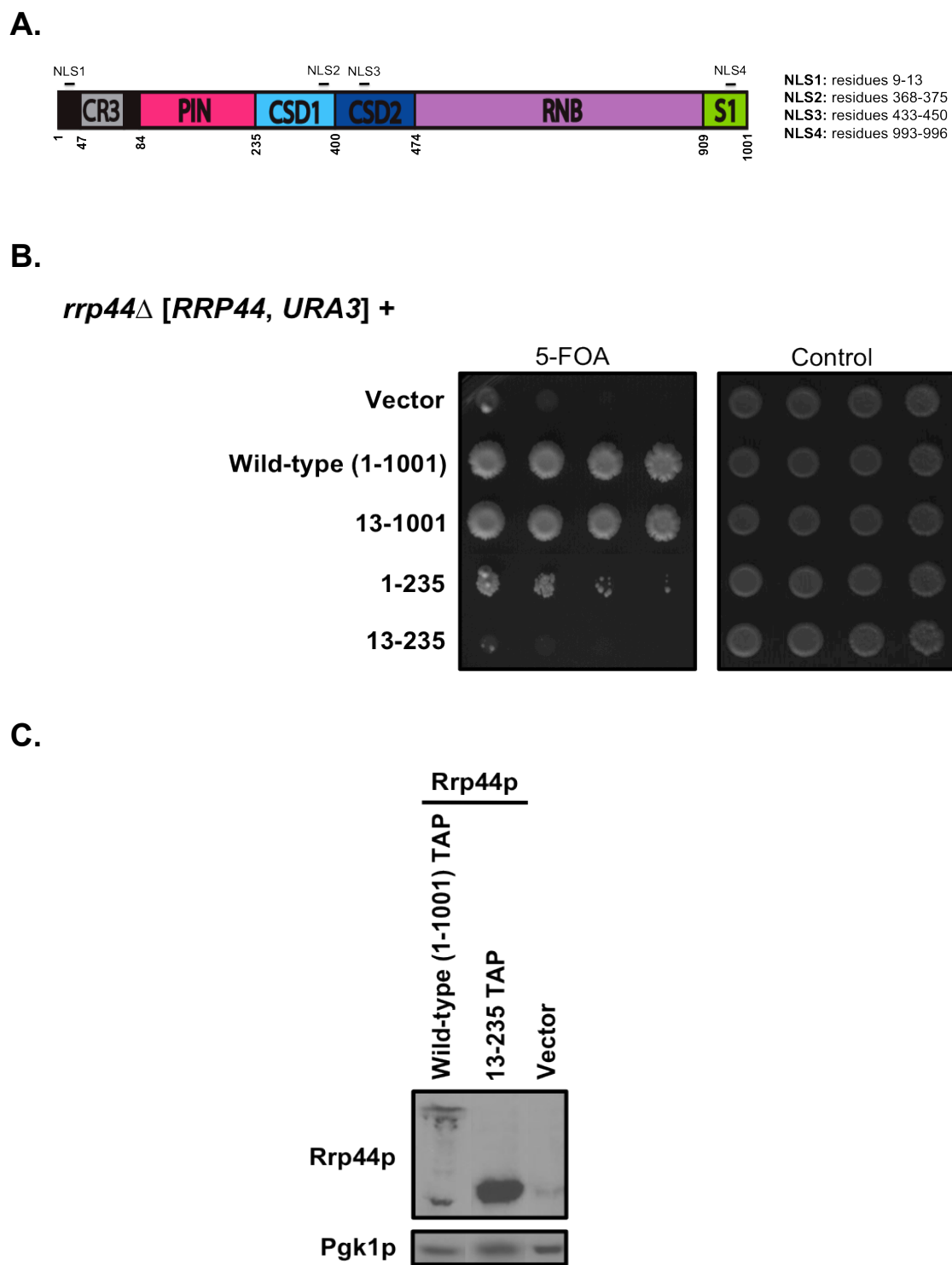


Figure 6.8: At least one of the putative localization signals (NLSs) of Rrp44p is needed for viability.

Figure 6.8: At least one of the putative localization signals (NLSs) of Rrp44p is needed for viability. **A**, A schematic representation of the putative NLSs of Rrp44p. **B**, An *rrp44* deletion strain complemented by full-length *RRP44* on a plasmid with a *URA3* marker was transformed with plasmids encoding each of the depicted truncations. Transformants were spotted onto media containing 5-FOA and control media (SC –LEU –URA + 2% dextrose), and grown for several days. Growth of the *rrp44* truncations was compared to two controls, empty vector and wild-type *RRP44*. Growth on 5-FOA indicates that truncated Rrp44p can carry out the essential function of Rrp44p. **C**, Whole-cell lysate was obtained from a strain containing the C-terminally TAP-tagged 13-235 Rrp44p truncation described in (A) and was analyzed by Western blot analysis with antibodies specific to Protein A to detect Rrp44p and to Pgk1p as a loading control. Expression of the Rrp44p truncated protein was compared to wild-type Rrp44p.

DISCUSSION

Residues 34 to 46 are needed for the essential function of Rrp44p.

The results shown in this Chapter indicate that while the first 33 residues of Rrp44p are not essential, conserved residues 34 to 46 of the linker region are required for viability. Further analysis showed that although deletion of the linker results in a lack of cell growth, mutating six of the most conserved residues of this region had no effect on cell viability. These differences in growth may be because the residues of the linker region participate in redundant protein-protein interactions that are required for viability. In support of this, the N-terminus of Rrp44p is needed for interaction with the exosome (see Chapter 5; Lehner and Sanderson, 2004; Schneider et al., 2009; Wang et al., 2007).

An alternate explanation for why the 47-1001 truncation fails to support viability is that this truncated protein may be non-functional. Although this N-terminal truncation is expressed, there is a possibility that the protein is misfolded, and thus, causes a decrease in protein function.

The N-terminus is one of many regions of Rrp44p that are needed for exosome interaction.

Since both the 34-1001 and 47-1001 truncations do not interact with the exosome, the data presented here can be interpreted in one of two ways. It may be that only the first 33 residues of Rrp44p are needed to bind the core exosome. Alternatively, this region and the linker region (residues 1 to 46) may be required for exosome interaction (see Chapter 7). In support of this, four of the most conserved residues of the linker region have been implicated in exosome interaction (Bonneau et al., 2009).

Because Rrp44p loosely associates with the exosome (Allmang et al., 1999b), interaction of the N-terminal truncations of Rrp44p with the exosome was tested under low and high salt conditions (50 mM and 1 M, respectively). The inability of both truncations to interact with the exosome, even under low salt conditions, suggests that at least the first 33 residues of Rrp44p are necessary for exosome association.

The observation that the 34-1001 truncation has no obvious growth defect, but is unable to interact with the exosome, may mean that association of Rrp44p with the exosome is not required for viability. In support of this, Rrp44p does not co-purify with exosomes in humans, *Arabidopsis*, or trypanosomes (Allmang et al., 1999b; Chekanova et al., 2002; Estevez et al., 2001). Additionally, human Rrp44p can complement a yeast *rrp44* mutant (Shiomi et al., 1998). Alternatively, the interaction between Rrp44p and the exosome may be essential, with multiple regions of Rrp44p redundant in exosome interaction. In support of this, it has been previously shown that, in addition to the N-terminus of Rrp44p, the CR3 region (Lehner and Sanderson,

2004; Schneider et al., 2009), nuclease domains, and CSDs (Wang et al., 2007) of Rrp44p are needed for exosome interaction. Therefore, it seems that the N-terminus is just one of many regions/domains that mediates the interaction between Rrp44p and the exosome.

The functional interaction between the N-terminus and the nuclease domains of Rrp44p

The first 33 residues, and at least three of the six conserved residues of the linker region (E38, D44, and I45), are not needed for endonuclease activity. Mutations of the other three linker residues (Y40, L41, and R42), however, are synthetically lethal with a C-terminal truncation that lacks the exonucleolytic RNB domain of Rrp44p but not with the D551N mutant that abolishes Rrp44p exonuclease activity. A mutant that is synthetically lethal with an Rrp44p mutant that lacks exonuclease activity may be in a residue or region that is needed for endonuclease activity. This genetic interaction can occur because cells require at least one nuclease activity of Rrp44p to survive. Therefore, although the results of these growth assays differ, it is possible that Y40, L41, and R42 are needed for the endonuclease activity of Rrp44p (see Chapter 7).

Another explanation as to why the Y40A, L41A, and R42A mutants are synthetically lethal with a C-terminal truncation that lacks the RNB domain is that these linker residues and the RNB domain make redundant contacts with the core exosome or exosome cofactor. If the interaction between Rrp44p and the exosome/exosome cofactor is required for viability, mutating these linker residues alone, or in combination with D551N, would still allow Rrp44p to associate with the exosome/exosome cofactor and, therefore, would not affect viability. In contrast, mutating these residues in a truncation that lacks the RNB domain may mean that Rrp44p is no longer able to interact with the exosome/exosome cofactor, resulting in a synthetic lethal phenotype (see Chapter 7).

While the functional interaction between the N-terminus of Rrp44p and the endonucleolytic PIN domain is still unclear, the data presented in this Chapter indicate that the first 33 residues of Rrp44p are not needed for exonuclease activity. Specifically, two different growth assays show that there is no genetic interaction between these residues and the RNB domain of Rrp44p.

The essential function of Rrp44p is likely to be in the nucleus.

Although the essential function of Rrp44p and the exosome is not yet known, the finding that Rrp44p requires at least one putative NLS for viability suggests that the essential function of Rrp44p is in the nucleus. Despite the lack of growth in a strain containing a truncation that lacks all four putative NLSs, 13-235, the data presented here do not exclude the possibility that this

truncated protein can still localize to the nucleus. Even if this truncated protein does localize to the nucleus, it would still be unclear why this truncation is unable to support cell growth.

In support of the essential function of Rrp44p being in the nucleus, none of the known cytoplasmic exosome functions are essential. The data presented here do not exclude the possibility, however, that an unidentified cytoplasmic exosome function is required for viability. In contrast to the known functions of cytoplasmic exosome, the end products of RNA processing by the nuclear exosome are required for viability, specifically the formation of the 5.8S rRNA. Several studies, however, suggest that 5.8S rRNA processing is not the essential function of Rrp44p, namely because when Rrp44p is depleted or the exonuclease activity of Rrp44p is abolished, 5.8S rRNA is still produced. Likewise, depleting Rrp44p results in defects in snRNA, snoRNA, and tRNA processing, however, these mature RNAs are always produced (Allmang et al., 1999a; Allmang et al., 1999b; de la Cruz et al., 1998; Dziembowski et al., 2007; LaCava et al., 2005; Lebreton et al., 2008; Mitchell et al., 1997; Mitchell et al., 1996; Schaeffer et al., 2009; Schneider et al., 2009; van Hoof et al., 2000a). This suggests that there are redundant pathways to process these RNAs.

The nuclear exosome is also involved in the degradation of aberrantly processed RNAs, including rRNA, sn/snoRNA, tRNA and mRNA. It is possible that the essential function of Rrp44p is the degradation of aberrant RNAs in the nucleus. In support of this, the degradation of these RNAs requires the TRAMP complex, a nuclear cofactor complex that is also required for cell viability (Castano et al., 1996; Inoue et al., 2000; Liang et al., 1996).

Because the nuclear RNAs targeted for processing and degradation by Rrp44p are crucial in many aspects of gene expression, including splicing and translation, there are likely redundant pathways to ensure these nuclear RNAs are efficiently processed and rapidly degraded, when needed. Because Rrp44p is needed for the processing and degradation of a variety of nuclear RNAs, the essential function of Rrp44p may be a combination of nuclear RNA processing reactions and/or the degradation of multiple RNAs, instead of just one specific RNA substrate. For a more detailed discussion of the essential function of Rrp44p, see Chapter 7.

Chapter 7: Conclusions and Perspectives

SUMMARY AND FUTURE DIRECTIONS

The endoribonucleolytic PIN domain of Rrp44p is required for cell survival.

The N-terminus of Rrp44p contains a PIN domain, which prior to this study, had not been characterized in eukaryotic Rrp44p. PIN domain-containing proteins in other organisms have nuclease activity. Specifically, these enzymes coordinate a divalent cation and catalyze the cleavage of DNA or ssRNA (Arcus et al., 2004; Bleichert et al., 2006; Daines et al., 2007; Fatica et al., 2004; Glavan et al., 2006; Levin et al., 2004). Because Rrp44p has the conserved acidic residues of the PIN domain active site, it was hypothesized that the PIN domain of Rrp44p may have nuclease activity. Through a collaboration with Dr. Cecilia Arraiano's laboratory (Instituto de Tecnologia Química e Biológica [ITQB]; Lisbon, Portugal), the data presented here show that the PIN domain of Rrp44p has endoribonuclease activity. It is likely that this second nuclease active site was not previously identified because it is detectable under different conditions than the exoribonuclease activity of Rrp44p. Despite three separate studies that have identified the endonuclease activity of Rrp44p (Lebreton et al., 2008; Schaeffer et al., 2009; Schneider et al., 2009), the substrates of the PIN domain have not yet been identified. The endo- and exonuclease activities of Rrp44p either act on separate substrates or on some of the same substrates. Additionally, the endonuclease activity may prefer RNA substrates that have not yet been identified as exosome substrates.

Although the catalytic activity of the PIN domain is not required for viability, the C-terminus of the PIN domain, specifically residues 203 to 235, is needed for cell survival. This may be because this region is needed for interaction with the core exosome, an exosome cofactor, and/or an RNA substrate. In support of this, the PIN domain of Rrp44p has been shown to mediate interaction with the exosome (Schneider et al., 2009).

The exonuclease activity of Rrp44p is needed for a functional nuclear and cytoplasmic exosome.

When it was discovered in 2007, the catalytically active RNB domain of Rrp44p was shown to be responsible for the 3' to 5' exonuclease activity of the exosome (Dziembowski et al., 2007). In support of this, the data presented here also show that the exonuclease activity of Rrp44p is needed for exosome-mediated 3' to 5' mRNA degradation. Because of the importance of this nuclease activity in general mRNA turnover, these findings reveal that Rrp44p is a key post-transcriptional regulator of eukaryotic gene expression.

In addition to the important role the exonuclease activity of Rrp44p plays in the cytoplasm, the results presented here also indicate that the exonuclease activity is needed for

metabolism of the yeast 35S rRNA precursor in the nucleus. Specifically, the catalytic activity of the RNB domain of Rrp44p is needed for the degradation of the 5' ETS and for the processing of the 7S pre-rRNA to form the mature 5.8S rRNA species. Importantly, other studies corroborate these findings (Dziembowski et al., 2007; Lebreton et al., 2008; Schneider et al., 2009). In further support of a role for the exonuclease activity of Rrp44p in the nucleus, the catalytic RNB domain of Rrp44p genetically interacts with the nuclear exosome cofactor, Rrp6p. Rrp6p also possesses exoribonuclease activity and is needed for many of the RNA processing and degradation reactions that occur in the nucleus (Allmang et al., 1999a; Briggs et al., 1998; Butler, 2002; Fatica et al., 2000; van Hoof et al., 2000a). The genetic interaction between these two nucleases suggests that they may have overlapping functions, however, a recent study suggests that the physical interaction between Rrp44p and Rrp6p is needed for the metabolism of only a few Rrp6p substrates (Callahan and Butler, 2008).

Determining the RNA substrates of the endo- and exonuclease activities of Rrp44p

It is not yet known whether the endo- and exonuclease activities of Rrp44p are involved in the other RNA processing and degradation reactions of the exosome. Specifically, the nuclear exosome is involved in processing RNA precursors to form small, stable, mature RNA species, as well as in the degradation of improperly processed forms of these, and other, RNAs. In contrast, the cytoplasmic exosome is strictly involved in mRNA degradation, where it is involved in general mRNA decay to rid the cell of transcripts that are no longer needed, and in mRNA surveillance, where it degrades aberrant transcripts that can have potentially deleterious effects on the cell. Future studies of Rrp44p should focus on the involvement of its nuclease activities in these cellular RNA processing and degradation reactions.

Determining the cytoplasmic RNA substrates of Rrp44p

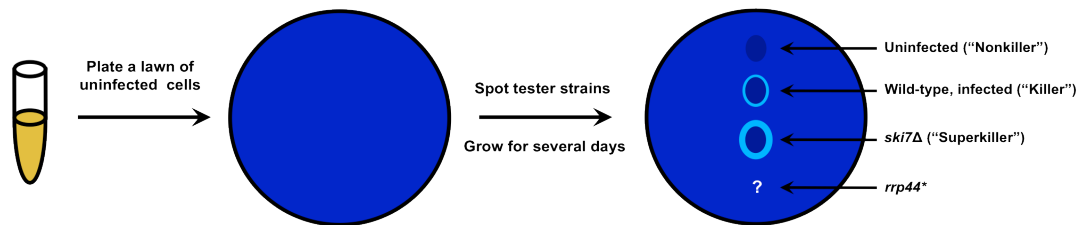
In addition to its role in general mRNA turnover, the cytoplasmic exosome is involved in RNA surveillance pathways, in which it rapidly degrades aberrant transcripts. An example of this is the nonsense-mediated decay (NMD) pathway. Here, transcripts that contain premature termination codons (PTCs) are targeted for degradation by the cytoplasmic exosome (Mitchell and Tollervey, 2003; Takahashi et al., 2003). To determine whether the nuclease activities of Rrp44p are involved in this RNA surveillance pathway, the stability of a representative PTC-containing transcript should be measured in strains containing the catalytically inactive nuclease point mutants. Briefly, the *GAL::pgk1pG*-PTC reporter should be transformed into *rrp44* deletion strains that are complemented by the D171A and D551N mutants. The *GAL::pgk1pG*-

PTC reporter contains a premature termination codon in the open reading frame of 3-phosphoglycerate kinase (*PGK1*) and a polyguanine (pG) tract to distinguish between the reporter and the endogenous transcript (Takahashi et al., 2003). Because this reporter is under the control of the galactose promoter, the *GAL::pgk1pG*-PTC transcript is expressed in media containing galactose, however, its transcription is rapidly shut off in media containing dextrose. Therefore, to determine the stability of the transcript, strains initially grown in galactose should be shifted to media containing dextrose. As a control, the stability of the *GAL::pgk1pG*-PTC transcript in these strains should be compared to the stability of the reporter in two control strains, a wild-type strain and a strain with a defective cytoplasmic exosome. RNA should then be isolated from these strains at various times and subjected to agarose formaldehyde gel electrophoresis, followed by Northern blot analysis. To determine whether strains containing the catalytically inactive nuclease point mutants are defective in NMD, blots should be hybridized with a probe specific to *pgk1pg*, and *SCR1* as a loading control. Stabilization of the *GAL::pgk1pG*-PTC transcript in strains containing the D171A and/or D551N mutants would suggest that the endo- and/or exonuclease activities of Rrp44p are needed for NMD.

The cytoplasmic exosome also has an antiviral function, whereby the exosome is thought to degrade unadenylated viral RNA, including the L-A virus and the M satellite RNA. M satellite RNA encodes a protein toxin, which is secreted from cells, and ultimately kills surrounding uninfected cells. The antiviral activity of the exosome was initially discovered in a screen for “superkiller” mutants, or mutants that are able to more efficiently kill surrounding cells (Toh and Wickner, 1980). This screen identified the *SKI* genes, several of which encode exosome subunits and cytoplasmic exosome cofactors, including Ski7p and the Ski complex. Because the Ski proteins are involved in mRNA degradation, it is thought that the exosomes antiviral activity stems from its ability to degrade viral RNAs (Brown et al., 2000; Meaux and van Hoof, 2006).

To test whether the nuclease activities of Rrp44p are needed for viral RNA degradation, and thus the antiviral activity of the exosome, a killer assay should be performed (Figure 7.1; Schaeffer et al., 2008). In this assay, infected, toxin-producing cells that contain the D171A or D551N mutant should be spotted on a lawn of uninfected cells. As a control, (i) an uninfected strain, (ii) a wild-type, toxin-producing strain, and (iii) a *ski7* deletion strain should also be spotted on the lawn of uninfected cells. The premise of this assay is that infected cells secrete the killer toxin that then kills the surrounding uninfected cells, thus creating a halo, or zone of clearance. In this assay, the uninfected control strain would not create a halo. In contrast, the *ski7* deletion strain, a known “superkiller” strain, would produce a halo larger than the wild-type,

A.



B.

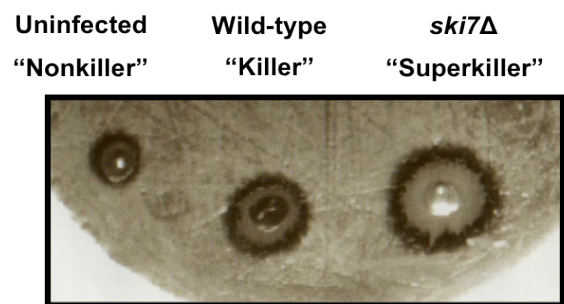


Figure 7.1: Schematic representation of the yeast killer assay.

Figure 7.1: Schematic representation of the yeast killer assay. The involvement of the nuclease activities of Rrp44p in viral RNA degradation should be analyzed using the killer assay. First, a lawn of uninfected cells should be plated. Next, tester strains, including (i) an uninfected strain (ii) a wild-type, infected strain (iii) a *ski7* deletion strain and (iv) infected strains containing the catalytically inactive *rrp44* nuclease mutants (*rrp44**; *rrp44* D171A or *rrp44* D551N) should be spotted on the lawn of uninfected cells. Infected cells secrete a killer toxin that then kills the surrounding uninfected cells, thus creating a halo, or zone of clearance. The *ski7* deletion strain, a known “superkiller” strain, produces a halo larger than the wild-type, infected strain.

infected strain. If the halo produced by the strain containing the D171A and/or D551N mutants is similar to the *ski7Δ* strain, this would suggest that the endo- and/or exonuclease activities of Rrp44p are required for viral RNA degradation and may, therefore, be needed for the antiviral function of the exosome.

Determining the nuclear RNA substrates of Rrp44p

The exonuclease activity of Rrp44p is required for 5.8S rRNA processing and for the degradation of the 5' ETS, whereas the endonuclease activity does not seem to be required for the metabolism of the 35S rRNA precursor. The data presented here, however, do not exclude the possibility that the nuclease activities of Rrp44p are needed for the other RNA processing and degradation reactions of the nuclear exosome (Table 7.1). In addition to processing and degrading the 35S rRNA precursor, the nuclear exosome processes and degrades snRNAs and three different types of snoRNAs, including those that are independently transcribed, snoRNAs that are intron-derived, and polycistronic snoRNAs (Allmang et al., 1999a; Allmang et al., 1999b; de la Cruz et al., 1998; Mitchell et al., 1997; van Hoof et al., 2000a). The nuclear exosome is also involved in RNA surveillance, whereby it rapidly degrades aberrantly processed forms of these RNAs (Egecioglu et al., 2006; LaCava et al., 2005). The exosome also maintains the fidelity of gene expression by degrading unspliced mRNAs (Bousquet-Antonelli et al., 2000) and cryptic unstable transcripts (CUTs), which are the result of transcription of intergenic regions (Davis and Ares, 2006).

To determine if the nuclease activities of Rrp44p are needed for these nuclear RNA processing and degradation reactions, RNA should be isolated from a strain containing the D171A mutant and a strain containing the D551N mutant. As a control, RNA should also be isolated from a wild-type strain and a strain with a defective nuclear exosome. The RNA from these strains should be subjected to denaturing urea-polyacrylamide gel electrophoresis, followed by Northern blot analysis. To determine if the Rrp44p nuclease activities are needed for the processing and/or degradation of snRNAs, snoRNAs, unspliced mRNA, and/or CUTs, blots should be hybridized with probes specific to known nuclear exosome substrates including: (i) three snRNAs, U1, U4, and U5 (ii) at least one of each of the different types of snoRNAs, including snR33, U18, and U14 (iii) the intron of a typically spliced mRNA, such as *RP51A* and (iv) a CUT, such as *SGRI*. The presence of RNA processing and/or degradation intermediates in strains containing the D171A and/or D551N mutants would suggest that the endo- and/or exonuclease activities of Rrp44p are needed for these nuclear RNA metabolism reactions.

Table 7.1: Strains and probes needed to test the role of the Rrp44p nucleases in nuclear RNA metabolism				
Nuclear exosome substrate	Role of the exosome in RNA metabolism	Strain to test role of Rrp44p endonuclease in RNA metabolism	Northern blot analysis with a probe specific to:	
snRNAs	Processing; degradation of aberrant snRNAs	<i>rrp44</i> D171A <i>rrp44</i> D551N	U1, U4, U5	
snoRNAs	-Independently transcribed snoRNAs (ITSnoRNAs) -Intron-derived snoRNAs (I-DsnoRNAs) -Polycistronic snoRNAs (PsnoRNAs)	<i>rrp44</i> D171A <i>rrp44</i> D551N	-snR33 (ITSnoRNA) -U18 (I-DsnoRNA) -U14 (PsnoRNA)	
Hypomodified initiator tRNA ^{Met}	Degradation	<i>rrp44</i> D171A, <i>trm6-504</i> [*] <i>rrp44</i> D551N, <i>trm6-504</i> [*]	tRNA _i ^{Met}	
Cryptic Unstable Transcripts (CUTs)	Degradation	<i>rrp44</i> D171A <i>rrp44</i> D551N	<i>SGR1</i> [°]	
mRNAs with improperly processed 3'-ends	Degradation	<i>rrp44</i> D171A, <i>rna14.1</i> [‡] <i>rrp44</i> D551N, <i>rna14.1</i> [‡]	<i>HSP104</i> [°]	
Unspliced mRNAs	Degradation	<i>rrp44</i> D171A <i>rrp44</i> D551N	<i>RP51A</i> [‡]	

^{*}*trm6* encodes m¹A methyltransferase, which is needed for proper tRNA_i^{Met} modification

[‡]*rna14* encodes CFI, a factor needed for 3'-end mRNA cleavage and polyadenylation

[°]*SGR1* is a non-coding, promoter-associated transcript

[°]*HSP104* encodes a heat shock protein and is a known substrate of CFI

[‡]*RP51A* encodes a ribosomal protein of the small (40S) subunit

In addition to degrading improperly processed snRNAs, and snoRNAs, the nuclear exosome also degrades improperly modified tRNAs, including the initiator tRNA_i^{Met} (Table 7.1). This hypomodified tRNA accumulates in cells that have a defective m¹A methyltransferase, which results in a lack methylation of adenosine at position 58 of the T ψ C loop (Kadaba et al., 2004; Kadaba et al., 2006). To test whether the endo- and/or exonuclease activities of Rrp44p are needed to degrade these transcripts, the D171A and D551N mutants should be separately combined with a temperature-sensitive mutant of the m¹A methyltransferase, *trm6-504*. RNA should be isolated from a wild-type strain, a strain containing only the *trm6-504* mutant, a strain containing the D171A *trm6-504* double mutant, and a strain containing the D551N *trm6-504* double mutant. The RNA from these strains should be subjected to denaturing urea-polyacrylamide gel electrophoresis, followed by Northern blot analysis with a probe specific to the initiator tRNA_i^{Met}. The presence of hypomodified tRNA_i^{Met} in strains containing the D171A and/or D551N mutants would suggest that the endo- and/or exonuclease activities of Rrp44p are needed for the degradation of improperly modified tRNAs.

Lastly, the nuclear exosome is involved in degrading mRNAs that have defects in 3'-end processing (Table 7.1; Libri et al., 2002; Torchet et al., 2002). These types of transcripts accumulate in cells that are defective in transcription termination, mRNA cleavage, or polyadenylation. To test whether the nucleases activities of Rrp44p are needed to degrade these transcripts, the D171A and the D551N mutants should be separately combined with a temperature-sensitive mutant of *rna14.1*, which encodes an mRNA cleavage and polyadenylation factor, CFI (Minvielle-Sebastia et al., 1994; Minvielle-Sebastia et al., 1991). A strain containing only the *rna14.1* mutant does not accumulate 3'-extended transcripts due to the rapid degradation of these aberrant mRNAs by the nuclear exosome. In contrast, a strain containing *rna14.1* and an exosome mutant, does accumulate these transcripts (Torchet et al., 2002). To determine whether the endo- and/or exonuclease activities of Rrp44p degrade these aberrant mRNAs, RNA should be isolated from a strain containing only the *rna14.1* mutant, a strain that contains the *rna14.1* mutant that also lacks a functional nuclear exosome, a strain containing the D171A *rna14.1* double mutant, and a strain containing the D551N *rna14.1* double mutant. The RNA from these strains should be subjected to denaturing urea-polyacrylamide gel electrophoresis, followed by Northern blot analysis. Specifically, blots should be hybridized with a probe specific to *HSP104*, a transcript known to be processed at its 3'-end by CFI (Libri et al., 2002). The presence of 3'-extended *HSP104* species in strains containing the D171A and/or D551N mutants would suggest that the endo- and/or exonuclease activities of Rrp44p are needed for the degradation of mRNAs that have defects in 3'-end processing.

Global approaches to determining the RNA substrates of Rrp44p

The approaches described above should be useful in determining if the nuclease activities of Rrp44p are needed to degrade specific RNA substrates, however, these approaches are limited to only known nuclear exosome substrates. Therefore, two more global approaches should be taken to determine the substrates of nuclease activities of Rrp44p. First, a tiling microarray should be performed on a wild-type strain, a strain containing the D171A mutant, and a strain containing the D551N mutant. Comparing the results from the catalytically inactive nuclease mutants to the wild-type strain will yield RNA substrates that are upregulated in the nuclease mutants, and are therefore, degraded by the Rrp44p endo- and/or exonuclease.

Another approach to detect the RNA substrates of the Rrp44p nucleases is to examine the genetic interactions that occur in a wild-type strain but do not occur in strains containing the D171A or D551N mutants. To do this, a heterozygote diploid-based synthetic lethality analysis with microarrays (dSLAM) should be performed (Figure 7.2; Pan et al., 2007). Specifically, the D171A and D551N mutants should be introduced, individually, into a population of approximately 6000 heterozygous diploid Yeast Knockout (YKO) mutants. The heterozygote diploid double mutants should then be sporulated and the resultant haploid double mutants should be studied for synthetic lethality by microarray analysis. Briefly, the unique 20 nucleotide sequences flanking the deletion cassette of the YKO mutants, termed “molecular barcodes”, should be PCR amplified with a Cy3-labeled primer from the haploid double mutants (Experiment) and a Cy5-labeled primer from the haploid single knockout mutants (Control). Both PCR products should be hybridized to a microarray chip containing each of the “molecular barcodes”. A synthetic lethal interaction should be observed by high Control-to-Experiment Cy5 hybridization signal intensity (Pan et al., 2004).

The CR3 region of Rrp44p may be a putative zinc binding domain.

The N-terminus of Rrp44p contains a CR3 region that contains three conserved cysteine residues (C47, C52, and C55). Despite extensive functional analyses of Rrp44p over the past three years, no information exists on the CR3 region in Rrp44p or in any other protein. Further complicating the study of this region is the lack of structural data on the N-terminus of Rrp44p. Although there are two published crystal structures of the catalytic subunit, neither contain the N-terminus in its entirety. Because of the lack of functional and structural data on this region, it is still not known how the CR3 region functions in Rrp44p, or the exosome, whether this region adopts a unique fold, or if this region consists of more than three cysteine residues.

Despite the lack of the entire N-terminus, the latest crystal structure of Rrp44p (Bonneau

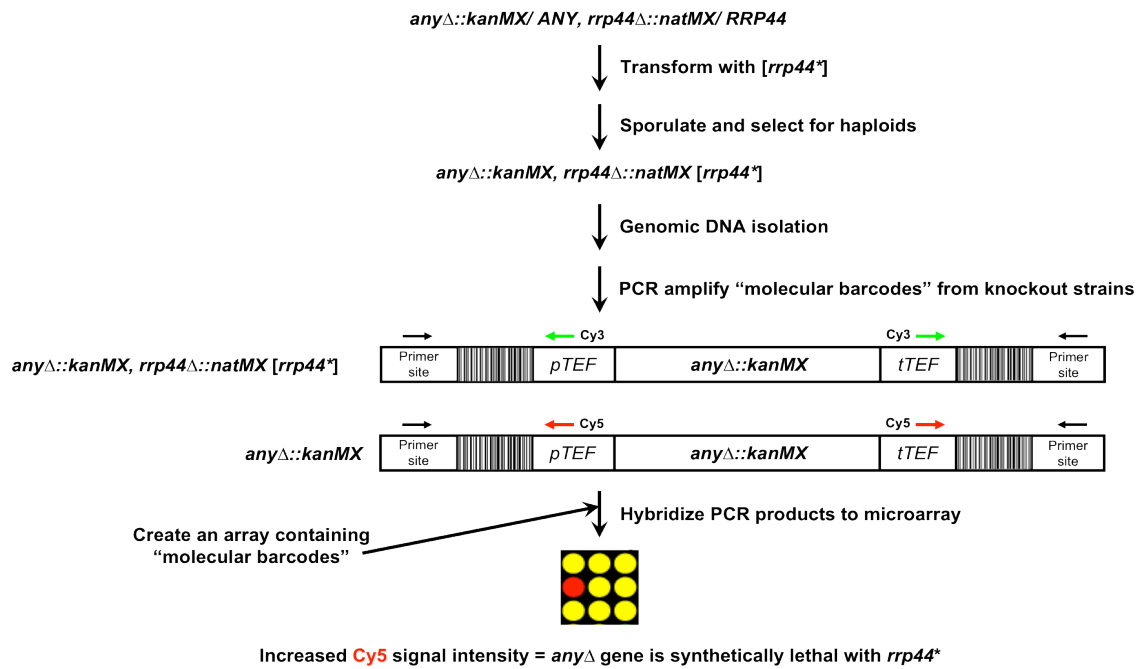


Figure 7.2: Schematic representation of the heterozygote diploid-based synthetic lethality analysis with microarrays (dSLAM) technique.

Figure 7.2: Schematic representation of the heterozygote diploid-based synthetic lethality analysis with microarrays (dSLAM) technique. The genetic interactions of the Rrp44p nucleases should be analyzed using dSLAM. Specifically, a plasmid encoding the catalytically inactive nuclease point mutations of *RRP44* (*rrp44**; *rrp44* D171A or *rrp44* D551N) should be transformed into a population of approximately 6000 heterozygous diploid Yeast Knockout (YKO) mutants. The heterozygote diploid double mutants should then be sporulated and haploids should be selected. To determine whether the double mutants are synthetically lethal, the unique 20-nucleotide sequences flanking the deletion cassette of the YKO mutants, termed “molecular barcodes”, should be PCR amplified. In the double mutants, a Cy3-labeled primer should be used for amplification whereas a Cy5-labeled primer should be used to PCR amplify the “molecular barcodes” in the single mutants. Both PCR products should be hybridized to a microarray chip containing each of the “molecular barcodes”. A synthetic lethal interaction should be observed by high Cy5 hybridization signal intensity.

et al., 2009) provided at least two interesting findings. First, the second and third residues of the CR3 region are modeled to form a disulfide bond, however, the data presented here suggest that the disulfide bond is not required for the function of Rrp44p, or the exosome. In addition, the electron density of the crystal structure of Rrp44p is not defined enough to determine whether the residues of the CR3 region form a disulfide bond with each other or with other residues of Rrp44p. The second interesting observation of the Rrp44p crystal structure is that the three cysteine residues of the CR3 region are in close proximity to histidine 184 (H184). This spatial organization is consistent with coordination of a zinc ion, however, no metal ion was present in the crystal structure. The lack of a metal ion may be an artifact of the protein purification conditions that were used, and does not exclude the possibility that the CR3 region and H184 coordinate a metal. To determine whether Rrp44p coordinates a zinc ion, a GST-fusion of full-length Rrp44p should be expressed and purified from *E. coli*. The purified protein sample should then be subjected to atomic absorption spectroscopy, a technique used to determine the concentration of a particular metal element in a sample (Sperling and Welz, 1999). Briefly, this technique transforms a liquid protein sample into an atomic gas and then analyzes the free atoms of the protein sample. As an internal control, this technique should detect the presence of Mn^{2+} , which is coordinated by the PIN domain active site, and Mg^{2+} , which is coordinated by the RNB domain active site. If these metals are not detected, the GST-fusion of Rrp44p should be purified using different purification conditions to ensure that the lack of metals is not due to the protein purification conditions.

The coordination of a zinc ion may be needed, directly or indirectly, for Rrp44p protein stabilization, interaction with the exosome, and/or for Rrp44p nuclease activity. Indeed, preliminary evidence suggests that endonuclease activity of Rrp44p is stimulated by the addition of zinc (Filipa Reis and Dr. Cecilia Arraiano, personal communication). In addition, mutation of the three cysteine residues of the CR3 region results in growth defects and defects in exosome interaction and function. However, the requirement for the CR3 region in exosome interaction may be indirect. If the coordination of a zinc atom between the CR3 region and H184 is needed for proper folding, the CR3 region in the triple CR3 mutant may not be folded correctly, which could also cause the upstream linker region to misfold. Since this region contains residues that contact the exosome (Y40, L41, R42, and D44), this misfolding could disrupt exosome interaction. Therefore, further studies are needed to determine whether the CR3 region is directly involved in exosome interaction or if the lack of interaction seen in the triple CR3 mutant is a secondary consequence of protein misfolding.

Several experiments, including *in vivo* genetic analysis and *in vitro* biochemical nuclease assays, also suggest that the CR3 region is needed for the nuclease activities of Rrp44p. Therefore, the overall conclusion is that the defects seen in the triple CR3 mutant may be explained by the lack of coordination of a zinc ion.

The CR3 region of Rrp44p may be needed for 3' to 5' mRNA degradation because this region may interact with a protein involved in 3' to 5' mRNA decay.

The observation that mutating the three cysteine residues of the CR3 region is synthetically lethal with the D171A mutant and a mutant that inactivates the 5' to 3' mRNA degradation pathway, suggests that the CR3 region is needed for exonuclease activity of Rrp44p. Although the CR3 region and the active site of the RNB domain are not in close proximity in the crystal structure, the CR3 region may be needed to interact with cytoplasmic exosome cofactors that are required for 3' to 5' mRNA degradation, including Ski7p and the Ski complex. It has been previously reported that Ski7p interacts with the exosome (Araki et al., 2001), however, an interaction between the exosome and the Ski complex has not yet been shown. To determine whether the CR3 region of Rrp44p interacts with these cofactors, Rrp44p and the cytoplasmic exosome cofactors should be C-terminally tagged. Specifically, Ski7p and members of the Ski complex, Ski2p, Ski3p, and Ski8p, should be C-terminally Myc-tagged, whereas wild-type Rrp44p and the triple CR3 mutant should be C-terminally TAP-tagged. Wild-type Rrp44p, the triple CR3 mutant, and a strain containing an empty vector should be purified. Proteins bound to Rrp44p should be eluted, subjected to SDS-PAGE, and Western blot analysis should be performed. Specifically, interactions between Rrp44p, Ski7p, and the Ski complex should be detected using antibodies specific to the Myc tag.

Targeted co-purification of proteins bound to Rrp44p followed by Western blot analysis is one way to detect interactions between Rrp44p and the cytoplasmic exosome cofactors, however, a limitation of this technique is that the individual proteins must be tagged. Therefore, a more global approach should be taken to determine what proteins bind to the CR3 region of Rrp44p. Specifically, C-terminally TAP-tagged wild-type Rrp44p and the triple CR3 mutant should be purified and subjected to SDS-PAGE. The SDS gel should then be stained with Coomassie Brilliant Blue. Any proteins that bind to wild-type Rrp44p, but not to the triple CR3 mutant, should be extracted and analyzed by mass spectrometry.

Another possible explanation for why the triple CR3 mutant is synthetically lethal with the D171A mutant is that the CR3, D171A mutant protein is not expressed. A potential decrease in expression, coupled with a loss of exosome interaction due to the triple CR3 mutation, may

explain the lack of complementation by the CR3, D171A mutant. To determine whether the lack of growth is an artifact of expression, the CR3, D171A mutant should be C-terminally TAP-tagged and protein expression should be analyzed using SDS-PAGE and Western blot analysis.

Nonstop mRNA degradation requires Rrp44p.

A functional nonstop mRNA degradation pathway may not require the nuclease activities of Rrp44p.

The data presented here suggest that the nuclease activities of Rrp44p are not needed for the degradation of nonstop transcripts. These findings can be explained by one of three possibilities. First, the nucleases may be redundant in nonstop decay. One method used to determine whether the nuclease activities of Rrp44p are redundant in this mRNA surveillance pathway was to measure the stability of a representative nonstop reporter in a strain that abolishes both of the nuclease activities of Rrp44p. Specifically, the *GAL::pgk1pG*-nonstop reporter was transformed into an *rrp44* deletion strain that was complemented by a plasmid encoding a temperature-sensitive allele of *rrp44* (*rrp44^{ts}*). This strain also contained a plasmid encoding the D171A or D551N point mutation of Rrp44p. These strains were initially grown at the permissive temperature (23°C) then shifted to the non-permissive temperature (37°C) for ten hours to inactivate the *rrp44^{ts}* allele. However, Northern blot analysis revealed that *GAL::pgk1pG*-nonstop transcript was still unstable after the ten hour shift to the non-permissive temperature, indicating that this time course was not sufficient to inactivate the *rrp44^{ts}* allele. Therefore, alternative means must be used to test whether nonstop transcripts are degraded when both the endo- and exonuclease activities of Rrp44p are abolished.

Another way to test the redundancy of the Rrp44p nucleases in nonstop decay is to test whether the double catalytically inactive D171A, D551N mutant can rescue the nonstop mRNA decay defect of the triple CR3 mutant using the *his3*-nonstop growth assay. Briefly, an *rrp44* deletion strain that is complemented by a plasmid encoding the triple CR3 mutation (*rrp44Δ* C47S, C52S, C55S) will be transformed with a plasmid encoding the double D171A, D551N point mutation. As a control, the *rrp44Δ* C47S, C52S, C55S strain will also be transformed with plasmids encoding wild-type *RRP44*, the D171A mutation alone, the D551N mutation alone, and an empty vector. The resulting strains will then be transformed with the *his3*-nonstop reporter, which contains a nonstop mutation in the *HIS3* open reading frame. The basis for this assay is that the *his3*-nonstop transcript will be stable in a strain that is defective in nonstop mRNA degradation, and will, therefore, be viable on media lacking histidine. Conversely, in a strain with a functional nonstop decay pathway the *his3*-nonstop transcript will be rapidly degraded,

resulting in a lack of viability on media lacking histidine. Since, independently, the nuclease activities of Rrp44p are not required for nonstop decay, wild-type Rrp44p, the D171A mutant, and the D551N mutant should rescue the nonstop decay defects caused by the triple CR3 mutant, which will result in a lack of growth of these strains on media lacking histidine. In contrast, if the nuclease activities of Rrp44p are redundant in nonstop decay, the double catalytically inactive mutant should not be able to rescue the triple CR3 mutant, and should, therefore, be viable on media lacking histidine.

A second explanation for why the endo- and exonuclease activities of Rrp44p are not needed to degrade nonstop transcripts is that nonstop decay is independent of the known catalytic activities of the cytoplasmic exosome. Under this hypothesis, a different cellular nuclease would be responsible for the degradation of nonstop transcripts. Interestingly, a recent genome-wide screen to identify factors involved in nonstop decay did not identify any additional nucleases (Wilson et al., 2007).

Lastly, abolishing the nuclease activities of Rrp44p may not have an effect on nonstop decay because Rrp6p, a known nuclear exosome cofactor, may be required for the degradation of nonstop transcripts. Although Rrp6p is presumed to be present only in the nucleus, a small fraction of the protein may associate with the cytoplasmic exosome. In this case, a small amount of Rrp6p activity may be sufficient for the degradation of nonstop transcripts. In support of this, the human homolog of Rrp6p localizes to the nucleus and the cytoplasm in human cell lines (Lejeune et al., 2003). Additionally, Rrp6p has been shown to interact with Ski7p, a cytoplasmic exosome cofactor required for general 3' to 5' mRNA degradation and nonstop mRNA decay (Collins et al., 2007; Peng et al., 2003). To determine whether Rrp6p is involved in nonstop decay, the stability of a representative nonstop transcript, *GAL::pgk1pG-nonstop*, should be measured in an *rrp6* deletion strain. As a control, the nonstop reporter should also be transformed into a wild-type strain and a *ski7* deletion strain. The aforementioned strains should be grown in liquid media containing galactose to induce expression of the reporter and then switched to media containing dextrose to shut off reporter transcription. RNA should then be isolated from the strains and subject to agarose formaldehyde gel electrophoresis. Northern blot analysis should then be performed using probes specific to *pgk1pG*, and *SCR1* as a loading control. If the *GAL::pgk1pG-nonstop* transcript has a similar half-life in the *rrp6* deletion strain as it does in the *ski7* deletion strain, Rrp6p may be involved in nonstop decay, which would suggest that it is not excluded from the cytoplasm.

The CR3 region of Rrp44p is needed for a functional nonstop mRNA decay pathway.

Nonstop transcripts are not efficiently degraded in the triple CR3 mutant, which suggests that the CR3 region is needed for nonstop decay. This result can be interpreted in one of two ways. In the first interpretation, the nuclease activities of Rrp44p are redundant in nonstop decay. In the second interpretation, the nuclease activities of Rrp44p are not required for nonstop decay, meaning that a nuclease activity that is independent of the exosome is responsible for the degradation of nonstop transcripts.

If the nuclease activities of Rrp44p are redundant in nonstop decay, the nonstop decay defects observed in the triple CR3 mutant may be because the triple CR3 mutant protein is expressed at lower levels than wild-type Rrp44p. Under this hypothesis, decreased expression of Rrp44p could result in decreased nuclease activity, which would result in the stabilization of nonstop transcripts. If the nuclease activities of Rrp44p are found to be redundant in nonstop decay, the stability of a representative nonstop transcript should be analyzed in a strain that overexpresses the triple CR3 mutant. Briefly, an *rrp44* deletion strain that is complemented by a plasmid that overexpresses the triple CR3 mutant should be transformed with the *GAL::pgk1pG*-nonstop transcript. As a control, the *GAL::pgk1pG*-nonstop reporter should also be transformed into a wild-type strain and in a *ski7* deletion strain. RNA should be isolated from these strains and subjected to agarose formaldehyde gel electrophoresis. The stability of the *GAL::pgk1pG*-nonstop transcript should be analyzed in these strains by performing Northern blot analysis. Specifically, blots should be hybridized with probes that are specific to *pgk1pG*, and *SCR1* as a loading control. If the decreased expression of the triple CR3 mutant is responsible for a non-functional nonstop decay pathway, the *GAL::pgk1pG*-nonstop transcript should be rapidly degraded in a strain that overexpresses the triple CR3 mutant. Alternatively, stabilization of the nonstop reporter in the strain that overexpresses the triple CR3 mutant would suggest that the CR3 region is truly needed for nonstop decay. If the nuclease activities of Rrp44p are redundant in nonstop decay, the latter result would not be surprising given that the CR3 region seems to be needed for the nuclease activities of Rrp44p.

The CR3 mutant could have defects in nonstop decay even if the nuclease activities of Rrp44p are not redundant for the degradation of nonstop transcripts. If the nuclease activities are not redundant, another nuclease is responsible for degrading nonstop transcripts. It may be that the CR3 region of Rrp44p interacts with this nuclease, and that this interaction is required for nonstop decay. This hypothesis could be tested by determining whether any cellular nucleases associate with wild-type Rrp44p but not with the triple CR3 mutant.

Lastly, regardless of whether the nuclease activities of Rrp44p are redundant in nonstop decay, the nonstop decay defects observed in the triple CR3 mutant may be because the CR3 region of Rrp44p is needed interact with the nonstop mRNA degradation machinery. Specifically, the CR3 region could be tested for its interaction with the cytoplasmic exosome cofactors, Ski7p and the Ski complex (see above).

The far N-terminus of Rrp44p is needed for viability and is only one of several regions of Rrp44p that is required for interaction with the exosome.

The N-terminus of Rrp44p consists of the far N-terminus, which includes residues 1 to 46, a CR3 region, and a PIN domain. The structure and function of the far N-terminus is not yet known. This region, however, does contain a linker region of conserved residues, specifically, residues 34 to 46. The results presented here indicate that this linker region is important for viability and that at least the first 33 residues of the far N-terminus of Rrp44p are needed for exosome interaction. What is not yet known is which residues of the linker region are needed for viability and whether the rest of the far N-terminus of Rrp44p mediates interaction with the exosome.

Determining which residues of the far N-terminus of Rrp44p are needed for viability

The data presented here indicate that the linker region, which consists of the highly conserved residues 34 to 46, is needed for viability. Interestingly, individually mutating six of the most conserved residues of this region has no effect on cell growth. This discrepancy may be because the residues of the linker region make redundant protein-protein interactions that are required for viability. For example, the linker region may be needed to interact with the exosome (Bonneau et al., 2009) and/or with an exosome cofactor. If this interaction is required for viability, mutation of one residue would not affect cell growth, however, deletion of the entire region would be lethal. In support of this, the data presented here and in other studies indicate that the N-terminus is needed for interaction with the exosome (Bonneau et al., 2009; Lehner and Sanderson, 2004; Schneider et al., 2009; Wang et al., 2007). To test whether these residues are redundant for viability, several combinations of linker mutants would have to be generated and tested for complementation of an *rrp44Δ* [*RRP44*, *URA3*] strain. The generation of these mutants would be laborious given that there are six highly conserved residues, with a large number of combinations that could be tested.

This experiment would determine whether the linker residues are redundant for viability, but would not establish whether these residues are redundant for an essential protein-protein

interaction. To test this would be difficult, given that a vital protein-protein interaction would have to be identified and combinations of linker mutants would have to be tested for the lack of this protein-protein interaction. Although the results presented here, along with several other studies, seem to indicate that multiple regions of Rrp44p are needed for association with the exosome, no one study has identified whether these regions are truly redundant for exosome interaction. Additionally, it is not yet known whether the interaction between Rrp44p and the yeast exosome is required for viability.

Determining which regions of the N-terminus of Rrp44p are needed for exosome interaction

The data shown here indicate that the first 33 residues of Rrp44p are needed for exosome interaction, however, the data do not exclude the possibility that the entire far N-terminus of Rrp44p is needed for association with the exosome. Specifically, the linker region, residues 34 to 46, may function with the first 33 residues to mediate the interaction between Rrp44p and the exosome.

Therefore, to determine if the linker region of Rrp44p is also required for exosome interaction, an exosome subunit, Rrp43p, should be C-terminally Myc-tagged. In addition, wild-type Rrp44p, the 34-1001 and 47-1001 truncations, and a truncation that lacks the conserved stretch of linker residues, residues 34 to 46, should be C-terminally TAP-tagged and then purified. Proteins bound to wild-type and truncated Rrp44p should be eluted and subjected to SDS-PAGE and Western blot analysis. Specifically, the interactions between Rrp44p and the exosome, namely Rrp43p, should be observed through the use of an antibody specific to the Myc tag. If only the first 33 residues of Rrp44p are required for exosome interaction, wild-type Rrp44p and a truncation lacking residues 34 to 46 would interact with the exosome. In contrast, if the entire far N-terminus, which includes residues 1 to 46, is required to interact with the exosome, an interaction between only wild-type Rrp44p and the exosome would be observed. If the residues of this region make redundant contacts with the exosome, and the interaction between Rrp44p and the exosome is essential, this result would explain why deletion of the first 46 residues of Rrp44p does not support viability.

Although the first 33 residues of the far N-terminus of Rrp44p are necessary for association with the exosome, this region does not appear to be sufficient for exosome interaction. Specifically, the data presented here and in other studies, indicate that the entire N-terminus of Rrp44p, including the uncharacterized far N-terminus, CR3 region, and PIN domain, are required for exosome interaction (Schneider et al., 2009). In addition, a cryo-EM structure of the yeast exosome suggests that the RNB domain of Rrp44p also makes contacts with the

exosome (Wang et al., 2007). Therefore, the N-terminus is likely one of many regions of Rrp44p that are needed for exosome interaction.

Determining the function of the three conserved residues of the far N-terminus of Rrp44p

Point mutations of three of the conserved residues of the far N-terminus (Y40A, L41A, and R42A) are synthetically lethal with a C-terminal truncation of Rrp44p that lacks the exonucleolytic RNB domain. Because at least one nuclease activity of Rrp44p is required for cell viability, this finding suggests that these residues may be needed for the endonuclease activity of Rrp44p. However, these three point mutants are not synthetically lethal with the D551N mutant, which suggests that these residues are not needed for PIN domain nuclease activity. To differentiate between these possibilities, the Y40A, L41A, and R42A point mutants, as well as wild-type Rrp44p, should be expressed and purified as GST-fusions from *E. coli*. The purified Rrp44p proteins should then be incubated with a ^{32}P 5'-end labeled A₃₀ oligonucleotide. RNA aliquots should be isolated at certain times and subjected to denaturing urea-polyacrylamide gel electrophoresis for single nucleotide separation. A PhosphorImager screen should then be used to detect endonucleolytic cleavage of the A₃₀ substrate.

The synthetic lethality observed when these three point mutations were generated in a C-terminal truncation of Rrp44p that lacks the RNB domain could also be because these three linker residues and the RNB domain make redundant contacts with the exosome and/or exosome cofactors. If the interaction between Rrp44p and the exosome/exosome cofactor are required for viability, mutating the linker residues alone, or in combination with D551N, would still allow for Rrp44p to make these vital protein-protein interactions, and would thus not affect viability. In contrast, mutating these conserved residues in a truncation that lacks the RNB domain may mean that Rrp44p is no longer able to participate these essential protein-protein interactions, resulting in a synthetic lethal phenotype. To determine whether these regions of Rrp44p are redundant for protein-protein interactions, the following proteins should be C-terminally TAP-tagged and purified: wild-type Rrp44p, the individual linker point mutants, the D551N mutant, and a mutant version of Rrp44p that combines a linker mutant with the D551N mutant. The purified proteins should be subject to SDS-PAGE, followed by staining of the SDS gel with Coomassie Brilliant Blue. Any proteins that bind to the individual linker mutants and the D551N mutant, but not to the combination mutant, should be extracted and analyzed by mass spectrometry.

Determining the essential function of Rrp44p

Thirteen years after the discovery of the exosome (Mitchell et al., 1997), its essential function(s) is not yet known. In addition, it is not clear why the ten subunits of the exosome are all required for viability. The essential function of Rrp44p is likely nuclear because none of the known cytoplasmic functions of the exosome are required for viability and removing the putative NLSs of Rrp44p results in a lack of cell growth. The data presented here, however, do not exclude the possibility that the Rrp44p truncation that lacks the putative NLSs (13-235) can still localize to the nucleus. To address this, the cellular localization of this truncated protein will be determined by GFP-tagging residues 13-235. In addition to being nuclear, the essential function of Rrp44p requires its nuclease activities, as abolishing both the endo- and exonuclease activities of Rrp44p results in a lack of cell growth.

Although it is possible that the essential function of the exosome is distinct from that of Rrp44p, the findings presented here suggest that the essential function of the exosome mirrors that of Rrp44p. Specifically, Rrp44p is the only catalytic subunit of the exosome, with two separate nuclease active sites. Since the exosome has only been shown to be involved in RNA metabolism, the essential function of the exosome most likely requires the nuclease activity of Rrp44p. Additionally, the likely nuclear essential function of the exosome would have to rely on the nuclear localization of Rrp44p since Rrp44p is the only subunit that contains a putative NLS.

The data presented here suggest that the essential function of Rrp44p and the exosome is nuclear, however, the nuclear exosome is involved in numerous RNA processing reactions. Because the products of these processing reactions are required for cell survival, it is impossible to know which of the processing reactions is/are needed for viability. The essential function of Rrp44p and the exosome is likely not the processing of rRNA, snRNA, or snoRNAs because when the exonuclease activity of Rrp44p is abolished or any of the exosome subunits are depleted, these mature RNAs are still produced (Allmang et al., 1999a; Allmang et al., 1999b; de la Cruz et al., 1998; Dziembowski et al., 2007; LaCava et al., 2005; Lebreton et al., 2008; Mitchell et al., 1997; Mitchell et al., 1996; Schaeffer et al., 2009; Schneider et al., 2009; van Hoof et al., 2000a). This is likely because there are redundant pathways for nuclear RNA processing.

The nuclear exosome is also involved in nuclear RNA surveillance, whereby it degrades aberrantly processed forms of RNA, as well as CUTs, and the 5' ETS of the 35S rRNA precursor. Therefore, it is possible that the essential function of Rrp44p, and the exosome, is the degradation of aberrant nuclear RNAs. In support of this, the degradation of these RNAs requires the TRAMP complex, a nuclear cofactor complex that is also essential for cell survival (Castano

et al., 1996; Inoue et al., 2000; Liang et al., 1996). To test whether the degradation of aberrant RNAs is required for viability is difficult given that these aberrant RNAs arise spontaneously and are present at low levels. Additionally, this would be difficult to test because there are so many aberrant RNAs that are degraded by the nuclear exosome. If the essential function of Rrp44p and the exosome is the degradation of aberrant RNAs, cell viability would likely require the efficient degradation of more than one aberrant RNA.

CONCLUDING REMARKS

This dissertation has contributed to the field of eukaryotic RNA metabolism by providing a detailed genetic and biochemical analysis of the catalytic subunit of the eukaryotic RNA degrading exosome, Rrp44p. Specifically, this work has described a novel nuclease activity of Rrp44p and has shown that the regions/domains of Rrp44p have specific functions in both the cytoplasmic and nuclear exosome. Further, this work has uncovered the roles of Rrp44p in eukaryotic RNA metabolism, as a post-transcriptional regulator of gene expression and as an RNA surveillance protein to maintain the fidelity of gene expression.

Finally, this dissertation work provides a foundation on which to develop further experiments to elucidate the RNA substrates of Rrp44p and to structurally, and functionally, define the CR3 region of Rrp44p.

Appendix: The essential function of Rrp44p cannot be carried out by other 3' to 5' exoribonucleases.

INTRODUCTION

The crystal structure of the eukaryotic exosome shows homology to two RNA degradation complexes, bacterial PNPase and the archaeal exosome. Specifically, PNPase and the archaeal and eukaryotic exosomes contain a PH ring that consists of six PH domains, or subunits (Liu et al., 2006), that are homologous to RNase PH (Zuo and Deutscher, 2001). In bacteria, RNase PH is a phosphorolytic 3' to 5' exoribonuclease that uses inorganic phosphate to process, or trim, tRNA precursors to form mature tRNA species (Wen et al., 2005). In PNPase and the archaeal exosome, three of the PH domains have 3' to 5' exoribonuclease activity (Buttner et al., 2005; Lorentzen and Conti, 2005; Navarro et al., 2008; Symmons et al., 2000). Because of the similarity between these RNA degradation complexes, it was initially thought that the PH ring of the eukaryotic exosome was the site of RNA degradation. However, recent studies have shown that despite the sequence and structural similarity to PNPase and the archaeal exosome, the six PH subunits of the eukaryotic exosome are catalytically inactive due to mutations the RNase PH-like active sites. Therefore, unlike PNPase and the archaeal exosome, the PH ring of the yeast and human exosome does not have exonuclease activity (Dziembowski et al., 2007; Liu et al., 2006).

Interestingly, however, a PH subunit in the *Arabidopsis thaliana* exosome, Rrp41p (AtRrp41p), displays phosphorolytic 3' to 5' exoribonuclease activity *in vitro* and can also rescue the rRNA processing and mRNA degradation defects of a yeast *rrp41* mutant. AtRrp41p can also interact with several of the yeast exosome subunits *in vivo* and *in vitro* (Chekanova et al., 2000), which suggests that this catalytically active subunit can participate in the evolutionarily conserved interactions that are needed for the structural integrity of the exosome.

Three of the ten exosome subunits bind to one side of the PH ring, forming a cap. These subunits contain multiple RNA binding domains and are, therefore, thought to bind the RNA substrates of the exosome. The last remaining subunit, Rrp44p, is the only catalytically active subunit of the yeast and *Drosophila* exosome (Dziembowski et al., 2007; Mamolen and Andrulis, 2009). This is also likely the case in humans, where the other nine exosome subunits are catalytically inactive (Liu et al., 2006). Rrp44p is homologous to bacterial RNase II and RNase R, two hydrolytic 3' to 5' exoribonucleases. Specifically, these enzymes use a water molecule to degrade mRNA from the 3'-end (Cannistraro and Kennell, 1994; Cheng and Deutscher, 2002).

Rrp44p has a similar domain organization and is, thus structurally similar, to RNase II and RNase R. Specifically, *E. coli* RNase II has 44% amino acid similarity and 22% amino acid identity to yeast Rrp44p, whereas *E. coli* RNase R has 48% amino acid similarity and 25%

amino acid identity to Rrp44p. These proteins have two consecutive N-terminal cold shock domains (CSDs), a central catalytic domain (RNB), that is responsible for the 3' to 5' exoribonuclease activity of the proteins, and a C-terminal S1 domain (Figure A.2A). The CSDs and S1 domain are putative RNA binding domains that are positioned on one side of the RNB domain. In all three proteins, ssRNA is threaded through the putative RNA binding domains, such that the 3'-end of the RNA rests in the catalytic site of the RNB domain (Bonneau et al., 2009; Frazao et al., 2006; Lorentzen et al., 2008; Zuo et al., 2006). Biochemically, Rrp44p is more similar to RNase R. Unlike RNase II, RNase R and Rrp44p are able to degrade structured RNAs and double stranded RNAs that contain a single stranded 3' overhang (Dziembowski et al., 2007; Vincent and Deutscher, 2006).

The eukaryotic exosome is involved in RNA processing and degradation in the nucleus and cytoplasm. Because Rrp44p is the catalytic subunit, Rrp44p is most likely responsible for these RNA processing and degradation reactions. Specifically in the nucleus, the pairing of Rrp44p with another hydrolytic 3' to 5' exoribonuclease, Rrp6p (Briggs et al., 1998), is required for efficient RNA processing of RNA precursors to form small, stable, mature RNAs (Allmang et al., 1999a; Allmang et al., 1999b; Mitchell et al., 1997; Mitchell et al., 1996), and for the rapid degradation of aberrantly processed RNAs (Bousquet-Antonelli et al., 2000; Davis and Ares, 2006; Kadaba et al., 2004; Libri et al., 2002; Torchet et al., 2002). Although these proteins are structurally dissimilar, it has recently been reported that these enzymes are functionally redundant (Lebreton et al., 2008).

In this Appendix, AtRrp41p, *E. coli* RNase II and RNase R, and *S. cerevisiae* Rrp6p, were tested individually for complementation of a yeast *rrp44* deletion strain. Here, the data indicate that none of these 3' to 5' exonucleases can carry out the essential function of Rrp44p.

RESULTS

***A. thaliana* Rrp41p cannot carry out the essential function of Rrp44p.**

Although none of the PH subunits of the yeast exosome are catalytically active, AtRrp41p has 3' to 5' exonuclease activity *in vitro*. In addition, AtRrp41p can complement the RNA processing and degradation defects of a yeast *rrp41* mutant (Chekanova et al., 2000). To determine whether AtRrp41p can carry out the essential function of Rrp44p, a plasmid containing *A. thaliana* *RRP41* under the control of the constitutively active glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter was generated and transformed into an *rrp44Δ* [*RRP44*, *URA3*] strain. Transformants were spotted onto media containing 5-FOA and grown for several days. Growth of AtRrp41p was compared to two controls, wild-type *RRP44* and empty vector. Similar to the empty vector control, AtRrp41p was not able to suppress the *rrp44* deletion (Figure A.1A). This lack of suppression was not due to a lack of expression, as AtRrp41p was able to complement a yeast *rrp41* deletion strain (Figure A.1B). This suggests that although AtRrp41p is catalytically active, it is not able to carry out the essential function of Rrp44p.

***E. coli* RNase II and RNase R cannot carry out the essential function of Rrp44p.**

E. coli RNase II and RNase R are structurally and functionally similar to yeast Rrp44p (Frazao et al., 2006; Zuo et al., 2006). To determine whether these bacterial exonucleases can carry out the essential function of Rrp44p, plasmids containing *E. coli* RNase II and RNase R under the control of the constitutively active GPD promoter were generated and transformed into an *rrp44Δ* [*RRP44*, *URA3*] strain. Transformants were spotted onto media containing 5-FOA and grown for several days. Growth of the bacterial exonucleases was compared to two controls, wild-type *RRP44* and empty vector. Despite their similarities to Rrp44p, overexpression of RNase II or RNase R did not suppress the *rrp44* deletion (Figure A.2B). This suggests that neither of these bacterial exonucleases can carry out the essential function of yeast Rrp44p.

The data presented in Chapter 3 indicate that cells lacking the exonuclease activity of Rrp44p have a slow growth phenotype, indicating that this nuclease activity is not needed for viability but rather for optimal cell growth. Although RNase II and RNase R cannot carry out the essential function of Rrp44p, the exonuclease activities of these proteins may be interchangeable with the exonuclease activity of Rrp44p. Under this hypothesis, coupling RNase II and/or RNase R *in trans* with a truncation of Rrp44p that lacks exonuclease activity should restore optimal cell growth. To test this, plasmids that overexpress RNase II and RNase R were individually transformed into an *rrp44Δ* [*RRP44*, *URA3*] strain that also contains a plasmid encoding a C-

A.

rrp44 Δ [*RRP44*, *URA3*] +

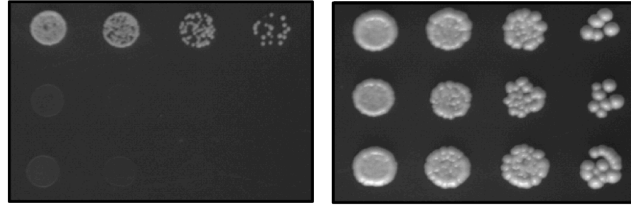


Overexpressed *A. thaliana* Rrp41p

Vector

5-FOA

Control



B.

rrp41 Δ [*RRP41*, *URA3*] +

5-FOA

Control

Vector

Overexpressed *A. thaliana* Rrp41p

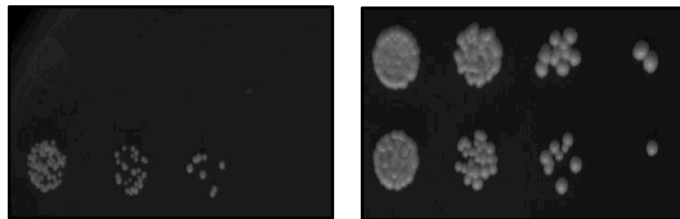


Figure A.1: *A. thaliana* Rrp41p does not suppress a yeast *rrp44* deletion.

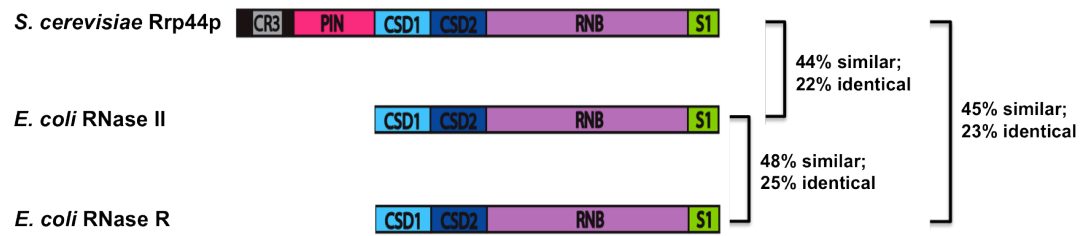
Figure A.1: *A. thaliana* Rrp41p does not suppress a yeast *rrp44* deletion. **A,** An *rrp44* deletion strain complemented by full-length *RRP44* on a plasmid with a *URA3* marker was transformed with a plasmid that overexpressed *A. thaliana* *RRP41*. Transformants were spotted onto media containing 5-FOA and control media (SC –HIS –URA + 2% dextrose), and grown for several days. Growth of *A. thaliana* *RRP41* was compared to two controls, wild-type *RRP44* and empty vector. Growth on 5-FOA would suggest that *A. thaliana* Rrp41p can carry out the essential function of Rrp44p. **B,** An *S. cerevisiae* *rrp41* deletion strain complemented by full-length *S. cerevisiae* *RRP41* on a plasmid with a *URA3* marker was transformed with a plasmid that overexpressed *A. thaliana* *RRP41*. Transformants were spotted onto media containing 5-FOA and control media (SC –HIS –URA + 2% dextrose), and grown for several days. Growth of *A. thaliana* *RRP41* was compared to empty vector. Growth on 5-FOA would suggest that *A. thaliana* Rrp41p can carry out the essential function of yeast Rrp41p.

terminal truncation that lacks the exonucleolytic RNB domain of Rrp44p. Transformants were then spotted onto media containing 5-FOA and grown for several days. Growth of the strains containing the bacterial exonucleases and truncated Rrp44p were compared to three controls, wild-type *RRP44*, a strain containing only the C-terminal truncation of Rrp44p, and empty vector. Despite the lack of suppression of an *rrp44* deletion strain, overexpressed RNase R was able to restore optimal growth in a strain that lacked Rrp44p exonuclease activity. In contrast, overexpressing RNase II *in trans* with the same C-terminal truncation of Rrp44p did not restore optimal growth (Figure A.2C). The lack of *in trans* suppression by RNase II is not due to a lack of protein expression, as RNase II is expressed in yeast (Figure A.2D). These results suggest that the exonuclease activity of *E. coli* RNase R, but not RNase II, may be able to substitute for yeast Rrp44p exonuclease activity. Additionally, these results are consistent with the 34-1001 truncation and triple CR3 mutant of Rrp44p being able to complement an *rrp44* deletion, but not being able to interact with the exosome.

***S. cerevisiae* Rrp6p cannot carry out the essential function of Rrp44p.**

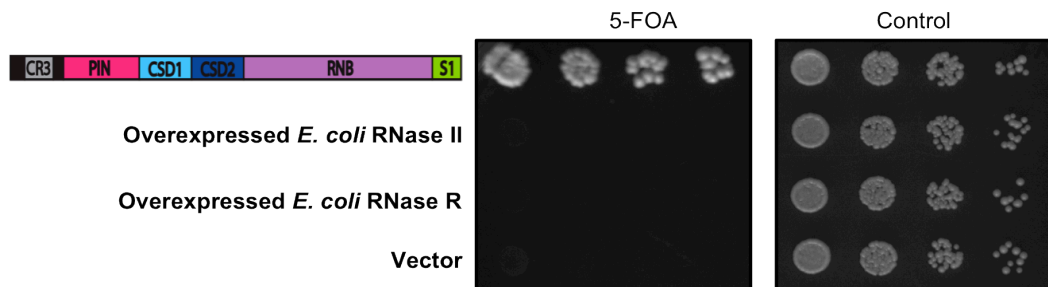
Rrp6p and Rrp44p share some overlapping functions in the nucleus (Chapter 3; Lebreton et al., 2008). Since the essential function of Rrp44p is likely nuclear, Rrp6p may be redundant with the essential function of Rrp44p. To test this, a plasmid encoding *S. cerevisiae* Rrp6p under the control of the constitutively active GPD promoter was generated and transformed into an *rrp44Δ* [*RRP44*, *URA3*] strain. Transformants were spotted onto media containing 5-FOA and grown for several days. Growth of yeast Rrp6p was compared to two controls, wild-type *RRP44* and empty vector. Similar to the empty vector control, Rrp6p did not support viability (Figure A.3A). The lack of suppression by yeast Rrp6p was not due to a lack of expression, as Rrp6p was able to complement an *rrp6* deletion strain (Figure A.3B). Therefore, although Rrp6p and Rrp44p may share some overlapping functions in the nucleus, none of these functions are the essential function of Rrp44p.

A.



B.

rrp44 Δ [*RRP44*, *URA3*] +



C.

rrp44 Δ [*RRP44*, *URA3*] +

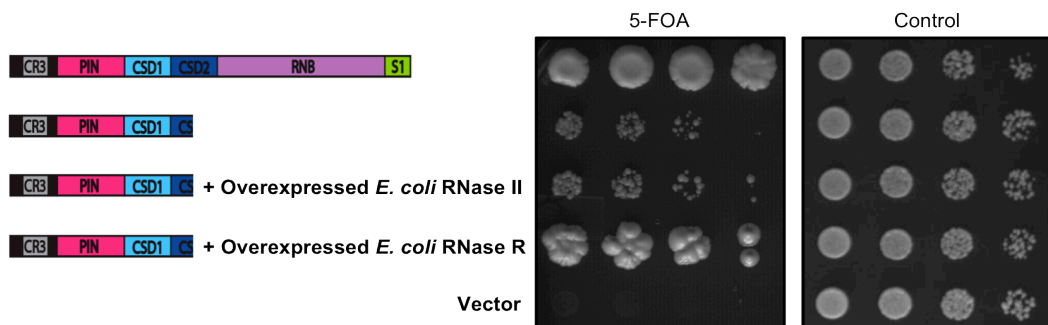


Figure A.2: *E. coli* RNase II or RNase R does not suppress a yeast *rrp44* deletion.

D.

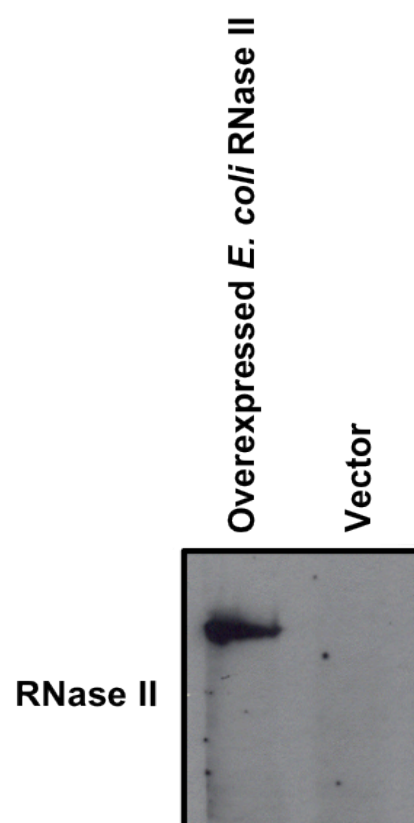
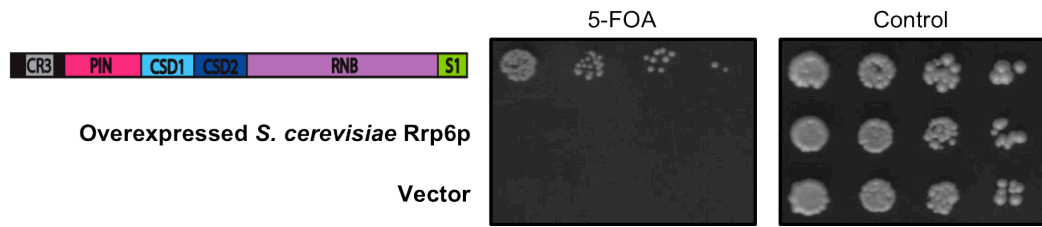


Figure A.2: *E. coli* RNase II or RNase R does not suppress a yeast *rrp44* deletion.

Figure A.2: *E. coli* RNase II or RNase R does not suppress a yeast *rrp44* deletion. **A,** A schematic representation of the domain organization of *S. cerevisiae* Rrp44p and *E. coli* RNase II and RNase R. Also shown are the percent amino acid similarity and identity between the three homologs. **B,** An *rrp44* deletion strain complemented by full-length *RRP44* on a plasmid with a *URA3* marker was transformed with plasmids that overexpressed *E. coli* RNase II and RNase R. Transformants were spotted onto media containing 5-FOA and control media (SC –HIS –URA + 2% dextrose), and grown for several days. Growth of the yeast strains containing *E. coli* RNase II and RNase R was compared to two controls, wild-type *RRP44* and empty vector. Growth on 5-FOA would suggest that the *E. coli* RNases can carry out the essential function of Rrp44p. **C,** An *rrp44* deletion strain complemented by full-length *RRP44* on a plasmid with a *URA3* marker was transformed with a C-terminal truncation of *rrp44* that lacks the region that is homologous to RNase II and RNase R and a plasmid that overexpressed *E. coli* RNase II or RNase R. Transformants were spotted onto media containing 5-FOA and control media (SC –HIS –URA + 2% dextrose), and grown for several days. Growth of the mutants was compared to two controls, wild-type *RRP44* and empty vector. Growth on 5-FOA indicates that the N-terminus of Rrp44p, *in trans* with an *E. coli* RNase, can carry out the essential function of Rrp44p. **C,** Whole-cell lysate was obtained from a strain containing a plasmid that overexpressed *E. coli* RNase II and was analyzed by Western blot analysis with antibodies specific to the His tag to detect RNase II. Expression of *E. coli* RNase II was compared to empty vector.

A.

*rrp44*Δ [*RRP44*, *URA3*] +



B.

*rrp6*Δ +

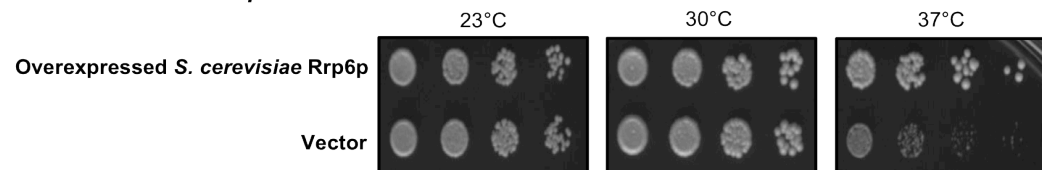


Figure A.3: *S. cereivisiae* Rrp6p does not suppress an *rrp44* deletion.

Figure A.3: *S. cerevisiae* Rrp6p does not suppress an *rrp44* deletion. An *rrp44* deletion strain complemented by full-length *RRP44* on a plasmid with a *URA3* marker was transformed with a plasmid that overexpressed *S. cerevisiae* *RRP6*. Transformants were spotted onto media containing 5-FOA and control media (SC –HIS –URA + 2% dextrose), and grown for several days. Growth of *RRP6* was compared to two controls, wild-type *RRP44* and empty vector. Growth on 5-FOA would suggest that *S. cerevisiae* Rrp6p can carry out the essential function of Rrp44p. **B,** An *S. cerevisiae* *rrp6* deletion strain was transformed with a plasmid that overexpressed *S. cerevisiae* *RRP6*. Transformants were spotted onto selection media and grown for several days at the indicated temperatures. Growth of *RRP6* was compared to empty vector. Growth at 37°C indicates that Rrp6p can rescue the temperature-sensitive phenotype of the *rrp6* deletion strain.

DISCUSSION

The data presented in this Appendix indicate that AtRrp41p, *E. coli* RNase II and RNase R, and yeast Rrp6p, were not able to carry out the essential function(s) of yeast Rrp44p. Because each of these enzymes is distinct, they were tested for suppression of an *rrp44* deletion strain for different reasons.

AtRrp41p was tested for suppression of an *rrp44* deletion strain because it has 3' to 5' exonuclease activity *in vitro* and can interact with the yeast exosome *in vivo* and *in vitro*. In addition, AtRrp41p is able to rescue the RNA processing defects of a yeast *rrp41* mutant (Chekanova et al., 2000), which suggests that it localizes to the nucleus. This assumed localization is important as the essential function of Rrp44p is likely nuclear. Despite these observations, AtRrp41p was not able to suppress an *rrp44* deletion. There are several possible explanations for this lack of suppression. First, it may be that AtRrp41p and yeast Rrp44p have different essential functions. For example, if AtRrp41p is required for RNA processing and yeast Rrp44p is required for the degradation of aberrant RNAs in the nucleus, overexpressing AtRrp41p in an *rrp44* deletion strain would not restore viability. Another explanation is that, although AtRrp41p can associate with yeast Rrp4p and Rrp44p (Chekanova et al., 2000), AtRrp41p may have weak or non-existent interactions with the exosome in the *rrp44* deletion strain.

E. coli RNase II and RNase R were tested for suppression of an *rrp44* deletion strain because these enzymes are biochemically similar to Rrp44p. More importantly for these suppression studies, Rrp44p, RNase II, and RNase R share a similar domain organization of the same three RNA binding domains and catalytic RNB domain (Bonneau et al., 2009; Frazao et al., 2006; Lorentzen et al., 2008; Zuo et al., 2006). If these domains of Rrp44p were required for the protein's essential function, RNase II and RNase R should be able to suppress an *rrp44* deletion. The data presented in Chapter 3, however, indicate that the N-terminus of Rrp44p, specifically the CR3 region and PIN domain, are required for viability. Since RNase II and RNase R do not have these regions, it is not surprising that these proteins are unable to carry out the essential function of Rrp44p. Unexpectedly, however, overexpressing RNase R *in trans* with a C-terminal truncation of Rrp44p that lacks exonuclease activity restored optimal growth. This suggests that Rrp44p functions as an exonuclease independent of its association with the exosome. In support of this, the triple CR3 mutant of Rrp44p and a truncation lacking the first 33 residues of Rrp44p can complement an *rrp44* deletion, however, these mutant proteins do not interact with the exosome. Similarly, although human Rrp44p is essential, its interaction with the exosome is not required for viability (Allmang et al., 1999b; Raijmakers et al., 2004). Another

study also indicates that the interaction between yeast Rrp44p and the exosome is not required for viability, since human Rrp44p can complement a yeast *rrp44* mutant (Shiomi et al., 1998). Therefore, a general theme that has emerged from these, and other, studies is that the interaction between Rrp44p and the exosome is not required for Rrp44p exonuclease activity or for cell survival.

In the *in trans* RNase R overexpression studies, a C-terminal truncation that consisted of residues 1-440 was used. Because this truncation includes a portion of the RNase II/R homology region, specifically CSD1 and a portion of CSD2, this truncation does not accurately address whether overexpressing RNase R *in trans* with the N-terminus of Rrp44p (including the CR3 region and PIN domain) can restore optimal cell growth. To better test this, an *rrp44* deletion strain that is complemented by a plasmid encoding only the regions of Rrp44p that are not homologous to RNase II/R (residues 1-235) should be transformed with a plasmid that overexpresses *E. coli* RNase R.

Lastly, yeast Rrp6p was tested for suppression of an *rrp44* deletion strain because these proteins share some overlapping functions, as demonstrated in Chapter 3 and in previous studies (Lebreton et al., 2008). Specifically, combining the catalytically inactive exonuclease point mutant of Rrp44p, D551N, with an *rrp6* deletion, results in a synthetic sick phenotype. Additionally, these proteins are known to be required for some of the same RNA processing and degradation reactions in the nucleus (Briggs et al., 1998). Despite this, yeast Rrp6p cannot carry out the essential function of Rrp44p. This may be because these proteins have different essential functions. In support of this, the functional interaction between Rrp44p and Rrp6p is important for only certain Rrp6p substrates (Callahan and Butler, 2008).

Because the exonucleolytic RNB domain of Rrp44p interacts with Rrp6, future studies should focus on whether the exonuclease activity of Rrp6p can substitute for the exonuclease activity of Rrp44p. Briefly, Rrp6p should be overexpressed in yeast strains that contain the D551N mutant or a C-terminal truncation of Rrp44p that lacks exonuclease activity. If overexpressing Rrp6p is able to restore optimal growth in these strains, this would suggest that overexpression of Rrp6p abrogates the need for the 3' to 5' exonuclease activity of Rrp44p in the nucleus.

Moreover, although AtRrp41p, *E. coli* RNase II and RNase R, and yeast Rrp6p are expressed in yeast and are catalytically active, these proteins cannot carry out the essential function of Rrp44p. This is likely because the exonuclease activity of the catalytic subunit is not needed for viability and may be because AtRrp41p and yeast Rrp6p have different essential

functions. Until the essential function(s) of Rrp44p is known, it will be difficult to find a single protein that is redundant for the essential function of the catalytic subunit of the exosome.

1. Allmang, C., Kufel, J., Chanfreau, G., Mitchell, P., Petfalski, E., and Tollervey, D. (1999a). Functions of the exosome in rRNA, snoRNA and snRNA synthesis. *EMBO J* 18, 5399-5410.
2. Allmang, C., Petfalski, E., Podtelejnikov, A., Mann, M., Tollervey, D., and Mitchell, P. (1999b). The yeast exosome and human PM-Scl are related complexes of 3' → 5' exonucleases. *Genes Dev* 13, 2148-2158.
3. Amblar, M., and Arraiano, C.M. (2005). A single mutation in *Escherichia coli* ribonuclease II inactivates the enzyme without affecting RNA binding. *FEBS J* 272, 363-374.
4. Ameri, A., Machiah, D.K., Tran, T.T., Channell, C., Crenshaw, V., Fernstrom, K., Khachidze, M., Duncan, A., Fuchs, S., and Howard, T.E. (2007). A nonstop mutation in the factor (F)X gene of a severely haemorrhagic patient with complete absence of coagulation FX. *Thromb Haemost* 98, 1165-1169.
5. Anantharaman, V., and Aravind, L. (2003). New connections in the prokaryotic toxin-antitoxin network: relationship with the eukaryotic nonsense-mediated RNA decay system. *Genome Biol* 4, R81.
6. Anderson, J.S., and Parker, R.P. (1998). The 3' to 5' degradation of yeast mRNAs is a general mechanism for mRNA turnover that requires the SKI2 DEVH box protein and 3' to 5' exonucleases of the exosome complex. *EMBO J* 17, 1497-1506.
7. Andrade, J.M., Pobre, V., Silva, I.J., Domingues, S., and Arraiano, C.M. (2009). The role of 3'-5' exoribonucleases in RNA degradation. *Prog Mol Biol Transl Sci* 85, 187-229.
8. Andrei, M.A., Ingelfinger, D., Heintzmann, R., Achsel, T., Rivera-Pomar, R., and Luhrmann, R. (2005). A role for eIF4E and eIF4E-transporter in targeting mRNPs to mammalian processing bodies. *RNA* 11, 717-727.
9. Araki, Y., Takahashi, S., Kobayashi, T., Kajiho, H., Hoshino, S., and Katada, T. (2001). Ski7p G protein interacts with the exosome and the Ski complex for 3'-to-5' mRNA decay in yeast. *EMBO J* 20, 4684-4693.
10. Arcus, V.L., Backbro, K., Roos, A., Daniel, E.L., and Baker, E.N. (2004). Distant structural homology leads to the functional characterization of an archaeal PIN domain as an exonuclease. *J Biol Chem* 279, 16471-16478.
11. Bashkirov, V.I., Scherthan, H., Solinger, J.A., Buerstedde, J.M., and Heyer, W.D. (1997). A mouse cytoplasmic exoribonuclease (mXRN1p) with preference for G4 tetraplex substrates. *J Cell Biol* 136, 761-773.

12. Benard, L., Carroll, K., Valle, R.C., Masison, D.C., and Wickner, R.B. (1999). The ski7 antiviral protein is an EF1-alpha homolog that blocks expression of non-Poly(A) mRNA in *Saccharomyces cerevisiae*. *J Virol* 73, 2893-2900.
13. Bleichert, F., Granneman, S., Osheim, Y.N., Beyer, A.L., and Baserga, S.J. (2006). The PINc domain protein Utp24, a putative nuclease, is required for the early cleavage steps in 18S rRNA maturation. *Proc Natl Acad Sci U S A* 103, 9464-9469.
14. Boeke, J.D., Trueheart, J., Natsoulis, G., and Fink, G.R. (1987). 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. *Methods Enzymol* 154, 164-175.
15. Bonneau, F., Basquin, J., Ebert, J., Lorentzen, E., and Conti, E. (2009). The yeast exosome functions as a macromolecular cage to channel RNA substrates for degradation. *Cell* 139, 547-559.
16. Bousquet-Antonelli, C., Presutti, C., and Tollervey, D. (2000). Identification of a regulated pathway for nuclear pre-mRNA turnover. *Cell* 102, 765-775.
17. Briggs, M.W., Burkard, K.T., and Butler, J.S. (1998). Rrp6p, the yeast homologue of the human PM-Scl 100-kDa autoantigen, is essential for efficient 5.8 S rRNA 3' end formation. *J Biol Chem* 273, 13255-13263.
18. Brown, J.T., Bai, X., and Johnson, A.W. (2000). The yeast antiviral proteins Ski2p, Ski3p, and Ski8p exist as a complex in vivo. *RNA* 6, 449-457.
19. Bucheli, M.E., He, X., Kaplan, C.D., Moore, C.L., and Buratowski, S. (2007). Polyadenylation site choice in yeast is affected by competition between Npl3 and polyadenylation factor CFI. *RNA* 13, 1756-1764.
20. Bulman, D.E., Gangopadhyay, S.B., Bechuck, K.G., Worton, R.G., and Ray, P.N. (1991). Point mutation in the human dystrophin gene: identification through western blot analysis. *Genomics* 10, 457-460.
21. Butler, J.S. (2002). The yin and yang of the exosome. *Trends Cell Biol* 12, 90-96.
22. Buttner, K., Wenig, K., and Hopfner, K.P. (2005). Structural framework for the mechanism of archaeal exosomes in RNA processing. *Mol Cell* 20, 461-471.
23. Callahan, K.P., and Butler, J.S. (2008). Evidence for core exosome independent function of the nuclear exoribonuclease Rrp6p. *Nucleic Acids Res* 36, 6645-6655.
24. Cannistraro, V.J., and Kennell, D. (1994). The processive reaction mechanism of ribonuclease II. *J Mol Biol* 243, 930-943.
25. Caponigro, G., Muhlrads, D., and Parker, R. (1993). A small segment of the MAT alpha 1 transcript promotes mRNA decay in *Saccharomyces cerevisiae*: a stimulatory role for rare codons. *Mol Cell Biol* 13, 5141-5148.

26. Carpousis, A.J. (2007). The RNA degradosome of *Escherichia coli*: an mRNA-degrading machine assembled on RNase E. *Annu Rev Microbiol* 61, 71-87.
27. Carpousis, A.J., Van Houwe, G., Ehretsmann, C., and Krisch, H.M. (1994). Copurification of *E. coli* RNAase E and PNPase: evidence for a specific association between two enzymes important in RNA processing and degradation. *Cell* 76, 889-900.
28. Castano, I.B., Heath-Pagliuso, S., Sadoff, B.U., Fitzhugh, D.J., and Christman, M.F. (1996). A novel family of TRF (DNA topoisomerase I-related function) genes required for proper nuclear segregation. *Nucleic Acids Res* 24, 2404-2410.
29. Chatr-Aryamontri, A., Angelini, M., Garelli, E., Tchernia, G., Ramenghi, U., Dianzani, I., and Loreni, F. (2004). Nonsense-mediated and nonstop decay of ribosomal protein S19 mRNA in Diamond-Blackfan anemia. *Hum Mutat* 24, 526-533.
30. Chekanova, J.A., Dutko, J.A., Mian, I.S., and Belostotsky, D.A. (2002). *Arabidopsis thaliana* exosome subunit AtRrp4p is a hydrolytic 3'-->5' exonuclease containing S1 and KH RNA-binding domains. *Nucleic Acids Res* 30, 695-700.
31. Chekanova, J.A., Shaw, R.J., Wills, M.A., and Belostotsky, D.A. (2000). Poly(A) tail-dependent exonuclease AtRrp41p from *Arabidopsis thaliana* rescues 5.8 S rRNA processing and mRNA decay defects of the yeast *ski6* mutant and is found in an exosome-sized complex in plant and yeast cells. *J Biol Chem* 275, 33158-33166.
32. Cheng, Z.F., and Deutscher, M.P. (2002). Purification and characterization of the *Escherichia coli* exoribonuclease RNase R. Comparison with RNase II. *J Biol Chem* 277, 21624-21629.
33. Clissold, P.M., and Ponting, C.P. (2000). PIN domains in nonsense-mediated mRNA decay and RNAi. *Curr Biol* 10, R888-890.
34. Coller, J., and Parker, R. (2004). Eukaryotic mRNA decapping. *Annu Rev Biochem* 73, 861-890.
35. Collins, S.R., Kemmeren, P., Zhao, X.C., Greenblatt, J.F., Spencer, F., Holstege, F.C., Weissman, J.S., and Krogan, N.J. (2007). Toward a comprehensive atlas of the physical interactome of *Saccharomyces cerevisiae*. *Mol Cell Proteomics* 6, 439-450.
36. Cougot, N., Babajko, S., and Seraphin, B. (2004). Cytoplasmic foci are sites of mRNA decay in human cells. *J Cell Biol* 165, 31-40.
37. Daines, D.A., Wu, M.H., and Yuan, S.Y. (2007). VapC-1 of nontypeable *Haemophilus influenzae* is a ribonuclease. *J Bacteriol* 189, 5041-5048.

38. Davis, C.A., and Ares, M., Jr. (2006). Accumulation of unstable promoter-associated transcripts upon loss of the nuclear exosome subunit Rrp6p in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A* *103*, 3262-3267.
39. de la Cruz, J., Kressler, D., Tollervey, D., and Linder, P. (1998). Dob1p (Mtr4p) is a putative ATP-dependent RNA helicase required for the 3' end formation of 5.8S rRNA in *Saccharomyces cerevisiae*. *EMBO J* *17*, 1128-1140.
40. Decker, C.J., and Parker, R. (1993). A turnover pathway for both stable and unstable mRNAs in yeast: evidence for a requirement for deadenylation. *Genes Dev* *7*, 1632-1643.
41. Deutscher, M.P., and Li, Z. (2001). Exoribonucleases and their multiple roles in RNA metabolism. *Prog Nucleic Acid Res Mol Biol* *66*, 67-105.
42. Doma, M.K., and Parker, R. (2006). Endonucleolytic cleavage of eukaryotic mRNAs with stalls in translation elongation. *Nature* *440*, 561-564.
43. Dreyfus, M., and Regnier, P. (2002). The poly(A) tail of mRNAs: bodyguard in eukaryotes, scavenger in bacteria. *Cell* *111*, 611-613.
44. Dreyfuss, G., Kim, V.N., and Kataoka, N. (2002). Messenger-RNA-binding proteins and the messages they carry. *Nat Rev Mol Cell Biol* *3*, 195-205.
45. Dziembowski, A., Lorentzen, E., Conti, E., and Seraphin, B. (2007). A single subunit, Dis3, is essentially responsible for yeast exosome core activity. *Nat Struct Mol Biol* *14*, 15-22.
46. Egecioglu, D.E., Henras, A.K., and Chanfreau, G.F. (2006). Contributions of Trf4p- and Trf5p-dependent polyadenylation to the processing and degradative functions of the yeast nuclear exosome. *RNA* *12*, 26-32.
47. Estevez, A.M., Kempf, T., and Clayton, C. (2001). The exosome of *Trypanosoma brucei*. *EMBO J* *20*, 3831-3839.
48. Fatica, A., Morlando, M., and Bozzoni, I. (2000). Yeast snoRNA accumulation relies on a cleavage-dependent/polyadenylation-independent 3'-processing apparatus. *EMBO J* *19*, 6218-6229.
49. Fatica, A., Tollervey, D., and Dlakic, M. (2004). PIN domain of Nob1p is required for D-site cleavage in 20S pre-rRNA. *RNA* *10*, 1698-1701.
50. Frazao, C., McVey, C.E., Amblar, M., Barbas, A., Vonnrhein, C., Arraiano, C.M., and Carrondo, M.A. (2006). Unravelling the dynamics of RNA degradation by ribonuclease II and its RNA-bound complex. *Nature* *443*, 110-114.

51. Frischmeyer, P.A., van Hoof, A., O'Donnell, K., Guerrerio, A.L., Parker, R., and Dietz, H.C. (2002). An mRNA surveillance mechanism that eliminates transcripts lacking termination codons. *Science* 295, 2258-2261.
52. Gatfield, D., and Izaurralde, E. (2004). Nonsense-mediated messenger RNA decay is initiated by endonucleolytic cleavage in *Drosophila*. *Nature* 429, 575-578.
53. Gerdes, K., Christensen, S.K., and Lobner-Olesen, A. (2005). Prokaryotic toxin-antitoxin stress response loci. *Nat Rev Microbiol* 3, 371-382.
54. Ghaemmaghami, S., Huh, W.K., Bower, K., Howson, R.W., Belle, A., Dephoure, N., O'Shea, E.K., and Weissman, J.S. (2003). Global analysis of protein expression in yeast. *Nature* 425, 737-741.
55. Giaever, G., Chu, A.M., Ni, L., Connelly, C., Riles, L., Veronneau, S., Dow, S., Lucau-Danila, A., Anderson, K., Andre, B., *et al.* (2002). Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature* 418, 387-391.
56. Glavan, F., Behm-Ansmant, I., Izaurralde, E., and Conti, E. (2006). Structures of the PIN domains of SMG6 and SMG5 reveal a nuclease within the mRNA surveillance complex. *EMBO J* 25, 5117-5125.
57. Graham, A.C., Davis, S.M., and Andrulis, E.D. (2009). Interdependent nucleocytoplasmic trafficking and interactions of Dis3 with Rrp6, the core exosome, and importin- α 3. *Traffic*.
58. Grishin, N.V. (2001). KH domain: one motif, two folds. *Nucleic Acids Res* 29, 638-643.
59. Guo, X., Ma, J., Sun, J., and Gao, G. (2007). The zinc-finger antiviral protein recruits the RNA processing exosome to degrade the target mRNA. *Proc Natl Acad Sci U S A* 104, 151-156.
60. Houseley, J., and Tollervey, D. (2006). Yeast Trf5p is a nuclear poly(A) polymerase. *EMBO Rep* 7, 205-211.
61. Hsu, C.L., and Stevens, A. (1993). Yeast cells lacking 5'→3' exoribonuclease 1 contain mRNA species that are poly(A) deficient and partially lack the 5' cap structure. *Mol Cell Biol* 13, 4826-4835.
62. Ingelfinger, D., Arndt-Jovin, D.J., Luhrmann, R., and Achsel, T. (2002). The human LSM1-7 proteins colocalize with the mRNA-degrading enzymes Dcp1/2 and Xrnl in distinct cytoplasmic foci. *RNA* 8, 1489-1501.
63. Inoue, K., Mizuno, T., Wada, K., and Hagiwara, M. (2000). Novel RING finger proteins, Air1p and Air2p, interact with Hmt1p and inhibit the arginine methylation of Npl3p. *J Biol Chem* 275, 32793-32799.

64. Johnson, A.W., and Kolodner, R.D. (1995). Synthetic lethality of *sep1* (*xrn1*) *ski2* and *sep1* (*xrn1*) *ski3* mutants of *Saccharomyces cerevisiae* is independent of killer virus and suggests a general role for these genes in translation control. *Mol Cell Biol* *15*, 2719-2727.
65. Kadaba, S., Krueger, A., Trice, T., Krecic, A.M., Hinnebusch, A.G., and Anderson, J. (2004). Nuclear surveillance and degradation of hypomodified initiator tRNA^{Met} in *S. cerevisiae*. *Genes Dev* *18*, 1227-1240.
66. Kadaba, S., Wang, X., and Anderson, J.T. (2006). Nuclear RNA surveillance in *Saccharomyces cerevisiae*: Trf4p-dependent polyadenylation of nascent hypomethylated tRNA and an aberrant form of 5S rRNA. *RNA* *12*, 508-521.
67. Kadowaki, T., Chen, S., Hitomi, M., Jacobs, E., Kumagai, C., Liang, S., Schneider, R., Singleton, D., Wisniewska, J., and Tartakoff, A.M. (1994). Isolation and characterization of *Saccharomyces cerevisiae* mRNA transport-defective (*mtr*) mutants. *J Cell Biol* *126*, 649-659.
68. Kerem, B.S., Zielenski, J., Markiewicz, D., Bozon, D., Gazit, E., Yahav, J., Kennedy, D., Riordan, J.R., Collins, F.S., Rommens, J.M., *et al.* (1990). Identification of mutations in regions corresponding to the two putative nucleotide (ATP)-binding folds of the cystic fibrosis gene. *Proc Natl Acad Sci U S A* *87*, 8447-8451.
69. LaCava, J., Houseley, J., Saveanu, C., Petfalski, E., Thompson, E., Jacquier, A., and Tollervey, D. (2005). RNA degradation by the exosome is promoted by a nuclear polyadenylation complex. *Cell* *121*, 713-724.
70. Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* *227*, 680-685.
71. Lebreton, A., Tomecki, R., Dziembowski, A., and Seraphin, B. (2008). Endonucleolytic RNA cleavage by a eukaryotic exosome. *Nature* *456*, 993-996.
72. Lehner, B., and Sanderson, C.M. (2004). A protein interaction framework for human mRNA degradation. *Genome Res* *14*, 1315-1323.
73. Lejeune, F., Li, X., and Maquat, L.E. (2003). Nonsense-mediated mRNA decay in mammalian cells involves decapping, deadenylating, and exonucleolytic activities. *Mol Cell* *12*, 675-687.
74. Levin, I., Schwarzenbacher, R., Page, R., Abdubek, P., Ambing, E., Biorac, T., Brinen, L.S., Campbell, J., Canaves, J.M., Chiu, H.J., *et al.* (2004). Crystal structure of a PIN (PilT N-terminus) domain (AF0591) from *Archaeoglobus fulgidus* at 1.90 Å resolution. *Proteins* *56*, 404-408.

75. Liang, S., Hitomi, M., Hu, Y.H., Liu, Y., and Tartakoff, A.M. (1996). A DEAD-box-family protein is required for nucleocytoplasmic transport of yeast mRNA. *Mol Cell Biol* 16, 5139-5146.
76. Libri, D., Dower, K., Boulay, J., Thomsen, R., Rosbash, M., and Jensen, T.H. (2002). Interactions between mRNA export commitment, 3'-end quality control, and nuclear degradation. *Mol Cell Biol* 22, 8254-8266.
77. Liu, J., Valencia-Sanchez, M.A., Hannon, G.J., and Parker, R. (2005). MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. *Nat Cell Biol* 7, 719-723.
78. Liu, Q., Greimann, J.C., and Lima, C.D. (2006). Reconstitution, activities, and structure of the eukaryotic RNA exosome. *Cell* 127, 1223-1237.
79. Longtine, M.S., McKenzie, A., 3rd, Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J.R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* 14, 953-961.
80. Lorentzen, E., Basquin, J., Tomecki, R., Dziembowski, A., and Conti, E. (2008). Structure of the active subunit of the yeast exosome core, Rrp44: diverse modes of substrate recruitment in the RNase II nuclease family. *Mol Cell* 29, 717-728.
81. Lorentzen, E., and Conti, E. (2005). Structural basis of 3' end RNA recognition and exoribonucleolytic cleavage by an exosome RNase PH core. *Mol Cell* 20, 473-481.
82. Makarova, K.S., Aravind, L., Galperin, M.Y., Grishin, N.V., Tatusov, R.L., Wolf, Y.I., and Koonin, E.V. (1999). Comparative genomics of the Archaea (Euryarchaeota): evolution of conserved protein families, the stable core, and the variable shell. *Genome Res* 9, 608-628.
83. Mamolen, M., and Andrulis, E.D. (2009). Characterization of the *Drosophila melanogaster* Dis3 ribonuclease. *Biochem Biophys Res Commun* 390, 529-534.
84. Meaux, S., and van Hoof, A. (2006). Yeast transcripts cleaved by an internal ribozyme provide new insight into the role of the cap and poly(A) tail in translation and mRNA decay. *RNA* 12, 1323-1337.
85. Milligan, L., Decourty, L., Saveanu, C., Rappsilber, J., Ceulemans, H., Jacquier, A., and Tollervey, D. (2008). A yeast exosome cofactor, Mpp6, functions in RNA surveillance and in the degradation of noncoding RNA transcripts. *Mol Cell Biol* 28, 5446-5457.
86. Minvielle-Sebastia, L., Preker, P.J., and Keller, W. (1994). RNA14 and RNA15 proteins as components of a yeast pre-mRNA 3'-end processing factor. *Science* 266, 1702-1705.

87. Minvielle-Sebastia, L., Winsor, B., Bonneaud, N., and Lacroute, F. (1991). Mutations in the yeast RNA14 and RNA15 genes result in an abnormal mRNA decay rate; sequence analysis reveals an RNA-binding domain in the RNA15 protein. *Mol Cell Biol* 11, 3075-3087.
88. Mitchell, P., Petfalski, E., Houalla, R., Podtelejnikov, A., Mann, M., and Tollervey, D. (2003). Rrp47p is an exosome-associated protein required for the 3' processing of stable RNAs. *Mol Cell Biol* 23, 6982-6992.
89. Mitchell, P., Petfalski, E., Shevchenko, A., Mann, M., and Tollervey, D. (1997). The exosome: a conserved eukaryotic RNA processing complex containing multiple 3'-->5' exoribonucleases. *Cell* 91, 457-466.
90. Mitchell, P., Petfalski, E., and Tollervey, D. (1996). The 3' end of yeast 5.8S rRNA is generated by an exonuclease processing mechanism. *Genes Dev* 10, 502-513.
91. Mitchell, P., and Tollervey, D. (2003). An NMD pathway in yeast involving accelerated deadenylation and exosome-mediated 3'-->5' degradation. *Mol Cell* 11, 1405-1413.
92. Moore, M.J. (2005). From birth to death: the complex lives of eukaryotic mRNAs. *Science* 309, 1514-1518.
93. Muhlrads, D., Decker, C.J., and Parker, R. (1994). Deadenylation of the unstable mRNA encoded by the yeast *MFA2* gene leads to decapping followed by 5'-->3' digestion of the transcript. *Genes Dev* 8, 855-866.
94. Muhlrads, D., and Parker, R. (1992). Mutations affecting stability and deadenylation of the yeast *MFA2* transcript. *Genes Dev* 6, 2100-2111.
95. Muhlrads, D., and Parker, R. (1994). Premature translational termination triggers mRNA decapping. *Nature* 370, 578-581.
96. Mukherjee, D., Gao, M., O'Connor, J.P., Rajmakers, R., Pruijn, G., Lutz, C.S., and Wilusz, J. (2002). The mammalian exosome mediates the efficient degradation of mRNAs that contain AU-rich elements. *EMBO J* 21, 165-174.
97. Navarro, M.V., Oliveira, C.C., Zanchin, N.I., and Guimaraes, B.G. (2008). Insights into the mechanism of progressive RNA degradation by the archaeal exosome. *J Biol Chem* 283, 14120-14131.
98. Nazar, R.N. (2004). Ribosomal RNA processing and ribosome biogenesis in eukaryotes. *IUBMB Life* 56, 457-465.
99. Pan, X., Yuan, D.S., Ooi, S.L., Wang, X., Sookhai-Mahadeo, S., Meluh, P., and Boeke, J.D. (2007). dSLAM analysis of genome-wide genetic interactions in *Saccharomyces cerevisiae*. *Methods* 41, 206-221.

100. Pan, X., Yuan, D.S., Xiang, D., Wang, X., Sookhai-Mahadeo, S., Bader, J.S., Hieter, P., Spencer, F., and Boeke, J.D. (2004). A robust toolkit for functional profiling of the yeast genome. *Mol Cell* 16, 487-496.
101. Peng, W.T., Robinson, M.D., Mnaimneh, S., Krogan, N.J., Cagney, G., Morris, Q., Davierwala, A.P., Grigull, J., Yang, X., Zhang, W., *et al.* (2003). A panoramic view of yeast noncoding RNA processing. *Cell* 113, 919-933.
102. Ponting, C.P., Schultz, J., Copley, R.R., Andrade, M.A., and Bork, P. (2000). Evolution of domain families. *Adv Protein Chem* 54, 185-244.
103. Py, B., Causton, H., Mudd, E.A., and Higgins, C.F. (1994). A protein complex mediating mRNA degradation in *Escherichia coli*. *Mol Microbiol* 14, 717-729.
104. Py, B., Higgins, C.F., Krisch, H.M., and Carpousis, A.J. (1996). A DEAD-box RNA helicase in the *Escherichia coli* RNA degradosome. *Nature* 381, 169-172.
105. Raijmakers, R., Schilders, G., and Pruijn, G.J. (2004). The exosome, a molecular machine for controlled RNA degradation in both nucleus and cytoplasm. *Eur J Cell Biol* 83, 175-183.
106. Ridley, S.P., Sommer, S.S., and Wickner, R.B. (1984). Superkiller mutations in *Saccharomyces cerevisiae* suppress exclusion of M2 double-stranded RNA by L-A-HN and confer cold sensitivity in the presence of M and L-A-HN. *Mol Cell Biol* 4, 761-770.
107. Rockmill, B., Lambie, E.J., and Roeder, G.S. (1991). Spore enrichment. *Methods Enzymol* 194, 146-149.
108. Rodgers, N.D., Wang, Z., and Kiledjian, M. (2002). Regulated alpha-globin mRNA decay is a cytoplasmic event proceeding through 3'-to-5' exosome-dependent decapping. *RNA* 8, 1526-1537.
109. Schaeffer, D., Meaux, S., Clark, A., and van Hoof, A. (2008). Determining *in vivo* activity of the yeast cytoplasmic exosome. *Methods Enzymol* 448, 227-239.
110. Schaeffer, D., Tsanova, B., Barbas, A., Reis, F.P., Dastidar, E.G., Sanchez-Rotunno, M., Arraiano, C.M., and van Hoof, A. (2009). The exosome contains domains with specific endoribonuclease, exoribonuclease and cytoplasmic mRNA decay activities. *Nat Struct Mol Biol* 16, 56-62.
111. Schilders, G., Raijmakers, R., Raats, J.M., and Pruijn, G.J. (2005). MPP6 is an exosome-associated RNA-binding protein involved in 5.8S rRNA maturation. *Nucleic Acids Res* 33, 6795-6804.

112. Schilders, G., van Dijk, E., and Pruijn, G.J. (2007). C1D and hMtr4p associate with the human exosome subunit PM/Scl-100 and are involved in pre-rRNA processing. *Nucleic Acids Res* 35, 2564-2572.
113. Schneider, C., Anderson, J.T., and Tollervey, D. (2007). The exosome subunit Rrp44 plays a direct role in RNA substrate recognition. *Mol Cell* 27, 324-331.
114. Schneider, C., Leung, E., Brown, J., and Tollervey, D. (2009). The N-terminal PIN domain of the exosome subunit Rrp44 harbors endonuclease activity and tethers Rrp44 to the yeast core exosome. *Nucleic Acids Res* 37, 1127-1140.
115. Shiomi, T., Fukushima, K., Suzuki, N., Nakashima, N., Noguchi, E., and Nishimoto, T. (1998). Human dis3p, which binds to either GTP- or GDP-Ran, complements *Saccharomyces cerevisiae* *dis3*. *J Biochem* 123, 883-890.
116. Shyu, A.B., Belasco, J.G., and Greenberg, M.E. (1991). Two distinct destabilizing elements in the c-fos message trigger deadenylation as a first step in rapid mRNA decay. *Genes Dev* 5, 221-231.
117. Sikorski, R.S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 122, 19-27.
118. Sperling, M.B., and Welz, B. (1999). Atomic Absorption Spectrometry (Weinheim, Wiley-VCH).
119. Suryanarayana, T., and Subramanian, A.R. (1984). Function of the repeating homologous sequences in nucleic acid binding domain of ribosomal protein S1. *Biochemistry* 23, 1047-1051.
120. Symmons, M.F., Jones, G.H., and Luisi, B.F. (2000). A duplicated fold is the structural basis for polynucleotide phosphorylase catalytic activity, processivity, and regulation. *Structure* 8, 1215-1226.
121. Takahashi, S., Araki, Y., Sakuno, T., and Katada, T. (2003). Interaction between Ski7p and Upf1p is required for nonsense-mediated 3'-to-5' mRNA decay in yeast. *EMBO J* 22, 3951-3959.
122. Thomson, E., and Tollervey, D. (2010). The final step in 5.8S rRNA processing is cytoplasmic in *Saccharomyces cerevisiae*. *Mol Cell Biol* 30, 976-984.
123. Toh, E.A., and Wickner, R.B. (1980). "Superkiller" mutations suppress chromosomal mutations affecting double-stranded RNA killer plasmid replication in *saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A* 77, 527-530.

124. Torchet, C., Bousquet-Antonelli, C., Milligan, L., Thompson, E., Kufel, J., and Tollervey, D. (2002). Processing of 3'-extended read-through transcripts by the exosome can generate functional mRNAs. *Mol Cell* 9, 1285-1296.
125. Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 76, 4350-4354.
126. Tucker, M., Valencia-Sanchez, M.A., Staples, R.R., Chen, J., Denis, C.L., and Parker, R. (2001). The transcription factor associated Ccr4 and Caf1 proteins are components of the major cytoplasmic mRNA deadenylase in *Saccharomyces cerevisiae*. *Cell* 104, 377-386.
127. van Hoof, A., Frischmeyer, P.A., Dietz, H.C., and Parker, R. (2002). Exosome-mediated recognition and degradation of mRNAs lacking a termination codon. *Science* 295, 2262-2264.
128. van Hoof, A., Lennertz, P., and Parker, R. (2000a). Yeast exosome mutants accumulate 3'-extended polyadenylated forms of U4 small nuclear RNA and small nucleolar RNAs. *Mol Cell Biol* 20, 441-452.
129. van Hoof, A., Staples, R.R., Baker, R.E., and Parker, R. (2000b). Function of the ski4p (Csl4p) and Ski7p proteins in 3'-to-5' degradation of mRNA. *Mol Cell Biol* 20, 8230-8243.
130. Vanacova, S., Wolf, J., Martin, G., Blank, D., Dettwiler, S., Friedlein, A., Langen, H., Keith, G., and Keller, W. (2005). A new yeast poly(A) polymerase complex involved in RNA quality control. *PLoS Biol* 3, e189.
131. Vincent, H.A., and Deutscher, M.P. (2006). Substrate recognition and catalysis by the exoribonuclease RNase R. *J Biol Chem* 281, 29769-29775.
132. Wall, D., and Kaiser, D. (1999). Type IV pili and cell motility. *Mol Microbiol* 32, 1-10.
133. Wang, H.W., Wang, J., Ding, F., Callahan, K., Bratkowski, M.A., Butler, J.S., Nogales, E., and Ke, A. (2007). Architecture of the yeast Rrp44 exosome complex suggests routes of RNA recruitment for 3' end processing. *Proc Natl Acad Sci U S A* 104, 16844-16849.
134. Wang, Z., and Kiledjian, M. (2001). Functional link between the mammalian exosome and mRNA decapping. *Cell* 107, 751-762.

135. Wen, T., Oussenko, I.A., Pellegrini, O., Bechhofer, D.H., and Condon, C. (2005). Ribonuclease PH plays a major role in the exonucleolytic maturation of CCA-containing tRNA precursors in *Bacillus subtilis*. *Nucleic Acids Res* 33, 3636-3643.
136. Widner, W.R., and Wickner, R.B. (1993). Evidence that the *SKI* antiviral system of *Saccharomyces cerevisiae* acts by blocking expression of viral mRNA. *Mol Cell Biol* 13, 4331-4341.
137. Wilson, M.A., Meaux, S., and van Hoof, A. (2007). A genomic screen in yeast reveals novel aspects of nonstop mRNA metabolism. *Genetics* 177, 773-784.
138. Wolin, S.L., and Matera, A.G. (1999). The trials and travels of tRNA. *Genes Dev* 13, 1-10.
139. Wyers, F., Rougemaille, M., Badis, G., Rousselle, J.C., Dufour, M.E., Boulay, J., Regnault, B., Devaux, F., Namane, A., Seraphin, B., *et al.* (2005). Cryptic pol II transcripts are degraded by a nuclear quality control pathway involving a new poly(A) polymerase. *Cell* 121, 725-737.
140. Yasuda, M., Shabbeer, J., Osawa, M., and Desnick, R.J. (2003). Fabry disease: novel alpha-galactosidase A 3'-terminal mutations result in multiple transcripts due to aberrant 3'-end formation. *Am J Hum Genet* 73, 162-173.
141. Zheng, D., Ezzeddine, N., Chen, C.Y., Zhu, W., He, X., and Shyu, A.B. (2008). Deadenylation is prerequisite for P-body formation and mRNA decay in mammalian cells. *J Cell Biol* 182, 89-101.
142. Zuo, Y., and Deutscher, M.P. (2001). Exoribonuclease superfamilies: structural analysis and phylogenetic distribution. *Nucleic Acids Res* 29, 1017-1026.
143. Zuo, Y., Vincent, H.A., Zhang, J., Wang, Y., Deutscher, M.P., and Malhotra, A. (2006). Structural basis for processivity and single-strand specificity of RNase II. *Mol Cell* 24, 149-156.

VITA

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