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Functional consequences of RNA exosome complex alteration by conformational changes and cofactor binding

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**FUNCTIONAL CONSEQUENCES OF RNA EXOSOME COMPLEX
ALTERATION BY CONFORMATIONAL CHANGES AND
COFACTOR BINDING**

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COFACTOR BINDING**

A

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DOCTOR OF PHILOSOPHY

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Houston, Texas

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Functional consequences of RNA exosome complex alteration by conformational changes and cofactor binding

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The RNA exosome is an essential 3'-5' ribonuclease that processes or degrades a variety of RNA species in eukaryotes. It is composed of nine structural cores and one catalytic subunit, Rrp44. Structural studies captured two different conformations of Rrp44, Rrp44^{ch} (channel) and Rrp44^{da} (direct-access). The Rrp44^{ch} appears to recruit RNA substrates from the central channel formed by the core subunits, while the substrate is directly recruited to Rrp44^{da} bypassing the central channel. Although *in vivo* function of the Rrp44^{ch}-exosome is extensively studied, the function or even the presence of the Rrp44^{da}-exosome in cell has not been tested. In this study, I show the first *in vivo* evidence that the Rrp44^{da} is important for the RNA exosome function. I also found that the Rrp44^{da} and Rrp44^{ch} have distinct substrates, indicating that the RNA exosome alternates its conformation to exert specific functions. Furthermore, RNA sequencing analysis suggests that Rrp44^{ch}-exosome indirectly regulates expression of genes encoding ribosomal proteins.

The substrate specificity of the RNA exosome is partly determined by its cofactors that bind substrates. Rrp6 is a ribonuclease that interacts with the RNA exosome in the nucleus. It functions not only as a nuclease but also as an adaptor protein that bridges the RNA exosome to other cofactors such as an RNA helicase, Mtr4. In this

study, I found that Rrp6 and Mtr4 function beyond known biochemical and structural interactions. Mtr4 seems to interact with the RNA exosome independent of the Rrp6 N-terminus. In addition, the C-terminal domain of Rrp6 has functions other than the exosome interaction. Moreover, another exosome cofactor, Mpp6, appears to mediate the interaction of the RNA exosome with other nuclear cofactors, and this function is redundant with Rrp6.

This work demonstrates that there are two different RNA exosome conformations present *in vivo*, and they have specific functions. Additionally, I show that there are multiple dynamic interactions among the RNA exosome with its cofactors, which ensures proper processing or degradation of transcripts.

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Chapter 1: Introduction, background, and significance

INTRODUCTION AND BACKGROUND

RNA surveillance maintains the fidelity of eukaryotic gene expression

Most genetic information is transcribed into RNA, and there are a variety of RNAs with fundamental biological roles in cell. Since RNAs are critical for cellular functions, they not only have to be synthesized and processed in the right place at the right time but also must be degraded when no longer required. Therefore, most RNA species undergo turnover processes.

In addition to normal decay, both mRNA and ncRNA undergo surveillance to avoid accumulation of aberrant forms as transcription is much more of an error prone process compared to DNA replication (Poveda et al., 2010). For example, aberrant mRNA can be translated into aberrant proteins that could be toxic to cells (Campioni et al., 2010). Aberrant mRNAs can be recognized by the translational machinery in the cytoplasm. mRNAs that have a premature termination codon due to mutations in their gene or errors during processing are recognized by the nonsense-mediated decay machinery during translation and degraded by ribonucleases (Baker and Parker, 2004). mRNAs that do not contain an in-frame stop codon due to mutations or error prone processing also are targeted by a surveillance pathway namely, nonstop decay (van Hoof et al., 2002). A ribosome is thought to stall at the 3'-end of mRNAs that do not contain a stop codon, and the stalled ribosome is recognized by the cytoplasmic RNA decay machinery. In addition, mRNAs that contain a series of rare codons or secondary structures that stall translating ribosome are degraded by the no-go decay pathway (Doma and Parker, 2006). ncRNAs often undergo post-transcriptional modifications for their proper function, and these modifications are also under surveillance. For example,

tRNA^{Met} that lacks a methylation at the A58 position is recognized by an RNA surveillance pathway and degraded (Kadaba et al., 2004). The RNA exosome is one of the ribonucleases that degrade aberrant RNAs during the surveillance pathway, and its function will be discussed in the next sections.

The RNA exosome is a major 3' exoribonuclease with diverse functions.

The RNA exosome is an enzyme that is involved in many of the surveillance processes described above. It not only degrades RNAs but also processes precursor RNAs to mature forms (Januszyk and Lima, 2014). The RNA exosome is an essential 3'-5' exoribonuclease complex that is involved in the degradation and processing of a variety of RNA species (Januszyk and Lima, 2014). It was discovered as a protein complex that is required for the maturation of 5.8S rRNA (Mitchell et al., 1997). Studies revealed that the exosome is present both in the nucleus and the cytoplasm, functioning in the nuclear RNA surveillance and the cytoplasmic mRNA turnover, respectively (Allmang et al., 1999b; Jacobs Anderson and Parker, 1998b) (Fig. 1.1). In the nucleus, it processes 3'-ends of various non-coding RNA (ncRNA) species such as ribosomal RNAs (rRNAs) and small nucleolar RNAs (snoRNAs), and degrades aberrant RNAs. In addition, it is involved in regular mRNA turnover and mRNA surveillance pathways in the cytoplasm. The regular cytoplasmic mRNA decay is governed by two pathways, the Xrn1-mediated 5'-3' decay pathway and the exosome-mediated 3'-5' decay pathway. The 3'-5' decay is initiated by deadenylation of 3'-end of mRNA, and the deadenylated 3'-end is susceptible to degradation by the exosome. In addition to normal mRNAs, the

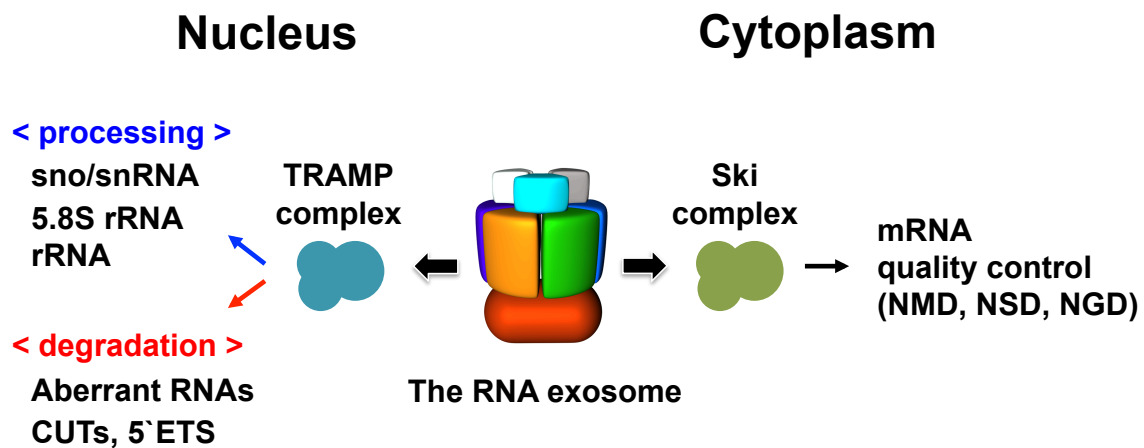


Figure 1.1 Function of the RNA exosome.

Large arrows indicate the interaction of the exosome with its cofactors, and small arrows show the RNA substrates that are processed or degraded by the exosome. CUTs, cryptic unstable transcripts; 5'ETS, 5' external transcribed spacer; NMD, nonsense mediated decay; NSD, non-stop decay; NGD, no-go decay.

RNA exosome degrades aberrant mRNAs: (1) mRNAs with premature stop codon (the nonsense-mediated decay (NMD) pathway), (2) mRNAs that lack termination codons (the non-stop decay (NSD) pathway), and (3) mRNAs with stalled ribosome (the no-go decay (NGD) pathway) (Chlebowski et al., 2013; Houseley and Tollervey, 2009).

Therefore, the RNA exosome is a critical enzyme for cellular RNA metabolism. However, we do not have a clear picture of how the exosome selects specific substrates and how it degrades or processes RNA species with such different characteristics.

The RNA exosome has a conserved ring-shaped structure.

The overall architecture of an RNA exosome-like complex is conserved in the three domains of life (Evguenieva-Hackenberg, 2010; Januszyk and Lima, 2014; Lykke-Andersen et al., 2009) (Fig. 1.2). In all three domains, the complex forms a ring-like structure that contains six RNase PH domains, three S1 (ribosomal protein S1) domains and generally three RNA-binding KH (ribonucleoprotein K homology) domains, although one or more of the KH domains can be replaced by a Zn-knuckle. The bacterial PNPase is a homotrimer, with each monomer contributing Two PH, one KH, and one S1 domain. (Fig. 1.2A) (Lin-Chao et al., 2007). The archaeal exosome is made from three copies of both Rrp41 and Rrp42 plus three copies of either Rrp4 or Csl4. Rrp41 and Rrp42 each contain a PH domain, while Rrp4 has S1 and KH domains, and Csl4 has S1 and Zn knuckle domains (Fig. 1.2B) (Nurmohamed et al., 2009).

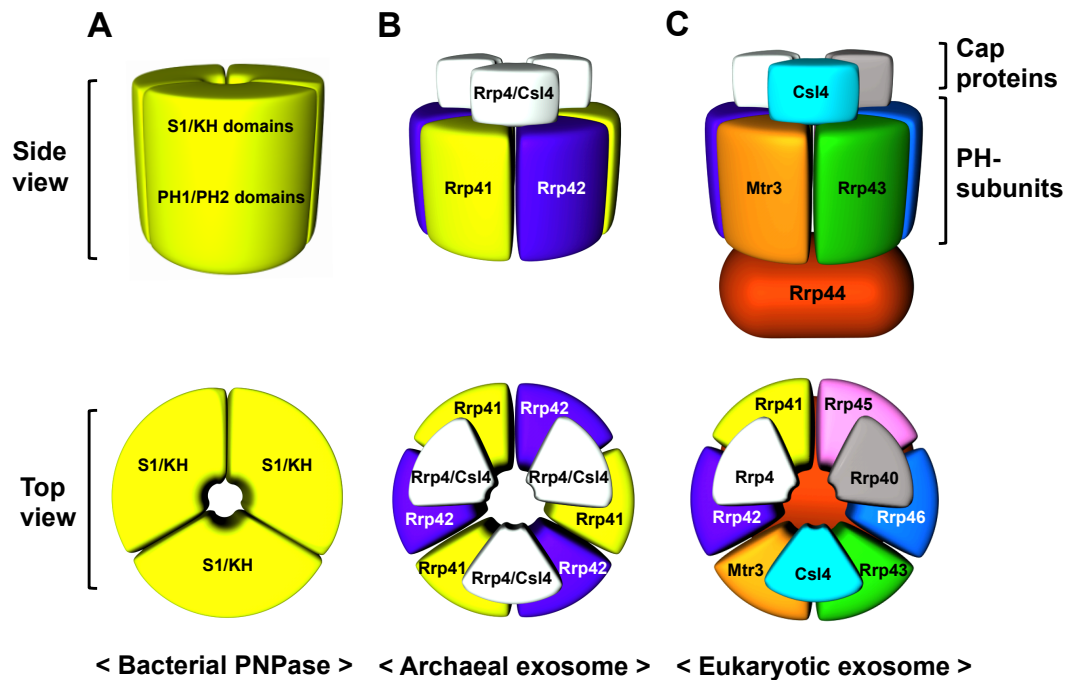


Figure 1.2. Structural organization of the exosome-like structures from three domains of life.

Schematic subunit organization of bacterial PNPase, archaeal exosome, and eukaryotic exosome. Models were generated by Cinema 4D software (Maxon) based on x-ray crystal structures of PNPase in *Escherichia coli* (A), the RNA exosome in *Sulfolobus solfataricus* (B), and the RNA exosome in *Saccharomyces cerevisiae* (C) (Lorentzen et al., 2005; Lu et al., 2010; Makino et al., 2013a; Nurmohamed et al., 2009; Shi et al., 2008).

Both the bacterial PNPase and the archaeal exosome harbor the catalytic activities inside of the PH-rings. Therefore, a single-stranded RNA is threaded into the central channel for degradation. The core of the eukaryotic RNA exosome comprises nine different essential subunits (Fig. 1.2C). Three RNA binding cap proteins are on top of the six RNase PH-like subunits forming the exosome core. Unlike the bacterial and archaeal counter parts, the core of the eukaryotic exosome is catalytically inert. Instead, it interacts with a tenth subunit, Rrp44, that contains both 3' exo- and endoribonuclease activities, and the activities of Rrp44 are regulated by the nine core subunits. (Lebreton et al., 2008; Lorentzen et al., 2008; Makino et al., 2013a; Schneider et al., 2009; Wasmuth and Lima, 2012) (Fig. 1.2C, red). Although the conserved structure suggests that RNAs are also threaded through the eukaryotic RNA exosome core to be degraded, chapters three and four of this thesis show that this is not true for all substrate RNAs.

The eukaryotic RNA exosome forms different structures by interacting with different catalytic subunits.

In contrast to the bacterial and archaeal counterparts, interestingly, the eukaryotic RNA exosome core is catalytically inert due to a point mutation in the catalytic residue (Dziembowski et al., 2007; Liu et al., 2006). Instead, it interacts with two families of catalytic subunits. The first family contains a single member in yeast, Rrp44, while other eukaryotes contain hDis3 and hDis3L members of this family. The second family consists of a single member, Rrp6, in many eukaryotes, including yeast and human. As an exception, one of the core subunits, AtRrp41, in the RNA exosome in plant, *Arabidopsis*

thaliana, retains the catalytic activity (Chekanova et al., 2000), and *A. thaliana* also possesses multiple Rrp6 homologs.

Rrp6 is a distributive 3'-5' exoribonuclease (Butler and Mitchell, 2011). It is exclusively localized to the nucleus functioning in the processing of RNAs such as 5.8S rRNA, snRNAs, and snoRNAs. It contains three domains: an N-terminal domain (NTD), an exoribonuclease domain (EXO), and a Helicase and RNase D C-terminal (HRDC) domain. It is homologous to RNase D from *E. coli*, and it has been suggested that the HRDC domain recruits RNA substrates to the EXO domain for catalysis. In addition, the C-terminal region of Rrp6 interacts with the exosome core (Makino et al., 2013a). Unlike the core components of the RNA exosome, Rrp6 is not essential for viability of budding yeast, but deletion of the *RRP6* gene yields a slow growth phenotype. However, *rrp6Δ* is synthetic lethal with exonuclease defective allele of *RRP44*, suggesting the redundancy between the exosome and Rrp6 in the nucleus (Schneider et al., 2009).

The yeast Rrp44 is present both in the nucleus and the cytoplasm, while human Dis3 is nuclear and Dis3L is cytoplasmic. These are processive 3'-5' exoribonucleases, while Rrp44 and Dis3 also process endoribonuclease activity (Lebreton et al., 2008; Schaeffer et al., 2009; Schneider et al., 2009). Each family member contains five domains: a PIN (PiIT N-terminus) domain, two CSD RNA-binding domains (Cold Shock Domain), an RNB catalytic domain (named after the *rnb* gene of *E. coli*), and a S1 RNA-binding domain (Fig. 1.3A). The exonuclease active site of the RNB domain is at the end of a long substrate binding cleft (Fig. 1.3C and D, yellow spheres in red circles). In contrast, the endonuclease site in the PIN domain is exposed to solvent that suggests the RNA substrates have access to the endonuclease site directly from the cytoplasm (Fig. 1.3C

and D, magenta spheres). While this PIN domain is conserved and catalytically active in Rrp44 and hDis3, Dis3L contains a catalytically inactive PIN domain.

Given that the RNA exosome core controls the activities of its catalytic subunits (Drazkowska et al., 2013; Wasmuth and Lima, 2012), it is necessary to study the interaction of the core with the catalytic subunits to study the function of the exosome. Previous studies found that the PIN domain of Rrp44 is essential for viability and for the interaction with the exosome core (Schaeffer et al., 2009; Schneider et al., 2009). The CR3 (Cysteine Rich with three cysteines) motif in the PIN domain coordinates a zinc ion to maintain a proper conformation of the YRD motif that is important for the interaction of Rrp44 with Rrp41, one of the core subunits (Schaeffer et al., 2012a) (Fig. 1.3B). Mutations in the CR3 motif reduce the interaction of Rrp44 with the core and significantly affect the growth of *Saccharomyces cerevisiae*. In addition, the mutations in the CR3 motif affect both the nuclear and cytoplasmic functions of the exosome (Schaeffer et al., 2012a; Schaeffer and van Hoof, 2011). However, the CR3 mutant still yields viable cells suggesting there are additional contact sites between Rrp44 and the core subunits.

The eukaryotic RNA exosome forms different conformations.

X-ray crystallographic studies revealed two different Rrp44 structures (Bonneau et al., 2009; Makino et al., 2013a) (Fig. 1.3C and D). In the two conformations of Rrp44, the PIN domain maintains the same position, but the CSD, RNB and S1 domains move. Since one of two structures was initially seen in a trimeric complex with Rrp45 and Rrp41 (Fig. 1.3C), one might think that it is merely a crystallization artifact or an

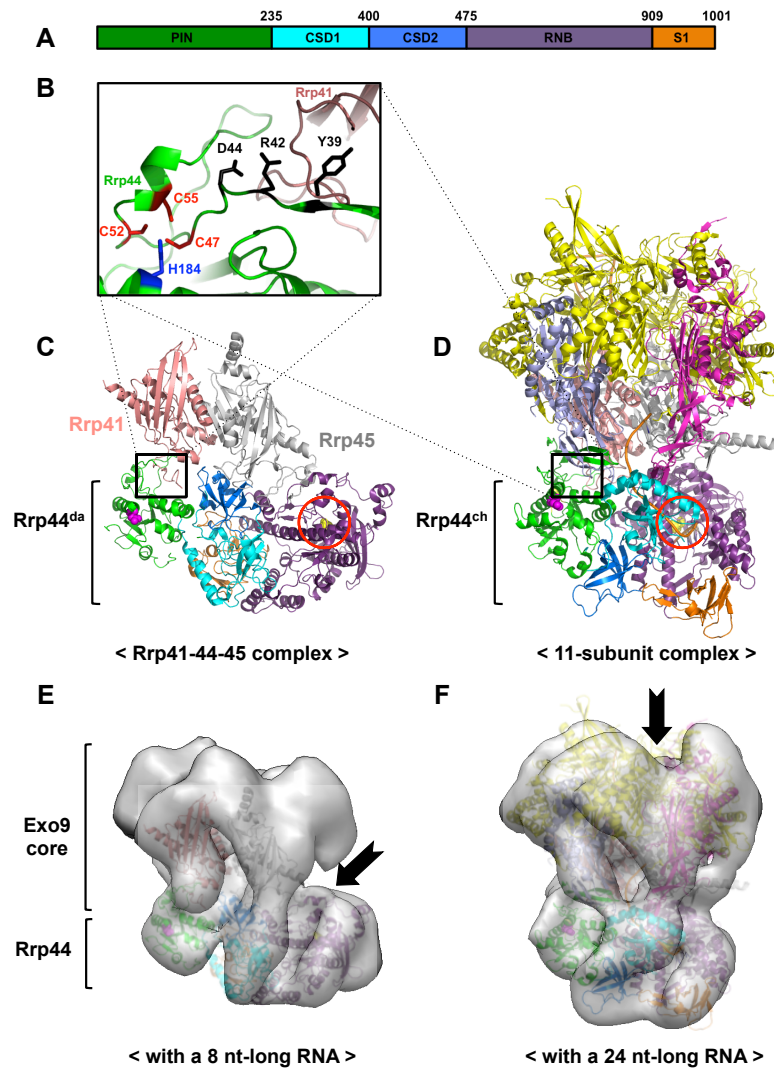


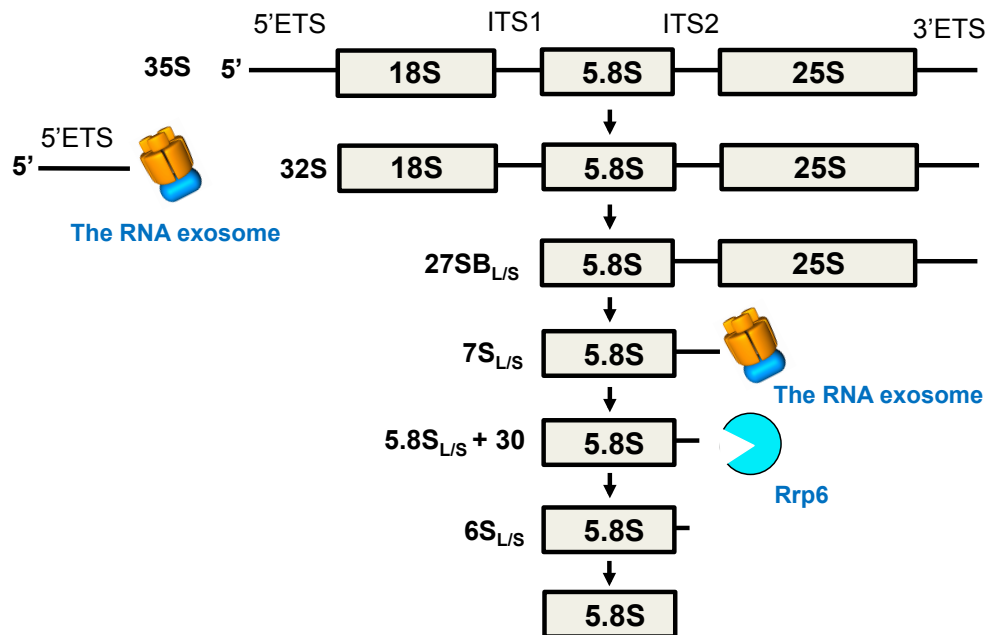
Figure 1.3. Two conformations of the RNA exosome.

(A) Schematic of the Rrp44 domains. (B) The CR3 motif (red and blue) and the YRD motif (black) that directly interacts with Rrp41 (salmon pink). (C)(D) The X-ray crystal structures of the two conformations of Rrp44 (Protein Data Bank: 2WP8, 4IFD) (Bonneau et al., 2009; Makino et al., 2013a). Five domains of Rrp44 are indicated as different colors for comparison. Subunits that do not directly contact with Rrp44 are depicted as yellow. Rrp41 (salmon pink) and Rrp45 (gray) directly interact with Rrp44 in both conformations. Rrp42 (slate) and Rrp43 (magenta) show their interaction with Rrp44 in the 11-subunit complex. The active site of endonuclease appears as magenta spheres, and yellow spheres in red circles indicate the exonuclease active site. (E)(F) Single-particle EM of the exosome with a 8 nt-long (Electron Microscopy Data Bank: EMD-2941) and a 24 nt-long RNA (EMD-2496) substrates, respectively (Liu et al., 2014). RNA entry sites are indicated by black arrows. Crystal structures of (C) and (D) are superimposed on the EM structures in (E) and (F), respectively. The cartoon versions of the X-ray crystal structures were generated by MacPyMol (Schrödinger, LLC).

assembly intermediate. However, surprisingly, a recent single-particle electron microscopy (EM) found that the exosome adopts two different conformations depending on the length of the substrates (Liu et al., 2014) (Fig. 1.3E and F). In addition, the two conformations of Rrp44 in the EM analysis fit to the two X-ray structures. Furthermore, a recent X-ray crystal structure showed that the Rrp44 conformation shown in figure 1.3C is actually present in a complete exosome structure with nine core subunits, suggesting that it could be a biologically relevant conformation (Makino et al., 2015). A striking difference between the two conformations is in the substrate entry site of Rrp44. One conformation appears to directly recruit substrates to Rrp44, while the other utilizes the central channel of the nine core subunits (Fig. 1.3E and F, arrows). It is tempting to speculate that the different conformations carry out different function, but prior to this work there was no evidence for this, or even that both conformations are present *in vivo*.

***In vivo* RNA exosome activity requires cofactors.**

How different RNA substrates are selectively degraded by the RNA exosome has been partly answered by identifying several cofactors that interact with the exosome. The cofactors appear to recognize substrates and deliver them to the RNA exosome for processing or degradation. In the nucleus, another exonuclease, Rrp6, interacts with the exosome core through its C-terminus (Butler and Mitchell, 2011). It cooperates with the exosome to process or degrade some nuclear substrates. For example, the RNA exosome core processes 7S rRNA into 5.8S rRNA + 30 nt processing intermediate, and the last 30 nt are processed by Rrp6 (Fig. 1.4). Rrp6 functions not only as a ribonuclease but also as an adaptor protein that mediates interaction of the RNA exosome with its cofactors such



Adapted from Granneman et al., *The EMBO Journal* 2011

Figure 1.4. Function of the RNA exosome in 35S rRNA processing pathway.

5' External Transcribed Spacer (5'ETS) is a byproduct of 35S rRNA processing and degraded by the RNA exosome. 7S rRNA is processed by the exosome yielding 5.8S + 30nt, and the extra 30 nucleotides are trimmed by Rrp6. ITS1/2: Internal Transcribed Spacer1/2.

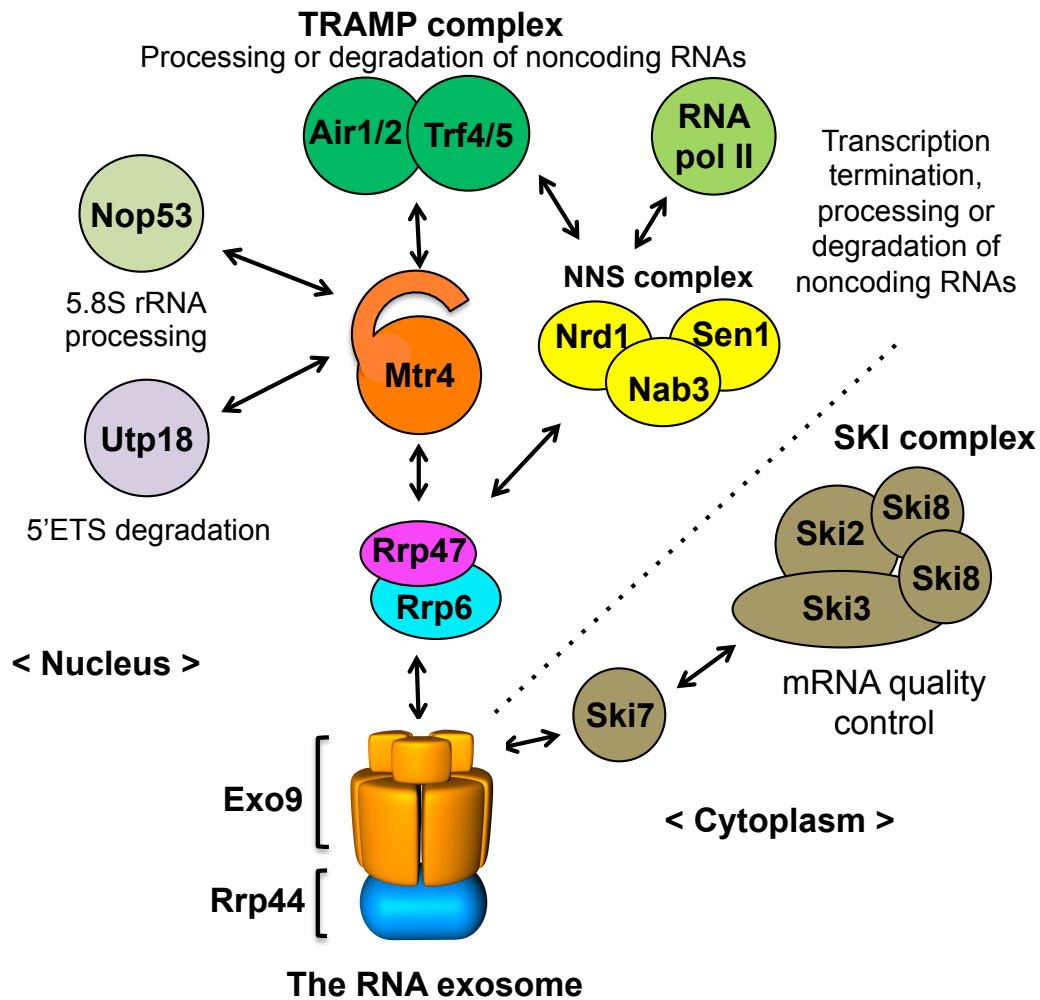


Figure 1.5. The interaction network of the RNA exosome with its cofactors in *Saccharomyces cerevisiae*.
Known direct interactions of the RNA exosome with cofactors are indicated by arrows.

as Rrp47 and Mtr4. Rrp47 is an RNA binding protein, and Rrp47 and Rrp6 mutually stabilize each other by the interaction (Feigenbutz et al., 2013a; Feigenbutz et al., 2013b).

An RNA helicase, Mtr4, is required for most of the nuclear functions of the RNA exosome (Butler and Mitchell, 2011). It has been shown that the N-termini of Rrp6 and Rrp47 interact with N-terminus of Mtr4 (Schuch et al., 2014) (Fig. 1.5). These results suggest that Rrp6 not only functions as an exoribonuclease but also bridges cofactors to the exosome. Mtr4 also interacts with other proteins that bind specific substrates, and those interactions are important for both Rrp6 and the exosome-dependent substrate degradation (Callahan and Butler, 2010; Klauer and van Hoof, 2013; Reis and Campbell, 2007; Wang et al., 2008). For example, Mtr4 interacts with ribosome associated proteins, Nop53 and Utp18, that recruit 7S rRNA and 5'ETS for processing and degradation, respectively (Thoms et al., 2015) (Fig. 1.5). Mtr4 also interacts with a poly(A) polymerase (Trf4 or Trf5) and a zinc knuckle RNA binding protein (Air1 or Air2) forming a TRAMP (Trf4/5-Air1/2-Mtr4 polyadenylation) complex. The TRAMP complexes are important for processing and/or degradation of noncoding RNAs (Callahan and Butler, 2010; Losh et al., 2015). Trf4/Trf5 oligoadenylates the 3'-end of the substrate generating a short 3' overhang. Then, Mtr4 binds to the 3' overhang and unwinds it for the exosome to degrade it (Butler and Mitchell, 2011; Chlebowski et al., 2013; Vanacova et al., 2005). The homologs of TRAMP components are also present in human such as hMTR4, hTRF4-1(POLS), hTRF4-2(PAPD5), and ZCCHC7 (a candidate of Air1/Air2 homolog), but whether they form a TRAMP-like complex has not been shown yet (Houseley and Tollervey, 2008; Shcherbik et al., 2010). Instead, hMTR4 forms the NEXT (Nuclear EXosome Targeting) complex with the putative RNA binding

protein (RBM7) and the Zn-knucle protein (ZCCHC8), and it has been shown that the NEXT complex degrades promoter upstream transcripts (PROMPTs) (Lubas et al., 2011).

The TRAMP complex also interacts with the NNS (Nab3-Nrd1-Sen1) transcription termination complex (Anderson and Wang, 2009; Butler and Mitchell, 2011) (Fig. 1.5). Nab3 and Nrd1 are RNA binding proteins, and Sen1 is an RNA helicase. The NNS complex functions in the termination of ncRNA transcription for processing or degradation by Nrd1-mediated interaction with RNA polymerase II (Vasiljeva et al., 2008a). Nrd1 also interacts with Trf4, one of the TRAMP complex subunits. In addition, one of the subunits of NNS complex, Nab3, was shown to interact with Rrp6, and the interaction is TRAMP independent (Fasken et al., 2015). Thus, the NNS complex potentially interacts with the RNA exosome through both the TRAMP complex and Rrp6. The NNS complex has not been detected in human. Specifically, humans do not possess the Nab3 homolog, and human homologs of Sen1 and Nrd1 (Senataxin and RBM17) do not participate in the transcription termination of ncRNA (O'Reilly et al., 2014; Suraweera et al., 2009).

In the cytoplasm, the exosome interacts with the superkiller (Ski) complex (Fig. 1.5). It comprises an RNA helicase (Ski2), a tetratricopeptide protein (Ski3), and two copies of a WD repeat protein (Ski8) (Araki et al., 2001; Liang et al., 1996; van Hoof et al., 2000c; Wang et al., 2005). The Ski complex is responsible for the cytoplasmic function of the RNA exosome, and Ski7 mediates the interaction of the Ski complex with the exosome (Fig. 1.5). After deadenylation by the Ccr4-Not deadenylase complex, normal mRNA can be degraded either from the 5' or the 3' end (Fig. 1.6). The 5'-3' exoribonuclease, Xrn1, degrades mRNA after decapping by the Dcp1/2 decapping

enzyme complex (Klauer and van Hoof, 2012). The RNA exosome is responsible for 3'-5' decay of mRNA.

The Ski complex is required not only for the regular 3'-5' mRNA decay pathway but also for the nonsense-mediated decay (NMD), non-stop decay, and no-go decay pathways (Doma and Parker, 2006; Jacobs Anderson and Parker, 1998b; Takahashi et al., 2003; van Hoof et al., 2000c). In nonstop decay, stalled ribosomes at the 3' end of mRNA appear to be recognized by the C-terminal domain of Ski7 that resembles the ribosome release factor, eRF3, released and degraded by the RNA exosome (van Hoof et al., 2002). In no-go decay, a ribosome stalled due to rare codons or stable secondary structures of mRNA is recognized by Dom34 and Hbs1 (a Ski7 paralog) for ribosome recycling and endonucleolytic cleavage of mRNA by yet unknown nuclease and the exosome-mediated degradation from the 3'-end (Tsuboi et al., 2012).

Therefore, the RNA exosome-cofactor interaction is required for the RNA exosome to selectively process or degrade RNA substrates. However, we still lack a complete picture of RNA exosome-cofactor interaction network. Therefore, it is essential to study how the RNA exosome interacts with its cofactors and the biological roles of the interaction to understand the function of the RNA exosome.

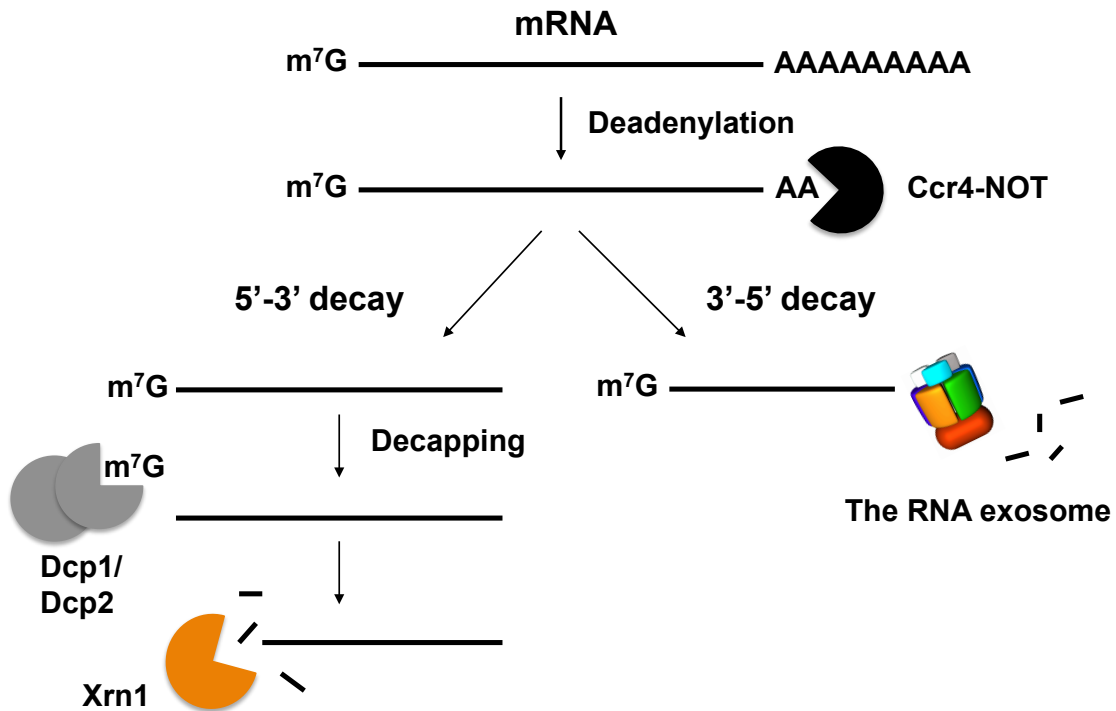


Figure 1.6. Normal RNA decay pathways.

mRNA that is destined to be degraded is deadenylated by the Ccr4-Not deadenylation complex. 5' m⁷G cap of deadenylated mRNA is removed by the Dcp1/2 decapping enzyme complex, and decapped 5'-end of mRNA is degraded by exoribonuclease Xrn1. The RNA exosome degrades mRNA from its 3'-end.

SIGNIFICANCE

Regulation of RNA turnover is critical for all living organisms. Considering that most of the genomic information is transcribed into RNA, there is no doubt that an enormous number of RNA species are present in cells. For the precise regulation of cellular processes, these RNA molecules need to be produced, properly processed, and degraded in the right place at the right time. The RNA exosome is a highly-regulated ribonuclease machinery that deals with the RNA processing and turnover in cells. Studies have identified numerous substrates of the exosome by inactivating its catalytic activities (Gudipati et al., 2012; Schneider et al., 2012). They provide valuable information for us to understand the function of the RNA exosome; however, our knowledge of how such different RNA species are specifically selected for processing and degradation is limited.

Given that the RNA exosome is a crucial enzyme that is involved in the cellular RNA surveillance, it is not surprising that the exosome is associated with human diseases (Fig. 1.7). Interestingly, however, different diseases arise depending on what exosome subunit is defective: (1) A whole genome sequencing analysis found that ~10 % of multiple myelomas harbor mutations in the hDIS3 gene (Chapman et al., 2011). A subsequent study revealed that these mutations affect the exoribonuclease activity of Rrp44/hDis3 (Tomecki et al., 2014), (2) Mutations in SKIV2L or TTC37, which encode the human homologs of Ski2 and Ski3, respectively, cause syndromic diarrhea also known as Trichohepatoenteric Syndrome (Fabre et al., 2012; Hartley et al., 2010), (3) Mutations in EXOSC3/Rrp40, which encodes one of the core subunits of the exosome, cause pontocerebellar hypoplasia and spinal motor neuron degeneration (Biancheri et al., 2013; Eggens et al., 2014; Halevy et al., 2014; Rudnik-Schoneborn et al., 2013;

Schwabova et al., 2013). Mutations in EXOSC8 (Rrp43 homolog) and EXOSC2 (Rrp4 homolog) are also associated with neurodegenerative disorders (Boczonadi et al., 2014; Di Donato et al., 2016). Furthermore, reduced activity of hDIS3 (Rrp44 homolog) has recently been suggested to be associated with pancreatic cancer (Hoskins et al., 2016b). These data suggest that the RNA exosome is more than a single enzyme, and each exosome component is involved in different functions of the exosome. However, current understanding of the RNA exosome function in its associated diseases is limited. Therefore, it is critical to investigate how the RNA exosome components interact with each other to elucidate the function of the exosome. In this study, we found that there are at least two different conformations of the RNA exosome present *in vivo*, and they have distinct functions. In addition, we investigated the interaction of the exosome with its cofactors and found that a nuclear subunit, Rrp6, functions beyond known biochemical activities. Furthermore, Mpp6 appears to function redundantly with Rrp6 mediating the interactions of the RNA exosome with its cofactors, indicating the redundant cofactor-exosome interactions. Taken together, this study provides further insights into the function of the RNA exosome.

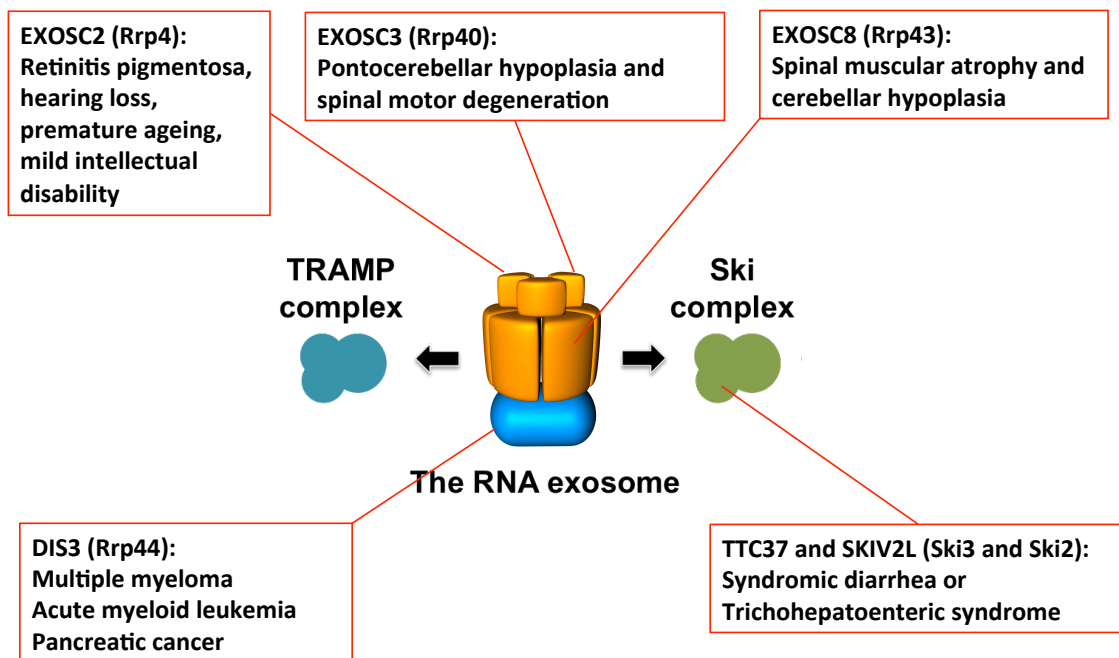


Figure 1.7 The RNA exosome subunits are associated with human diseases.
The human exosome subunit and its associated disease are indicated. Yeast subunit is shown in parenthesis.

Chapter 2: Materials and Methods

Strains, plasmids, and oligonucleotides

Yeast strains, plasmids, and oligonucleotides that are used in this study are described in Table 2.1, 2.2, and 2.3, respectively. Plasmids were generated by standard cloning methods and confirmed by DNA sequencing (GENEWIZ). Unless otherwise noted all plasmids are low copy and express the yeast genes from their endogenous promoter. The *rrp44-da* allele was generated by gene synthesis (GENEWIZ). Most of yeast strains used in this study are in the BY4741 background (Giaever et al., 2002) except for yAV1420 (Kadaba et al., 2004) and yAV1143 (Dunckley and Parker, 1999). Most yeast strains were generated by standard genetic crosses. The *leu2-Δ0* and *trp1Δ::hisG* alleles were generated as described previously (Alani et al., 1987; Brachmann et al., 1998). Yeast cells were grown in YPD (Yeast extract Peptone Dextrose) rich media or SC (Synthetic Complete; Sunrise Sciences) media with appropriate amino acids dropped out for auxotrophic selection. *E. coli* cells were grown in LB (Luria-Bertani) broth or on agar plates.

Site-directed mutagenesis

Site-directed mutagenesis was conducted using QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent). Briefly, a plasmid with a target gene was PCR amplified using appropriate mutagenesis primers. PCR reactions were digested by DpnI to remove parent plasmids and transformed into XL10 Gold ultracompetent cells. Plasmids were isolated from the resulting transformants, and the mutations were confirmed by DNA sequencing (GENEWIZ).

Yeast growth assays

For all growth assays of yeast strains in this study, cells were serially diluted in 96 well plates with 5-fold dilution. Then, the diluted cells were spotted on appropriate solid media, and growth was monitored.

To test the DNA damage response, appropriate amount of DNA damaging agents such as zeocin, 5-fluorouracil (5-FU), or 4-nitroquinoline 1-oxide (4NQO) were added when solid media were made. Growth assays were conducted as described above. For survival assay in the presence of zeocin, 13 replicates of each strain were inoculated into 5 ml YPD media containing 5µg/ml of zeocin or YPD without zeocin. After 20 hours of incubation, cells were serially diluted, and the 100 µl of diluted samples were plated into solid YPD media. Following incubation of the plates, colony forming units (CFU) of replicates were calculated. To avoid counting the jackpot culture, only median values among 13 replicates was taken for each strain. Then, CFU of strains in zeocin media was normalized to CFU of strains in YPD media to calculate survival rate. Resulting survival rate was normalized to wild-type to plot % survival of wild-type.

To test the RNA exosome-mediated normal RNA decay of the *rrp44* mutants, the *rrp44Δ dcp1-2* strain was transformed with a plasmid carrying a wild-type *RRP44* or mutant *RRP44* allele. Resulting transformants were serially diluted and spotted on solid media followed by incubation of the plates at 30°C and 37°C. Synthetic lethality was tested at 37°C as *dcp1-2* is a temperature sensitive allele of *DCPI*.

For testing tRNAi^{Met} degradation, the *trm6-504 gcn2 rrp44Δ* strain was transformed with wild-type or mutant *RRP44* alleles. The resulting transformants were serially diluted, spotted on a solid media, and incubated at room temperature and 36°C.

***HIS3* reporter assay for cytoplasmic exosome function**

To test the cytoplasmic mRNA surveillance, testing strains were transformed with a *his3-nonstop* reporter plasmid (pAV189) in which the *HIS3* gene lacks an in-frame stop codon or a *his3-Rz* reporter (pAV241) in which the *HIS3* gene carries a hammerhead ribosome cleavage site right before the stop codon. Resulting transformants were serially diluted and spotted on media lacking histidine to test the degradation of the *his3* reporter mRNA.

Cell lysis for protein isolation

Yeast cells were resuspended in IP50 buffer [50 mM NaCl, 50 mM Tris-HCl pH 7.5, 2 mM MgCl₂, 0.1% Triton X-100, 0.5 mM β -mercaptoethanol, and 0.1 mM PMSF, with complete EDTA-free protease inhibitors (Roche)] and vortexed with acid washed glass beads for 5 min at 4°C. Following centrifugation, supernatant was taken for further analyses. For the co-immunoprecipitation in the chapter 3, cells were lysed in IP0 (IP50 without NaCl).

Co-Immunoprecipitation

200 μ l of Cell lysates were incubated with 12 - 15 μ l IgG Sepharose 6 Fast Flow beads (Amersham Biosciences) and incubated at 4°C for 2 hours for Rrp44-TAP purification in the chapter 3 and overnight for Csl4-TAP, Mpp6-TAP, and Rrp4-TAP purifications in other chapters. Following incubation, beads were washed by IP50, unless otherwise specified, four times, and for each wash beads were agitated in wash buffer for

2 min. Washed beads were resuspended in the SDS-PAGE sample buffer followed by heating at 95°C for 5 min for elution. The eluted proteins were analyzed by western blot.

SDS-PAGE and Western blot analysis

For western blot analyses, protein samples were loaded on an 8% SDS-PAGE gel followed by transfer to a nitrocellulose membrane. The membrane was incubated in TBST (Tris-buffered saline with 0.1% Tween 20) with 5% nonfat milk for at least 30 min for blocking. Protein A antibody (rabbit, SigmaAldrich) was diluted 10000 times, anti-Myc antibody (mouse, a generous donation from Dr. Eric Wagner) was diluted 500 times, anti-Rrp6 antibody (rabbit, a generous donation from either Dr. David Tollervey (Mitchell et al., 2003)) was diluted 5000 times, anti-Rrp44 antibody (raised against full length GST-Rrp44 by NeoBioLab in rabbit. The GST-Rrp44 used was a generous donation from Dr. Cecilia Arraiano) was diluted 10000 times, anti-Mtr4 antibody (a generous gift from Dr. Patrick Linder (de la Cruz et al., 1998)) was diluted 10000 times, anti-Rad53 (rabbit, a generous donation from Dr. Jessica Tyler), anti-HA antibody (Roche) was diluted 5000 times, and Pgk1 antibody (mouse, Invitrogen) was diluted 5000 times into the blocking buffer. Membranes were incubated with the primary antibodies overnight at 4°C. Goat anti-mouse or anti-rabbit HRP-conjugated antibodies (Bio-Rad) were 1000 times diluted and used as secondary antibodies. Membranes were developed by ECL Prime Western Blotting Detection Reagent (Amercham), and pictures were taken by ImageQuant LAS 4000 mini biomolecular imager (GE Healthcare).

Overexpression of Rrp44

For *in vivo* competition assay (Chapter 3), various *RRP44* alleles were overexpressed under galactose inducible promoter. Briefly, cells were serially diluted and spotted on solid media containing either galactose or glucose as a sole carbon source. Growth was monitored after the spotting. To test the overexpression, overnight cultures were inoculated into fresh liquid media containing either galactose or glucose as a sole carbon source. Following incubation of the cultures for 4 hours, cells were harvested and all lysates were analyzed by western blot.

Northern blot analysis

Overnight cultures were inoculated into fresh media and grown until they reached mid-log phase at 30°C. The mid-log phase cells were harvested, and total RNA was isolated by standard phenol/chloroform extraction method (Caponigro et al., 1993). 10 µg of total RNA was subjected to 6% polyacrylamide (19:1) 8M urea gel electrophoresis. Then, RNAs were transferred to Zeta-Probe GT Blotting membrane (Bio-Rad). Blotted membrane was probed with appropriate ³²P-radiolabeled oligonucleotides. Blots were imaged on a STORM 860 or Typhoon 7000IP PhosphoImager and quantitated using ImageQuant software.

For testing tRNAi^{Met} degradation in the chapter 3, exponentially growing cells were transferred from 30°C to 37°C and incubated for 4 hours before the harvest. RNA isolation and northern blots were conducted as described above.

RNA sequencing analysis

For RNA sequencing analysis, total RNA was isolated using hot phenol protocol. rRNA depletion (ribozero), paired-end library construction with 50 nt reads lengths, and sequencing were conducted by the Nex-Gen Core in the University of Texas Medical Branch at Galveston. The quality of resulting reads were checked by FastQC (Andrews, 2010) and mapped to the genome of *Saccharomyces cerevisiae* (cer3) by Tophat (Trapnell et al., 2012). Mapped reads were used for differential expression analyses by Cufflinks, Cuffcompare, and Cuffdiff. The results were visualized by R package, CummeRbund (L. Goff, 2013). BEDtools software was used to identify genes that have nearby or overlapping CUTs or SUTs (Quinlan and Hall, 2010).

Localization of Rrp6

GFP-RRP6 constructs were transformed into the *rrp6Δ* or *rrp6Δrrp47* strain. Exponentially growing cells were subjected to fluorescence and differential interference contrast (DIC) microscopy (OLYMPUS BX60). Images were taken by HCSImageLive software and analyzed by ImageJ software.

Table 2.1: Yeast strains used		
Name	Genotype	reference
BY4741	<i>MATa/leu2-Δ0/ura3-Δ0/his3-Δ1/met15-Δ0</i>	Giaever et al., 2002
yAV1115	<i>MATa/leu2-Δ0/ura3-Δ0/his3-Δ1/rrp44Δ::NEO [RRP44, URA3]</i>	Schaeffer et al., 2009
yAV1143	<i>MATa/trp1/ura3-52/leu2-3,112/dcp1-2ts::TRP1/rrp44::NEO [RRP44, URA3]</i>	Schaeffer and van Hoof, 2011
yAV1117	<i>MATa/leu2-Δ0/ura3-Δ0/his3-Δ1/met15-Δ0 /RRP43::myc::HIS3</i>	Schaeffer et al., 2012
yAV1137	<i>MATa/leu2-Δ0/ura3-Δ0/his3-Δ1/rrp44Δ::HYG/rrp6Δ::NEO [RRP44, URA3]</i>	Schaeffer et al., 2012
yAV1234	<i>MATa/ura3-Δ0/leu2-Δ0/his3-Δ1/rrp41Δ::NEO [RRP41, URA3] [RRP41, LEU2]</i>	Wasmuth and Lima, 2012
yAV1244	<i>MATa/ura3-Δ0/leu2-Δ0/his3-Δ1/rrp45Δ::NEO [RRP45, URA3] [RRP45, LEU2]</i>	Wasmuth and Lima, 2012
yAV1634	<i>MATa/ura3-Δ0/leu2-Δ0/his3-Δ1/rrp44Δ::NEO/trp1Δ::hisG [RRP44, LEU2]</i>	This study
yAV1642	<i>MATa/ura3-Δ0/leu2-Δ0/his3-Δ1/rrp44Δ::NEO/rrp41Δ::NEO/trp1Δ::hisG [RRP44, URA3] [RRP41, TRP1]</i>	This study
yAV1751	<i>MATa/ura3-Δ0/leu2-Δ0/his3-Δ1/rrp44Δ::NEO/rrp45Δ::NEO/trp1Δ::hisG [RRP44, LEU2] [RRP41, URA3]</i>	This study
yAV1795	<i>MATa/ura3-Δ0/leu2-Δ0/his3-Δ1/rrp44Δ::NEO/rrp41Δ::NEO/rrp45Δ::NEO/trp1Δ::hisG [RRP44, LEU2] [RRP41, TRP1] [RRP45, URA3]</i>	This study
yAV1796	<i>MATa/ura3-Δ0/leu2-Δ0/his3-Δ1/rrp44Δ::NEO/rrp41Δ::NEO/rrp45Δ::NEO/trp1Δ::hisG [RRP44, LEU2] [rrp41-M, TRP1] [RRP45, URA3]</i>	This study
yAV1797	<i>MATa/ura3-Δ0/leu2-Δ0/his3-Δ1/rrp44Δ::NEO/rrp41Δ::NEO/rrp45Δ::NEO/trp1Δ::hisG [rrp44-da, LEU2] [RRP41, TRP1] [RRP45, URA3]</i>	This study
yAV1798	<i>MATa/ura3-Δ0/leu2-Δ0/his3-Δ1/rrp44Δ::NEO/rrp41Δ::NEO/rrp45Δ::NEO/trp1Δ::hisG [rrp44-da, LEU2] [rrp41-M, TRP1] [RRP45, URA3]</i>	This study
yAV1713	<i>MATa/ura3-Δ0/leu2-Δ0/his3-Δ1/rrp44Δ::NEO/rrp41Δ::NEO/trp1Δ::hisG [RRP44, LEU2] [RRP41, URA3]</i>	This study
yAV1714	<i>MATa/ura3-Δ0/leu2-Δ0/his3-Δ1/rrp44Δ::NEO/rrp41Δ::NEO/trp1Δ::hisG [rrp44-da, LEU2] [RRP41, URA3]</i>	This study
yAV1715	<i>MATa/ura3-Δ0/leu2-Δ0/his3-Δ1/rrp44Δ::NEO/rrp41Δ::NEO/trp1Δ::hisG [rrp44-CR3, LEU2] [RRP41, URA3]</i>	This study
	<i>MATa/ura3-Δ0/leu2-Δ0/his3-Δ1/rrp44Δ::NEO/rrp41Δ::NEO/trp1Δ::hisG [rrp44-exo-</i>	

yAV1717	<i>MATα/ura3-Δ0/leu2-Δ0/his3-Δ1/rrp44Δ::NEO/rrp41Δ::NEO/trp1Δ::hisG [rrp44-endo-, LEU2] [RRP41, URA3]</i>	This study
yAV1718	<i>MATα/ura3-Δ0/leu2-Δ0/his3-Δ1/rrp44Δ::NEO/rrp41Δ::NEO/trp1Δ::hisG [rrp44-endo-5A, LEU2] [RRP41, URA3]</i>	This study
yAV1420	<i>MATα/rrp44-20/trm6-504/gcn2-101/his1-29/ura3-52/ino1 (HIS4-lacZ, ura3-52)</i>	Kadaba et al., 2004
yAV1422	<i>MATα/rrp44-20/trm6-504/gcn2-101/his1-29/ura3-52/ino1/leu2-Δ0 (HIS4-lacZ, ura3-52)</i>	This study
yAV1966	<i>MATα/rrp44Δ/trm6-504/gcn2-101/his1-29/ura3-52/ino1/leu2-Δ0 (HIS4-lacZ, ura3-52) [RRP44, URA3]</i>	This study
yAV284	<i>MATα/leu2-Δ0/ura3-Δ0/his3-Δ1/met15-Δ0/ski7Δ::NEO</i>	Knockout library (Research Genetics)
yAV952	<i>MATα/leu2-Δ0/ura3-Δ0/his3-Δ1/met15-Δ0/xrn1Δ::NEO</i>	Knockout library (Research Genetics)
yAV756	<i>MATα/leu2-Δ0/ura3-Δ0/his3-Δ1/met15-Δ0/dcp2Δ::NEO</i>	Wilson et al 2007
yAV1980	<i>MATα/leu2-Δ0/ura3-Δ0/his3-Δ1/met15-Δ0/mpp6Δ::NEO</i>	Knockout library (Research Genetics)
yAV2044	<i>MATα/leu2-Δ0/ura3-Δ0/his3-Δ1/met15-Δ0/rrp6Δ::NEO/rex1Δ::NEO [RRP6, URA3]</i>	This study
yAV1195	<i>MATα/leu2-Δ0/ura3-Δ0/his3-Δ1/met15-Δ0/mtr4Δ::HYG [MTR4, URA3]</i>	Klauer and van Hoof, 2012
yAV1233	<i>MATα/leu2-Δ0/ura3-Δ0/his3-Δ1/met15-Δ0/mtr4Δ::HYG/rrp6Δ::NEO [MTR4, URA3]</i>	Klauer and van Hoof, 2012
yAV1979	<i>MATα/leu2-Δ0/ura3-Δ0/his3-Δ1/met15-Δ0/rrp47Δ::NEO</i>	Knockout library (Research Genetics)
yAV2000	<i>MATα/leu2-Δ0/ura3-Δ0/his3-Δ1/met15-Δ0/mpp6Δ::NEO/rrp6ΔNEO [MPP6, URA3]</i>	This study
yAV1981	<i>MATα/leu2-Δ0/ura3-Δ0/his3-Δ1/met15-Δ0/rex1Δ::NEO</i>	Knockout library (Research Genetics)

Table 2.2: plasmids used				
Name	Short description	Description	Marker	reference
pAV952	pNKY1009	for generation of <i>trp1Δ::hisG</i> strain		Alani et al., 1987
pAV948	pAD1	for generation of <i>leu2Δ0</i> strains		Brachmann et al., 1998
pAV883	da fragment	da fragment (R439A, R440A, H466A, L500A, D602A) synthesized by GENEWIZ	NA	This study
pAV241	HIS3-Rz-stop	HIS3-Hammerhead ribozyme-stop reporter	URA3	Meaux and van Hoof, 2006
pAV958	rrp44-CR3	RRP44 promoter, residues 1-1001 (C47S, C52S, C55S)	LEU2	Schaeffer et al., 2009
pAV503	rrp44-endo-	RRP44 promoter, residues 1-1001 (D171A)	LEU2	Schaeffer et al., 2009
pAV501	rrp44-exo-	RRP44 promoter, residues 1-1001 (D551N)	LEU2	Schaeffer et al., 2009
pAV344	RRP44	RRP44 promoter, residues 1-1001	LEU2	Schaeffer et al., 2009
pAV777	rrp44-yrd	RRP44 promoter, residues 1-1001 (Y40A, R42A, D44A)	LEU2	Schaeffer and van Hoof, 2011
pAV189	HIS3-nonstop	HIS3 nonstop reporter	URA3	van Hoof et al., 2002
pAV361	RRP44	RRP44 promoter, residues 1-1001	URA3	Schaeffer et al., 2009
pAV1085	rrp41-L	RRP41 promoter, rrp41-L (GESEGESEGE inserted after K62)	LEU2	Wasmuth and Lima, 2012
pAV1043	RRP41	RRP41 promoter, residue 1-246	URA3	Wasmuth and Lima, 2012
pAV1039	RRP41	RRP41 promoter, residue 1-246	LEU2	Wasmuth and Lima, 2012
pAV959	rrp44-CR3-TAP	RRP44 promoter, residues 1-1001 (C47S, C52S, C55S), TAP	LEU2	This study
pAV921	rrp44-da-TAP	RRP44 promoter, residues 1-1001 (R439A, R440A, H466A, L500A, D602A), TAP	LEU2	This study
pAV920	rrp44-yrd-TAP	RRP44 promoter, residues 1-1001 (Y40A, R42A, D44A), TAP	LEU2	This study
pAV917	RRP44-TAP	RRP44 promoter, residues 1-1001, TAP	LEU2	This study
pAV912	rrp44-CR3-da	RRP44 promoter, residues 1-1001 (C47S, C52S, C55S, R439A, R440A, H466A, L500A, D602A)	LEU2	This study

pAV911	rrp44-endo-da	RRP44 promoter, residues 1-1001 (D171A, R439A, R440A, H466A, L500A, D602A)	LEU2	This study
pAV910	rrp44-da	RRP44 promoter, residues 1-1001 (R439A, R440A, H466A, L500A, D602A)	LEU2	This study
pAV1109	rrp45-L	RRP45 promoter, rrp45-L (GESEGESEGEL inserted after G94)	TRP1	This study
pAV1089	rrp41-L	RRP41 promoter, rrp41-L (GESEGESEGEL inserted after K62)	TRP1	This study
pAV1079	rrp44-da-endo-exo-	GAL promoter, residues 1-1001 (D171A, R439A, R440A, H466A, L500A, D551N, D602A)	MET15	This study
pAV1077	rrp44-CR3-endo-exo-	GAL promoter, residues 1-1001 (C47S, C52S, C55S, D171A, D551N)	MET15	This study
pAV1076	rrp44-CR3	GAL promoter, residues 1-1001 (C47S, C52S, C55S)	MET15	This study
pAV1074	rrp44-da	GAL promoter, residues 1-1001 (R439A, R440A, H466A, L500A, D602A)	MET15	This study
pAV1058	rrp44-endo-exo-	GAL promoter, residues 1-1001 (D171A, D551N)	MET15	This study
pAV1053	RRP44	GAL promoter, residues 1-1001	MET15	This study
pAV1049	RRP41	RRP41 promoter, residue 1-246	TRP1	This study
pAV1033	rrp44-endo- Δ exo	GAL promoter, residues 1-235 (D171A)	MET15	This study
pAV1032	rrp44- Δ exo	GAL promoter, residues 1-235	MET15	This study
pAV1029	rrp44-exo-da	RRP44 promoter, residues 1-1001 (R439A, R440A, H466A, L500A, D551N, D602A)	LEU2	This study
pAV1044	RRP45	RRP45 promoter, residue 1-305	URA3	Wasmuth and Lima, 2012
pAV1065	RRP45	RRP45 promoter, residue 1-305	TRP1	This study
pAV1160	RRP44-TAP	GAL promoter, residues 1-1001, TAP	MET15	This study
pAV1161	rrp44-exo-TAP	GAL promoter, residues 1-1001 (D551N), TAP	MET15	This study
pAV1162	rrp44- Δ exo-TAP	GAL promoter, residues 1-235, TAP	MET15	This study
pAV1162	rrp44-endo- Δ exo-TAP	GAL promoter, residues 1-235 (D171A), TAP	MET15	This study
pAV1138	RRP6-2xMyc	RRP6 promoter, residues 1-733, 2xMyc	HIS3	This study

pAV1153	GFP	RRP6 promoter, GFP alone	HIS3	This study
pAV1145	rrp6D238N-2xMyc	RRP6 promoter, residues 1-733 (D238N), 2xMyc	HIS3	This study
pAV1156	GFP-rrp6 Δ N Δ C-2xMyc	RRP6 promoter, GFP and residues 129-519, 2xMyc	HIS3	This study
pAV1154	GFP-rrp6 Δ N	RRP6 promoter, GFP and residues 129-733, 2xMyc	HIS3	This study
pAV584	CSL4-TAP	CSL4 promoter, residues 1-292, TAP	LEU2	Borislava Tsanova; unpublished
pAV647	CSL4 Δ C-TAP	CSL4 promoter, residues 1-250, TAP	LEU2	Borislava Tsanova; unpublished
pAV648	CSL4 Δ N-TAP	CSL4 promoter, residues 111-292, TAP	LEU2	Borislava Tsanova; unpublished
pAV670	CSL4-FLAG	CSL4 promoter, residues 1-292, FLAG	LEU2	Borislava Tsanova; unpublished
pAV667	CSL4 Δ C-FLAG	CSL4 promoter, residues 1-250, FLAG	LEU2	Borislava Tsanova; unpublished
pAV668	CSL4 Δ N-FLAG	CSL4 promoter, residues 111-292, FLAG	LEU2	Borislava Tsanova; unpublished
pAV674	mtr4-archless	MTR4 promoter, residues 1-614, linker, 879-1073	LEU2	Jackson et al., 2011
pAV673	MTR4	MTR4 promoter, residues 1-1073	LEU2	Jackson et al., 2011
pAV707	mtr4 Δ 1-89	MTR4 promoter, residues 90-1073	LEU2	Ale Klauer; unpublished
pAV706	mtr4 Δ 1-12	MTR4 promoter, residues 13-1073	LEU2	Ale Klauer; unpublished
pAV673	MTR4	MTR4 promoter, residues 1-1073	URA3	Jackson et al., 2011
pAV1227	rrp6-I14E_R18E-2xMyc	RRP6 promoter, residues 1-733 (I14E, R18E), 2xMyc	HIS3	This study
pAV1228	Mtr4-F7A_F10A	MTR4 promoter, residues 1-1073 (F7A, F10A)	LEU2	This study
pAV1223	rrp6 Δ 520-733_Ski7-116-226-2xMyc	RRP6 promoter, residues 520-733, Ski7 residues 116-226, 2xMyc	HIS3	This study
pAV1224	ski7 Δ 117-225_rrp6-540-	SKI7 promoter, residues 117-225, Rrp6 residues 540-620, 3HA	LEU2	This study

	620-3HA			
pAV1225	ski7 Δ 117-225-3HA	SKI7 promoter, residues 117-225, 3HA	LEU2	This study
pAV1232	GFP-rrp6 Δ NLS	RRP6 promoter, GFP, residues 1-699	URA3	This study
pAV1233	GFP-rrp6 Δ EIR	RRP6 promoter, GFP, residues 1-539, 603-733	URA3	This study
pAV1234	GFP-rrp6 Δ EIR Δ NLS	RRP6 promoter, GFP, residues 1-539, 603-699	URA3	This study
pAV811	GFP-RRP6	RRP6 promoter, GFP, residues 1-733	URA3	Phillips and Butler 2003
pAV1168	MPP6	MPP6 promoter, residues 1-186	URA3	This study
pAV1155	MPP6	MPP6 promoter, residues 1-186	HIS3	This study
pAV1206	MPP6-TAP	MPP6 promoter, residues 1-186, TAP, ADH 3'UTR	URA3	This study

Table 2.3: Oligonucleotides			
oAV	Name	Seq	Description
224	SRP probe	gtctagccgcgaggaagg	Northern blot probes
910	U14 (snR128)	tcctaccgtggaaactgcg	Northern blot probes
911	pre U14	gatactacagtatacgatcactc	Northern blot probes
1151	5'ETS probe	cgaacgacaagcctactcg	Northern blot probes
1233	probe for 7S pre-rRNA	tgagaaggaaatgacgct	Northern blot probes
1234	tRNAiMET probe	tcggtttcgatccgaggacatcagggttatga	Northern blot probes
1235	tRNAcaaleu probe	tgggttctaagagattcgaac	Northern blot probes
1278	5S probe	tcgcgtatggtcacccactaca	Northern blot probes
849	snR33	aggaaccgactcaaaccgg	Northern blot probes
908	pre-snR33	aagttttgcaaatcgattgtcc	Northern blot probes
777	5.8s rRNA		Northern blot probes
1520	snR85_probe	aattacggctctaagaaacgatgaat	Northern blot probes
1036	snR38		Northern blot probes
482	CCCCSS	gatcggacatcccaagtctttctagaagtagtaccaagagtc cgcaaatgtcg	RRP44 to rrp44-CR3, XbaI site added
565	phos D209N sense II	5phos/ctccaggatgtgttcatatcaacgatgccctacatgc g	RRP44 to rrp44-D551N
566	D209N antisense II	cgcattgtagggcacgttgatatcaacacatcctggag	RRP44 to rrp44-D551N
569	CCCCSS antisense	cgacaatttgccgactcttggtactacttctagaaagacttgg gatgtccgatc	RRP44 to rrp44-CR3, XbaI site added
572	D171A sense	cgattaatgacagaaacgcgcgcgtataaggaaaacctgt caatgg	site-directed mutagenesis of RRP44 to rrp44-D171A
573	D171A antisense	ccatgacaggttttcttatagcgcgcgcttctgtcattaatc g	site-directed mutagenesis of RRP44 to rrp44-D171A
847	Y40AR42Ad44A_Fw_MluI	cgtaaagaaacacgcggttagcttcggctatcccatgtcttgcg	RRP44 to rrp44-yrd, MluI site added
848	Y40AR42Ad44A_Rev_MluI	cgaaagacatgggatagccgaagctaacgcgtgttctcttac g	RRP44 to rrp44-yrd, MluI site added
1419	rrp6_D238N_sense	aaaatacgaaagagattgccgttaattcttgagcatcacgatta	site-directed mutagenesis of RRP6 to

		tag	rrp6D238N
1420	rrp6_D238N_antisense	ctataatcgtgatgctcaagattaacggcaatctcttcgtat	site-directed mutagenesis of RRP6 to rrp6D238N
1547	Mtr4-F7A_F10A_F	aggatggattctactgatctggccgatgtgccgaggaaca cctgttgagct	site-directed mutagenesis of MTR4 to mtr4-F7A_F10A
1548	Mtr4-F7A_F10A_R	agctcaacaggtgttcctcggcaacatcgccagatcagta gaatccatcct	site-directed mutagenesis of MTR4 to mtr4-F7A_F10A
1549	Rrp6-I14E_R18E_F	tccggatgtactttatctagggtggagaatgtggtggaggca gcatcatcgtagccagcaag	site-directed mutagenesis of RRP6 to rrp6-I14E_R18E
1550	Rrp6-I14E_R18E_R	cttgactggctaacgatgatgctgcctccaccacattctccac cctagataaaagtacatccgga	site-directed mutagenesis of RRP6 to rrp6-I14E_R18E
1163	3Rrp44-F	tagaggcaggtgcctgaacttagcttctctgaggttaaggt ccatattg	cloning TAP-tagged RRP44 by homologous recombination
1164	TAP3UTR-R	cgtaatcagactcactatagggcgaattgggtaccgggccc cccctcgagtgccggtagaggtgtggtcaataa	cloning TAP-tagged RRP44 by homologous recombination
1339	41F	atgcactagtaagtggagaattgtttgttattt	Cloning of RRP41 (SpeI/XhoI)
1340	41R	atgcctcgagttcatagctgaggagtataagc	Cloning of RRP41 (SpeI/XhoI)
1341	45F	atgcactagttgctgaaagagaattactgatg	Cloning of RRP45 (SpeI/XhoI)
1342	45R	atgcctcgagatgacgatgacgaagttttgt	Cloning of RRP45 (SpeI/XhoI)
1462	RRP44_up_SpeI	atcactagtatacattgtgagggaacca	Cloning RRP44-TAP into p411GAL1
1463	RRP44_down	aaccggggatccgtcgacctttaacaataattctgccttac g	Cloning RRP44-TAP into p411GAL1
1464	TAP_up	ggtcgacggatccccgggtt	Cloning RRP44-TAP into p411GAL1
1465	TAP_down_XhoI	atcctcgagtaagaaatcgcttatttagaagt	Cloning RRP44-TAP into p411GAL1
1415	RRP6_110up_F	aagtcgaccccaaaaatagagggcatcg	Cloning RRP6-2xMyc into pRS413
1416	RRP6_350down_R	aagaattctgacaccgtc	Cloning RRP6-2xMyc into pRS413
1417	RRP6 Δ 1-128_R	cataacgcacttattgggtgc	Cloning rrp6 Δ N-2xMyc into pRS413
1418	RRP6_3end_F	tctcagaggaggacctgtaattatgtaaaacaagcgtattttt	Cloning RRP6-2xMyc into pRS413
1421	rrp6 Δ 1-128_F	gcaccaataagtgcgttatgagtcgaaaaacctcagttga	Cloning rrp6 Δ N-2xMyc into pRS413
1422	rrp6 Δ 520-733_R	ttacaggtcctctctgagatcagcttctgctccaggtcctct ctgagatcagcttctgctcctcagctcagcttctcattgggtg	Cloning rrp6 Δ C-2xMyc into pRS413

		ttctt	
1423	RRP6-3end_R	ttacaggtcctcctctgagatcagcttctgctccaggtcctctgagatcagcttctgctcctcagctcccttttaaatgacagattcttac	Cloning RRP6-2xMyc into pRS413
1441	GFP_F	gcaccaataagtgcgttatgagtaaaggagaagaactttt	Cloning GFP-rrp6 Δ N-2xMyc into pRS413
1442	GFP_R	ggggatccactagtcttaga	Cloning GFP-rrp6 Δ N-2xMyc into pRS413
1443	RRP6_3UTR_F	tctagaactagtgatcccctgattatgtaaacaagcgtattt	Cloning RRP6-2xMyc into pRS413
1444	rrp6 Δ N_F	tctagaactagtgatccccagtcgaaaaacctcagttga	Cloning rrp6 Δ N-2xMyc into pRS413
1509	rrp6 Δ C-ski7N_6	atc gcggccgc tgaagaaaagaattcctgacacc	Cloning rrp6 Δ 520-733_Ski7-116-226-2xMyc into pRS413
1510	ski7 Δ N-rrp6C_6	atc gcggccgc tgccggtagaggtgtggtc	Cloning ski7 Δ 117-225_rrp6-540-620-3HA into pRS415
1534	Ski7_1_116_R	atcaggcgtattccttttggtag	Cloning ski7 Δ 117-225_rrp6-540-620-3HA into pRS415
1533	Ski7_116_225_F	gaacaccaatgaggaagctactgatgataaactcaacttagaagagtc	Cloning rrp6 Δ 520-733_Ski7-116-226-2xMyc into pRS413
1535	Rrp6_540_620_F_1	ctacaaaaggaaatcgctgatcctcaaatccgtgatgttatgga	Cloning rrp6 Δ 520-733_Ski7-116-226-2xMyc in pRS413
1536	Rrp6_540_620_F_2	acagattgatatttaagttttctaattttctatttcgaatatgatacctccaagtt	Cloning rrp6 Δ 520-733_Ski7-116-226-2xMyc in pRS413
1537	Ski7_226_747_F	gaaaaattagaaaacttaaatatcaaatctgt	Cloning ski7 Δ 117-225_rrp6-540-620-3HA into pRS415
1538	Ski7_226_747_F_2	ctacaaaaggaaatcgctgatgaaaaattagaaaacttaaatatcaaatctgt	Cloning ski7 Δ 117-225_rrp6-540-620-3HA into pRS415
1541	Ski7_116_225_R	tcacaggtcctcctctgagatcagcttctgctccaggtcctcctctgagatcagcttctgctcctcagctcattgaaagcattaagtgggccga	Cloning ski7 Δ 117-225-3HA into pRS415
1560	GFP-Rrp6_F (NotI)	atgc gcggccgc ggttcgaatcccttagctctc	Cloning GFP-RRP6 into pRS416
1561	GFP-Rrp6_R (SacI)	atgc gagctc aagccttcgagcgtcccaaaa	Cloning GFP-RRP6 into pRS416
1562	Rrp6-delta-NLS_R	ttgtgtctattattttggccagg	Cloning GFP-rrp6 Δ NLS into pRS416

1563	Rrp6-delta-EIR_R	aacacttatagtctccaacaatattc	Cloning GFP-RRP6 Δ EIR into pRS416
1564	Rrp6-delta-EIR_F (overlapping region with Rrp6-delta-EIR-R)	gaatattgttgagactataagtgttgagaaacctctcggtgtt cctg	Cloning GFP-RRP6 Δ EIR into pRS416
1565	Rrp6-delta-NLS_F (overlapping region with Rrp6-delta-NLS_R)	cctggccaaaataatagacaacaatgattatgtaaaacaagc gtattttttatt	Cloning GFP-rrp6 Δ NLS into pRS416
1445	MPP6_300up_F	atcgaattcacgaatgtaggcttctttacat	Cloning of MPP6 into pRS416
1446	MPP6_300dn_R	atcgctcgacgagagaaagttaggtagc	Cloning of MPP6 into pRS413
1467	MPP6_300up_F(SpeI)	atc actagt acgaatgtaggcttctttacat	Cloning of MPP6-TAP into pRS416
1530	MPP6-TAP_ADH3_R	atcctcgagatctatattaccctgttatccc	Cloning of MPP6-TAP into pRS416

Chapter 3: RNA exosome channeling and direct-access conformations have distinct *in vivo* functions.

NOTE: This chapter is derived from work that was published: “RNA exosome channeling and direct-access conformations have distinct *in vivo* functions” Cell Reports Vol. 16, Issue 12, p3348-3358, 20 September 2016. I am a primary author of this paper. I conducted all the experiments described in this chapter and retain copyright of my work.

INTRODUCTION

The exo-9 core of the RNA exosome in association with Rrp44 has been studied by X-ray crystallography and electron microscopy (EM) (Bonneau et al., 2009; Liu et al., 2014; Liu et al., 2016b; Makino et al., 2013a; Makino et al., 2015; Malet et al., 2010; Wang et al., 2007). These studies revealed that the ability of RNA to bind inside the exo-9 ring is conserved between eukaryotic, bacterial and archaeal enzymes. However, since there is no active site in the ring, an important difference is that in the eukaryotic enzyme the RNA substrate is thought to pass all the way through the exo-9 ring to access the Rrp44 active site. This channeling of RNA substrates *in vitro* requires a long (30nt) unstructured 3' end (Bonneau et al., 2009; Liu et al., 2014; Makino et al., 2013a; Malet et al., 2010). A different conformation of Rrp44 has also been described by both X-ray crystallography and EM (Bonneau et al., 2009; Liu et al., 2014; Liu et al., 2016b; Makino et al., 2013a; Makino et al., 2015). This second conformation is seen *in vitro* in either the absence of RNA, or with very short (<12 nts) RNAs of unclear physiological relevance, and has been suggested to be important *in vivo* for acting on RNAs with highly structured 3' ends (Liu et al., 2014). In this alternative conformation, RNA is thought to access the exonuclease active site of Rrp44 without going through the central channel. For convenience, I will refer to these two conformations as Rrp44^{ch} (channel) RNA exosome conformation and Rrp44^{da} (direct access) RNA exosome conformation, indicating the substrate recruitment site. The exo-9 core of the RNA exosome contains the same proteins for both conformations and undergoes only minor conformational change (Liu et al., 2014). Thus, the only significant difference between the direct access and channel conformations of the RNA exosome is a large rotation of the exonuclease domain of

Rrp44, while the endonuclease domain and the exo-9 core are essentially identical in both conformations. The importance of the Rrp44^{ch} conformation is supported by the observation that mutations that sterically and electrostatically interfere with RNA channeling through exo-9 cause specific RNA processing and growth defects (Drazkowska et al., 2013; Malet et al., 2010; Wasmuth and Lima, 2012), but whether the Rrp44^{da}-exosome indeed allows direct access of substrates to the active site *in vivo*, whether the direct access conformation is only adopted when the RNA exosome is not RNA bound, or whether it is formed at all *in vivo* is not clear.

The large rotation of the exoribonuclease domain results in a different side of Rrp44 facing exo-9 in the two conformations. In this study, I show that the amino acid residues at the interface between Rrp44^{da} and exo-9 are important for association between the Rrp44 and exo-9, and thus that the direct access conformation is relevant *in vivo*. Furthermore, I show that specific RNA processing and degradation effects result from disrupting the rrp44^{da}-exosome and thus provide the first evidence that this conformation is used for specific RNA exosome functions. Shortly after the RNA exosome was discovered and before any relevant data were generated, two models for its overall function were proposed (Mitchell and Tollervey, 2000; van Hoof and Parker, 1999). The proteasome-like model proposed that to be degraded a substrate had to access a central channel of an overall ring-shape structure, and many aspect of this model have since proven correct (Makino et al., 2013b). The now dis-favored allosteric activation model was suggested as an alternative and one important aspect of it was that the RNA exosome adopts different conformations for different functions. Here, I provide the first evidence of the importance of alternative conformation. Our results unite key tenets of both models,

with a channel conformation that closely fits the proteasome-like model and an alternative conformation that is required for specific functions.

RESULTS

Overexpressing Rrp44 alleles suggest that the exonuclease domain contributes to interaction with the RNA exosome core.

The PIN domain of Rrp44 has been shown to be important for the interaction with the exosome core (Schaeffer et al., 2012a; Schneider et al., 2009). However, disruption of the Rrp44-exosome interaction by mutating the CR3 or YRD motif that are critical interaction sites in the PIN domain did not result in lethality of budding yeast, suggesting that there are additional interactions between the exosome and Rrp44.

To investigate whether the exonuclease domain of Rrp44 had any non-catalytic functions, we compared the effect of a mutation in the exonuclease active site (*rrp44-exo⁻*) to the effect of completely deleting the exonuclease domain (*rrp44-Δexo*). While both the *rrp44-exo⁻* and *rrp44-Δexo* mutations cause a slow growth phenotype as previously reported (Dziembowski et al., 2007; Lebreton et al., 2008; Schaeffer et al., 2009; Schneider et al., 2009), the phenotype caused by deletion of exonuclease domain is much more severe (Fig. 3.1A). These results indicate that the exonuclease domain has a noncatalytic function, although they do not indicate what the noncatalytic function might be.

To better understand which interactions are critical for RNA exosome function *in vivo*, I choose to overexpress catalytically inactive Rrp44. I reasoned (Fig. 3.1B) that the catalytically inactive subunit of a complex should displace the endogenous subunit and result in a dominant-negative phenotype. To validate this reasoning, I used a galactose-inducible promoter to conditionally overexpress either wild-type Rrp44, a catalytically inactive Rrp44 (Rrp44-endo⁻-exo⁻) that lacks both endo and exonuclease activities. As

expected, the catalytically inactive Rrp44 caused slow growth (Fig. 3.1C). I also overexpressed an additional Rrp44 (Rrp44-CR3-endo⁻-exo⁻) that combines the catalytic site mutations with a mutation previously shown to inhibit RNA exosome association (Schaeffer et al., 2012). This catalytically inactive Rrp44 that was unable to interact with exo9 had no effect on growth. Western blot analysis indicated that each mutant was overexpressed to a similar extent (Fig 3.1D). These results suggest that overexpression of the catalytically inactive mutant is indeed detrimental because it displaces the endogenous Rrp44 from the RNA exosome.

The endonuclease domain of Rrp44 has previously been shown to be a major exosome interacting domain. To test whether the exonuclease domain of Rrp44 is also important for the interaction with the RNA exosome core, I overexpressed just the catalytically inactive endonuclease domain (Rrp44-endo⁻-Δexo). Both Rrp44-endo⁻-Δexo and Rrp44-endo⁻-exo⁻ lack exonuclease activity, but in the latter the exonuclease domain is available to serve non-catalytic functions, while this domain is entirely deleted in the former. If the endonuclease domain were the only critical interaction site between Rrp44 and the core, overexpression of Rrp44-endo⁻-Δexo and Rrp44-endo⁻-exo⁻ would similarly displace the endogenous Rrp44 and cause growth inhibition. However, I did not observe any dominant negative growth defect when Rrp44-endo⁻-Δexo was overexpressed (Fig. 3.1E). The expression level of *rrp44-Δexo* cannot be directly compared to full length Rrp44 alleles because the antibody was raised against full length Rrp44. Therefore, I used C-terminal TAP-tagged variants of *RRP44* alleles to assess the expression levels by Protein A antibody. Overexpression of Rrp44-endo⁻-Δexo-TAP gives a similar result to untagged variants (Fig. 3.1E). Western blot analysis with anti-TAP antibodies indicated

that the full length Rrp44-endo⁻-exo⁻ and Rrp44-endo⁻-Δexo are expressed at similar levels (Fig. 3.1E). Overexpression of any of the full length Rrp44 constructs also caused the accumulation of degradation intermediates, but these degradation products are similar for wild-type Rrp44 and any of the mutants tested (Fig. 3.1C and E). These degradation products detected by the antibody to the C-terminal TAP tag do not possess the N-terminal region and are unlikely to efficiently associate with the exo-9 core. The results from these overexpression experiments are consistent with the exonuclease domain of Rrp44 contributing to exo-9 interaction, and led us to further pursue this hypothesis as described in the next section.

Identification of residues in the exonuclease domain of Rrp44 that contribute to interaction with the RNA exosome core.

Comparison of multiple X-ray crystallography and EM studies of the RNA exosome suggests that the endonuclease domain does not undergo major conformational changes between the direct access conformation and the channel conformation of the RNA exosome (Liu et al., 2014; Liu et al., 2016b; Makino et al., 2013a; Makino et al., 2015) (Fig. 3.2A, B, and C). In contrast, the exonuclease domain forms two distinct Rrp44-exosome interfaces with the core subunits Rrp41 and Rrp45 (Fig. 3.2B and C). Initial experiments suggested that the exonuclease domain has non-catalytic functions, possibly including contributing to exo-9 interaction (Fig. 3.1 and 3.3). Structural studies indicate that such exonuclease domain interaction with exo-9 would be specific to one or the other conformation. Five conserved residues of Rrp44 (R439, R440, H466, L500, D602) appear to be important for exonuclease

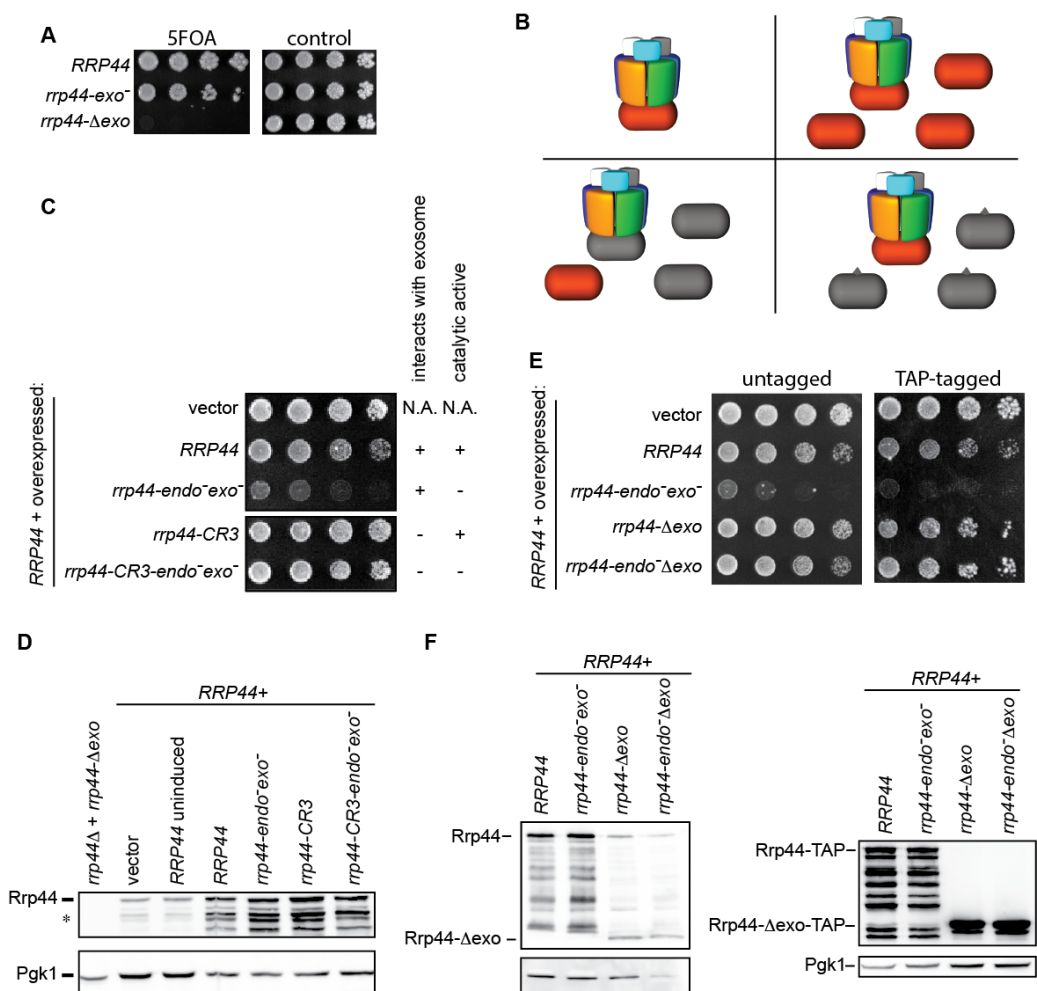


Figure 3.1. The exonuclease domain of Rrp44 contributes to the exosome interaction.

(A) The effect on growth of deleting the exonuclease domain of Rrp44 (*rrp44-Δexo*) is much more severe than the effect of a point mutation inactivating the exonuclease domain (*rrp44-exo⁻*) (B) Rationale behind overexpression experiments in C and E. Top left, normal RNA exosome. Top right: Overexpressing wild-type Rrp44 should not affect RNA exosome function. Bottom left: Overexpressing inactive Rrp44 should displace wild-type Rrp44 and inhibit RNA exosome function dominant negatively. Bottom right: Overexpressing inactive Rrp44 unable to interact with the RNA exosome should not displace wild-type Rrp44 and should not affect RNA exosome activity. Red oval: Active Rrp44. Grey oval: Catalytically inactive Rrp44. Grey triangle: Mutation that interferes with RNA exosome binding. (C) Overexpression of catalytically inactive Rrp44 causes a dominant negative phenotype. Wild-type yeast strains carrying various

Figure 3.1. continued

alleles of *RRP44* under a galactose inducible promoter were serially diluted and spotted on solid media containing galactose. No growth defect was observed on media containing glucose (data not shown). The white line between the third and fourth row indicates that lanes containing other irrelevant mutants were cut from the image. The five rows shown are from the same plate. **D, F, G.** Western blot analysis indicates mutant *RRP44* or *RRP44-TAP* alleles are overexpressed to similar extents. Cell lysates were subjected to western blot with α -Rrp44 (untagged Rrp44), α -Protein A (TAP-tagged Rrp44) and α -Pgk1 (loading control) antibodies. The position of full length Rrp44 is indicated, as is the position of several degradation products (*). The first lane of panel D contains a lysate from a strain that expresses only a truncated Rrp44. The absence of the signals for both full length Rrp44 and degradation products in this lane indicate that these signals are specific. The third lane of panel D is from the same strain as lane four but grown in glucose containing (noninducing) medium. Note that in the strain that contains endogenous Rrp44 and overexpressed *rrp44- Δ exo* (panel F lane 3) the band intensities for the two proteins are approximately equal even though the truncated Rrp44 likely lacks many of the epitopes recognized by the polyclonal α -Rrp44 antisera. **(E)** Overexpression of catalytically inactive endonuclease domain (*rrp44-endo- Δ exo*) does not cause a dominant negative phenotype.

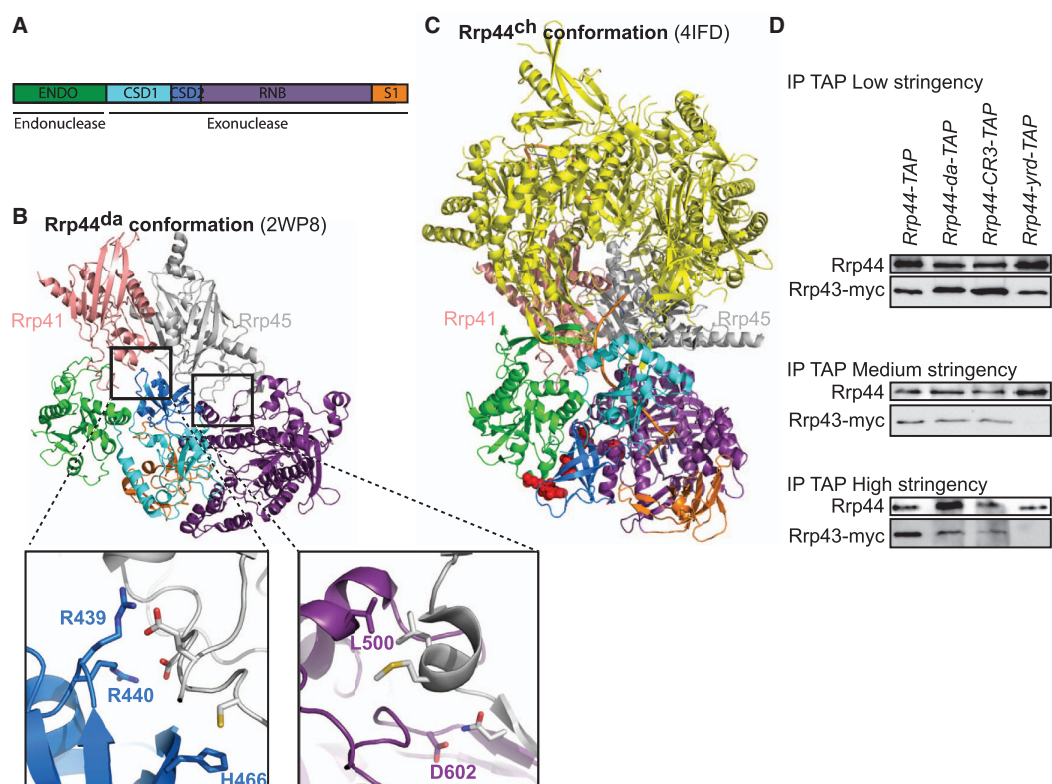


Figure 3.2. Identification of residues important for the da conformation of the RNA exosome.

(A) Domain organization of Rrp44. endonuclease domain: ENDO; cold shock domain 1/2: CSD1/2; RNase II family catalytic domain: RNB; S1 RNA binding domain: S1. The domains are color coded as in (B) and (C). (B) Five conserved residues (R439, R440, H466, L500, and D602) that are important for the formation of the Rrp44^{da}-conformation. (C) The five residues (shown as red spheres) are located on the bottom and exposed to solvent in the Rrp44^{ch}-conformation. The cartoon versions of the X-ray crystal structures were generated by MacPyMol (Schrodinger). (D) Mutations in the five residues of (B) disrupt the Rrp44-exosome interaction. The TAP-tagged variants of Rrp44 were immunoprecipitated at different wash conditions, and western blot was conducted by using anti-Protein A and anti-Myc antibodies.

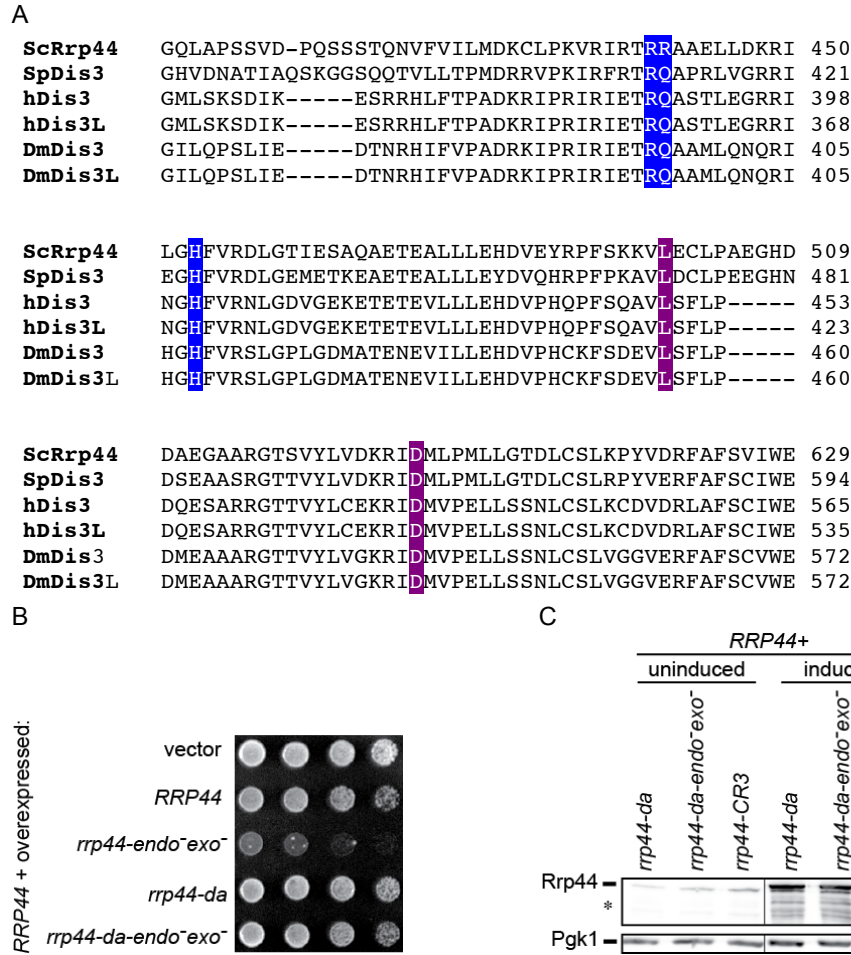


Figure 3.3. The five residues mutated in *rrp44-da* are important for the exosome interaction.

(A) Sequence alignment of part of Rrp44 with homologs from *Schizosaccharomyces pombe* (SpDis3), human (hDis3 and hDis3L) and *Drosophila melanogaster* (DmDis3 and DmDis3L). Blue and purple boxes highlight residues in the CSD2 domain and RNB domain, respectively, that are mutated in the *rrp44-da* allele. (B) *rrp44-da-endo⁻exo⁻* is not dominant-negative when overexpressed. This result is consistent with the results from co-immunoprecipitation that show that the *rrp44-da* mutation affects interaction with the RNA exosome core. Wild-type yeast strains carrying *RRP44* variants under a galactose inducible promoter were serially diluted and spotted on solid media containing galactose. No growth defect was observed on media containing glucose (data not shown). (C) The *rrp44-da*, and *rrp44-da-endo⁻exo⁻* mutants were successfully overexpressed. The black line between the third and fourth lane indicates that a lane containing an irrelevant mutant was cut from the image.

domain interaction with *exo-9* in the direct access conformation (Fig. 3.2B and 3.3A). Mutation of these residues would specifically disrupt the interaction of Rrp44^{da} with the RNA exosome core because they do not seem to participate in the Rrp44^{ch}-exosome core interaction and are largely solvent exposed in Rrp44^{ch} (Fig. 3.1C, red spheres). Therefore, we constructed a mutant allele of *RRP44*, *rrp44-da*, in which these five residues are changed to alanine and used co-immunoprecipitation to test for effects on *exo-9* interaction. Rrp44-TAP variants were immunoprecipitated from a yeast strain, which expresses Myc-tagged Rrp43 (one of the *exo-9* subunits), and western blot was performed to detect co-precipitation of Rrp43-Myc (Fig. 3.2D). Furthermore, we performed these experiments under low, medium and high stringency conditions (no NaCl, 50mM NaCl and 1M NaCl). Under the high stringency condition, similar to what we used previously (Schaeffer et al., 2012b), the amount of Rrp43-Myc that co-purified with Rrp44-da-TAP or the previously analyzed Rrp44-CR3-TAP was reproducibly reduced compared to wild-type Rrp44-TAP, suggesting that residues in both the endonuclease and exonuclease domains are important for the interaction of Rrp44 with the RNA exosome core (Fig. 3.2D). The CR3 motif within the endonuclease domain forms a zinc coordination site that is important for the proper positioning of the YRD motif that directly interacts with the *exo-9* core (Makino et al., 2013a; Schaeffer et al., 2012b). Rrp44-yrd-TAP, in which the YRD motif is changed to alanines, showed no detectable co-purified Rrp43-Myc at high stringency, consistent with the idea that the YRD motif directly interacts, while the CR3 motif has a less important role by positioning the YRD residues. Under medium stringency conditions, wild-type Rrp44, Rrp44-CR3 and Rrp44-da reproducibly co-immunoprecipitated approximately equal

amounts of Rrp43-myc, while Rrp44-yrd copurified strongly reduced amounts. Finally, under low stringency conditions, all three mutant forms of Rrp44-TAP co-purified Rrp43-myc. These data indicate that residues in both the endonuclease and exonuclease domains contribute to interaction with the core RNA exosome, although the contribution of the R439, R440, H466, L500, and D602 residues in the exonuclease domain is not as important as the contribution of the YRD motif in the endonuclease domain. In addition, the contribution of the R439, R440, H466, L500, and D602 residues for exo-9 interaction suggests the presence of the direct access conformation *in vivo*.

The RNA exosome direct access conformation is required for its normal function.

To determine whether the Rrp44^{da} conformation is required for the function of the RNA exosome *in vivo*, we tested the growth of the *rrp44-da* mutant by a plasmid shuffle assay. Briefly, an *RRP44* deletion strain that carries a wild-type *RRP44* allele on a plasmid with a *URA3* marker was transformed with a second plasmid carrying a wild-type *RRP44* or *rrp44* mutant alleles and a *LEU2* marker. Resulting transformants were plated on 5FOA containing media that selects for cells that have lost the *RRP44*, *URA3* plasmid as well as on control media. The strain transformed with *rrp44-CR3* or *rrp44-yrd* grew slowly after losing the wild-type *RRP44* gene, which is consistent with previous studies (Schaeffer et al., 2012b) (Fig. 3.4). Importantly, *rrp44-da* also caused a growth defect compared to wild type, although this growth defect was less severe than that of *rrp44-CR3* or *rrp44-yrd*. The slow growth of the *rrp44-da* strain is not due to reduced expression of the mutant allele because western blot analysis shows that the mutant and wild-type allele expressed from a plasmid are expressed at similar levels to

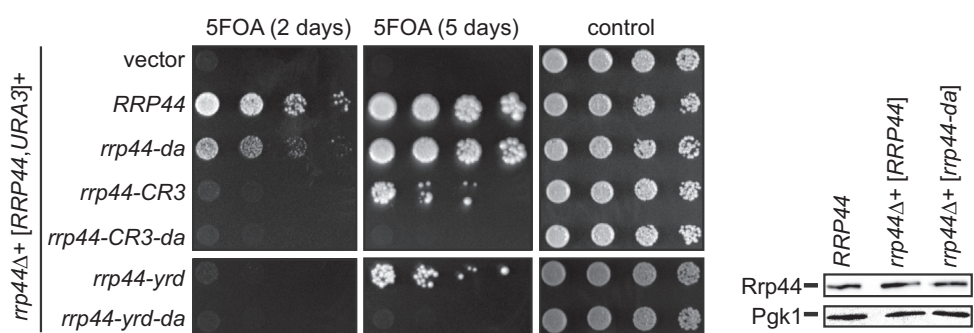


Figure 3.4. The Rrp44^{da}-exosome interface and the CR3/YRD RNA exosome interface are partially redundant.

rrp44-da causes a slow growth phenotype and is synthetic lethal with *rrp44-CR3* and *rrp44-YRD*. An *rrp44Δ* strain carrying a wild-type *RRP44* allele in a *URA3* plasmid was transformed with *LEU2* plasmids carrying *RRP44* variants. The transformants were serially diluted and spotted on 5FOA and SC-LEU-URA (control) media. The Western blot indicates that the plasmid-encoded *rrp44-da* allele is expressed at the same level as endogenous Rrp44 (first lane) or plasmid-encoded wild-type Rrp44 (second lane).

each other and to the endogenous Rrp44 (Fig. 3.4). The slow growth of the *rrp44-da* strain suggests that the direct access conformation is required for RNA exosome function. Interestingly, combining the *rrp44-da* mutation with either *rrp44-CR3* or *rrp44-yrd* resulted in lethality (Fig. 3.4), suggesting that the two contact sites with *exo-9* are partially redundant (see discussion).

Because the *rrp44-CR3-da* and *rrp44-yrd-da* alleles were lethal, we could not assess whether these proteins are expressed at the normal level. I thus repeated the analysis with TAP-tagged plasmids. The plasmid shuffle assay with *RRP44-TAP* variants confirmed that *rrp44-CR3-da* and *rrp44-yrd-da* alleles were lethal. Importantly, when introduced into a wild-type strain (that contains the endogenous *RRP44* gene) the Rrp44-CR3-da-TAP and Rrp44-YRD-da-TAP proteins were detected by western blot, ruling out the possibility that the lethal phenotype is due to the lack of expression (Fig. 3.6). Taken together, these data suggest that both the five residues mutated in *rrp44-da* and the CR3/YRD motif are important for the function of the RNA exosome, and that they are partially redundant. In addition, the importance of the five residues indicates that the Rrp44^{da}-exosome contributes to the essential function of the RNA exosome.

The RNA exosome direct access conformation utilizes both the *exo-* and *endoribonuclease* activities.

Since the RNA exosome processes both *exo-* and *endonuclease* activities, we tested what activities require the Rrp44^{da}-exosome conformation. For this experiment, we used well-characterized mutations that generate an RNA exosome with only *endonuclease* activity (*rrp44-exo⁻*) or an RNA exosome with only *exonuclease* activity

(*rrp44-endo*⁻) (Dziembowski et al., 2007; Lebreton et al., 2008; Schaeffer et al., 2009; Schneider et al., 2009). Introduction of the *rrp44-da* mutation into the RNA exosome that has only endonuclease activity resulted in lethality (Fig. 3.5A). This result suggests that the Rrp44^{da} exo-9 interface is required for endonuclease activity. Similarly, introducing the *rrp44-da* mutation into the RNA exosome that has only exonuclease activity resulted in severe growth defect (Fig. 3.5A). Therefore, the Rrp44^{da} conformation is also required for exonuclease activity. A similar experiment using TAP-tagged variants showed the same lethal phenotype, and the expression level of the TAP-tagged proteins was comparable to TAP-tagged wild-type Rrp44, suggesting that the lethality is not due to failure to express the variant (Fig. 3.6). This result indicates that disrupting the direct access RNA exosome conformation affects both the exo- and endonuclease activities of the RNA exosome.

The RNA exosome direct access conformation is important for nuclear functions but may be dispensable in the cytoplasm

The RNA exosome is present in both the nucleus and cytoplasm (Januszyk and Lima, 2014). The nuclear form of the RNA exosome is essential, while the cytoplasmic form is not (Jacobs Anderson and Parker, 1998a; Mitchell et al., 1997). Thus, the analysis of growth and viability described above assesses the essential nuclear function of the RNA exosome and suggests that the Rrp44^{da} conformation is important for nuclear function of the RNA exosome. This result predicts that the *rrp44-da* mutation may show genetic interactions with mutations of nuclear RNA exosome cofactors, such as Rrp6. Rrp6 is an additional exonuclease that associates with the RNA exosome in the nucleus,

but also has non-catalytic roles including mediating interactions with additional cofactors such as Rrp47 and Mtr4 (Butler and Mitchell, 2011; Feigenbutz et al., 2013a; Schuch et al., 2014). As expected, the *rrp44-da* mutation shows a synthetic growth defect with *rrp6Δ* (Fig. 3.5B) confirming that the Rrp44^{da} conformation is important for the nuclear functions of the RNA exosome.

The cytoplasmic RNA exosome functions in one of two general mRNA decay pathways, and the cytoplasmic RNA exosome is not essential because of redundancy between these pathways (Jacobs Anderson and Parker, 1998a). To investigate whether the Rrp44^{da} conformation is also important for the function of the cytoplasmic RNA exosome, we carried out two experiments. First, we tested for genetic interactions with the *dcp1-2* mutation. The cytoplasmic RNA exosome becomes essential if the alternative pathway is inactivated. *dcp1-2* is a temperature-sensitive mutation in the alternative pathway, such that the cytoplasmic RNA exosome is essential in a *dcp1-2* strain incubated at the restrictive temperature (Schaeffer and van Hoof, 2011). *rrp44-da* did not show a significant growth defect when combined with *dcp1-2* (Fig. 3.5C). This result is in contrast with *rrp44-CR3*, which is synthetic lethal with *dcp1-2* at the restrictive temperature as previously shown (Schaeffer et al., 2012b). The *rrp44-yrd* mutation is also synthetic lethal with *dcp1-2* as expected. This result suggests that the Rrp44^{da} conformation is not essential for mRNA degradation by the cytoplasmic RNA exosome.

In addition to its function in general mRNA decay, the cytoplasmic RNA exosome is required for the rapid degradation of specific aberrant mRNAs (Frischmeyer et al., 2002; Klauer and van Hoof, 2012; Meaux and Van Hoof, 2006; van Hoof et al., 2002). Thus, in the second experiment, I tested the effect of the *rrp44-da* mutation on this

mRNA quality control function. The *his3-nonstop* reporter mRNA lacks a stop codon and therefore is rapidly degraded by the cytoplasmic RNA exosome (van Hoof et al., 2002). Mutations that inactivate the cytoplasmic RNA exosome stabilize the *his3-nonstop* mRNA, which allows the cell to synthesize sufficient histidine to grow in the absence of added histidine. As previously reported (Schaeffer et al., 2012b; Schaeffer and van Hoof, 2011), the *rrp44-CR3* mutation allows a *his3-nonstop* strain to grow in the absence of added histidine, indicating a defect in cytoplasmic RNA exosome function (Fig. 3.5D, left two panels). As expected, the *rrp44-yrd* mutation has the same effect. In contrast, the *rrp44-da* mutation does not affect the *his3-nonstop* reporter mRNA, suggesting that the Rrp44^{da} conformation is not required for nonstop mRNA degradation by the cytoplasmic RNA exosome.

I repeated the assay for mRNA quality control defects with a different reporter mRNA (Fig. 3.5D, right two panels). The *his3-RZ* reporter contains a hammerhead ribozyme and therefore generates a truncated mRNA that lacks a poly(A) tail (Meaux and Van Hoof, 2006). Such mRNA cleavage fragments are also degraded by the cytoplasmic RNA exosome, regardless of whether they contain a stop codon or not. As with *his3-nonstop*, mutations that inactivate the cytoplasmic RNA exosome stabilize the *his3-RZ* mRNA, which allows the cell to synthesize sufficient histidine to grow in the absence of added histidine. As previously reported, the *rrp44-CR3* mutation allows a *his3-RZ* strain to grow in the absence of added histidine, and as expected, the *rrp44-yrd* mutation has the same effect. However, the *rrp44-da* mutation does not have this effect. Together, genetic analyses suggest that the Rrp44^{da}-exosome conformation functions in the nucleus but is dispensable for cytoplasmic RNA exosome functions.

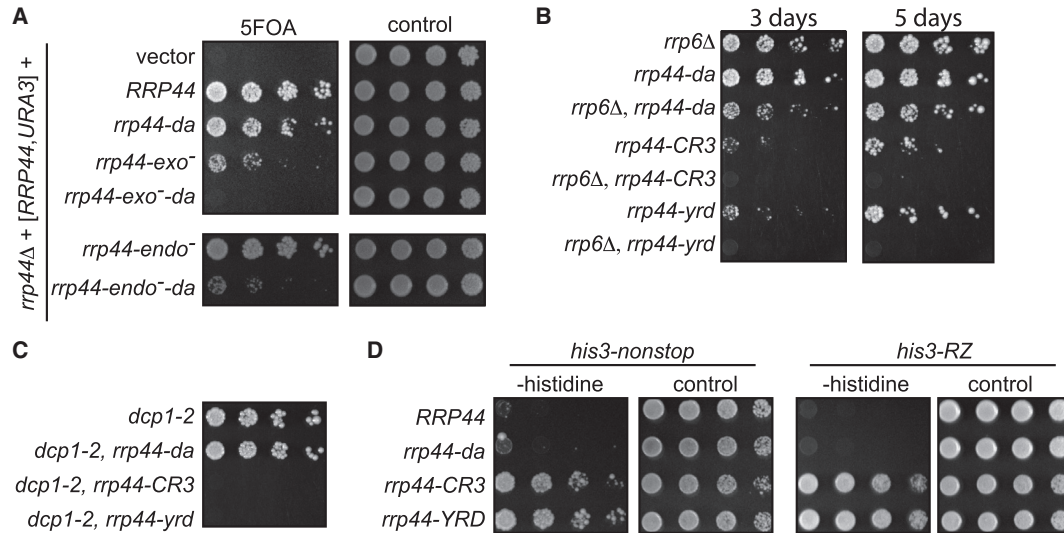


Figure 3.5. The Rrp44^{da}-exosome utilizes both the exo- and endonuclease activities and functions in the nucleus.

(A) *rrp44-da* is synthetic lethal with *rrp44-exo* and *rrp44-endo*. An *rrp44Δ* strain carrying a wild-type *RRP44* allele in a *URA3* plasmid was transformed with *LEU2* plasmids carrying *RRP44* variants. The transformants were serially diluted and spotted on 5FOA and SC-LEU-URA (control) media. (B and C) Synthetic growth defect of *rrp44-da* with *rrp6Δ* and *dcp1-2*. *rrp44Δ rrp6Δ* or *rrp44Δ dcp1-2* strains carrying a wild-type *RRP44* allele in a *URA3* plasmid were transformed with *LEU2* plasmids carrying a wild-type *RRP44*, *rrp44-da*, *rrp44-CR3*, or *rrp44-yrd*. The transformants were serially diluted and spotted on 5FOA. (D) Strains carrying *RRP44* variants were transformed with reporter constructs encoding aberrant *HIS3* mRNAs. The transformants were serially diluted and spotted on media lacking histidine or control plates containing histidine. The *his3-nonstop* reporter is the *HIS3* gene with its stop codon removed. The *his3-RZ* reporter has a hammerhead ribozyme cleavage site immediately upstream of stop codon of the *HIS3* gene.

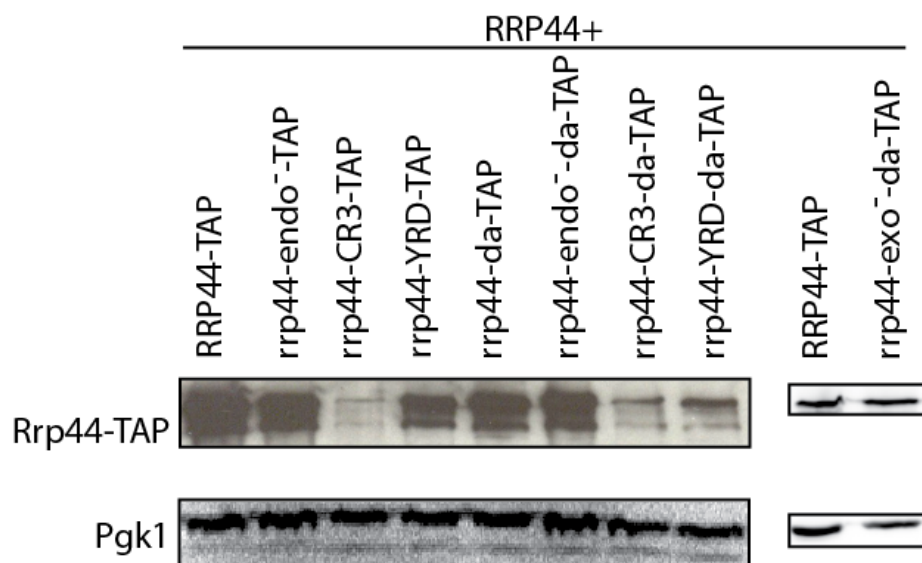


Figure 3.6. The lethality of *rrp44-CR3-da*, *rrp44-yrd-da*, and *rrp44-exo⁻-da* is not correlated with their expression level.

Specifically, the lethal *rrp44-CR3-da-TAP*, and *rrp44-yrd-da-TAP* expressed at higher levels than *rrp44-CR3-TAP*, the slow growing *rrp44-endo⁻-da-TAP* is expressed similar to either single mutant (*rrp44-endo⁻-TAP* and *rrp44-da-TAP*) and the lethal *rrp44-exo⁻-da-TAP* is expressed similar to wild-type *RRP44-TAP*.

The RNA exosome direct access conformation is required for specific RNA degradation events, but makes minor contributions to others.

It has been previously shown that unmodified initiator tRNA (tRNAi^{Met}) binds to the direct access conformation of the RNA exosome *in vitro* (Liu et al., 2014) and that the RNA exosome degrades hypomodified tRNAi^{Met} *in vivo* (Kadaba et al., 2004). I therefore hypothesized that the direct access conformation of the RNA exosome may be especially important for the degradation of hypomodified tRNAi^{Met} *in vivo*. Thus, we tested the hypomodified tRNAi^{Met} level by northern blot analysis as previously described. This analysis used a *trm6-504 rrp44*Δ strain transformed with plasmids encoding either wild-type or mutant Rrp44. The *trm6-504* mutation causes a defect in ^{m1}A58 methylation and thus causes the hypomodification that triggers exosome-mediated degradation of tRNAi^{Met} in the strain containing the wild-type *RRP44* gene. In contrast, tRNAi^{Met} accumulates to approximately two-fold higher levels in the *rrp44-da* strain (Figure 3.7A). Although the *rrp44-da* mutation only caused a 2-fold increase, this increase was highly reproducible and similar to previous reports (Kadaba et al., 2004; Wang et al., 2008). The *rrp44-exo*⁻ mutation caused similarly high tRNAi^{Met} levels, while the *rrp44-endo*⁻ mutation had no effect, suggesting that the exonuclease is the major activity responsible for hypomodified tRNAi^{Met} degradation. The *rrp44-CR3* and *rrp44-yrd* mutations also increased hypomodified tRNAi^{Met} levels, but this effect was reproducibly smaller than the effect of the *rrp44-da* mutation (see discussion).

I confirmed the role of the direct access conformation in pre-tRNAi^{Met} degradation using a growth assay. The temperature-sensitive growth of a *trm6-504* strain is caused by reduced tRNAi^{met} level and therefore this temperature sensitivity is

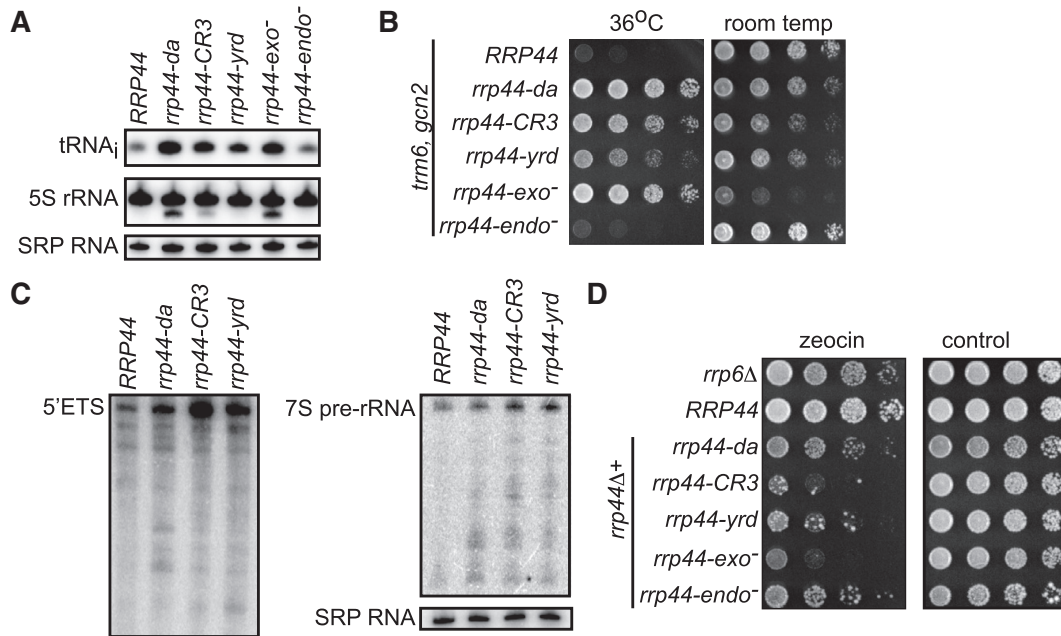


Figure 3.7. The Rrp44^{da}-exosome is required for specific functions of the RNA exosome.

(A) Total RNA isolated from *trm6*, *gcn2*, *rrp44Δ* strains carrying wild-type or mutant alleles of *RRP44* in a *LEU2* plasmid were subjected to northern blot. Shown is a representative result of two independent biological replicates. **(B)** Strains used in (A) were serially diluted and spotted on SC-LEU. **(C)** Total RNAs were isolated from *rrp44Δ* strains carrying a wild-type or mutant *RRP44* allele followed by northern blot probing 5'ETS and 7S pre- rRNA. The SRP RNA serves as a loading control. **(D)** *rrp6Δ* and *rrp44Δ* strains carrying wild-type or mutant *RRP44* alleles were serially diluted and spotted on media containing 10 mg zeocin/ml and YPD media as a control.

suppressed by *rrp44* mutations that affect tRNAi^{Met} degradation. The *trm6-504* strain with a wild-type *RRP44* allele failed to grow at 36 degrees (Fig. 3.7B). This growth phenotype was strongly suppressed by *rrp44-exo⁻* and *rrp44-da*, while *rrp44-CR3* and *rrp44-yrd* were slightly less effective at restoring growth. This growth phenotype mirrors the effects seen by northern blot confirming that the exonuclease activity and direct access conformation of Rrp44 are required for the rapid degradation of hypomodified tRNAi^{Met}.

The effect of Rrp44 on hypomodified tRNAi^{Met} was initially found in a strain that carries the *rrp44-20* point mutation. This same *rrp44-20* mutation also causes the accumulation of a truncated 5S rRNA (Kadaba et al., 2004). I therefore next analyzed the effect of the same *RRP44* mutations on 5S rRNA and the results mirrored what we observed for hypomodified tRNAi^{Met}. Specifically, the *rrp44-da* and *rrp44-exo⁻* strains reproducibly accumulated relatively high amounts of the truncated 5S rRNA, while the *rrp44-CR3* and *rrp44-yrd* mutations had a much smaller effect and the *rrp44-endo⁻* mutation had no effect (Fig. 3.7A).

To investigate the role of the direct access conformation on other specific nuclear RNA exosome functions, we next tested the effect of *rrp44-da* on the 5'ETS and 5.8S rRNA, two prototypical RNA exosome substrates. The RNA exosome degrades the 5' external transcribed spacer (5'ETS) that is generated from 35S pre-rRNA processing events (de la Cruz et al., 1998). The RNA exosome is also involved in the maturation of 5.8S rRNA by processing the 3'-end of 7S pre-rRNA (Allmang et al., 1999a). Using northern blot analysis, we reproducibly observed a 2-fold increase of the full-length 5'ETS and an accumulation of its degradation intermediates in *rrp44-da* compared to

wild type (Fig. 3.6C, left panel). In addition, *rrp44-da* showed a minor accumulation of the processing intermediates of 7S pre-rRNA (Fig. 3.7C, right panel). Importantly, these defects are not as severe as the defects in *rrp44-CR3* or *rrp44-yrd* (e.g. 6-fold increase in 5'ETS), indicating that the Rrp44^{da}-exosome has a minor contribution to the degradation of 5'ETS and processing of 7S rRNA to 5.8S rRNA (see below).

Recent studies have implicated the nuclear RNA exosome as important for the DNA damage response both in the budding yeast and HeLa cells (Hieronymus et al., 2004; Manfrini et al., 2015; Marin-Vicente et al., 2015). Specifically, the RNA exosome cofactors, Rrp6, Trf4, and the NEXT (Nuclear Exosome Targeting) complex were implicated in the DNA damage response (Gavalda et al., 2013; Hieronymus et al., 2004; Manfrini et al., 2015). I therefore tested whether mutations in the catalytic subunit of the RNA exosome itself cause sensitivity to zeocin, an agent that induces double-strand breaks (Chankova et al., 2007). As reported previously *rrp6Δ* strain was sensitive to zeocin (Manfrini et al., 2015) (Fig. 3.7D). I found that *rrp44-CR3* and *rrp44-exo⁻* strains are extremely sensitive to zeocin, while the *rrp44-da* strain showed sensitivity similar to *rrp6Δ*. This shows that the exonuclease activity of Rrp44 is required for the DNA damage response, but the direct access conformation is less critical.

Taken together, the observations that *rrp44-da* has stronger effects on tRNAi^{Met} and 5S rRNA than the *rrp44-CR3* and *rrp44-yrd* mutations, while *rrp44-CR3* and *rrp44-yrd* has stronger effects of growth, RNA exosome interaction, other RNA degradation reactions, and zeocin sensitivity suggest that the effects on tRNAi^{Met} and 5S rRNA reflect a specific requirement of the direct access conformation for these two RNA exosome functions (see discussion).

The balance between the two RNA exosome conformations is required for growth.

I next sought to identify the relationship between the two conformations, Rrp44^{da}- and Rrp44^{ch}-exosome, since the EM studies suggest dynamic conformational change between them (Liu et al., 2014; Liu et al., 2016b). Instead of being maintained by protein contacts, the Rrp44^{ch} conformation is thought to be stabilized by simultaneous interactions of long RNAs with the channel and exonuclease domain (Liu et al., 2016b). Specifically, the interaction surface between the exonuclease domain and the RNA exosome core is much larger and electrostatically more favorable in the direct access conformation than in the channel conformation (Liu et al., 2016b). Because of this reliance on RNA to stabilize the channel conformation, we could not identify specific Rrp44 residues required for the channel conformation. As an alternative way to disrupt channeling through the exosome core, we took advantage of the previously reported and characterized channel occluding mutations of Rrp41 and Rrp45 (*rrp41-L* and *rrp45-L*), in which an 11-amino acid residue insertion physically and electrostatically blocks the central channel of the RNA exosome (Wasmuth and Lima, 2012). *rrp41-L* and *rrp45-L* have slow growing and lethal phenotypes, respectively, which suggests that the central channel is essential. To test the relationship between the two RNA exosome conformations, we tested the genetic interaction between the *rrp44-da* and *rrp41-L* or *rrp45-L* mutations (Fig. 3.8A). Strikingly, *rrp44-da* suppressed the slow growing phenotype of *rrp41-L*. This suppression is specific for the *rrp44-da* allele as *rrp44-exo*⁻ and *rrp44-endo*⁻ do not have a significant effect, and *rrp44-CR3* is synthetic lethal with *rrp41-L* (and thus has the opposite effect of the *rrp44-da* allele). Similarly, the *rrp44-da* mutation suppressed the lethality of the *rrp45-L* channel occluding mutation (Fig. 3.8B).

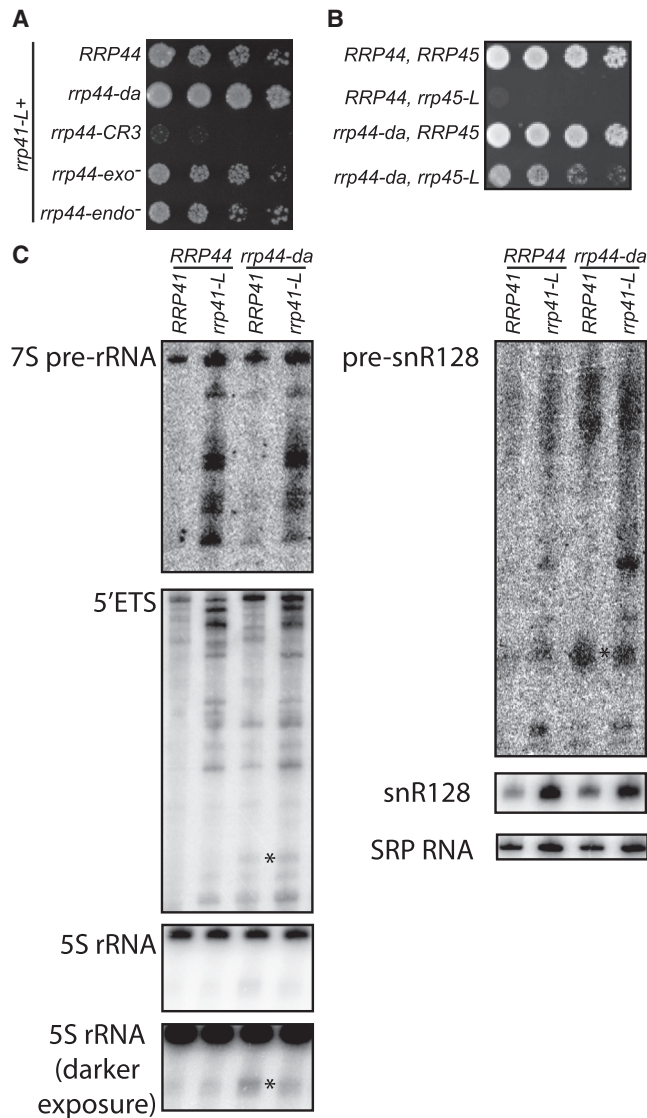


Figure 3.8. The balance between two RNA exosome conformations is required for growth.

(A and B) *rrp44-da* suppresses the growth defect of *rrp41-L* and *rrp45-L*. *rrp44Δrrp41Δ* or *rrp44Δrrp45Δ* strains carrying *RRP44* variants in a *LEU2* plasmid and a wild-type *RRP41* or *RRP45* in a *URA3* plasmid were transformed with a *TRP1* plasmid carrying the *rrp41-L* or *rrp45-L* allele. The transformants were serially diluted and spotted on 5FOA media. **(C)** *rrp44Δrrp41Δ* strains carrying *RRP44* and *RRP41* variants were subjected to total RNA isolation followed by northern blot analysis probing 7S pre-rRNA, 5'ETS, 5S rRNA, snR128 snoRNA, and SRP RNA. The asterisk (*) indicates 5'ETS, 5S, and snR128 RNA species specifically and reproducibly detected in the *rrp44-da* strain.

I conclude that a proper balance between two conformations is important for the essential function of the RNA exosome (see discussion).

Having generated strains with either the direct access conformation or the channel disrupted, we further compared the role of the two conformations in specific RNA exosome functions by northern blotting for known RNA exosome substrates. In addition, we tested whether the suppression of *rrp41-L* growth phenotype by *rrp44-da* was accompanied by restoration of RNA processing and degradation defects. For 7S pre-rRNA to 5.8S rRNA processing we detected intermediates in the *rrp41-L* strain and lower levels of the same intermediates in the *rrp44-da* strain (Fig. 3.8C). This confirms the conclusion that the direct access route makes a much smaller contribution to 5.8S processing than the channel route. Furthermore, the processing defect seen in the *rrp41-L* *rrp44-da* double mutant closely resembled that seen in *rrp41-L*, indicating that suppression of the *rrp41-L* growth phenotype is not accompanied by suppression of this rRNA processing defect.

As described above, the *rrp44-da* strain accumulated full-length 5'ETS as well as some degradation intermediates. The *rrp41-L* strain also accumulated 5'ETS degradation intermediates but not the full length 5'ETS. The *rrp41-L* strain accumulated much higher levels of degradation intermediates than the *rrp44-da* strain, again confirming that the channel route is the major degradation route for 5'ETS. Several intermediates were specific for *rrp41-L*, while one specific intermediate was only detected in *rrp44-da* reproducibly, although at low levels (Fig. 3.8C, asterisk). Rather than suppressing the *rrp41-L* phenotype, the effect of combining *rrp41-L* with *rrp44-da* appeared additive,

such that both sets of intermediates from the single mutants and the accumulation of full length 5'ETS were seen in the double mutant.

Although, as pointed out above, for some RNA exosome substrates we saw no suppression of the *rrp41-L* phenotype by *rrp44-da*, for other substrates we did see a suppression that correlates with the suppression of the growth phenotype. Specifically, the RNA subunit of the signal recognition particle (SRP) is commonly used as a loading control, but we noted that it was reproducibly 3-fold more abundant in the *rrp41-L* mutant than in the *RRP41* control strain, consistent with a recent report that this RNA is also a substrate for the RNA exosome (Leung et al., 2014). The *rrp41-L rrp44-da* double mutant strain accumulated only 2-fold more SRP than the *RRP41, RRP44* control strain (Fig. 3.8C). Thus, the increased growth rate of this double mutant correlates with a smaller defect in the processing of this particular RNA. I noticed a similar trend with the *snR128* snoRNA. 3' extended species of this snoRNA accumulate in RNA exosome mutants, and we observed this phenotype for the *rrp41-L* strain as well. Strikingly however, the mature *snR128* also over accumulated in the *rrp41-L* strain, and this over accumulation was slightly, but reproducibly less severe in the *rrp41-L rrp44-da* double mutant (Fig. 3.8C). Overall, these data indicate that although the *rrp44-da* mutation suppresses the growth phenotype of the blocked RNA exosome channel in *rrp41-L*, most of the RNA processing defects in *rrp41-L* are not suppressed. I did see some minor suppression of SRP and *snR128* defects, but whether this suppression is cause or effect of the suppression of the growth defect is not yet clear. In addition, the comparison of the *rrp41-L* and *rrp44-da* strains confirmed the above conclusion that the direct access

conformation of the RNA exosome is required for a few specific functions while the channel conformation is required for many other functions.

DISCUSSION

Structural studies have captured the RNA exosome in two conformations *in vitro* (Liu et al., 2014; Makino et al., 2015). One conformation is consistent with RNA threading through the central channel of the exo-9 core to access the exonuclease active site, while in the other conformation RNA substrates directly access the active site, bypassing the channel. Here, we provide the first evidence that the Rrp44^{da}-exosome is present *in vivo*, and that it has specific functions. I identified and mutated five residues in the exonuclease domain that interact with exo-9 in the direct access conformation, but are facing the solvent in the channel conformation. I show that mutation of these five residues reduces the co-immunoprecipitation of Rrp44 with Rrp43 and causes a slow growth phenotype. I conclude that the direct access conformation of the RNA exosome exists *in vivo* and contributes to RNA exosome function.

Several observations suggest that the direct access conformation of the exosome has specific but limited functions. Most importantly, the severity of RNA exosome defects seen in different *RRP44* alleles cannot be explained by quantitative differences in Rrp44 activity with some alleles more severely affecting overall exosome activity and others having a smaller effect. For example, the *rrp44-da* mutation has a smaller effect than *rrp44-yrd* on growth, cytoplasmic RNA exosome functions, and most nuclear RNA exosome functions. In contrast, the *rrp44-da* mutation has a larger effect than *rrp44-yrd* on degradation of hypomodified tRNA^{iMet} and truncated 5S rRNA. In fact, for these latter two functions, the severity of the defect in *rrp44-da* is similar to that seen in the catalytically inactive *rrp44-exo⁻* mutant. Second, while the *rrp44-da*, *rrp44-CR3* and *rrp44-yrd* alleles all affect Rrp43 co-immunoprecipitation, the severity of these defects

does not correlate well with growth and RNA degradation defects. Specifically, the *rrp44-da* and *rrp44-CR3* mutations have similar effects on RNA exosome core interactions and *rrp44-yrd* has a larger effect. This is in contrast to the growth defects that are milder for *rrp44-da* and more severe for *rrp44-CR3* and *rrp44-yrd*. Because of this disconnect between the effect on RNA-exosome binding and growth, we conclude that the effects seen for *rrp44-da* are not simply due to reduced interaction with the RNA exosome core. Third, there is an allele specific suppression of the *rrp41-L* growth phenotype. Specifically, the slow growth phenotype of the *rrp41-L* mutant is suppressed by the *rrp44-da* mutation, but enhanced by the *rrp44-CR3* mutation. Such an allele-specific interaction is difficult to explain by both *rrp44* alleles reducing overall RNA exosome function, but is readily explained by one of the alleles disrupting a specific function. Based on all of these data, we conclude that the *rrp44-da* allele disrupts a specific aspect of RNA exosome function. Based on the structural studies and the effect on RNA exosome core co-immunoprecipitation, the most likely explanation is that the *rrp44-da* allele specifically disrupts the direct access conformation of the RNA exosome.

By analyzing a variety of previously characterized RNA exosome functions either by Northern blot or growth phenotypes, we show that the channeling and direct access conformations of the RNA exosome have distinct functions. Using channel-occluding mutations and qRT-PCR, it has been previously shown that the channeling conformation is required for the degradation of CUTs and 5' ETS, and processing of 5.8S rRNA and U4 snRNA by the nuclear exosome and mRNAs by the cytoplasmic exosome (Drazkowska et al., 2013; Wasmuth and Lima, 2012). I confirm the 5.8S rRNA processing and 5' ETS degradation defects and show that channel-occluding mutations

also lead to defects in snoRNA processing. I also noted that the RNA subunit of SRP was more abundant in the channel-occluding mutant, consistent with a recently described role of the RNA exosome in SRP quality control (Leung et al., 2014). Most of the substrates affected by the channel occluding mutations were affected much less strongly by the direct access mutation. Conversely, the direct access mutant accumulated a truncated 5S rRNA form, while the channel occluding mutation had no effect on 5S rRNA. I also found that the direct access mutant of Rrp44 was completely inactive in degrading hypomodified tRNA^{iMet}.

Although we identified distinct functions for the direct access and channeling conformations, both appear to be important for degradation of some substrates, such as 5'ETS, although the pattern of intermediates that accumulate in the two mutants is distinct. This may be because the 5'ETS can be degraded by either pathway, or because 5'ETS degradation is initiated by the direct access conformation and then finished by channeling through exo-9. This switch between access routes would require that the 3' end of 5'ETS dissociates from the Rrp44 catalytic site, a possibility consistent with oligoadenylation by TRAMP at internal sites (Schneider et al., 2012).

Surprisingly, the growth defect of channel occluding mutations, *rrp41-L* and *rrp45-L*, is suppressed by disruption of the direct access route (Fig. 3.8A and B). While many of the defects seen in the single mutants are not reversed in the double mutant, accumulation of full-length *snR128* snoRNA, and SRP RNA in *rrp41-L* is decreased in the *rrp41-L rrp44-da* double mutant. A possible explanation is that when one conformation of the RNA exosome is inhibited, the other conformation inappropriately acts on these RNAs. Partially disrupting both conformations could suppress phenotypes

by interfering with the inappropriate action of the alternative conformation.

Strikingly, the defects (in initiator tRNA and 5S rRNA) we describe for *rrp44-da* closely resemble those described previously for the *rrp44-20* allele (Kadaba et al., 2004). The single amino acid substitution in *rrp44-20* (Gly₈₃₃ - Asp) is positioned within the RNA binding channel of the exonuclease domain near the -5 nucleotide (numbering from the active site). Although this part of the RNA-binding channel is shared between the direct access route and the channel route, the mutation appears to have a larger effect on direct access-dependent substrates. I suggest that introduction of a bulky, negative charged residue at this position has a more disruptive effect on the short (12 nt) RNA path of the direct-access route than the much longer (30 nt) path through the channel. Our results raise the possibility that defects in the two different conformations cause different human diseases. Specifically, multiple myeloma genomes often contain mutations in the Rrp44 exonuclease domain, but not in other RNA exosome subunits (Weissbach et al., 2015). In contrast, pontocerebellar hypoplasia is caused by point mutations in the *exo-9* core (EXOSC3 and EXOSC8; Boczonadi et al., 2014; Wan et al., 2012). The residues mutated in *rrp44-da* are highly conserved in the human ortholog (Fig. 3.3), suggesting that the direct access conformation is also important in humans. Thus, we speculate that defects in the direct access function of DIS3 might contribute to the development of multiple myeloma, while defects in the channel-dependent functions may lead to pontocerebellar hypoplasia. The specific mutations in multiple myeloma may either directly affect the ability to adopt the direct access conformation, analogous to *rrp44-da*, or affect RNA interactions more severely in the short direct access route than in the longer channel route, as we propose for *rrp44-20*.

Genetic analyses suggest that the direct access conformation of the RNA exosome is important for both exo- and endoribonuclease activities. How RNA substrates access the endonuclease active site in general is unknown. Multiple crystal structures indicate the endonuclease domain is static with the active site readily accessible from the solvent (Bonneau et al., 2009; Makino et al., 2013a; Makino et al., 2015). However, biochemical analyses indicate that channel occluding mutations also affect the endonuclease activity of the RNA exosome (Drazkowska et al., 2013; Wasmuth and Lima, 2012). This suggests that there may be an additional uncharacterized conformation of Rrp44 that orients the endonuclease site towards the channel. One explanation why the *rrp44-da* mutation inhibits endonuclease activity is that this hypothetical conformational change of the endonuclease domain likely breaks the contact of exo-9 with the YRD motif of Rrp44 and therefore integrity of the RNA exosome in this hypothetical conformation would depend more heavily on interactions between exo-9 and the exonuclease domain of Rrp44.

Regardless of whether substrates utilize the direct access route or the channel route, the RNA exosome function requires additional proteins that are thought to mediate substrate specificity. Mutations in the TRAMP subunit Trf4 also affect both the degradation of hypomethylated tRNA^{iMet} and the accumulation of truncated 5S rRNA (Kadaba et al., 2004; Kadaba et al., 2006), suggesting that TRAMP is capable of delivering RNA substrates to the direct access route, in addition to its better characterized ability to deliver channel-dependent substrates. Thus, the route the RNA takes in the RNA exosome may not be dictated by the cofactor that delivers it to the substrate.

In summary, we show that the direct access conformation of the RNA exosome is present *in vivo* and functions on specific substrates in the nucleus. A major difference between the two conformations is the length of the paths, which is ~30nt and 12nt, respectively (Bonneau et al., 2009; Liu et al., 2014; Makino et al., 2015; Malet et al., 2010). The longer RNA binding path through a channel is likely to increase continuous binding to long single stranded RNAs and thus processivity of the RNA exosome, while direct access may be more suitable for structured RNAs or RNAs that are part of large RNPs that don't fit through the central channel (such as tRNAⁱ^{Met} and 5S rRNA). The access point utilized by a particular substrate could therefore result in distinct outcomes in the processing or degradation reactions. Taken together, we propose that the RNA exosome adopts different conformations to accommodate RNA substrates with vastly different characteristics. This resembles the allosteric activation model that was proposed soon after the discovery of the RNA exosome, but has since lost favor (Mitchell and Tollervey, 2000).

Chapter 4: RNA sequencing analysis suggests that the direct-access conformation of the RNA exosome is important for snoRNA processing and/or degradation.

INTRODUCTION

As discussed in chapter 3, there are two different conformations of the RNA exosome present *in vivo*, and they appear to have distinct functions. Specific disruption of each conformation followed by testing several known substrates of the RNA exosome allowed us to identify substrates of each conformation. The results suggested that the direct access conformation is required for the degradation of specific substrates such as tRNAi^{Met} and truncated 5S rRNA (Han and van Hoof, 2016) (Fig. 3.7), and the channel-through conformation appears to be critical for most function of the exosome. In addition, they cooperate to degrade certain substrates such as the 5'ETS. To gain insight into how these two conformations function in cell, I conducted high-throughput RNA sequencing analysis. I observed over 1858 transcripts are differentially regulated when the channel-through conformation is disrupted, suggesting that the channel-through conformation is critical for the RNA exosome function. Disruption of the direct access conformation did not result in global change of transcript levels. However, I found that snoRNAs are more abundant specifically when the direct access conformation is disrupted. These results are in line with the conclusion of chapter 3 that the two conformations have distinct *in vivo* functions.

RESULTS

The channel-through conformation of the RNA exosome globally regulates transcripts.

Total RNA was isolated from biological duplicates of the wild type, *rrp44-da*, *rrp41-L*, and *rrp44-da rrp41-L* strains. Ribosomal RNA was depleted (ribozero) and subjected to RNA sequencing analysis (50 nts paired end reads). RNA sequencing reads were mapped to the genome of *Saccharomyces cerevisiae*. I obtained over 18 million reads of each sample with more than 96% overall mapping rate.

Differential expression analysis was conducted with the Tuxedo protocol (Trapnell et al., 2012) and further analyzed by the R package, CummeRbund (L. Goff, 2013). Scatter plots of transcript levels in all tested strains showed that biological replicates are similar to each other, indicating that the results are reproducible (Fig. 4.1). It appears that there are large changes in *rrp41-L*, while *rrp44-da* is similar to wild type. Interestingly, *rrp44-da rrp41-L* also showed large changes in transcript levels similar to *rrp41-L* even though the double mutant grows better than *rrp41-L* (Fig. 3.8). Numerous transcripts are differentially regulated with more abundant transcripts when the channel-through conformation is disrupted (*rrp41-L*) (Fig. 4.1 and 4.2). I found that 1858 transcripts are significantly regulated in *rrp41-L*. Direct targets of the RNA exosome are expected to be accumulated in the mutants, and indeed we detect 1367 transcripts that are more abundant and only 491 depleted. The abundant transcripts include 333 CUTs and SUTs (Cryptic Unstable Transcript and Stable Unannotated Transcript) which are known targets of the nuclear RNA exosome (Fox et al., 2015; Xu et al., 2009). The abundant transcripts also include 53 transcripts that are antisense to known genes but were not

previously annotated as CUTs or SUTs. Depleted transcripts in *rrp41-L* are mostly mRNAs, and include only 2 CUT/SUTs and 1 antisense transcript that overlaps with a coding region. Since these are depleted in the mutant, they are potentially indirect targets. Thus, mRNAs are not an important target of the channel through conformation of the RNA exosome. These results suggest that the channel conformation of the RNA exosome degrades CUT/SUTs and antisense transcripts. Considering the number of transcripts that are more abundant in *rrp41-L*, the Rrp44^{ch}-exosome appears to globally affect the "dark matter of the transcriptome".

It has been shown that the expression of noncoding antisense RNAs is correlated to the expression of nearby or overlapping mRNAs (Camblong et al., 2007; Uhler et al., 2007; Xu et al., 2009). Since, many CUTs and SUTs are increased and many mRNAs are decreased in *rrp41-L*, I wondered if increased CUTs or SUTs are responsible for decreased mRNA levels. Thus, I asked whether depleted mRNAs in *rrp41-L* have increased CUTs or SUTs nearby. Interestingly, 141 of 488 decreased mRNAs have at least one CUT/SUT that is increased within 5kb up- or downstream. Narrowing down the distance to 1kb yielded 69 decreased mRNAs in close proximity to increased CUTs/SUTs. This proximity suggests that down regulation of some mRNAs is partly due to the effect of increased CUT/SUTs in *rrp41-L*.

It is also possible that depleted mRNAs are either the cause or consequence of slow growth of the *rrp41-L* strain. If this is the case, the faster growing *rrp44-da rrp41-L* strain would have higher mRNA expression levels than slow growing *rrp41-L*. Indeed, volcano plots of differentially expressed transcripts showed that decreased transcripts in *rrp41-L* are less severely affected in *rrp44-da rrp41-L* (Fig. 4.2). Interestingly, gene

ontology analysis of depleted genes in *rrp41-L* and *rrp44-da rrp41-L* showed that the structural constituents of ribosome are specifically enriched in the *rrp41-L* strain, indicating that low level of genes encoding ribosomal proteins correlates with slow growth of the *rrp41-L* strain (Table 4.1). More interestingly, mRNAs that encode ribosomal proteins are highly enriched in the list of mRNAs that are near increased CUT/SUTs. 17 out of 141 mRNAs that have CUT/SUTs within 5kb up- or downstream encode ribosomal proteins. They were more enriched (12 out of 69 mRNAs) when the distance between CUT/SUTs and mRNAs was narrowed down to 1kb. In addition, 4 of the 141 mRNAs that have nearby CUT/SUTs encode translation elongation factors. These data indicate the possible CUT/SUTs-mediated regulation of ribosomal protein expression. Taken together, the results suggest that the Rrp44^{ch}-exosome degrades CUT/SUTs which potentially regulate the expression of mRNAs including those encoding ribosomal proteins. However, not all downregulated mRNAs have nearby CUT/SUTs, indicating that other mechanisms contribute to downregulation of some mRNAs.

Disruption of Rrp44^{da} conformation also shows that more transcripts are accumulated than those that are decreased (Fig. 4.2). I found that 66 transcripts are significantly regulated. Of these 56 are increased in the mutant and only 10 are decreased. The much smaller number of affected transcripts confirms our previous conclusion that the direct access conformation of the RNA exosome is specialized for a few specific targets. Increased transcripts include 4 snoRNAs (small nucleolar RNAs; snR39B, snR66, snR13 and snR71), 1 snRNA (U6 snRNA) 7 mRNAs (POS5, NRD1,

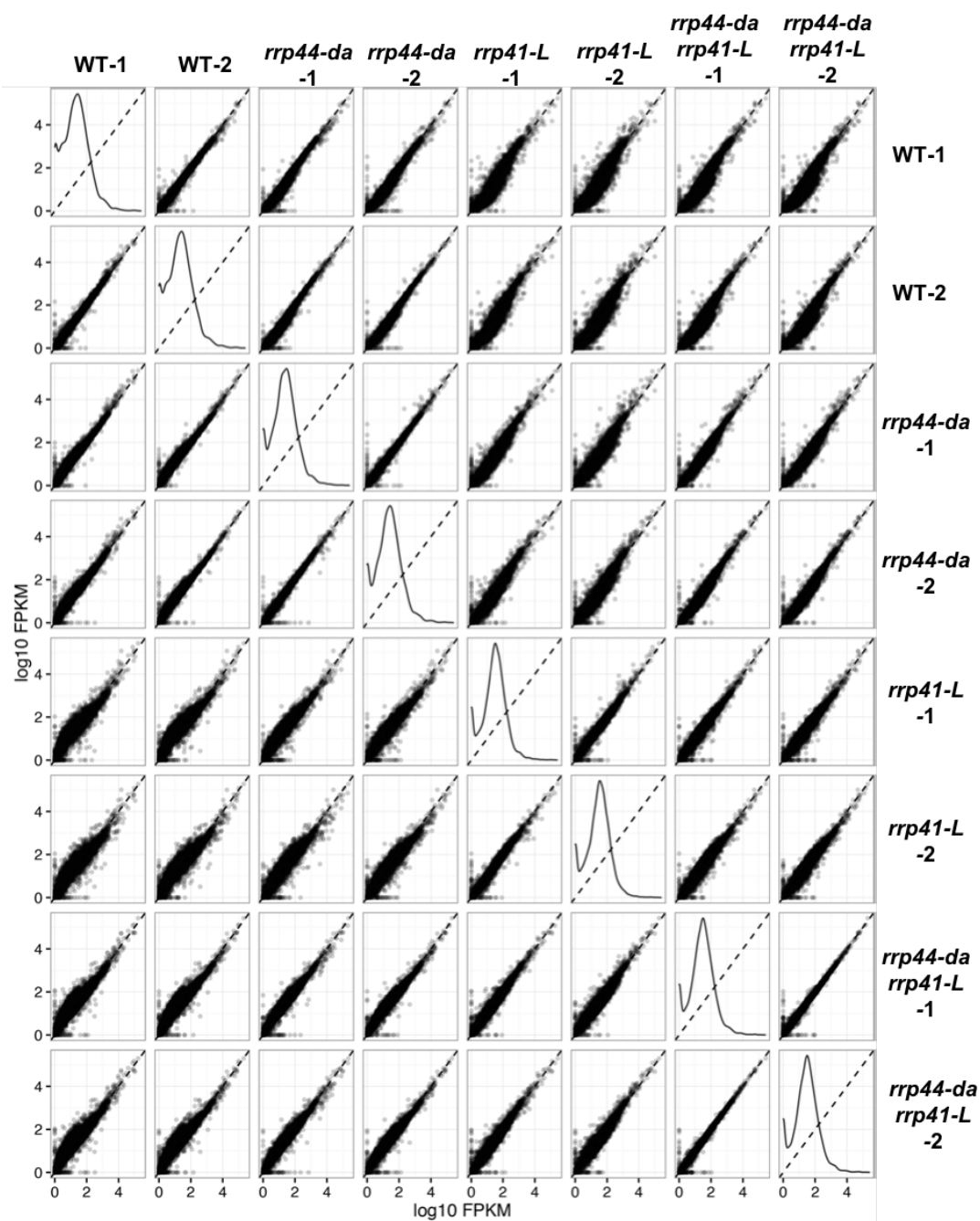


Figure 4.1. Scatter plot of transcript expression levels in all tested strains.

Scatter plots were generated by R package, CummeRbund using datasets generated by Cuffdiff. Each strain was analyzed in biological duplicates.

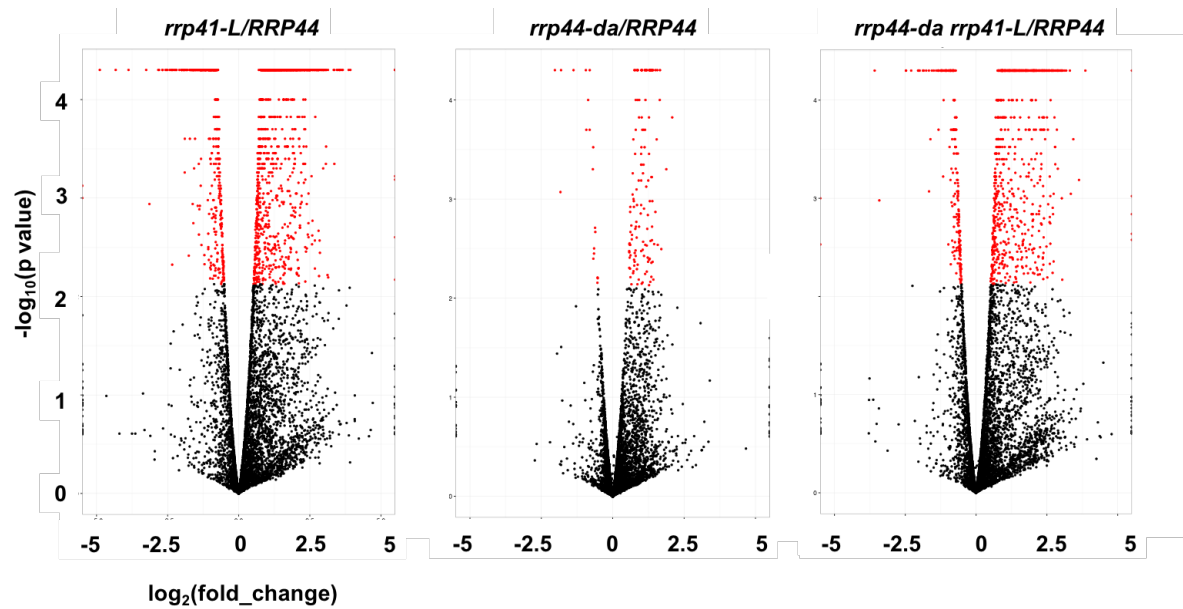


Figure 4.2. Differential transcript levels in *rrp44-da*, *rrp41-L*, and *rrp44-da rrp41-L* mutant strains.

Transcript levels were normalized to expression levels in wild-type. Volcano plots are drawn by R package, CummeRbund.

Downregulated genes in <i>rrp41-L</i>	Cluster frequency	P-value
structural constituent of ribosome	52 out of 431 genes, 12.1%	6.06E-15
structural molecule activity	59 out of 431 genes, 13.7%	4.67E-11
RNA pseudouridylation guide activity	12 out of 431 genes, 2.8%	7.55E-06
rRNA pseudouridylation guide activity	12 out of 431 genes, 2.8%	7.55E-06
base pairing with rRNA	19 out of 431 genes, 4.4%	1.03E-05
RNA modification guide activity	18 out of 431 genes, 4.2%	3.71E-05
rRNA modification guide activity	18 out of 431 genes, 4.2%	3.71E-05
transferase activity, transferring glycosyl groups	21 out of 431 genes, 4.9%	5.17E-05
carbon-oxygen lyase activity	13 out of 431 genes, 3.0%	0.00013
rRNA binding	21 out of 431 genes, 4.9%	0.00025
Downregulated genes in <i>rrp44-da rrp41-L</i>	Cluster frequency	P-value
RNA pseudouridylation guide activity	11 out of 219 genes, 5.0%	4.37E-08
rRNA pseudouridylation guide activity	11 out of 219 genes, 5.0%	4.37E-08
RNA modification guide activity	15 out of 219 genes, 6.8%	4.48E-07
rRNA modification guide activity	15 out of 219 genes, 6.8%	4.48E-07
base pairing with rRNA	15 out of 219 genes, 6.8%	6.75E-07
rRNA binding	17 out of 219 genes, 7.8%	2.74E-06
carbon-oxygen lyase activity	9 out of 219 genes, 4.1%	0.00056
mRNA binding	18 out of 219 genes, 8.2%	0.00085
poly(A) RNA binding	18 out of 219 genes, 8.2%	0.00093
ligase activity	13 out of 219 genes, 5.9%	0.00164
Table 4.1. Functional Gene Ontology Analysis of downregulated genes. Downregulated genes in <i>rrp41-L</i> or <i>rrp44-da rrp41-L</i> were subjected to GO (Gene Ontology) analysis through GO term finder in Saccharomyces Genome Database.		

XBP1, FUS1, SAG1, SDL1, and MFalpha2), and CUT/SUTs. The 10 decreased transcripts include eight cellular mRNAs (PHO5, YNL0404W, PHO89, TIR1, YHR202W, HIS4, PHO12 and CPA2) and two mRNAs derived from a single TY1 transposable element (YJR027W and YJR029W). Considering the small number of transcripts misregulated in *rrp44-da* compared to *rrp41-L*, the central channel of the RNA exosome appears to be the major path of substrate recruitment while the direct access conformation rather has specific substrates. It is consistent with the previous northern blot results shown in chapter 3 (Fig. 3.7 and 3.8). It is also possible that we observe only partial disruption of the direct access conformation in *rrp44-da*. Considering the partial redundancy between Rrp6 and the Rrp44^{da}-exosome (Fig. 3.5B), deletion of *RRP6* could further improve the detection of specific substrates of the Rrp44^{da}-exosome.

Previously, we showed that simultaneous disruption of both conformations in *rrp44-da rrp41-L* yields a faster growth phenotype than *rrp41-L* (Fig. 3.8). Interestingly, we found that the number of misregulated genes in the double mutant is lower than that in the *rrp41-L* strain. 1101 transcripts were significantly regulated in *rrp44-da rrp41-L* with 861 up and 240 downregulated transcripts. As mentioned above, unlike *rrp41-L*, genes that encode ribosomal proteins are not downregulated in *rrp44-da rrp41-L* (Table 4.1). This result is consistent with the growth assay in figure 3.8, and it supports our hypothesis that the balance between the two conformations is important for the optimal function of the RNA exosome.

Rrp44^{da}-exosome is important for snoRNA processing and/or degradation.

44 of the 56 increased transcripts in *rrp44-da* are CUT/SUTs. However, they do not appear to be specific substrates of the Rrp44^{da}-exosome since most of them are more highly expressed in *rrp41-L* (data not shown), indicating the redundancy between the two conformations. However, it appears that snoRNAs are specifically enriched in the list as we found four increased snoRNAs. Hierarchical clustering analysis of differentially expressed transcripts in *rrp44-da* also showed that snoRNAs are the most affected transcripts among them compared to wild-type or *rrp41-L* (Fig. 4.3).

snoRNAs are divided into two families, H/ACA box containing and C/D box containing and can be either intron-derived, independently transcribed or polycistronically transcribed. The four snoRNAs that are significantly affected in the *rrp44-da* mutant are all C/D independently transcribed snoRNAs. To examine whether other (kinds of) snoRNAs were also affected without reaching significance individually, I examined all snoRNA levels in the *rrp44-da*, *rrp41-L*, and *rrp44-da rrp41-L* strains (Fig. 4.4). Interestingly, many other snoRNAs were expressed at higher levels in the *rrp44-da* mutant with no obvious pattern of what type of snoRNA was/was not affected. This result was in contrast to the *rrp41-L* mutant where many snoRNAs tended to be expressed at lower levels. This result and the fact that four of the snoRNAs are statistically significantly upregulated suggest that the direct access conformation of the RNA exosome has a role in snoRNA processing or degradation.

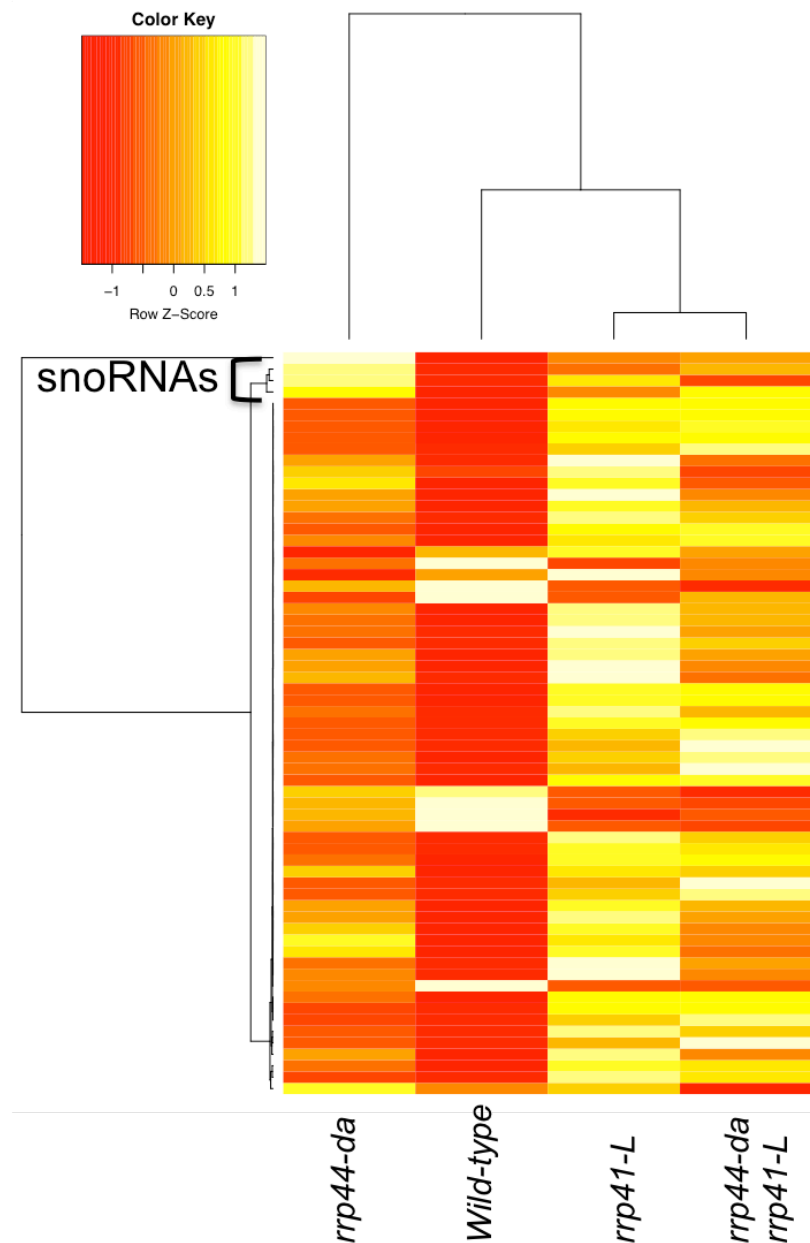


Figure 4.3. Hierarchical clustering of transcripts that are differentially expressed in *rrp44-da*.

Generation of heat map and hierarchical clustering were conducted by R package, CummeRbund. Only the 66 transcripts that are significantly affected by *rrp44-da* were included. Transcripts are color coded by z-score. The z-score is the difference in expression level of that transcript from the average level for that transcript in all four strains divided by the standard deviation. Thus, yellow indicates higher expression and red indicated lower expression than in the other strains. The four affected snoRNAs are among the transcripts with the highest z-score.

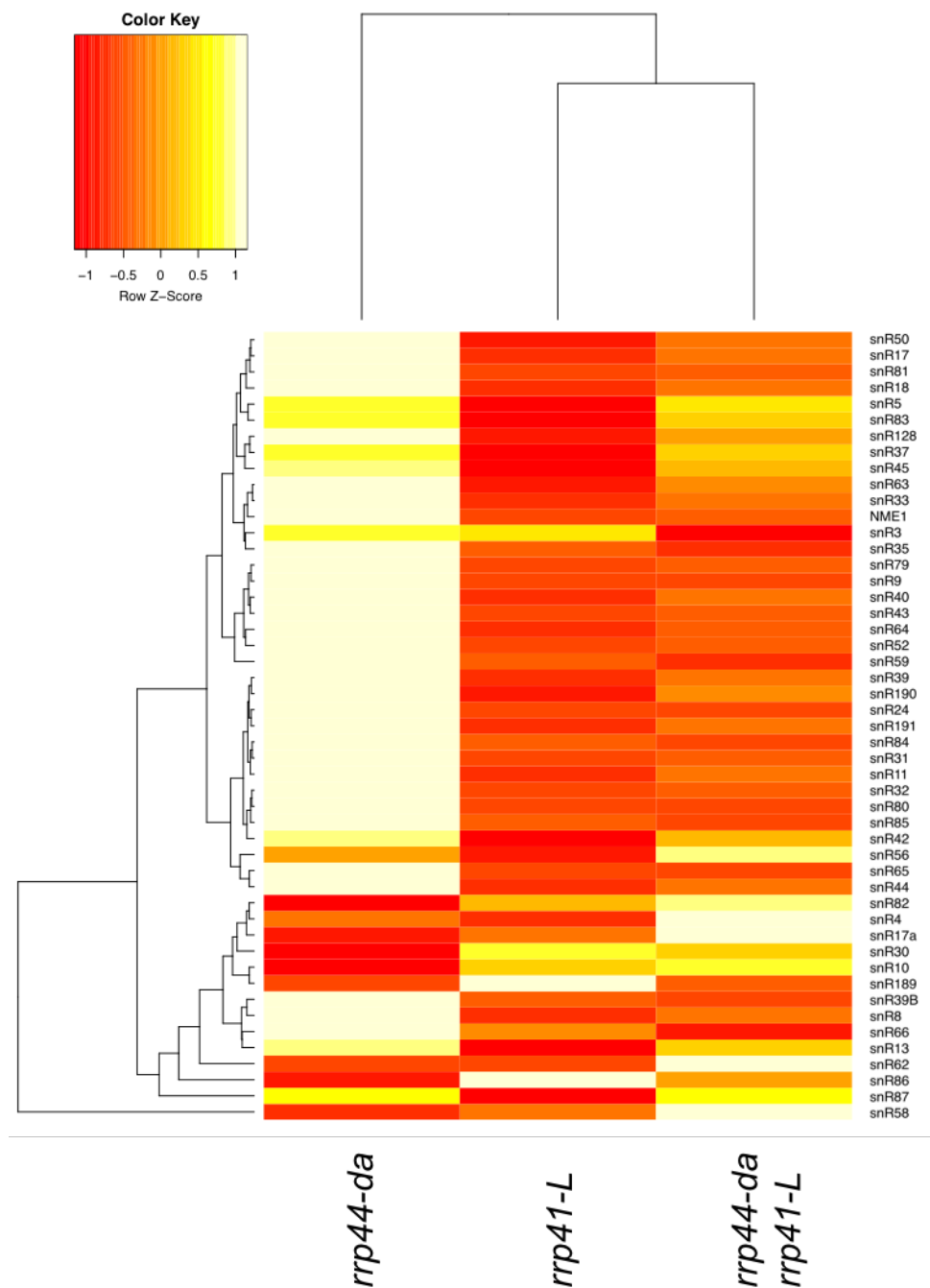


Figure 4.4. Hierarchical clustering of snoRNA expression level.

All yeast snoRNAs were analyzed. Generation of heat map and hierarchical clustering were conducted by R package, CummeRbund. Overexpressed transcripts are color coded as yellow and lower expression is shown as red. Expression level of snoRNAs are normalized to wild type before the analysis.

DISCUSSION

In the chapter 3, I found that there are two different RNA exosome conformations, Rrp44^{da} and Rrp44^{ch}, present in *vivo*, and they have distinct functions. While northern blot analysis gave us valuable information regarding the functions of them, it is technically limited because only few RNA species can be tested at once. High-throughput RNA sequencing provides a global view of transcriptome and has been used to find substrates of the RNA exosome either using an *RRP6* null strain or *rrp44* mutant alleles in combination with UV crosslinking substrates to exosome subunits (Schneider et al., 2012). Thus, to understand how the Rrp44^{da}-exosome functions in cells, I employed RNA sequencing analysis of *rrp44-da* and *rrp41-L* strains. The results show that there is a global misregulation of the transcriptome in the *rrp41-L* strain, suggesting that the channel conformation of the RNA exosome is important for many RNA exosome functions. This result is consistent with a previous report that used UV-crosslinking and analysis of cDNA (CRAC) (Schneider et al., 2012). The mRNAs that were affected indirectly by *rrp41-L* were enriched for ribosomal protein encoding mRNAs that mapped near directly affected CUTs. The expression of ribosomal proteins is coregulated with rRNA transcription and processing through a number of transcriptional and post-transcriptional mechanisms. The effect on ribosomal protein genes of nearby CUTs that accumulate if the RNA exosome is compromised may reflect an additional coregulation mechanism.

Interestingly, only a small number of transcripts was affected in the *rrp44-da* strain, and I found that snoRNAs are specifically enriched. This result indicates that the Rrp44^{da}-exosome is important for snoRNA degradation and/or processing. The study that

used CRAC to identify the RNA exosome substrates found that snoRNA species specifically cross-linked to Rrp44, not the core subunits (Schneider et al., 2012). In addition, a subsequent CRAC analysis was recently conducted to identify substrates of the Rrp44^{da}-exosome, and snoRNAs were consistently identified as substrates of the direct access route of the RNA exosome (Delan-Forino et al., 2017). Especially, the study showed that the 3'-end processing appears to require the Rrp44^{da}-exosome, and other regions are regulated by Rrp44^{ch}-exosome. These data indicate that snoRNAs are specifically degraded or processed by the direct access conformation of the RNA exosome, while there is still redundancy between the two conformations. However, the results of our RNA sequencing and the recent CRAC analyses are inconsistent with our northern blot results that showed snoRNAs are mainly accumulated in the *rrp41-L* strain (Fig. 3.7 and 3.8). Since there is a global misregulation of transcripts in *rrp41-L* and the same amount of total RNAs are loaded in northern blot analysis, it is possible that accumulation of certain RNA species is overrepresented. The *rrp41-L* strain is slow growing, perhaps due to inappropriate ribosome biogenesis since it requires the RNA exosome. If the amount of ribosomal RNA is low in *rrp41-L*, which is majority of RNA species in cell, other RNA species could be overrepresented in northern blot analysis. Indeed, we found that genes encoding ribosomal proteins are downregulated in *rrp41-L*. In the RNA sequencing analysis, ribosomal RNAs were depleted before the sequencing library construction. In addition, CRAC analyses normalize reads to purified protein level. Thus, these two high throughput analyses could avoid the possible problem in normalization. However, a cautious interpretation is still required when a global misregulation of transcript are analyzed.

Although I found that mainly snoRNAs are specific substrates of the Rrp44^{da}-exosome, other RNA species could be missed due to the limitation of the method. In the recent CRAC analysis, it was found that pre-mRNAs are preferentially bound by Rrp44^{da}-exosome, while cytoplasmic mRNAs are bound by the Rrp44^{ch}-exosome. It is possible that mutations in *rrp44-da* do not fully disrupt the direct access pathway, and we only observe effects of partial disruption. It is also possible that the preferential binding of a small subset of these transcripts does not result in major changes in RNA level. Considering the Rrp44^{da}-exosome degrades aberrant RNAs such as hypomodified tRNA^{iMet} and truncated form of 5S rRNA, these substrates could remain undetected in the RNA sequencing analysis due to possible masking effect of abundant normal species (Han and van Hoof, 2016) (Fig. 3.7). In addition, small RNA species can be missed in total RNA sequencing. Thus, size selection before the sequencing library construction may increase the chance of detection. Furthermore, tRNAs are highly modified, and these modifications often hinder reverse transcriptase reaction during cDNA synthesis (Zheng et al., 2015). Poorly detected tRNA reads are also mapped to multiple locations in the genome since many of them are encoded by multiple genes, and this limits the analysis. Recently, a tRNA profiling method that overcomes this limitation by skipping cDNA synthesis step has been successfully used (Goodarzi et al., 2016). This method would improve the detection of Rrp44^{da}-substrates since it appears to degrade or process short, structured substrates.

Among the mRNAs that are affected in the *rrp44-da* mutant are three mRNAs that encode proteins involved in phosphate acquisition (the alkaline phosphatases Pho5 and Pho12, and the phosphate importer Pho89). These genes have previously been shown

to be affected by *rrp6Δ*, *trf4Δ* and *trf5Δ* (San Paolo et al., 2009; Uhler et al., 2007), suggesting that the few mRNAs we identified as affected by *rrp44-da* are not random noise, but are true effects. Similarly, *NRD1* is among the mRNAs overexpressed in the *rrp44-da* mutant. Nrd1 is an exosome cofactor as part of the NNS complex and the RNA exosome has previously been suggested to be involved in *NRD1* mRNA processing from a 3' extended precursor (Fox et al., 2015). Finally, among the upregulated CUTs is *SRG1*, a transcript previously shown to be affected by *rrp6Δ* and *trf4Δ* (Fox et al., 2015; Thompson and Parker, 2007). These results suggest that the mRNAs that are affected in our *rrp44-da* mutant are likely authentic direct or indirect targets of the RNA exosome.

Taken together, we confirm that the *rrp44-da* mutant has defects in a few specific RNA exosome functions, with the complementary strengths of northern blotting (chapter 3) and RNAseq (this chapter) identifying specific functions, including degradation or processing of aberrant tRNAi^{Met}, 5S rRNA, snoRNAs and a few CUTs and mRNAs. Considering that northern blot and RNAseq identified different and relatively few species, it is possible that other RNAs are also targeted by the direct access conformation of the RNA exosome.

Chapter 5: The RNA exosome is important for DNA damage response.

INTRODUCTION

Response to DNA damage is critical for cells to maintain genome integrity, which is challenged by many cellular and environmental factors (Finn et al., 2012).

Physiological sources of DNA damage include reactive oxygen species produced by cellular metabolism or response to a pathogen, and errors made during DNA replication. Environmental factors include ionizing radiation, ultraviolet, and carcinogenic agents. Therefore, cells need to respond to DNA damage that constantly occurs and repair it properly to maintain genome integrity. As discussed in chapter 3, the RNA exosome is important for the DNA damage response (Fig. 3.7).

RNA exosome cofactors have been associated with the DNA damage response before. For example, loss of Trf4 or Trf5, nuclear cofactors of the RNA exosome, or Rrp6, an RNase associated with the RNA exosome in the nucleus, increase replication dependent histone mRNA level (Reis and Campbell, 2007). Trf4 has been shown to control ribosomal DNA copy number by regulating non-coding RNAs from telomeres and rDNA (ribosomal DNA) regions (Houseley et al., 2007). In addition, Trf4 is known to resolve R-loops, DNA/RNA hybrids that form during transcription and are one of the major sources of DNA mutations (Gavalda et al., 2013). Furthermore, mutations in another exosome cofactor, the Nrd1/Nab3/Sen1 complex, which is important for transcription termination of non-coding RNAs, leads to decreased silencing of the rDNA locus (Vasiljeva et al., 2008b). These results strongly suggest that the RNA exosome is important for maintaining genome integrity.

Recently, Rrp6 has emerged as an important player of the DNA damage response. EXOSC10, the human homolog of Rrp6, is required for DNA double-strand breaks by

homologous recombination (Marin-Vicente et al., 2015). Following DNA double-strand breaks, single stranded DNA (ssDNA) is generated by a nuclease, and the ssDNA is coated by the ssDNA-binding protein, RPA (Replication Protein A) (Manfrini et al., 2015). This RPA-coated ssDNA is recognized by the checkpoint kinase Mec1/ATR, which promotes DNA repair by homologous recombination. A study found that Rrp6 is one of the regulators of RPA-coating of ssDNA (Manfrini et al., 2015). These results indicate that RNA processing enzymes are required to maintain genome stability even though the mechanism of their action is unclear.

Since Rrp6 is one of the nuclear subunits of the RNA exosome, I wondered whether the RNA exosome core is also involved in the DNA damage response. As shown in chapter 3, I found that Rrp44 mutations yield strong sensitivity to the DNA damaging agent zeocin (Fig. 3.7). Since zeocin is known to induce double strand breaks, the zeocin sensitivity of the *rrp44* mutant strains suggests that the RNA exosome core also play a role in the DNA damage response (Chankova et al., 2007). In this chapter, I further investigated the RNA exosome-mediated DNA damage response. I further confirmed zeocin sensitivity of the *rrp44* mutant strains by a survival assay. In addition, growth assays of the *rrp44* mutant strains indicates that the RNA exosome is important for growth in the presence of DNA damaging agents that act by different mechanisms. These results strongly suggest that the RNA exosome core is indeed required for the DNA damage response. Surprisingly, specific mutations affect sensitivity to specific agents, suggesting that the RNA exosome affects DNA damage sensitivity through several mechanisms.

RESULTS

Rrp44 is important for survival of *Saccharomyces cerevisiae* in the presence of zeocin.

I found that Rrp44 is important for growth of yeast on solid media containing zeocin, suggesting that the RNA exosome core is required for repair of DNA double strand breaks (Fig. 3.7). To further confirm this result, I conducted a survival assay. Briefly, wild-type, *rrp6* Δ , and *rrp44* mutant strains were grown in YPD overnight. The overnight cultures were diluted into YPD containing 5 μ g/ml zeocin and grown for 20 hours followed by plating the cells on YPD agar and determining colony forming units (CFUs). The median CFU from 13 replicate zeocin exposures was used to determine the sensitivity of each strain. Unlike average CFU, the median is less sensitive to the occurrence of mutations that inflate the CFU measurement. (Pope et al., 2008). The median CFUs for each mutant were then normalized to median CFU for wild type. *rrp6* Δ showed a 40% survival, confirming that Rrp6 is important for DNA damage response (Fig. 5.1). Interestingly, I found that *rrp44-da* survived only 14% relative to wild-type in media containing zeocin. Considering that growth of *rrp6* Δ on zeocin-containing media was similar to that of *rrp44-da* in the assay in figure 3.7, it appears that Rrp44^{da}-exosome is more important for survival of cells than for growth. However, it is possible that the growth assay on solid media is not sensitive enough detect the difference. I also found that *rrp44-CR3* and *rrp44-exo*⁻ are extremely sensitive to zeocin as they showed only 0.5% survival relative to wild type (Fig. 5.1). Taken together with the growth assay in figure 3.7, this result strongly suggests that the RNA exosome, especially

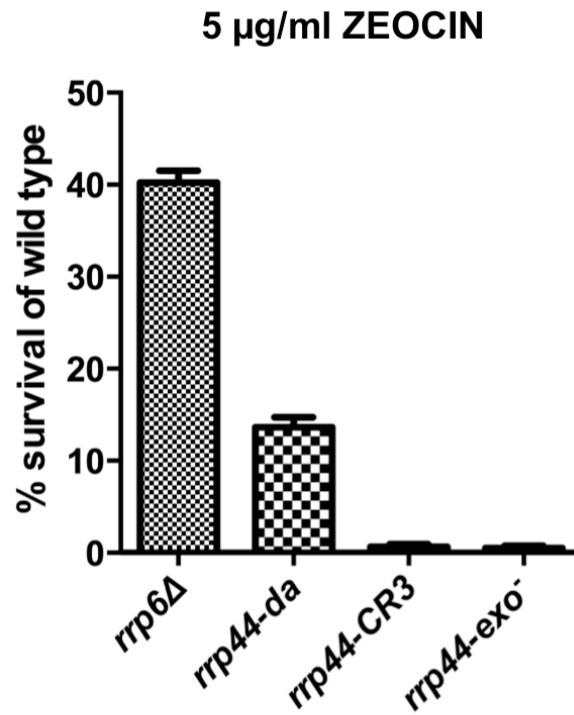


Figure 5.1. Rrp44 is important for survival of *Saccharomyces cerevisiae* in the presence of zeocin.

Overnight cultures of wild type, *rrp6* Δ , and *rrp44* mutant strains were diluted into YPD containing 5 μ g/ml ZEOCIN and grown for 20 hours. Colony forming units of each strain were calculated, and survival of mutants relative to wild type was measured.

the exonuclease activity of Rrp44, is required for survival in the presence of DNA damaging agents.

Rrp44 is required for 5-FU and 4NQO resistance.

Since I found that Rrp44 is required for DNA damage response induced by zeocin, I wondered whether the *rrp44* mutations also affect the sensitivity of yeast to other DNA damaging agents. Zeocin intercalates into DNA and causes double stranded DNA breaks. To test whether the sensitivity was specific to this type of DNA damage or more general, I tested 5-Fluorouracil (5-FU) and 4-Nitroquinoline N-oxide (4NQO), which act by different mechanisms.

5-FU is a thymidylate synthetase inhibitor, and inhibition of this enzyme results in dTTP depletion. Due to dTTP depletion, more dUTP gets incorporated into DNA. Repair of the U-containing DNA by base excision repair results in DNA damage (Seiple et al., 2006). Deletion of *RRP6* has been shown to increase 5-FU sensitivity of yeast, and it was suggested that the sensitivity is both based on its incorporation of uracil into DNA and 5-FU into RNA (Hoskins and Scott Butler, 2007). Subsequent studies also found that 5-FU also incorporate into RNA, and 5-FU containing RNAs are less susceptible for Rrp6-mediated degradation (Hoskins and Butler, 2008; Silverstein et al., 2011). In addition to *RRP6*, the genes encoding exosome core subunits, *RRP41*, *RRP44*, and *RRP46* showed drug-induced haploinsufficiency, suggesting potential roles of the RNA exosome in the 5-FU resistance (Lum et al., 2004). Therefore, to test whether the core RNA exosome is also involved in 5-FU resistance, growth assays of the *rrp44*-mutants were conducted in media containing 5-FU (Fig. 5.2, upper panel). I observed a result similar to that of the

zeocin sensitivity assay in figure 3.7. As shown previously, *rrp6Δ* showed sensitivity to 5-FU (Hoskins and Scott Butler, 2007). All of the *rrp44* mutations except for the endonuclease defective mutant of Rrp44, *rrp44-endo⁻*, were sensitive to 5-FU, suggesting that the exonuclease activity is required for the 5-FU response. Interestingly, *rrp44-da* showed a similar 5-FU sensitivity to *rrp44-CR3* and *rrp44-yrd* in contrast to that *rrp44-da* has less zeocin sensitivity compared to *rrp44-CR4/yrd*, indicating the presence of distinct mechanisms that confer resistance to different agents (Fig. 3.7 and 5.2). In addition, deletion of the cytoplasmic exosome cofactor *SKI7* did not show any growth defect in the media containing 5-FU. These results indicate that the Rrp44 exonuclease activity of the nuclear exosome is required for 5-FU resistance of yeast.

In addition to 5-FU, I tested the sensitivity of mutant strains to 4NQO, which is another known DNA damaging agent (Jones et al., 1989). It has been suggested that 4NQO mimics UV-induced damage, but the mechanism of action is still unclear (Ikenaga et al., 1975). Growth assays showed that *rrp44-CR3* and *rrp44-yrd* are sensitive to 4NQO (Fig. 5.2, lower panel). However, I did not observe a growth defect of *rrp6Δ*, indicating that Rrp6 and Rrp44 have distinct roles in DNA damage response. In addition, *rrp44-da* also did not show a detectable growth defect, suggesting that 4NQO response requires the channel-through conformation of the RNA exosome. Furthermore, surprisingly, *rrp44-exo⁻* showed wild-type level sensitivity to 4NQO unlike *rrp44-CR3* and *rrp44-yrd*. Since the Rrp44-exosome core interaction is disrupted in *rrp44-CR3* and *rrp44-yrd*, this result suggests that 4NQO response does not require the exonuclease activity but the interaction of Rrp44 with the core exosome. It also suggests a potential structural role of the exosome in the 4NQO response. Considering that the growth of *ski7Δ* was comparable to

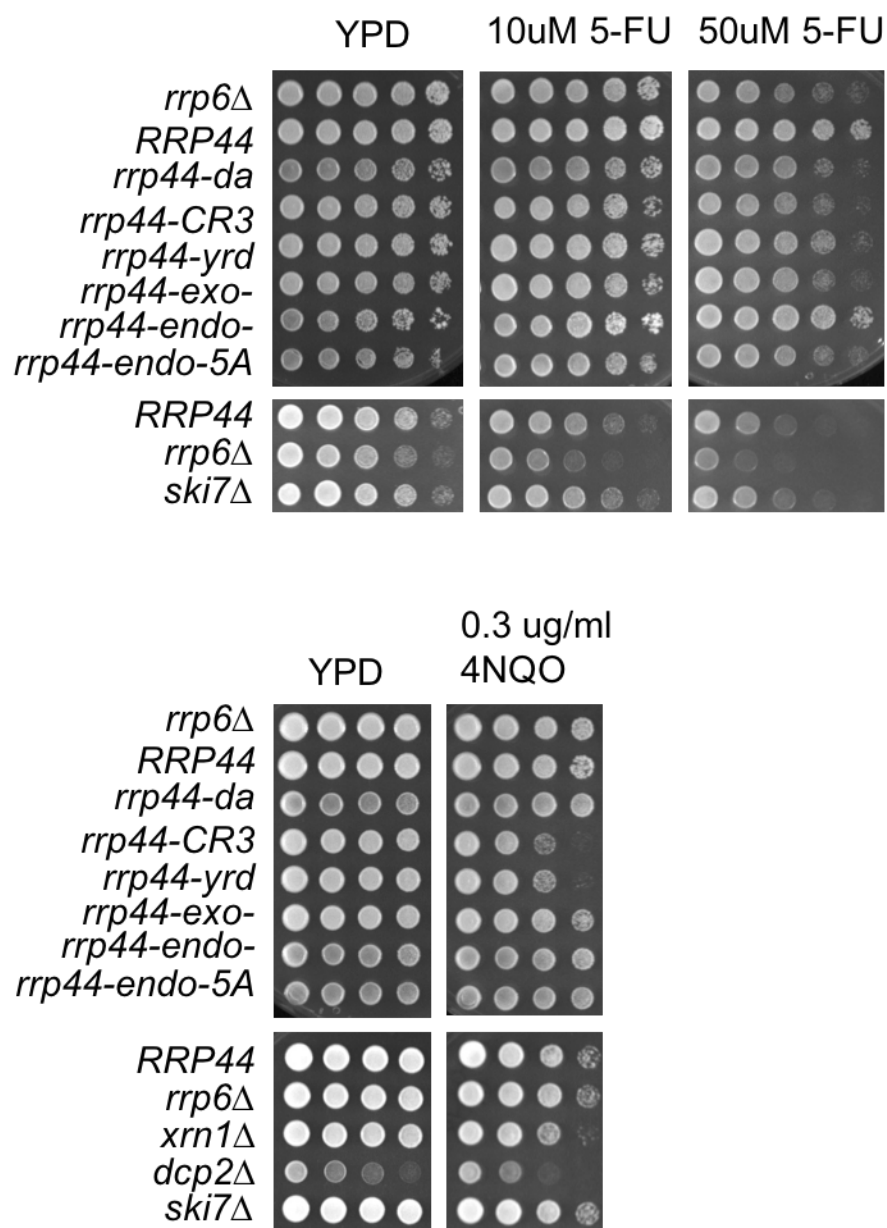


Figure 5.2. Rrp44 is required for 5-FU and 4NQO resistance.

Cells of each strain were serially diluted and spotted on YPD agar containing 5-Fluorouracil or 4-Nitroquinoline N-oxide.

wild type, 4NQO response requires nuclear function of the RNA exosome. Taken together, these data suggest that the RNA exosome is required for the DNA damage response induced by a variety of DNA damaging agents. In addition, The RNA exosome-mediated DNA damage response appears to require the nuclear function of the exosome, and Rrp6 and Rrp44 seem to have both redundant and distinct roles in it.

The RNA exosome may be not required for DNA damage checkpoint activation upon zeocin exposure.

Rrp6 has been shown to promote RPA-coating of ssDNA after DNA double strand breaks, and the RPA-coating of ssDNA activates checkpoint kinase Mec1/ATR (Manfrini et al., 2015). Mec1/ATR activation can be monitored by phosphorylation Rad53, which controls DNA damage checkpoint upon phosphorylation. Since I observed that Rrp44 is important for DNA damage response, I tested whether *rrp44* mutations affects checkpoint activation. To test this, wild type or mutant strains were exposed to zeocin, and cells were harvested every 30 min. To test Rad53 phosphorylation, total protein lysates of harvested cells were subjected to western blot using anti-Rad53 antibody. Decreased electrophoretic mobility of Rad53 indicates its phosphorylation. The result showed that Rad53 phosphorylation is initiated between 60 and 90 min after the zeocin exposure in wild type. *rrp6Δ*, *rrp44-da*, and *rrp44-CR3* showed similar activation. This result suggests that the RNA exosome is not involved in the checkpoint activation. However, it is also possible that potential difference in the Rad53 protein levels complicate the interpretation. Further analysis using a phospho-specific antibody and the *mec1Δ* strain as a negative control would be required to make a decisive conclusion.

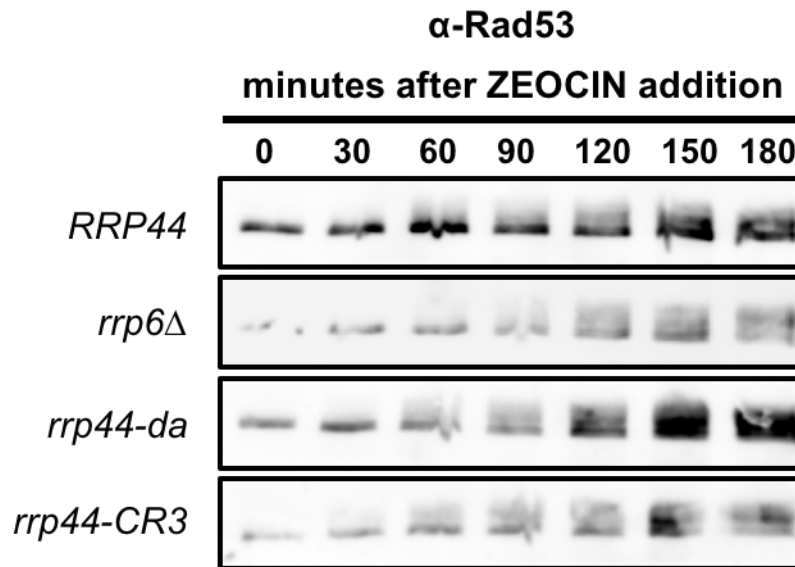


Figure 5.3. The RNA exosome does not promote checkpoint activation upon zeocin exposure.

Wild-type, *rrp6Δ*, and *rrp44* mutant strains were grown until mid-log phase, and exposed to 5μg/ml zeocin. Cells were harvested every 30 min. Total proteins were isolated from harvested cells and subjected to western blot using anti-Rad53 antibody.

DISCUSSION

The DNA damage response is critical for cells to maintain genome integrity. Genome integrity is intimately related to human diseases such as cancer, neurodegenerative disorders, and immune deficiencies (Jackson and Bartek, 2009). Chromosome translocation is directly related to lymphoid tumors, and most carcinogens are known DNA damaging agents (Bassing and Alt, 2004; Hoeijmakers, 2001). DNA mutations in neurons are associated with neurodegenerative disorders such as Alzheimer's, Huntington's, and Parkinson's diseases (Rass et al., 2007). Therefore, it is important to understand cellular DNA damage response. Trf4, a subunit of the TRAMP complex, resolves R-loops formed during transcription, suggesting a potential role of the RNA exosome in DNA damage response as R-loops are a source of DNA damage (Gavalda et al., 2013). Recently, the RNA exosome core was also shown to be important for removing R-loops during non-coding RNA transcription, and defects in the exosome function led to mutational asymmetry in the immunoglobulin locus in B cell (Lim et al., 2017). Since formation of R-loops exposes the nontemplate strand of DNA, which is vulnerable to damage, it is critical to properly remove R-loops to minimize DNA mutations (Santos-Pereira and Aguilera, 2015). Therefore, the above results suggest that the RNA exosome may resolve R-loops formed during transcription by using ribonuclease activities, and this activity may contribute to preventing DNA damage.

In this study, I found that the RNA exosome core is required for DNA damage response induced by zeocin, 5-FU (5-Fluorouracil), and 4NQO (4-Nitroquinoline N-oxide) (Fig. 5.1 and 5.2). Rrp6 is shown to be important for checkpoint activation by promoting Mec1/ATR kinase activation (Manfrini et al., 2015). However, I did not

observe any checkpoint activation defect in *rrp6Δ* and *rrp44* mutants. This result is inconsistent with the previous report that showed deletion of *RRP6* results in delayed checkpoint activation (Manfrini et al., 2015). The study induced DNA double strand breaks by expressing HO endonuclease that cleaves the *MAT* locus, while I induced DNA damage by adding DNA damaging agents. The different methods could attribute to different results. Therefore, further investigation is required to conclude whether the RNA exosome core is also required for checkpoint activation.

DNA double strand breaks yield single strand DNA (ssDNA) at the site of breaks after nuclease-mediated resection for repair. Then, the ssDNA is coated by single strand DNA-binding protein, RPA, which induces Mec1/ATR kinase activation (Finn et al., 2012). Since the RNA exosome and its cofactors have been shown to resolve R-loops, it is intriguing to see whether there is any transcription or RNA/DNA hybrid formation involved in ssDNA region during DNA damage repair. Indeed, a study found that small RNAs are produced from DNA double strand breaks, and they proposed that these small RNAs recruit important proteins to the site of DNA damage (Wei et al., 2012). Thus, it is possible that the RNA exosome resolve RNA/DNA hybrids and degrade those small RNAs at the site of DNA damage after the protein recruitment.

Interestingly, I observed that different *RRP44* alleles differ in their sensitivity to three DNA-damaging agents that I used. Both *RRP6* and *RRP44* appear to be required for survival of yeast in the presence of zeocin or 5-FU. The channel-through conformation of the exosome seems to be more important than the direct access conformation for the zeocin resistance since *rrp44-da* showed a less severe phenotype compared to *rrp44-CR3* and *rrp44-yrd* in the presence of zeocin. However, *rrp44-da* showed a similar growth

defect to *rrp44-CR3* and *rrp44-yrd* in 5-FU containing media. Furthermore, either Rrp6 or the exonuclease activity of Rrp44 does not appear to be required for 4NQO resistance. 4NQO could reduce the growth only when the Rrp44-exosome interaction is disrupted in *rrp44-CR3* and *rrp44-yrd*. These data strongly indicate that there are distinct mechanisms to respond to different DNA-damaging agents, and potential structural roles of the RNA exosome in 4NQO resistance. Thus, there are other unknown mechanisms that confer resistance to DNA-damaging agents besides that the exonuclease activity of the RNA exosome resolves R-loop to protect DNA from damaging agents.

In summary, I have shown that the RNA exosome core is important for the response to DNA damages induced by zeocin, 5-FU, and 4NQO. Our data suggest that the exonuclease function of the Rrp44 is critical for the resistance of yeast to DNA-damaging agents such as zeocin and 5-FU, and it involves the nuclear function of the RNA exosome. However, the exonuclease activity of Rrp44 or Rrp6 is not required for the 4NQO resistance, suggesting possible structural roles of the RNA exosome in DNA damage response. It is possible that mutation in Rrp44 indirectly affects mRNA levels that are coding important genes for DNA damage response. However, since the cytoplasmic function of the exosome is not required for the DNA damage response, it is likely that the nuclear function of the exosome is directly involved in the process. Further investigation is required to understand the molecular mechanism of the RNA exosome-mediated DNA damage response.

Chapter 6: Genetic analyses suggest that Rrp6 and Mtr4 function beyond known biochemical activities and physical interactions.

INTRODUCTION

The core of the RNA exosome is composed of nine structural and one catalytic subunit, Rrp44 (Dis3) (Makino et al., 2013a; Makino et al., 2015; Zinder et al., 2016). While this RNA exosome core has detectable RNase activity *in vitro*, its specificity is very limited. Furthermore, *in vivo* activity requires a number of other proteins, named RNA exosome cofactors. The cofactors for the nuclear and cytoplasmic RNA exosome are different, and even within each compartment specific cofactors may be required for specific RNA exosome functions. (Butler and Mitchell, 2011; Losh and van Hoof, 2015; Zinder and Lima, 2017). For example, Ski7 is required for cytoplasmic RNA exosome functions, but completely dispensable for its nuclear functions. Furthermore, the C-terminal domain of Ski7 is required for the degradation of mRNAs that lack a stop codon, but not other mRNAs. Therefore to understand RNA exosome function, it is critical to understand the roles of the cofactors.

The nuclear RNA exosome directly interacts with the Rrp6 cofactor and Rrp6 in turn interacts with other cofactors. One prominent role of the C-terminal domain of Rrp6 is to interact with the RNA exosome core and X-ray crystallography has revealed the details of this interaction. (Fig. 6.1A and B) (Makino et al., 2013a; Makino et al., 2015; Wasmuth et al., 2014). In addition, another region in the C-terminus, termed the lasso, can bind RNA *in vitro*, but it is unclear how it contributes to RNA exosome functions *in vivo*. The N-terminus of Rrp6 interacts with Rrp47 and the Rrp6/47 complex interacts with the RNA helicase Mtr4. One important role of Rrp6 is therefore thought to be to bridge interactions of other cofactors with the RNA exosome. A puzzling observation is that both the nuclear RNA exosome and Mtr4 are essential for viability, but Rrp6 and

Rrp47 are not. This reflects that we incompletely understand the role of these protein-protein interactions *in vivo*.

The protein-protein interactions of Rrp6 suggest that an important role of Rrp6 is to recruit Mtr4. Mtr4 is required for all nuclear functions of the RNA exosome and in turn interacts with many other RNA cofactors that have more specialized functions. In addition to the typical RNA helicase domains, Mtr4 contains an arch domain and a short N-terminal domain. The helicase core, arch, and N-terminus each interact with specific other RNA exosome cofactors. Specifically, the N-terminus interacts with Rrp6/47 and the arch domain interacts with Nop53 and Utp18. The Mtr4-Nop53 interaction is required for the exosome mediated processing of 7S pre-rRNA into 5.8S rRNA, while the Mtr4-Utp18 interaction is required for 5'ETS degradation, respectively (Klauer and van Hoof, 2013; Taylor et al., 2014; Thoms et al., 2015; Weir et al., 2010). The RNA helicase core of Mtr4 interacts with a poly(A) polymerase (Trf4 or Trf5) to form the TRAMP complex (Trf4/5-Air1/2-Mtr4 polyadenylation complex) (LaCava et al., 2005; Losh et al., 2015; Schuch et al., 2014; Vanacova et al., 2005). This interaction is important for pre-snoRNA processing or degradation, but does not affect 5.8S rRNA maturation or 5'ETS degradation (Losh et al., 2015). Therefore, the RNA exosome, Rrp6, Rrp47, and Mtr4 appear to form the basal nuclear RNA exosome machinery required for all its functions, with other cofactors interacting with the basal machinery for specific functions.

In addition to Rrp6 being an RNA exosome co-factor that mediates protein-protein interaction, it is also a 3' to 5' exoribonuclease, and its RNase activity is thought to be important for the degradation or processing of some RNAs. For example, the 3' end processing of 7S pre-rRNA to 5.8S rRNA is thought to occur in three sequential steps. In

the first step the 300 nts 7S species is processed to a 5.8S+30 intermediate of 190 nts. This 5.8S+30 species is then processed to a 6S intermediate, and finally to 5.8S (of 160nts). Mutations that either delete *RRP6* or inactivate its RNase activity result in the accumulation of 5.8S+30, while mutations in other RNA exosome subunits such as the catalytic subunit Rrp44 result in the accumulation of 7S pre-rRNA (Briggs et al., 1998; Mitchell et al., 1997). Therefore, the RNase activity of Rrp44 appears to process 7S into 5.8S+30, while the RNase activity of Rrp6 then further processes 5.8S+30. An important unanswered question is to what extent the overall function of Rrp6 reflects its catalytic activity or its protein-protein interaction activities. Furthermore, it is also not clear whether the Rrp6 catalytic activity *in vivo* occurs as part of the RNA exosome and with RNA exosome cofactors, or independent of the RNA exosome machinery.

In this study, we aimed to thoroughly investigate the cofactor-exosome interactions mainly focusing on how Rrp6 mediates the interaction of Mtr4 and the exosome core. While the N-termini of Rrp6 and Mtr4 have been shown to interact (Schuch et al., 2014), the biological significance of the interactions has not been fully tested and our genetic analyses suggest that the N-termini of Rrp6 and Mtr4 function beyond the known Rrp6-Mtr4 interaction. I also show that the catalytic domain of Rrp6 is sufficient for some of its functions, suggesting it may act independently of both the RNA exosome and its cofactors. Finally, we show that the exosome interacting domains of Ski7 and Rrp6 are interchangeable. However, unlike the exosome interacting domain of Ski7, the C-terminal domain of Rrp6 has other functions than the exosome interaction, which is consistent with a recent study that showed that a part of the C-terminal domain, lasso, enhances the *in vitro* exosome activity (Wasmuth and Lima, 2017). Our genetic

analyses suggest that the C-terminal domain of Rrp6 together with a largely unstudied cofactor Mpp6 may mediate additional interactions of the RNA exosome with other cofactors. Furthermore, interaction of Rrp6 with the exosome or other cofactors appears to be important for its nuclear localization as deletion of interacting domains and nuclear localization signal yields a localization defect. Together these results greatly expand our understanding of the role of Rrp6.

RESULTS

To study how Rrp6 mediates interactions of the RNA exosome and its biological significance, we first aimed to test whether known interactions are important for the exosome function. X-ray crystallography and biochemical analyses showed that the C-terminus of Rrp6 interacts with the RNA exosome core, and the N-termini of Rrp6 and Rrp47 form a complex with the N-terminus of Mtr4 (Fig. 6.1A and B) (Makino et al., 2015; Schuch et al., 2014). To understand the function of the interactions, we generated the N- or C-terminal truncation mutants of Rrp6 in which known interaction domains are deleted (Fig. 6.1A). I could not obtain stable expression of the N-terminal truncated Rrp6 as losing the interaction with Rrp47 is shown to destabilize the Rrp6 protein (Feigenbutz et al., 2013a). However, we were able to gain stable expression by fusing GFP to the N-terminal truncation mutant of Rrp6 (Fig. 6.1E). Expression levels of the mutant Rrp6 proteins are comparable to wild type, indicating that the growth defects are not due to low expression (Fig. 6.1E).

I tested the *in vivo* interactions of Rrp6 truncation mutants. Exhaustive attempts to confirm that Rrp6 Δ N was defective in Mtr4 interaction were unsuccessful. Despite trying different tagging strategies and/or using antibodies against the native proteins, I could not reproducibly show that wild-type Mtr4 and Rrp6 co-immunoprecipitate, precluding any attempts to show that the interaction is disrupted in Rrp6 Δ N. In contrast, the interaction between the RNA exosome and Rrp6 was readily confirmed by immunoprecipitation. Pull down of TAP-tagged exosome core subunit Csl4 resulted in co-purification of wild-type, catalytically dead Rrp6, and Rrp6 Δ N, but Rrp6 Δ C failed to co-purify (Fig. 6.2). As a control, in all of the strains the Rrp44 exosome subunit co-

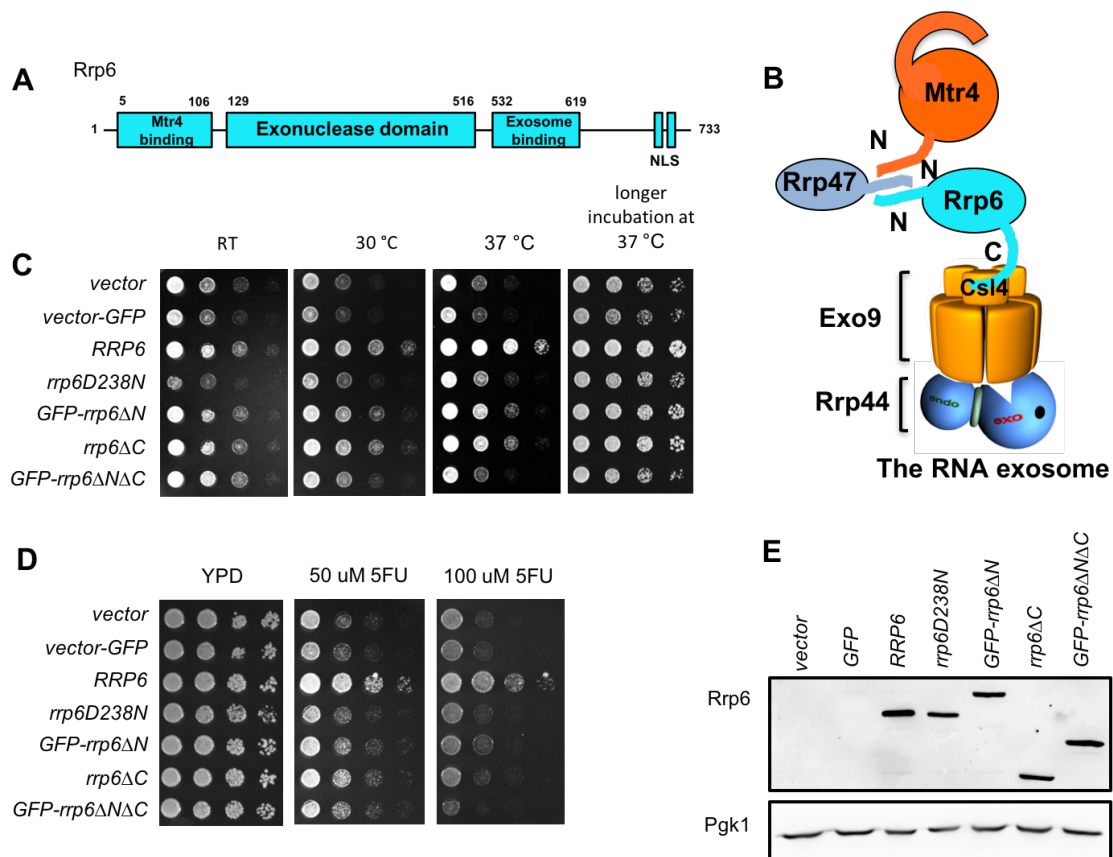


Figure 6.1. Interaction with the RNA exosome and other cofactors are important for Rrp6 function.

(A) Primary structure of Rrp6. It contains an N-terminal Mtr4 interacting domain, an exoribonuclease domain, a C-terminal exosome binding domain, and a Nuclear Localization Signal (NLS). (B) Schematic of the RNA exosome-cofactor interactions. The N-termini of Mtr4, Rrp47, and Rrp6 interact with each other. The C-terminal domain of Rrp6 interacts with the exosome core. (C) *rrp6Δ* carrying different *RRP6-2xMyc* alleles were serially diluted and spotted on solid media. (D) The same strains from (C) were spotted on media containing 5-Fluorouracil. (E) Expression of different Rrp6 mutant proteins was tested by western blot using anti-Myc antibody.

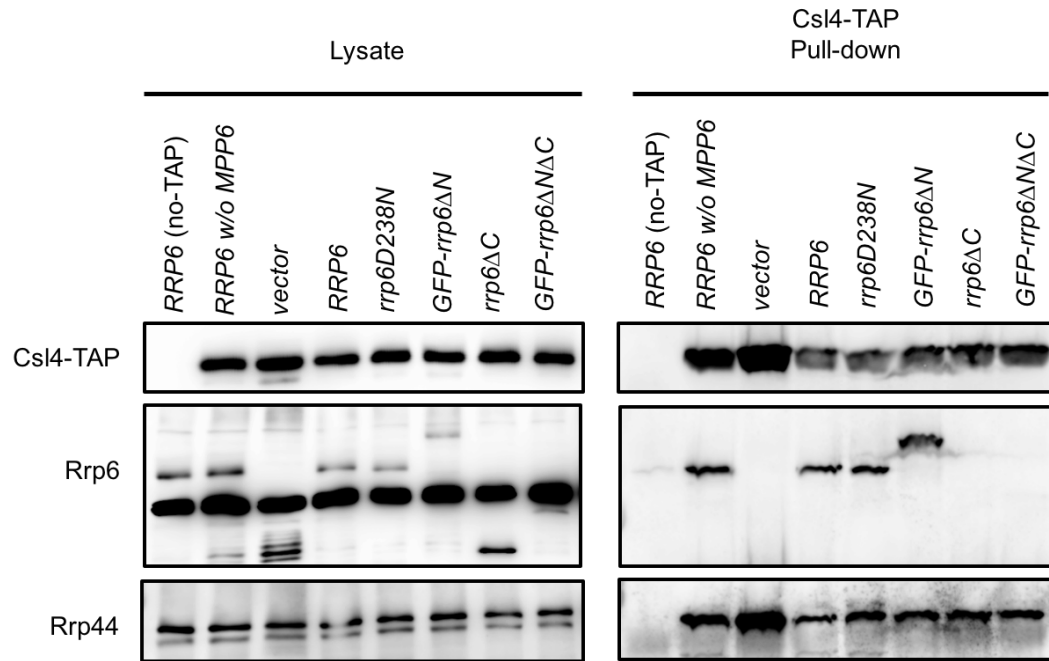


Figure 6.2. The C-terminal domain of Rrp6 is required for the interaction of Rrp6 with the RNA exosome core.

Tap-tagged Csl4, one of the exosome core subunits, was immunoprecipitated, and co-purification of Rrp6 was tested by western blot using anti-Rrp6 antibody. Csl4-TAP and Rrp44 were detected by anti-Protein A and anti-Rrp44 antibodies, respectively. The prominent band detected with anti-Rrp6 antiserum in the total lysate but not in the pull-down is an unidentified yeast protein that is also detected in lysates from *rrp6Δ*.

precipitated with Csl4-TAP, indicating that the RNA exosome was successfully purified. These results confirm previous studies that showed the C-terminal domain is important for the exosome interaction (Callahan and Butler, 2008; Makino et al., 2013a; Makino et al., 2015; Wasmuth et al., 2014).

To confirm that the N- and C-terminal Rrp6 truncation mutants disrupted some physiological function we tested their ability to support growth. Both *rrp6ΔN* and *rrp6ΔC* strains grew better than *rrp6Δ*, but not as well as wild-type, suggesting that interaction of Rrp6 with either exosome or Mtr4 is important for some but not all RNA exosome function (Fig. 6.1C). Since Rrp6 has been shown to be important for 5-FU resistance of yeast, we also tested 5-FU sensitivity of the mutants (Hoskins and Scott Butler, 2007). 5-FU sensitivity of mutant strains showed that the interaction domains are important for Rrp6 functions (Fig. 6.1D). Thus, the truncation mutants we generated confirmed the previous reports that show the N- and C-terminal domains are important for the Rrp6 function (Callahan and Butler, 2008; Makino et al., 2013a; Makino et al., 2015; Wasmuth et al., 2014). In addition, this characterization of our mutant alleles confirmed their utility to investigate how Rrp6 genetically interacts with the RNA exosome and other cofactors.

The exonuclease activity of Rrp6 is redundant with exonuclease activity of Rrp44 and Rex1.

It has been shown that Rrp6 has some functional redundancy with two other 3' exoribonucleases, but it has not been determined whether the exoribonuclease of Rrp6 is redundant with these other RNases. Specifically, *rrp6Δ* is synthetic lethal or shows synthetic growth defect with exonuclease defective allele of *RRP44* (Dziembowski et al.,

2007; Schneider et al., 2009). To test whether the catalytic activity or structural roles of Rrp6 are redundant with Rrp44, we tested genetic interaction of the *RRP6* mutant alleles with the exonuclease defective mutant of Rrp44, *rrp44-exo⁻*. For this, we conducted a plasmid shuffle assay (Fig. 6.3) (Schaeffer et al., 2012a). Briefly, wild-type or mutant *RRP6* strains that also contained an *RRP44* deletion and wild-type *RRP44* on a *URA3* plasmid were transformed with a plasmid carrying a *rrp44-exo⁻* allele in a *LEU2* plasmid. Resulting transformants were plated on the media containing 5FOA (5-Fluoroorotic acid), to select for cells that lost the *RRP44/URA3* plasmid. Catalytically inactive mutant of Rrp6, *rrp6D238N*, showed synthetic lethal phenotype with *rrp44-exo⁻*, suggesting that the exonuclease activities of Rrp6 and Rrp44 are redundant.

I also tested the genetic interaction of the *RRP6* alleles with *REX1* null mutation. Rex1 and Rrp6 are both member of the RNase D family and have some functional overlap. Both Rex1 and the RNA exosome function in tRNAi^{Met} and 5S rRNA processing (Kadaba et al., 2004; Ozanick et al., 2009; Piper et al., 1983). In addition, *rrp6Δ* is synthetic lethal with *rex1Δ* (van Hoof et al., 2000a). Thus, to understand what function of Rrp6 is redundant with Rex1, we tested genetic interaction of the *RRP6* alleles with *rex1Δ* (Fig. 6.3, bottom right panel). The result showed that *rex1Δ* is synthetic lethal with *rrp6D238N*, suggesting that the exonuclease activity of Rrp6 is redundant with Rex1. Taken together, these data indicate that catalytic functions of Rrp6 overlap with those of Rrp44 and Rex1.

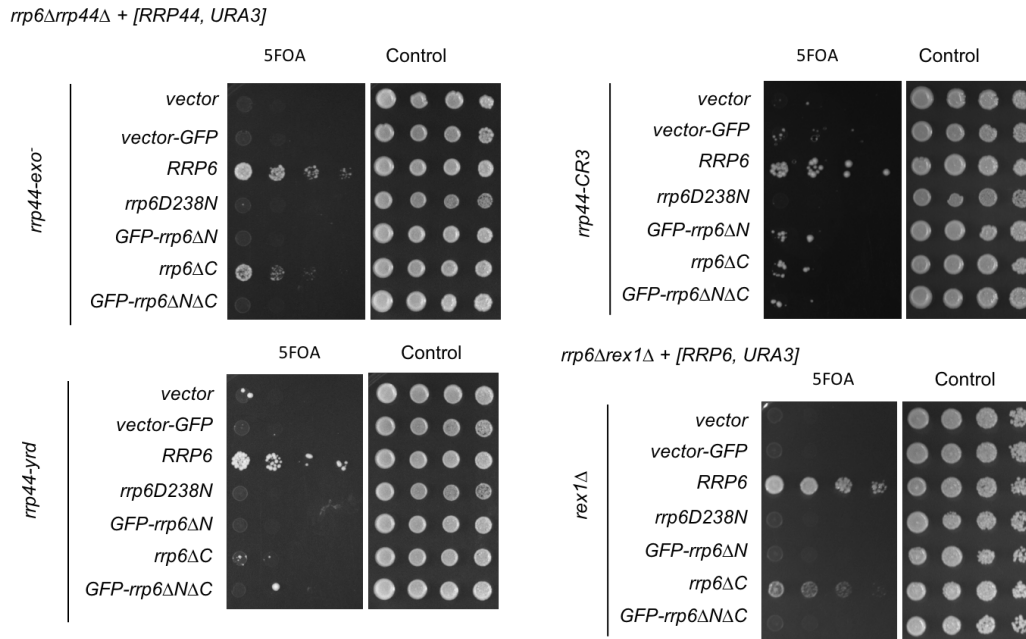


Figure 6.3. The exonuclease activity of Rrp6 is redundant with exonuclease activity of Rrp44 and Rex1.

To test genetic interaction of *RRP6* with *RRP44*, an *rrp6Δrrp44Δ* strain that carries a wild-type *RRP44* allele on a *URA3* plasmid was transformed with different *RRP44* and *RRP6* alleles on *LEU2* and *HIS3* plasmids, respectively. Cells were serially diluted and spotted on media containing 5-FOA or control media. To test the genetic interaction of *REX1* with *RRP6*, an *rrp6Δrex1Δ* strain carrying a wild-type *RRP6* allele on a *URA3* plasmid was transformed with different *RRP6* alleles. Transformants were spotted on 5-FOA containing media or control media (bottom right panel).

Redundancy between the exonuclease activities of Rrp6 and Rrp44 is independent of the Rrp6-exosome core interaction.

Genetic analyses of additional *RRP6* alleles showed that the C-terminal truncation of Rrp6 does not show synthetic lethal phenotype with *rrp44-exo⁻*, but slow growth. This result indicates that the redundancy between Rrp6 and Rrp44 is independent of the Rrp6-exosome core interaction (Fig. 6.3). Interestingly, unlike *rrp6ΔC*, *rrp6ΔN* is synthetic lethal with *rrp44-exo⁻*. This observation suggests that Rrp6 has exosome core-independent function and is consistent with previous reports (Callahan and Butler, 2008; Graham et al., 2009; Gudipati et al., 2012; Kiss and Andrulis, 2010). Furthermore, this exosome-independent function still requires the domain that interacts with the RNA exosome cofactors Mtr4 and Rrp47. The N-terminal truncation of Rrp6 is also synthetic lethal with *rex1Δ*, while the C-terminal deletion of Rrp6 is viable with a slight growth defect (Fig. 6.3, bottom right panel). This result is similar to the interaction of *RRP6* alleles with *rrp44-exo⁻*, indicating that the exosome core independent function of Rrp6 is redundant with Rex1.

I also tested genetic interaction of *RRP6* mutant alleles with *rrp44-yrd* and *rrp44-CR3* in which the Rrp44-exosome core interaction is weakened (Han and van Hoof, 2016; Schaeffer et al., 2012a). These *rrp44* mutations also showed synthetic growth defect with all *rrp6* mutant alleles tested (Fig. 6.3). This result indicates that Rrp44 requires its interaction with the exosome core to function as previously reported. In addition, the Rrp6 independent function of the RNA exosome requires the Rrp44-exosome interaction, suggested by a growth defect of *rrp44-yrd* and *rrp44-CR3* with *rrp6ΔC*.

Taken together, these data show that Rrp6 has exosome-independent functions that require the Mtr4 and Rrp47 interaction domain. Since Mtr4 interacts with other exosome cofactors such as Trf4/5 and Air1/2 that interact with specific substrates, it appears that those substrates can be redundantly processed or degraded by Rrp6 and Rrp44.

The function of the N-terminus of Rrp6 extends beyond interacting with Mtr4.

Since the N-terminal domain of Rrp6 has been shown to interact with the N-terminus of Mtr4, we attempted to test the significance of the interaction *in vivo* (Schuch et al., 2014). Briefly, we tested the genetic interaction of the N-terminal truncation mutant alleles of *MTR4* with the *rrp6* mutant we generated in figure 6.1 (Fig. 6.4). A previous graduate student had generated two of N-terminal truncation mutants of Mtr4, *mtr4Δ1-12* in which highly conserved N-terminal 12 residues are deleted, and *mtr4Δ1-89* in which the complete N-terminus is deleted, but the core helicase domain remains (Fig. 6.4B; Klauer and van Hoof, unpublished). The first 14 N-terminal residues of Mtr4 were subsequently shown to interact with Rrp6 in the X-ray crystal structure, while *in vitro* binding studies showed that the N-terminal 80 residues are important for the Rrp6 interaction (Schuch et al., 2014). For comparison, we also used an *mtr4* mutant in which the Arch-domain is deleted (Jackson et al., 2010). The Mtr4 mutant proteins are expressed comparable to wild-type protein (Fig. 6.4B, right panel). As shown previously, *mtr4-archless* caused a severe growth defect (Klauer and van Hoof, 2013) (Fig. 6.4B, left panel). In comparison, *mtr4Δ1-89* caused a milder growth defect, while *mtr4Δ1-12* caused no growth defect.

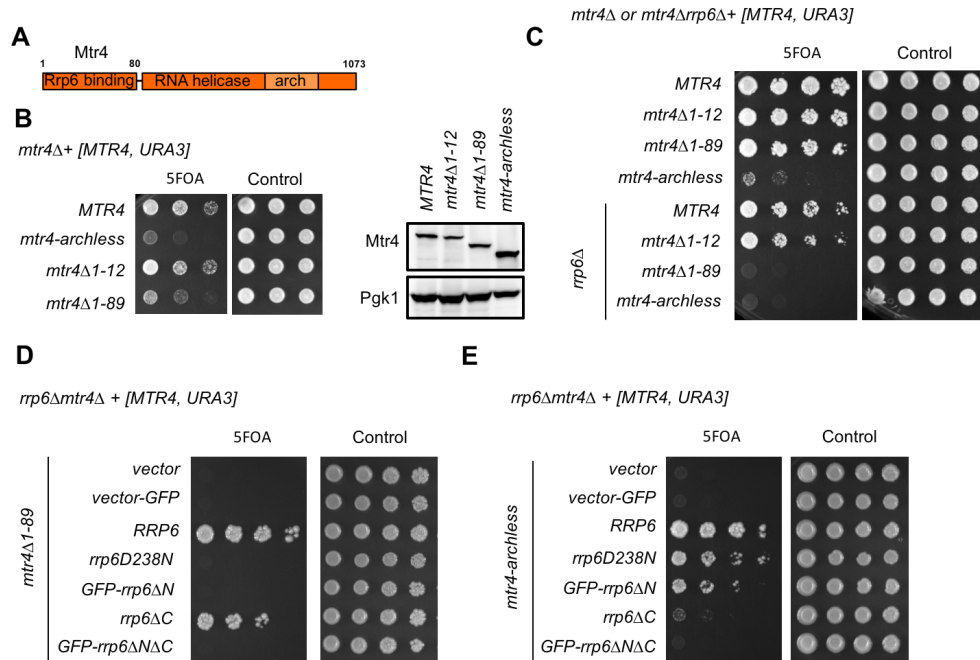


Figure 6.4. The function of the N-terminus of Rrp6 extends beyond interacting with Mtr4.

(A) Schematic domain organization of Mtr4. The N-terminal domain interacts with Rrp6/Rrp47. It contains helicase core followed by characteristic Arch domain. (B) Growth of N-terminal truncation mutants of Mtr4. *mtr4Δ* carrying a wild-type *MTR4* allele in a *URA3* plasmid was transformed with wild-type or mutant alleles of *MTR4*. Serially diluted transformants were spotted on 5-FOA containing media. (C) Growth of N-terminal truncation and *mtr4-archless* mutants of Mtr4 in the presence or absence of *RRP6* was tested. Spotted cells were incubated longer than the plates on the panel (B). (D)(E) Genetic interaction of *mtr4Δ1-89* and *mtr4-archless* with *rrp6* mutant alleles was tested.

The different effects of growth *mtr4Δ1-89* and *mtr4Δ1-12* raise two possibilities. Either, the entire N-terminal 89 residues are important for the Rrp6 interaction and the interaction is important for the exosome function, or the N-terminal Mtr4 domain has more than one function as that the first twelve residues are interacting with Rrp6 while the rest of N-terminal domain has some other critical function. To attempt to distinguish between these possibilities, we combined each *MTR4* allele with *rrp6Δ*. Importantly, this showed that *rrp6Δ* is synthetic lethal with *mtr4Δ1-89* (Fig. 6.4C). The observation that even in the complete absence of Rrp6, deletion of residues 1-89 of Mtr4 affected growth strongly indicates that the N-terminus of Mtr4 has functions beyond interacting with Rrp6.

To test what functions of Rrp6 are redundant with Mtr4, we tested the genetic interaction of each mutant *mtr4* with the *rrp6* alleles we generated (Fig. 6.4D and E). Interestingly, *rrp6ΔN* showed a synthetic growth defect with *mtr4Δ1-89*. If the sole function of the N-termini of Mtr4 and Rrp6 is the interaction with one another, disrupting the same function would not result in synthetic phenotype. This result strongly suggests that the N-termini of both Rrp6 and Mtr4 have functions that are independent of their known interaction. *mtr4Δ1-89* also showed a genetic growth defect with *rrp6D238N*, but not with *rrp6ΔC*. One possible explanation of these genetic interactions is that the N-terminus of Mtr4 is required for delivery to the exoribonuclease activity of Rrp44 through the direct access route, bypassing the Rrp6-exosome interaction.

In addition, the *mtr4-archless* showed synthetic growth defect with *rrp6ΔC* (Fig. 6.4E). Since the C-terminus of Rrp6 interacts with the core exosome, one explanation is that Mtr4 interacts with the core exosome through the Arch-domain either directly or indirectly, and the interaction is Rrp6 independent. Consistent with this possibility is that

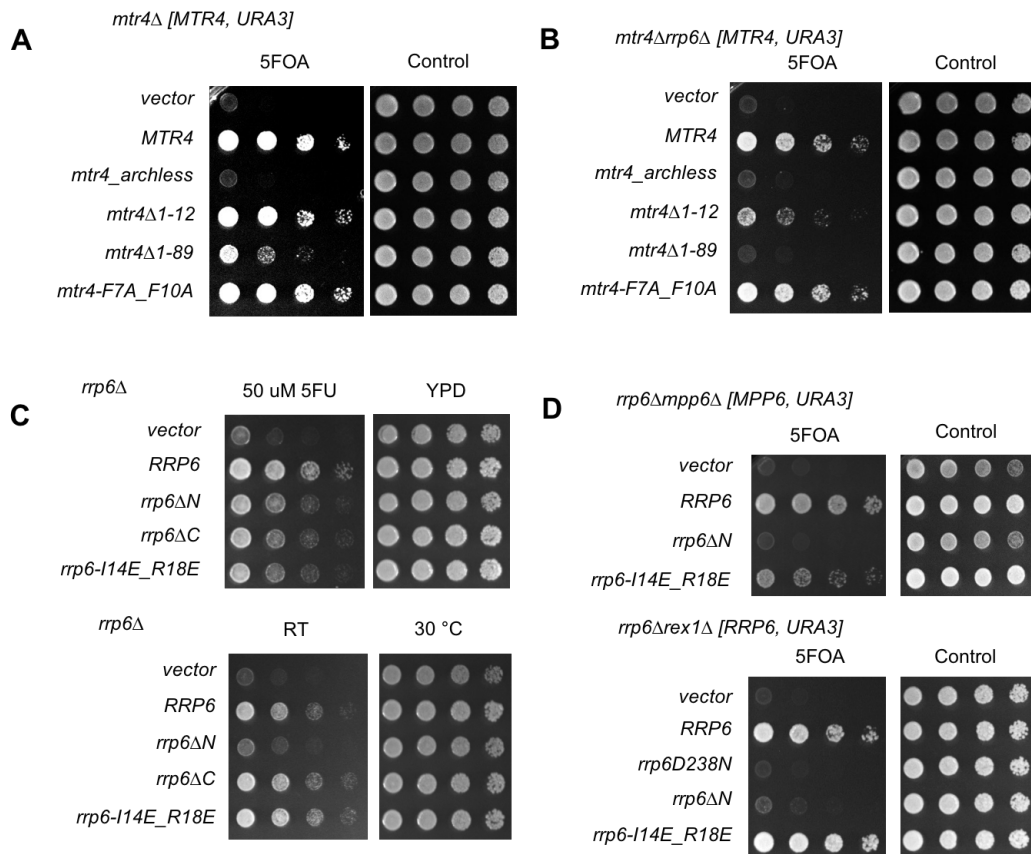


Figure 6.5. The function of the N-terminus of Rrp6 and Mtr4 extends beyond interacting with Mtr4.

(A) Growth of *mtr4-F7A_F10A* in which the interaction with Rrp6 is specifically disrupted was tested. (B) *mtr4-F7A_F10A* did not show synthetic growth defect with *rrp6Δ*. (C) 5-FU resistance and growth of *rrp6-I14E_R18E* in which the interaction with Mtr4 is disrupted were tested. (D) Genetic interaction of *rrp6-I14E_R18E* with *mpp6Δ* and *rex1Δ* was tested by plasmid shuffle assay.

rrp6 Δ *N* and *rrp6-D238N* are not synthetic lethal with *mtr4-archless*. (Figure 6.4E; see discussion).

To independently test the importance of the Rrp6-Mtr4 interaction we used point mutations previously shown to disrupt the interaction. Previous structural and biochemical studies identified important interacting residues of Mtr4 and Rrp6, and mutations of those residues abolished the interactions with each other *in vitro* (Schuch et al., 2014). Mtr4-F7A_F10A has been shown to reduce *in vivo* interaction with Rrp6 compared to wild-type Mtr4 (Schuch et al., 2014). Unlike *mtr4* Δ 1-89, the *mtr4-F7A_F10A* had little effect on growth. Importantly, the *mtr4-F7A_F10A* mutant did not show any growth defect in *rrp6* null background unlike *mtr4* Δ 1-89. These results are consistent with the finding that F7 and F10 are important for Rrp6 interaction (Fig. 6.5B), and that other residues in the N-terminus of Mtr4 have some other function that is Rrp6 independent.

I next tested point mutations in the N-terminus of Rrp6 that disrupt the Mtr4 interaction and compared their effect to *rrp6* Δ *N*. *rrp6-I14E_R18E* has been shown to disrupt the Mtr4 interaction (Schuch et al., 2014). Interestingly, this point mutation in the N terminus of Rrp6 showed some phenotypic similarities with *rrp6* Δ *N*, but also many differences. Specifically, both showed a similar 5-FU sensitivity, suggesting that the Rrp6-Mtr4 interaction is important for 5-FU resistance (Fig. 6.5C). However, the point mutant showed normal growth at room temperature, while *rrp6* Δ *N* is defective. In addition, it did not show synthetic lethal phenotype with *mpp6* Δ or *rex1* Δ unlike *rrp6* Δ *N*. The expression level of Rrp6-I14E_R18E was comparable to wild type, indicating that the 5-FU sensitivity is not due to the low expression (Fig. 6.9). Taken together, these data

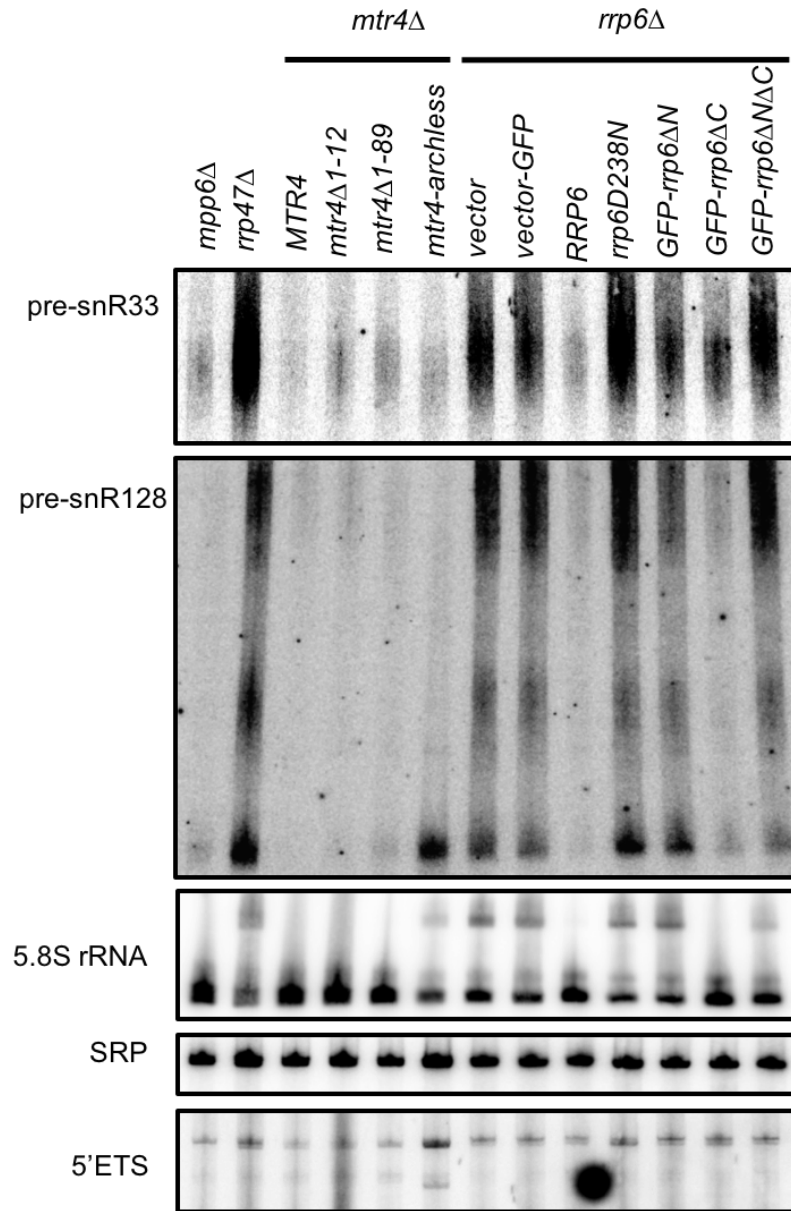


Figure 6.6. Analysis of RNA processing defects of the *mtr4* and *rrp6* mutants further support the Mtr4 independent function of Rrp6 N-terminal domain. Total RNA from each mid-log phase cells of each strain was isolated, and northern blot was conducted probing indicated RNA species.

indicate that the N-terminal domains of Rrp6 and Mtr4 have other functions than the interaction with one another (see discussion).

Analyses of RNA processing further support the Mtr4 independent function of the Rrp6 N-terminal domain.

To start to investigate the molecular defects underlying the observed growth defects, we isolated total RNA from viable *mtr4* and *rrp6* mutants and conducted northern blot analyses probing select known RNA exosome substrates (Fig. 6.6). Rrp6 is known to degrade or processes small nucleolar RNAs (snoRNAs) and trims 30 nt from the 3' of 5.8S rRNA precursor (Butler and Mitchell, 2010; Gudipati et al., 2012; Phillips and Butler, 2003). It has previously been shown that 3' extended and polyadenylated snoRNA species accumulate in *rrp6Δ* and other RNA exosome mutants (Allmang et al., 1999a; van Hoof et al., 2000b). Our northern blot showed these defects for pre-snR33 and pre-snR128 in *rrp6Δ* and *rrp47Δ* (Fig. 6.6). It has been shown that most phenotypes observed in *rrp47Δ* is due to loss of Rrp6, which is an interaction partner of Rrp47 (Feigenbutz et al., 2013a). Thus, *rrp47Δ* would show a similar result to *RRP6* null. *rrp6D238N*, a catalytically inactive form of Rrp6, also showed a severe defect in snoRNA processing, while *rrp6ΔC* showed a milder defect. This result suggests that Rrp6 has two roles in snoRNA processing: It acts as a ribonuclease on snoRNAs and to deliver them to the RNA exosome, and presumably to Rrp44. This result is consistent with the growth assay in figure 6.3 that shows the redundancy between the catalytic activities of the Rrp6 and Rrp44.

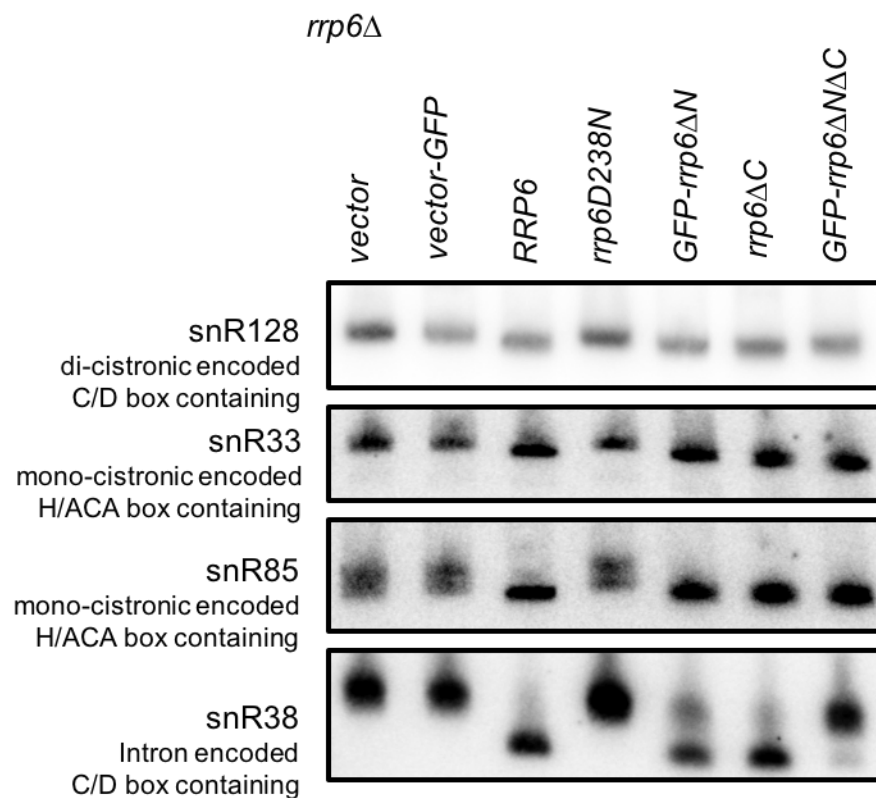


Figure 6.7. The catalytic domain is sufficient for the final trimming of some snoRNAs.

Total RNA was isolated from the *rrp6Δ* strain that carries different *RRP6* alleles followed by northern blot probing indicated snoRNAs.

Interestingly, the *mtr4Δ1-89* did not show a defect in snoRNA processing, while *rrp6ΔN* is defective. I also observed that while *mtr4-archless* accumulated 5.8S+30 as previously reported and as expected from the arch-Nop53 interaction (Klauer and van Hoof, 2013; Thoms et al., 2015), the *mtr4Δ1-89* allele did not have this defect. In contrast to *mtr4Δ1-89*, the *rrp6ΔN* mutation accumulated 5.8S+30. These different effects of *mtr4Δ1-89* and *rrp6ΔN* on snoRNA and 5.8S rRNA processing are also consistent with the growth assay in figure 6.3, and confirm that the N-terminus of Rrp6 has functions other than the Mtr4 interaction.

The catalytic domain of Rrp6 is sufficient for the final trimming of most snoRNAs.

In addition to the long 3' extended and polyadenylated snoRNA precursors that accumulate in *rrp6Δ* and other RNA exosome mutants, the *rrp6Δ* strain also accumulates snoRNAs that are extended by just a few nucleotides (Allmang et al., 1999a; van Hoof et al., 2000b). In our northern blot analysis, we observed a different pattern of accumulation for these than for the longer species. Specifically, the *rrp6ΔN* and *rrp6ΔC*, and even the *rrp6ΔNΔC* mutants did not accumulate these snoRNAs with short extensions for the snR128, snR33, and snR85 snoRNAs. The catalytically inactive allele, *rrp6D238N*, showed a processing defect in these snoRNAs (Fig. 6.7). This suggests that the catalytic domain of Rrp6 is sufficient for the final trimming of these snoRNAs. Strikingly, we found that the final trimming of a different snoRNA was largely unaffected by either *rrp6ΔN* or *rrp6ΔC*, but was defective when both domains were deleted. This suggests that the final trimming of snoRNAs may not occur the same for all snoRNAs. snR38 is

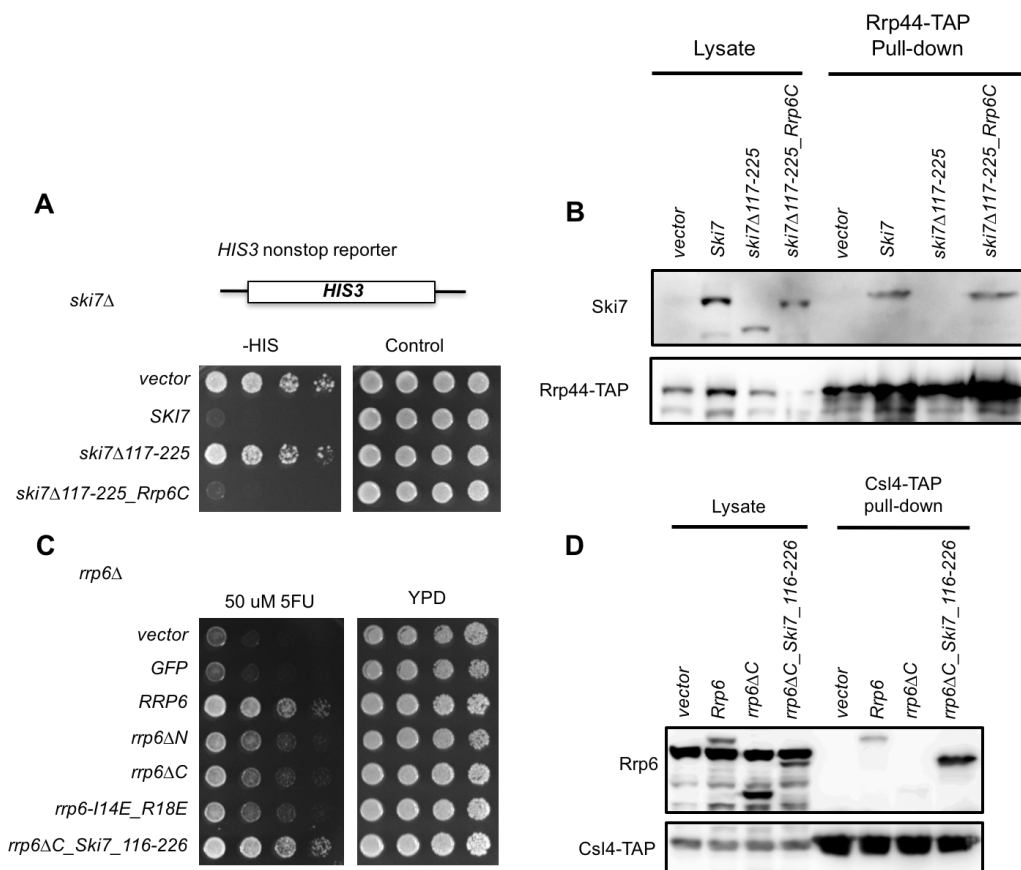


Figure 6.8. The exosome interacting domains of Rrp6 and Ski7 are interchangeable.

(A) *ski7Δ* carrying *HIS3* gene that does not contain in-frame stop codon was transformed with *SKI7* variants followed by spotting on media lacking histidine. *ski7Δ117-225* has the exosome interaction domain deleted. In *ski7Δ117-225_Rrp6C*, the exosome interacting region of Ski7 is replaced by the C-terminus of Rrp6. (B) Immunoprecipitation of Rrp44-TAP by IgG-coated beads was conducted to test the interaction of the Ski7 variants with the exosome core. Pulled-down fraction was subjected to western blot using anti-HA (for Ski7-3HA) and anti-Protein A antibodies. (C) The C-terminal truncation mutant of Rrp6 was fused to the exosome interaction domain of Ski7. 5-FU sensitivity of the variant was tested. (D) Interaction of Rrp6ΔC_Ski7_116-226 with the exosome core was tested by immunoprecipitation. TAP-tagged Csl4, one of the exosome core subunits, were pulled down followed by western blot using anti-Rrp6 and anti-Protein A antibodies.

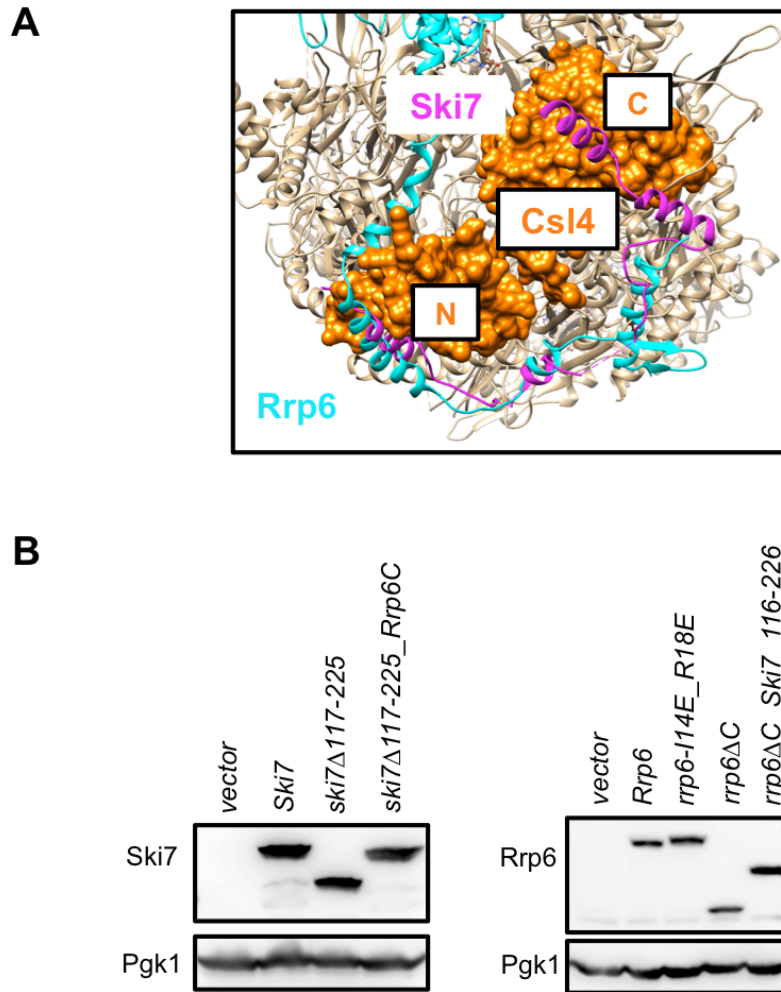


Figure 6.9. Interaction of Rrp6 and Ski7 with the exosome core.

(A) Cartoon version of the x-ray crystal structure of the RNA exosome (5C0W and 5JEA). C-terminal domain of Rrp6 and N-terminal domain of Ski7 are interacting with the exosome core, and the interaction is overlapping. Orange: Csl4; Magenta: Ski7; Cyan: Rrp6. **(B)** Expression level of Ski7 and Rrp6 variants are comparable to wild-type level. Anti-HA, anti-Rrp6, and anti-Pgk1 antibodies were used to detect Ski7-3HA, Rrp6, and Pgk1 (loading control).

the smallest snoRNA and the only intron encoded snoRNA we tested. Whether either of these characteristics determines the trimming requirement will require further testing.

The exosome interacting domains of Rrp6 and Ski7 are largely interchangeable

The above results reveal that its C-terminal domain is important for Rrp6 function, possibly through mediating RNA exosome interactions. However this C-terminal domain has two other suggested functions. It has also been suggested to function to mediate RNA binding and it contains a predicted NLS (nuclear localization sequence). Structural analyses of the cytoplasmic exosome showed that while Ski7 and Rrp6 show no sequence similarity, they interact with overlapping regions of the RNA exosome core (Fig. 6.9A) (Kowalinski et al., 2016; Liu et al., 2016a). I reasoned that if the function of the C-terminal domain of Rrp6 is to mediate exosome interaction, then it might be interchangeable with the N-terminal RNA exosome interacting domain of Ski7. For this, we first tested whether the C-terminal domain of Rrp6 could functionally replace the exosome interacting domain of Ski7 by testing cytoplasmic decay of the *his3-nonstop* reporter mRNA (van Hoof et al., 2002). Deleting the RNA exosome interacting region of Ski7 (*ski7Δ117-225*) resulted accumulation of *his3-nonstop* mRNA as reflected in the ability to grow on media lacking histidine (Fig. 6.8A). I next generated, a chimeric protein in which residues 117-225 of Ski7 were replaced by residues 540 to 620 of Rrp6 (*Ski7Δ117-225_Rrp6C*). Strikingly, *Ski7Δ117-225_Rrp6C* was fully capable of complementing the *ski7Δ* strain for nonstop mRNA degradation. In addition, the *Ski7Δ117-225* was expressed but failed to coprecipitate with Rrp44-TAP, and this coIP defect was restored for *Ski7Δ117-225_Rrp6C* (Fig. 6.8B). These results

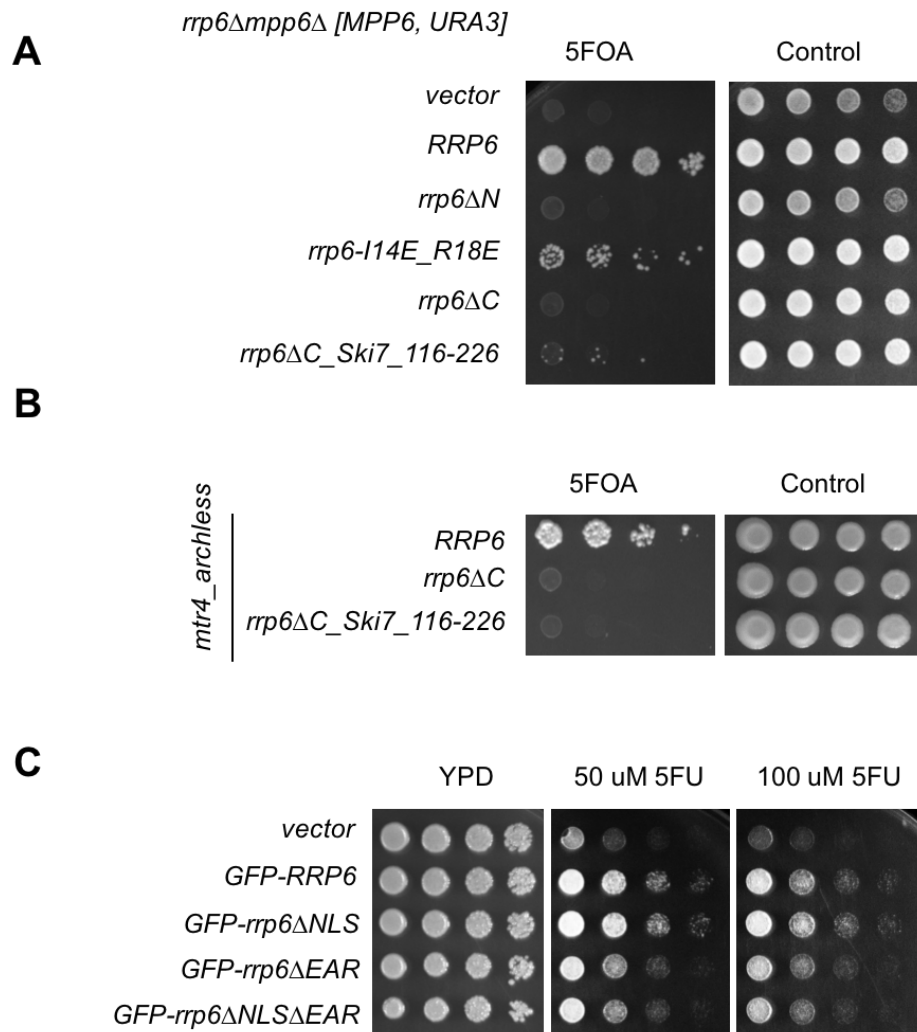


Figure 6.10. The C-terminal domain of Rrp6 and Mpp6 may mediate additional interactions with cofactors.

(A) Genetic interaction of *mpp6Δ* with the *rrp6* mutant alleles was tested by plasmid shuffle assay. The exosome interaction region of Ski7 did not rescue synthetic growth phenotype of *rrp6ΔC* with *mpp6Δ* (B) The exosome interaction region of Ski7 did not complement the C-terminal deletion of Rrp6. (C) Rrp6 variants that do not contain nuclear localization signal (NLS), exosome association region (EAR), or both were generated followed by testing 5-FU sensitivity.

confirm that the Ski7-RNA exosome interaction is required for nonstop mRNA decay (van Hoof et al., 2002). More interestingly they show that the RNA exosome interaction regions of Rrp6 and Ski7 are interchangeable.

Next, we fused the exosome interacting domain of Ski7 to the C-terminal deletion mutant of Rrp6 and tested 5-FU resistance. I found the wild-type level 5-FU resistance of *rrp6ΔC-Ski7_116-226*, while *rrp6ΔC* shows sensitivity, suggesting that the exosome interacting domain of Ski7 functionally replace the C-terminal domain of Rrp6 (Fig. 6.8C). Immunoprecipitation of Csl4-TAP, one of the exosome subunits, showed the physical interaction of Rrp6ΔC-Ski7_116-226 with the exosome (Fig. 6.8D).

Taken together, the co-IP data suggest that the exosome interacting domains of Rrp6 and Ski7 are interchangeable for mediating RNA exosome interaction *in vivo*. Furthermore, the observation that the two regions are genetically interchangeable indicates that the two swapped domains have the same physiological function, which is RNA exosome interaction.

The C-terminal domain of Rrp6 and Mpp6 may mediate additional interactions with cofactors.

Since the C-terminal domain of Rrp6 is about twice as long as the exosome association region of Ski7, it is possible that the extra 100 residues have other functions. Thus, we further tested whether the exosome interacting domain of Ski7 can fully complement the C-terminal deletion of Rrp6.

rrp6Δ has been showed to be synthetic lethal with *mpp6Δ* (Milligan et al., 2008). Mpp6 (M-phase phosphoprotein) has been shown to interact with Rrp6 and Mtr4 in yeast

and human, respectively (Kim et al., 2016a; Schilders et al., 2007). In addition, knockdown of *MPP6* in human epithelial type 2 cells showed defect in 5.8S rRNA processing (Schilders et al., 2005). I found that either the deletion of N-terminus or C-terminus of Rrp6 is synthetic lethal of *MPP6* deletion, suggesting that the redundancy between Rrp6 and Mpp6 is the interaction of the exosome and cofactors (Fig. 6.10A). Unlike 5-FU sensitivity in figure 6.8, the exosome interacting domain of Ski7 did not rescue the synthetic growth defect of *rrp6ΔC* with *mpp6Δ* (Fig. 6.10A).

As shown in figure 6.4 *rrp6ΔC* is also synthetic lethal with *mtr4-archless*. This synthetic lethality was not rescued by the exosome interaction domain of Ski7 (Fig. 6.10B). Expression levels of Rrp6ΔC and Rrp6ΔC-Ski7_116-226 were comparable to wild-type Rrp6, indicating that growth defect is not due to reduced expression (Fig. 6.9B).

These results suggest that the C-terminal domain of Rrp6 has other functions than the exosome interaction and that these functions are redundant with Mpp6 or the Arch domain of Mtr4.

Exosome/cofactor interaction and nuclear localization signals of Rrp6 are redundant.

The C-terminal domain of Rrp6 contains putative nuclear localization signals (NLS) (Fig. 6.1A), and a previous report showed that deletion of NLS impairs nuclear localization of Rrp6 (Phillips and Butler, 2003). I wondered whether the absence of NLS in the C-terminal deletion mutant attribute to the phenotype we observed. To test whether the 5-FU sensitive phenotype of *rrp6ΔC* is attributed to the exosome interaction or NLS,

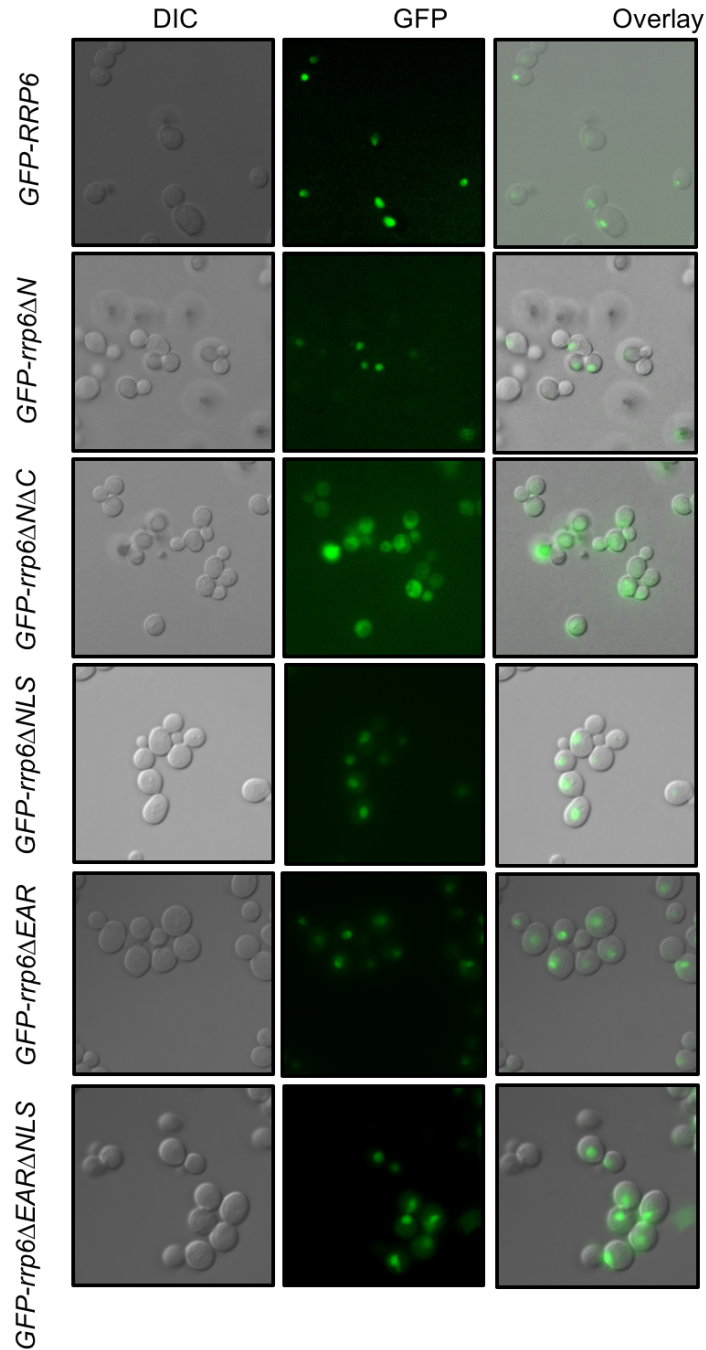


Figure 6.11. Localization of GFP-fused Rrp6 variants.

rrp6Δ strain carrying various *RRP6* alleles was grown until mid-log phase and localization was tested by fluorescence microscopy. Differential interference contrast (DIC) image and GFP fluorescence image were merged by using ImageJ software.

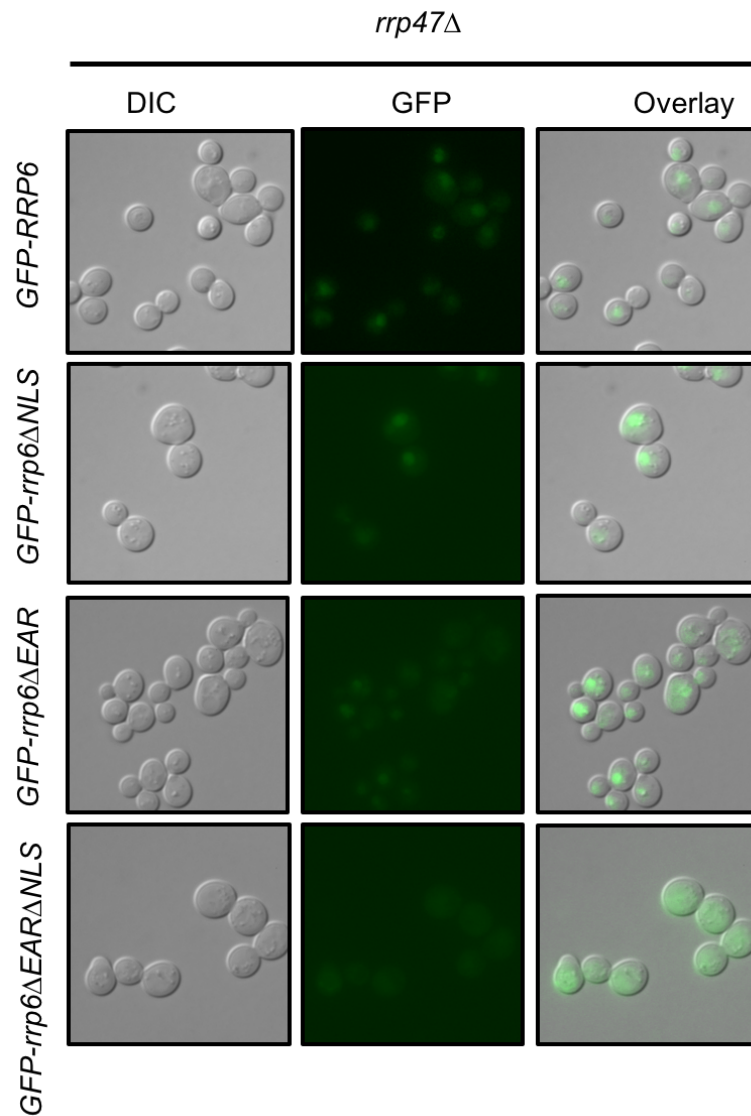


Figure 6.12. Localization of GFP-fused Rrp6 variants in *rrp47Δ*. *rrp6Δrrp47Δ* strain carrying various *RRP6* alleles was grown until mid-log phase and localization was tested by fluorescence microscopy. Differential interference contrast (DIC) image and GFP fluorescence image were merged by using ImageJ software.

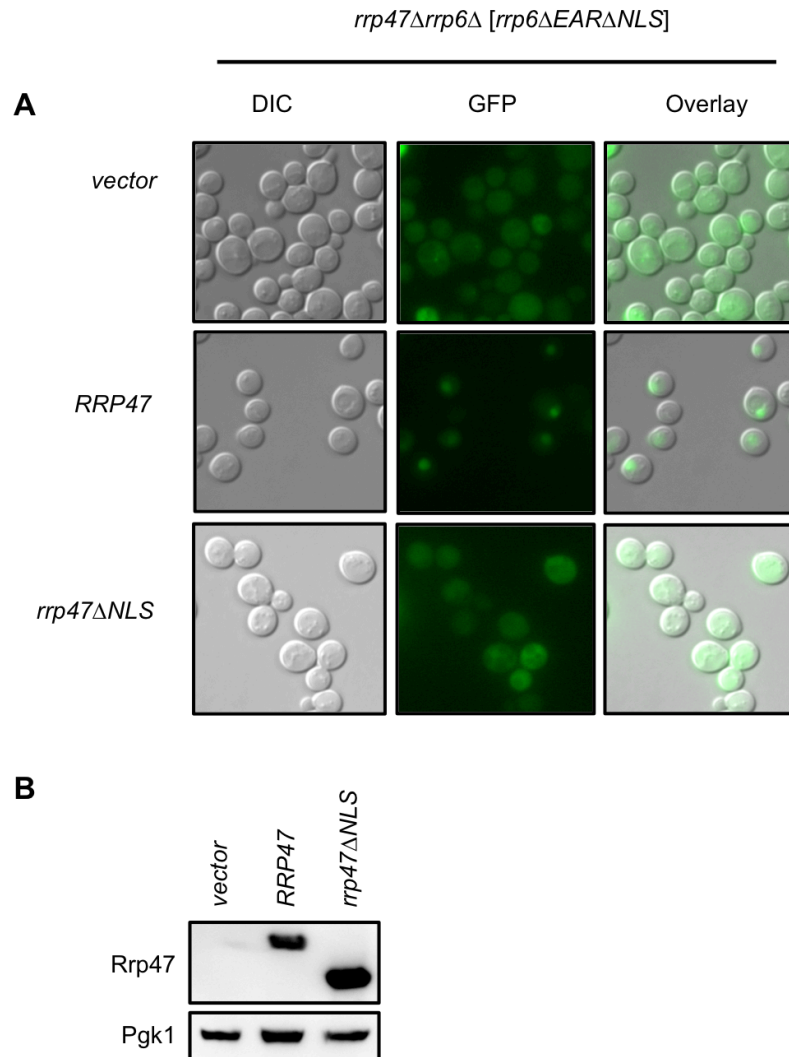


Figure 6.13. Localization of GFP-fused Rrp6 variants in *rrp47Δrrp6Δ* carrying *rrp6ΔEARΔNLS*.

rrp6Δrrp47Δ strain carrying a *rrp6ΔEARΔNLS* allele was transformed with a vector, RRP47, or *rrp47ΔNLS* plasmid. Resulting transformants were grown until mid-log phase and localization was tested by fluorescence microscopy. Differential interference contract (DIC) image and GFP fluorescence image were merged by using ImageJ software.

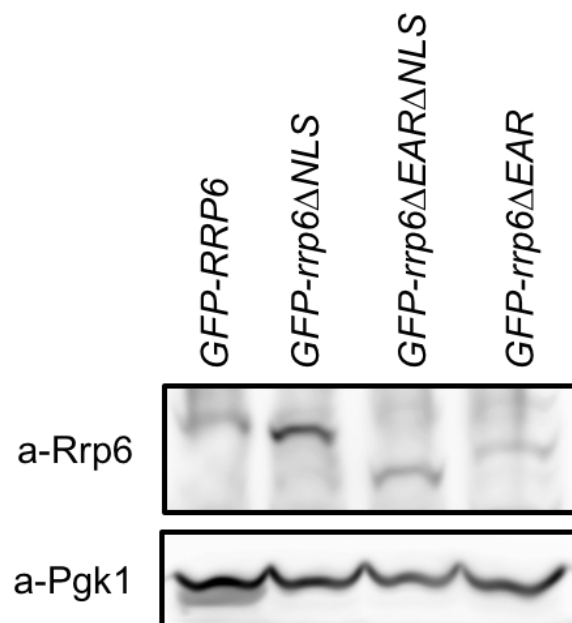


Figure 6.14. Expression level of GFP-fused Rrp6 variants in *rrp47Δ*.

Total protein was isolated from each strain followed by western blot using anti-Rrp6 and anti-Pgk1 antibody (loading control).

GFP-fused *RRP6* alleles in which either NLS or known exosome association region (EAR) is deleted were constructed and tested for 5-FU resistance (Fig. 6.10C). Deletion of two putative NLSs did not affect 5-FU sensitivity, while precise deletion of EAR showed a growth defect on 5-FU containing media. This result indicates that the exosome interaction is important for 5-FU sensitivity but the NLS is not.

I next analyzed the localization of GFP-fused Rrp6 proteins by fluorescence microscopy and found that NLS deletion (*GFP-Rrp6* Δ NLS) caused only a minor defect in nuclear localization (Fig. 6.11). Interestingly, Rrp6 was mislocalized when both the N- and C-terminal domains of Rrp6 were simultaneously deleted. However, a simultaneous deletion of both EAR and NLS only showed minor localization defect in wild-type background, while its nuclear localization is completely defective in *rrp47* null background (Fig. 6.11 and 6.12). The defective localization of *GFP-Rrp6* Δ EAR Δ NLS in *rrp47* null background was restored when a plasmid copy of *RRP47* was introduced (Fig. 6.13A). Interestingly, deletion of a potential NLS of Rrp47 did not restore the nuclear localization of *GFP-Rrp6* Δ EAR Δ NLS in *rrp47* null background, indicating that nuclear localization of Rrp47 contributes to the localization of Rrp6. Rrp47 Δ NLS protein was expressed even higher than wild type (Fig. 6.13B). Thus, the localization defect of *GFP-Rrp6* Δ EAR Δ NLS is not due to the lack of expression of Rrp47 Δ NLS. These data suggest that the exosome/cofactor interactions and NLS are redundant in the localization of Rrp6. This result is consistent to the recent study that suggested there are multiple pathways of Rrp6 nuclear localization (Gonzales-Zubiate et al., 2017). Western blot analysis for the GFP-fused Rrp6 variants showed that GFP-fused proteins are expressed and intact in *rrp47* Δ , indicating that cytoplasmic localization of *GFP-Rrp6* Δ EAR Δ NLS is not due to

the nonspecific GFP cleavage from the construct (Fig. 6.14). Although Rrp6 Δ N Δ C shows cytoplasmic localization, substantial amount of protein appears to be present in the nucleus since we observed functional snoRNA processing of *rrp6* Δ N Δ C (Fig. 6.7).

DISCUSSION

Structural studies provided insights into how the RNA exosome and its cofactors such as Rrp6, Rrp47, Mtr4, and Ski7 interact with one another (Kowalinski et al., 2016; Liu et al., 2016a; Makino et al., 2015; Schuch et al., 2014; Wasmuth et al., 2014; Wasmuth and Lima, 2017). Rrp6 interacts with the exosome via its C-terminal domain in the nucleus and with the N-termini of Mtr4 and Rrp47 via its N-terminal domain. Thus, Mtr4 and Rrp47 appear to interact with the exosome core through Rrp6 (Fig. 6.1B). However, the biological significance of the interaction has not been fully tested. In this study, we thoroughly investigated the known structural, biochemical interactions of the RNA exosome with its cofactors, Rrp6 and Mtr4. I found that deleting the Mtr4-interacting domain of Rrp6 resulted in RNA processing defects not seen upon deletion of the Rrp6-interacting domain of Mtr4. Furthermore, we showed that deleting the Mtr4-interacting domain of Rrp6 is synthetic lethal with deleting the Rrp6-interacting domain of Mtr4. I conclude that there are still unknown interactions among Rrp6, Mtr4, and the RNA exosome. Similarly, we show that the exosome-interaction function of the Rrp6-C-terminal domain can be replaced by the equivalent domain of Ski7. However, restoring exosome interaction to *rrp6* Δ C suppresses only some growth phenotypes suggesting that the C-terminus of Rrp6 also has unknown functions.

Our genetic analyses showed that both the catalytic activity and the exosome/cofactor interactions are important for Rrp6 function (Fig. 6.1C and D). This finding is consistent to the previous reports that showed either N-terminal or C-terminal truncation of Rrp6 yields RNA processing defects or growth defect of yeast (Callahan and Butler, 2008; Stead et al., 2007). Rrp6 was shown to be functionally redundant with

Rrp44, an essential catalytic subunit of the RNA exosome (Dziembowski et al., 2007; Schneider et al., 2009) but it was previously unknown what functions of Rrp6 were redundant with Rrp44. Genetic interaction of the different *RRP6* alleles with the exonuclease defective mutant of *RRP44*, *rrp44-exo⁻* showed that the catalytic activities of Rrp6 and Rrp44 are redundant (Fig. 6.3). Interestingly, *rrp6ΔC* was not synthetically lethal with *rrp44-exo⁻*. Northern blot analysis indicated that the final trimming of many snoRNAs requires the catalytic activity of Rrp6, but not the interaction domains of Rrp6. Combined these findings suggest that RNA-exosome independent catalytic activities of Rrp6 are redundant with the exonuclease activity of the Rrp44. This result is in contrast with current models that suggest that after delivery to the RNA exosome substrates can be directed to either exoribonuclease.

Comparing the effects of point mutations and truncations allowed us to clarify some of the functions of Mtr4 and Rrp6 alleles. For example, we found that deleting the N-terminal domain of Rrp6 or mutating Mtr4-interacting domains had similar effects on 5-FU sensitivity, but different effects on growth under normal conditions or synthetic lethality with *mpp6Δ* or *rex1Δ*. Therefore we can conclude that the Mtr4-Rrp6 interaction is critical for 5FU resistance, but that other functions of Rrp6 are important for growth under these other conditions. An important first step in identifying what aspects of *Rrp6ΔN* are important in the absence of Rex1 or Mpp6 will be to identify point mutations in the N-terminus that are synthetic lethal with *rex1Δ* or *mpp6Δ*.

Similar to our approach of comparing point mutations and interaction domain deletions, the exchange of the RNA exosome interacting regions of Rrp6 and Ski7 also provided insight into what functions of a domain can be ascribed to what activity of that

domain. Specifically, replacing the RNA exosome interacting region of Ski7 with that from Rrp6 appeared to result in a fully functional Ski7, suggesting that this region of Ski7 has no unique functions. In contrast, although we could restore co-immunoprecipitation and some growth phenotypes to *rrp6ΔC* with a region from Ski7, other phenotypes of *rrp6ΔC* were not complemented. Especially, the synthetic lethal phenotype of *rrp6ΔC* with *mpp6Δ* or *mtr4-archless* was not rescued by the exosome interacting region of Ski7 (Fig. 6.10A and B). These data suggest that the C-terminal domain of Rrp6 has other functions than the exosome interaction, and those unknown functions are redundant with Mpp6 and the Arch domain of Mtr4. Recent structural study found that the C-terminal domain contains a region named “lasso” that enhances the RNase activity of the RNA exosome *in vitro* (Wasmuth and Lima, 2017). Therefore, it is possible that the additional function of the Rrp6 C-terminus is attributed to the lasso, and further analysis of *in vivo* function of lasso would be informative.

It was previously shown that Rrp6 mediates some interactions between Mtr4 and the RNA exosome. However, unlike Mtr4 and the RNA exosome, Rrp6 is not essential. One explanation of these previous findings is that there may be other interactions between Mtr4 and the RNA exosome. *mtr4-archless* was previously shown to be synthetically lethal with *rrp6Δ*. Here we show that *mtr4-archless* is synthetically lethal with *rrp6ΔC*, but not with other alleles of *RRP6*. This suggests that Mtr4 may interact with the RNA exosome through its Arch domain, either directly or indirectly (Fig. 6.4E). In addition, the N-terminal truncation mutant of Mtr4 did not result in significant RNA processing defects (Fig. 6.6), implying that the N-terminal region is not strictly required for the interaction with the RNA exosome. Interestingly, *mtr4-F7A_F10A* or *rrp6-*

I14E_R18E in which the Mtr4-Rrp6 interaction is specifically disrupted, has previously been shown to be lethal only when Mtr4 was fused to GFP (Schuch et al., 2014). These data indicate that the C-terminal GFP hinders additional interactions of Mtr4 with Rrp6 or the exosome. Taken together with our genetic analyses, we conclude that the N-terminal domain of Mtr4 is not the only site for RNA exosome interaction, but other regions such as the arch domain also mediate interaction with the RNA exosome. Further analyses are required to test whether the interaction is a direct protein-protein contact or an indirect interaction through another cofactor.

To understand how localization of Rrp6 related to its various functions, we used GFP fusions. The results suggest that three different interactions of Rrp6 redundantly determine its localization: The N-terminal interaction with Rrp47, the interaction with the RNA exosome, and the NLS, which presumably interacts directly with a karyopherin. Requirement of the nuclear localization of Rrp47 in *GFP-Rrp6ΔEARΔNLS* localization supports the hypothesis that the Rrp47 interaction contributes to the nuclear localization of Rrp6 (Fig. 6.13). This conforms and extends a recent study that suggested multiple pathways of Rrp6 nuclear localization (Gonzales-Zubiate et al., 2017). While Gonzales-Zubiate suggested that Rrp6 contains multiple regions that directly interact with specific karyopherins, we favor the hypothesis that interaction with Rrp47 and the RNA exosome creates a complex with multiple NLSs. Strikingly, although localization of Rrp6 is very redundant, it does not appear strictly required for all of its functions because *GFP-Rrp6ΔNΔC* is largely diffuse in the cytoplasm but fully function in the final trimming of many snoRNAs (Fig. 6.7).

In summary, we showed that the N-termini of Rrp6 and Mtr4 have functions beyond known biochemical activities and physical interactions. In addition, Mtr4 appears to interact with the RNA exosome independently of its N-terminal domain. Furthermore, the C-terminal domain of Rrp6 has functions in addition to exosome interaction. Taken together, we propose that there are multiple dynamic pathways of the RNA exosome-cofactor interactions, and the multitude of interactions allows the RNA exosome to complete its various processing or degradation functions that are critical cellular processes. Further investigation of interactions and additional functions will provide further insight into the function of the RNA exosome.

Chapter 7: Mpp6 and Rrp6 redundantly mediate the exosome-cofactor interactions.

INTRODUCTION

Known RNA exosome-cofactor interactions show that Rrp6 is a central cofactor that mediates the interaction of the RNA exosome with other nuclear cofactors (Fig. 1.5). However, interestingly, deletion of *RRP6* yields viable cells, while deletion of *TRF4/TRF5*, *MTR4*, or other nuclear cofactors results in lethality or severe growth defect (Castano et al., 1996; Giaever et al., 2002). These results suggest that either the exosome-cofactor interactions are not essential or there are other factors that mediate the interactions besides Rrp6.

Indeed, there is another nuclear cofactor of the RNA exosome, Mpp6 (M-Phase Phosphoprotein 6), that was identified in a synthetic lethal screen with *rrp47Δ*, and deletion of *MPP6* is also synthetic lethal with *RRP6* null (Milligan et al., 2008). Interestingly, immunoprecipitation of Mpp6-TAP followed by Mass spectrometry identified all of the exosome core components and nuclear cofactors (Milligan et al., 2008). Mpp6 does not contain any sequence similarity with known domains. Although, it has two conserved motifs with unknown function, overall amino acid sequence is poorly conserved even within *Saccharomycetaceae*. *In silico* secondary structure analyses by PONDR and PSIPRED indicate that Mpp6 is a largely disordered protein, suggesting its major function is protein-protein interaction (Jones, 1999; Obradovic et al., 2003)(Fig. 7.1). Given that Mpp6 is an RNA binding protein with no nuclease activity, it appears to deliver substrates or other cofactors to Rrp6 or the core exosome (Milligan et al., 2008; Schilders et al., 2005). However, its interaction with cofactors and the exosome is not completely understood. In addition, *MPP6* null mutation affects noncoding RNA

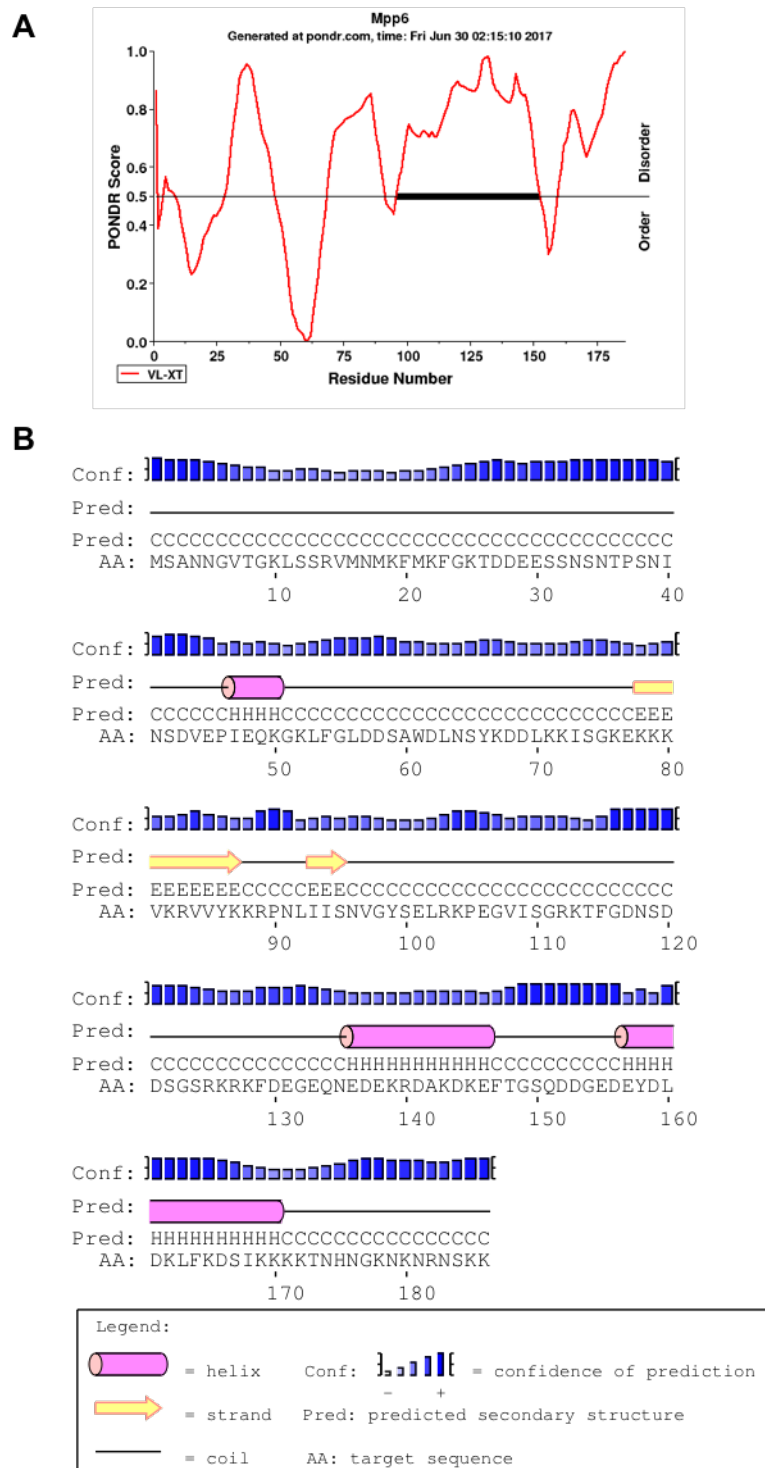


Figure 7.1. Mpp6 is a largely disordered protein.
Secondary structure analyses by POND-R (A) and PSIPRED (B) predict that Mpp6 is highly disordered.

processing, but it did not result in severe RNA processing defects perhaps because of redundancy with Rrp6 or Rrp47 (Milligan et al., 2008) (Fig. 6.6, Lane 1).

An *in vitro* reconstitution study of recombinant proteins showed that Mpp6 interacts with the exosome core but not with Rrp6 (Schuch et al., 2014). In addition, cross-linking and mass spectrometry analysis indicated that Mpp6 interacts with Rrp40, a core subunit of the exosome (Shi et al., 2015). However, another *in vitro* study suggested the interaction of Mpp6 with Rrp6, and studies of human homologs showed that Mpp6, Mtr4, and Rrp47 form a heterotrimeric complex *in vitro* (Kim et al., 2016a; Schilders et al., 2007).

In vivo functional and interaction studies may be complicated by redundant functions and interactions of cofactors. Therefore, in this study, we aim to more completely understand the interactions of the exosome with Mpp6 and investigate the functional significance of the interactions. Previous studies mentioned above showed that Mpp6 potentially interacts with both the core exosome and Rrp6 or Mtr4, and its deletion is synthetic lethal with *rrp6Δ*. Therefore, our central hypothesis is that Mpp6 mediates the interaction of the RNA exosome with its nuclear cofactors to deliver exosome substrates, and this function is largely redundant with Rrp6. Our genetic analyses suggest that the redundancy between Rrp6 and Mpp6 is the exosome/cofactor interactions. Unlike the *in vitro* reconstitution experiment, Mpp6 stably interacts with Rrp6 rather than the RNA exosome core, and the C-terminal domain of Rrp6 appears to contribute to the Mpp6 interaction. In addition, we found that the C-terminal domain of Csl4, one of the core exosome subunits, is important for the Mpp6 interaction.

RESULTS

Functional redundancy between Mpp6 and Rrp6 is recruitment of cofactors/substrates to the RNA exosome.

Synthetic lethal phenotype of *mpp6Δ* with *rrp6Δ* suggests that they are functionally redundant (Milligan et al., 2008) (Fig. 7.2). To investigate what function is redundant, we used *RRP6* alleles that are lacking interaction with either the RNA exosome or other cofactors (discussed in chapter 6). Interestingly, we found that *mpp6Δ* is synthetic lethal with the *rrp6* mutant in which Mtr4 interaction or the exosome interaction domains are deleted (Fig. 7.2). In contrast, combining *mpp6Δ* with a catalytically inactive Rrp6 was viable, although slow growing. These results are consistent with the idea that the redundant function between Mpp6 and Rrp6 is cofactor/substrates recruitment to the RNA exosome. It is still possible that the redundancy includes yet known functions since the N-terminal domain of Rrp6 appears to have more than Mtr4/Rrp47 interaction role as shown in chapter 6. Importantly, specific disruption of the Rrp6-Mtr4 interaction by point mutations in Rrp6 showed a growth defect in *mpp6Δ*, indicating that Mpp6 mediates the exosome-Mtr4 interaction redundantly with Rrp6 (Fig. 6.10A). Taken together, these data suggest that Mpp6 is an RNA exosome cofactor that mediates the interaction of the exosome with other cofactors, and that its function is redundant with Rrp6.

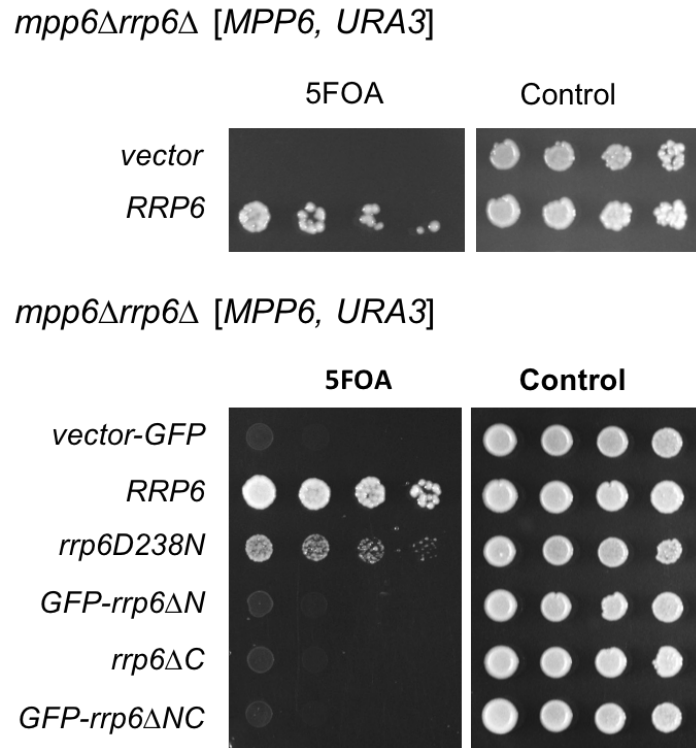


Figure 7.2. Functional redundancy between Mpp6 and Rrp6 is recruitment of cofactors/substrates to the RNA exosome. *mpp6Δ* is synthetic lethal with *rrp6Δ* (upper panel). Genetic interaction of various *RRP6* alleles with *mpp6Δ* is tested by plasmid shuffle assay.

The C-terminal domain of Rrp6 contributes to the Mpp6 interaction.

To study the *in vivo* interaction of Mpp6 with the RNA exosome or cofactors, we conducted co-immunoprecipitation (co-IP). Since Rrp6 appears to redundantly mediate the exosome/cofactor interactions, co-IP of Mpp6-TAP was conducted from strains with different *RRP6* alleles in which either the interaction with the RNA exosome or other cofactors is disrupted. Interestingly, we did not observe any Rrp44 co-purified by Mpp6-TAP in the absence of Rrp6 (Fig. 7.3, compare lane 1 and 2 on the right panel). This result suggests that Rrp6 is critical for the interaction of Mpp6 with the exosome core. In addition, the coIP of Rrp6 Δ C with Mpp6 is reduced. This suggests that either the C-terminal domain of Rrp6 itself, or the RNA exosome contributes to the Mpp6 interaction. (Fig. 7.3, compare lane 2 and 4 in the right panel). One possible explanation is that the RNA exosome core, Rrp6, and Mpp6 mutually stabilize complex formation.

To test whether Mpp6 stabilizes the Rrp6-RNA exosome interaction we use co-immunoprecipitation. In the previous co-IP experiment, deletion of *MPP6* did not affect the exosome-Rrp6 interaction (Fig. 6.2, compare lane 2 and 4 on the right panel). To further extend this result, we conducted co-IP using more stringent wash condition. TAP-tagged Csl4, one of the core subunits, was immunoprecipitated in the absence or presence of *MPP6*. Washes were performed with 50 mM NaCl (low stringency) or 500 mM NaCl (high stringency) (Fig. 7.4). Even with the more stringent co-IP, we did not observe a reproducible difference in the RNA exosome-Rrp6 interaction. This result indicates that Mpp6 is not required for the exosome-Rrp6 interaction. Taken together, these data suggest that the C-terminal domain of Rrp6 is important for both the exosome and Mpp6 interactions, and Rrp6 functions to stabilize the Mpp6-exosome interaction.

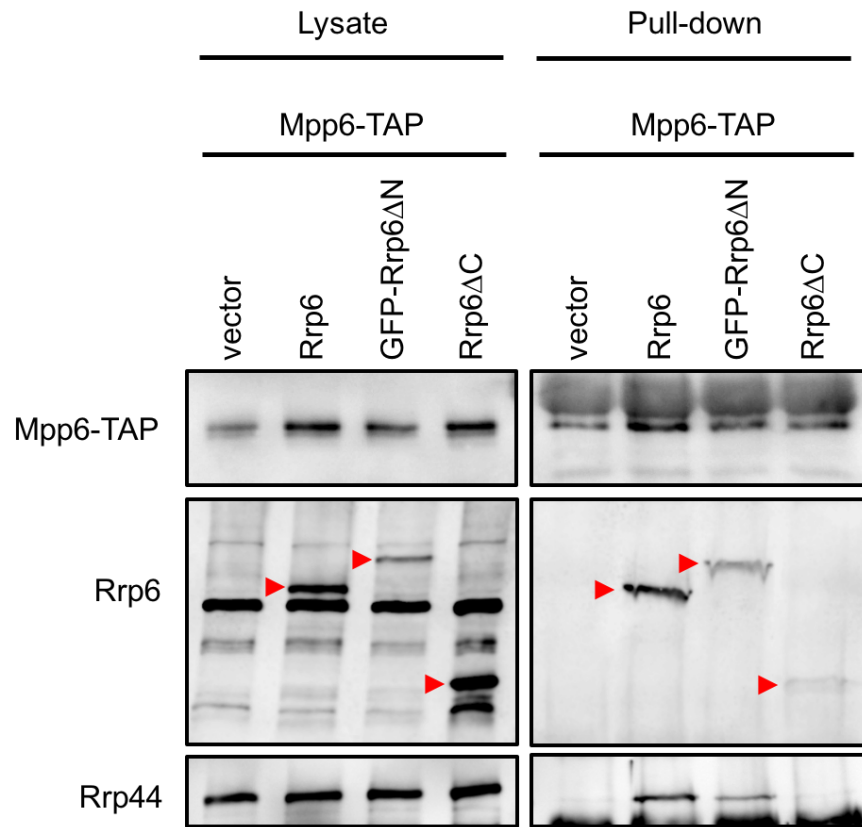


Figure 7.3. The C-terminal domain of Rrp6 contributes to the Mpp6 interaction.

Cell lysates from each strain were incubated with IgG Sepharose beads and incubated for overnight. Washed beads were loaded on SDS-PAGE for western blot.

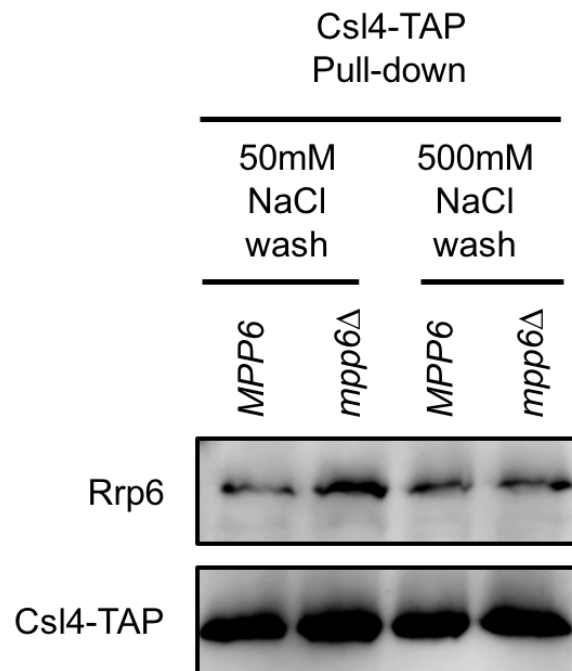


Figure 7.4. Mpp6 is not required for the exosome-Rrp6 interaction.

Csl4-TAP was immunoprecipitated using IgG Sepharose beads. After the incubation of lysates with the beads, samples were washed by either 50 mM NaCl buffer or 500 mM NaCl buffer.

Genetic interactions between *CSL4* and *MPP6*.

Although the co-IP experiment in figure 7.2 suggests that Mpp6 does not directly interact with the RNA exosome, considerable other data do support such an interaction. Specifically, Mpp6 cross-links to Rrp40, one of the core subunits (Shi et al., 2015), and purified recombinant exosome core interacts with Mpp6 (Schuch et al., 2014). Furthermore, the synthetic lethal phenotype of *mpp6Δ* with *rrp6ΔC*, in which the exosome interacting domain is deleted, suggests that Mpp6 interacts with the exosome core (Fig. 7.2).

I therefore looked for genetic interactions between mutations in Rrp6 or Mpp6 and Csl4, the only RNA exosome subunit with non-essential domains. The X-ray structure indicates that the N-terminal domain of Csl4 makes a major contribution to the Rrp6 binding site. I thus used an N-terminally truncated Csl4 to destabilize the Rrp6-Csl4 interaction (Schuch et al., 2014) (Fig. 7.5A). In parallel, we also used a C-terminal truncation of Csl4. The C-terminal domain of Csl4 is positioned between Rrp40 and the N-terminal domain of Csl4, and thus also may interact with Rrp6, Mpp6, or both. As shown before, truncation of either C-terminal or N-terminal domain did not affect the growth of yeast (Schaeffer et al., 2009). Furthermore, the expression level of the mutant Csl4 proteins is comparable to wild-type protein (Fig. 7.5B, Control and 7.5D). Interestingly, we found that the combinations of *csl4ΔC* with *rrp6Δ* and *csl4ΔN* with *mpp6Δ* were synthetic lethal, while the other combinations were not (Fig. 7.5B). This is consistent with the idea that Mpp6 becomes critical when the Rrp6 interaction with Csl4 is destabilized. Conversely, these data suggest that the C-terminal domain of Csl4 may contribute to the Mpp6 interaction site. An interaction between the C-terminal domain of

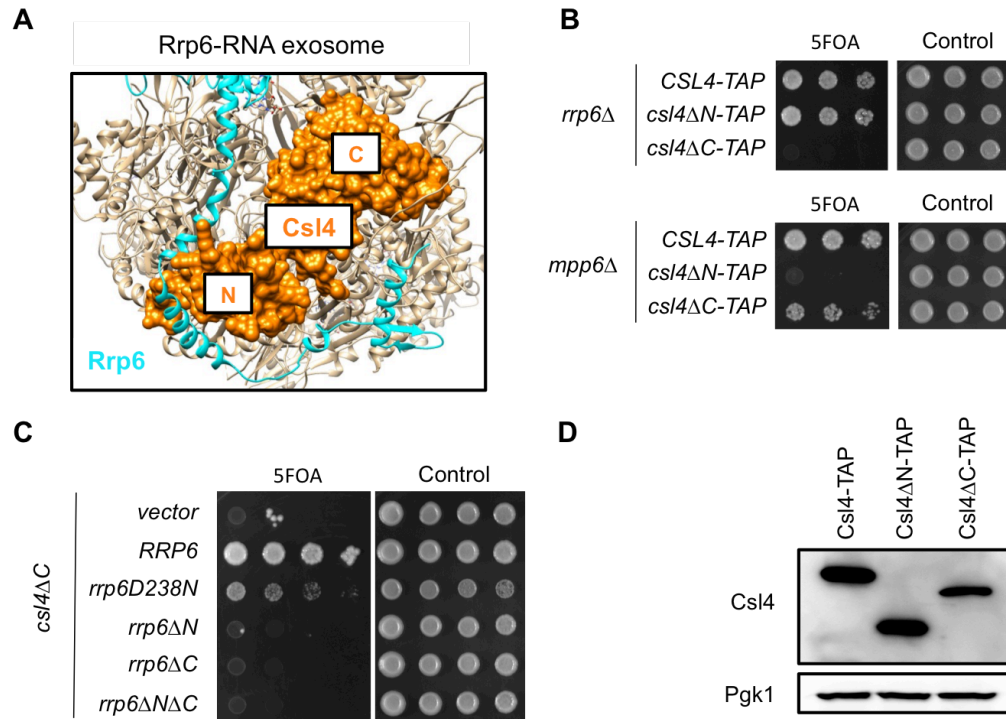


Figure 7.5. The C-terminal domain of Csl4 is important for the Mpp6 interaction.

(A) Cartoon version of the x-ray crystal structure of the RNA exosome with Rrp6 (4IFD). The C-terminal domain of Rrp6 interacts with core subunits including Csl4. (B) Top panel: *rrp6Δcsl4Δmpp6Δ* strain carrying a wild-type *CSL4* allele in a *URA3* plasmid and a wild-type *MPP6* in a *HIS3* plasmid was transformed with indicated *CSL4* alleles and spotted on media containing 5FOA; Bottom panel: the same strain used in the top panel carrying a wild-type *RRP6* instead of *MPP6* in a *HIS3* plasmid was used for the growth assay. (C) *rrp6Δcsl4Δ* strain carrying a wild-type *CSL4* allele in a *URA3* plasmid was transformed with a *csl4ΔC* allele and indicated *RRP6* alleles in *LEU2* and *HIS3* plasmid, respectively, followed by plasmid shuffle assay. (D) Expression level of Csl4 mutant proteins were tested by western blot using anti-Protein A antibody. Pgk1 was used as a loading control.

Csl4 with Mpp6 predicts that *csl4ΔC* and *mpp6Δ* might have similar genetic interactions with other mutations. To test this, we assayed for genetic interactions between *csl4ΔC* and various *RRP6* alleles. Indeed, the genetic interaction we observed are essentially identical (compare fig 7.2 and 7.5C). These data strongly indicate that the redundant function of Rrp6 with the C-terminus of Csl4 is Mpp6-mediated role. Thus, it is likely that the C-terminal domain of Csl4 interacts with Mpp6.

Since the genetic and structural analyses suggested that the N-terminal domain of Csl4 is important for Rrp6 interaction, we attempted to test *in vivo* physical interaction of Csl4 with Rrp6 (Fig. 7.6). As expected, the N-terminal truncated Csl4 did not interact with Rrp6. I also observed that the interaction of the C-terminal truncated Csl4 with Rrp6 is lost. However, both *Csl4ΔC* and *Csl4ΔN* failed to coIP Rrp44, suggesting that we were unable to immunoprecipitate a stable RNA exosome complex from these strains. This instability of the RNA exosome complex prevents us from meaningfully interpreting the failure to coIP Rrp6.

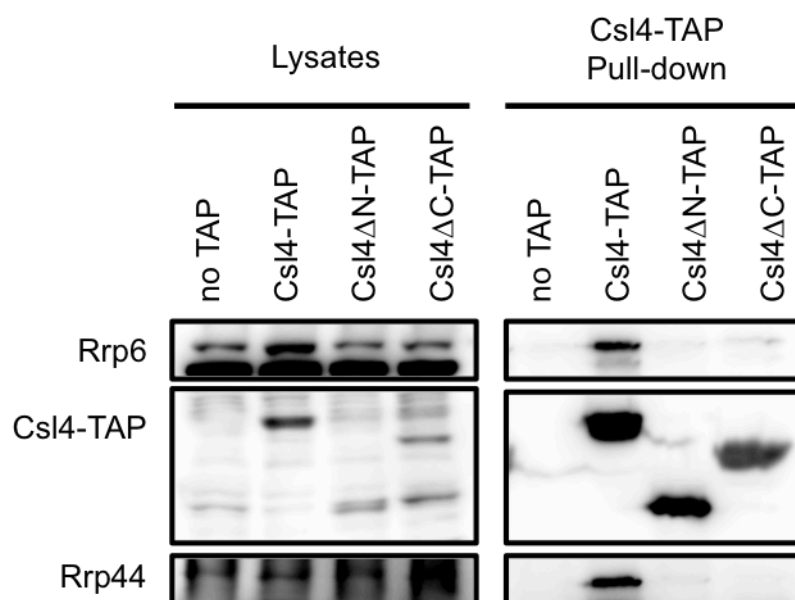


Figure 7.6. Both the N- and C-terminal domains of Csl4 are important for the Rrp6 interaction.

csl4 Δ that carries a wild-type *CLS4* or TAP-tagged *CSL4* alleles in a *LEU2* plasmid was used. Cell lysates from indicated strains were incubated with IgG Sepharose beads for overnight followed by 3 times of wash. Bound proteins were analyzed by western blot using anti-Rrp6, anti-Protein A, and anti-Rrp44 antibodies.

DISCUSSION

Mpp6 is the least understood exosome cofactor among those that are identified, perhaps partially because deletion of *MPP6* does not result in severe RNA processing defect (Butler and Mitchell, 2011; Milligan et al., 2008; Schilders et al., 2005) (Fig. 6.6, lane1). However, synthetic lethality of *mpp6Δ* with *rrp6Δ* or *rrp47Δ* suggests that Mpp6 is an important protein that functions in the RNA exosome-mediated RNA processing and degradation (Garland et al., 2013) (Fig. 7.2). In addition, it has been shown that Nrd1, one of the NNS transcription termination complex, and Rrp6 interact with Mpp6 competitively (Kim et al., 2016b). The NNS (Nrd1/Nab3/Sen1) complex terminates noncoding RNA transcription, and interacts with the TRAMP complex or Rrp6 to process noncoding RNAs (Fasken et al., 2015; Tudek et al., 2014). Considering that the NNS complex interacts with both Rrp6 and Mpp6, it is possible that Mpp6 has Rrp6-like function, which is mediating the cofactor-exosome interaction.

In this study, we found that Mpp6 physically interacts with Rrp6 *in vivo*, and the C-terminus of Rrp6 contributes to the Mpp6 interaction (Fig. 7.3). The yeast two-hybrid assay suggested that a C-terminal region of Mpp6 interacts with Rrp6 *in vivo*, and the C-terminal region is sufficient to displace the exonuclease domain of Rrp6 from Nrd1 *in vitro* (Kim et al., 2016b). This result indicates that the exonuclease domain of Rrp6 interacts with the C-terminal domain of Mpp6. Thus, together with our data, it appears that both the exonuclease domain and C-terminus of Rrp6 contributes to the Mpp6 interaction. Partial loss of Mpp6 interaction of Rrp6ΔC could be because the interaction of the exonuclease domain of Rrp6 with Mpp6 is still retained (Fig. 7.3).

Although *in vitro* analyses showed that Mpp6 directly interacts with the exosome core, our *in vivo* pull-down experiment indicates that Rrp6 is required for the stable Mpp6-exosome interaction (Schuch et al., 2014; Shi et al., 2015) (Fig. 7.3). Moreover, our genetic analyses of *mpp6Δ* with various *rrp6* mutants strongly suggest that Mpp6 mediates the interaction of cofactors/RNA substrates with the RNA exosome independent of Rrp6. In addition, genetic interactions suggest that Mpp6 interacts with the exosome core via the C-terminal domain of Csl4. *In vivo* and *in vitro* co-IP experiments that test the interaction of Csl4ΔC with Mpp6 would provide valuable information about the Csl4-Mpp6 interaction. It is possible that the Mpp6-exosome interaction is transient and only occurs under certain circumstances such as when substrates are present. Because Mpp6 appears to consist mainly of intrinsically disordered regions it may simultaneously interact with Csl4, Rrp40, Rrp6 and perhaps other proteins. It has been shown that RNase treatment reduces co-purification of Rrp44 when Nrd1 is immunoprecipitated (Kim et al., 2016b). Since one way that Nrd1 interact with Rrp44 is through Mpp6, it is possible that the Mpp6-exosome core interaction is substrate dependent. However, the above mentioned biochemical study did not involve RNA substrates since they used highly purified proteins (Schuch et al., 2014). I speculate that inconsistencies we observe between *in vitro* and *in vivo* studies could be due to absence or presence of other Mpp6 interacting proteins. Since Mpp6 interacts with multiple RNA exosome cofactors, some interactions would occur only transiently when it is required. In addition, competitive interactions among cofactors could hinder detection of stable *in vivo* interaction in co-IP. Finally, Mpp6 is a heavily phosphorylated protein with at least 9 different phosphorylation sites detected by mass spec (see yeastgenome.org, unipep.org and

phosphogrid.org) suggesting that its interactions may be regulated *in vivo* in ways that are not reflected in studies with recombinant proteins.

Functional analysis of Mpp6 is complicated because *mpp6Δ* does not result in significant RNA processing defects due to its functional redundancy with Rrp6. In addition, disruption of those redundant functions by also deleting other cofactors such as Rrp6 results in lethality (Fig. 7.2), making it impossible to assay individual RNA processing/degradation reactions. Therefore, conditional inactivation of *RRP6* or *MPP6* allele should prove useful to examine RNA processing defects of mutant strains, and it would provide a better understanding Mpp6 function. Furthermore, mapping Mpp6 residues that are important for specific interactions with Nrd1, Rrp6, Mtr4, and the exosome core would help to understand the complex network of the exosome cofactor interactions. The identification of multiple synthetic lethal interactions here should facilitate creating *MPP6* alleles that disrupt individual physical interactions

In summary, this study suggests that Rrp6 and Mpp6 redundantly mediate the RNA exosome-cofactor interactions. It appears that Mpp6 stably interacts with Rrp6, while it may more transiently interact with the exosome core *in vivo*, likely in part through the C-terminal domain of Csl4 (Fig. 7.7). As discussed in chapter 6, the RNA exosome-cofactor interactions are largely redundant, and this study provides further insight into the redundancies between Rrp6 and Mpp6. Further investigation how Mpp6 interacts with the exosome and other cofactors such as Mtr4 would give a clear picture of the RNA exosome function in the nucleus.

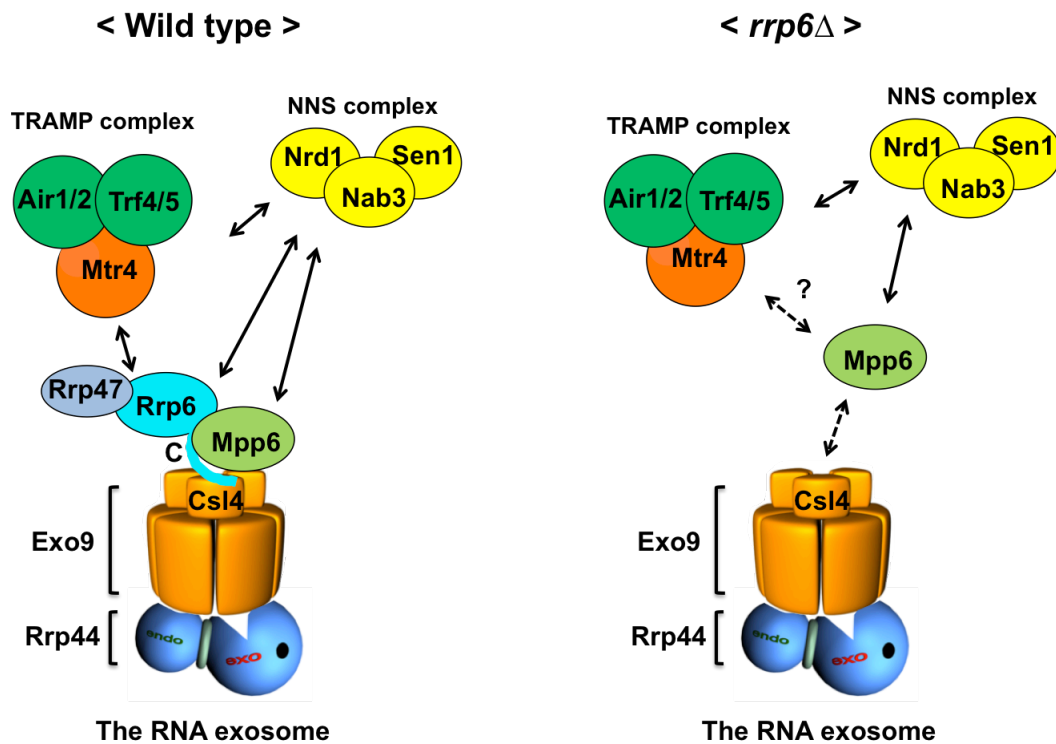


Figure 7.7. Models of the RNA exosome-cofactor interactions.

Mpp6 stably associates with Rrp6 by binding to the catalytic domain and the C-terminal domain of Rrp6. In the absence of Rrp6, Mpp6 could mediate the interaction of other cofactors such as the NNS complex with the RNA exosome.

Chapter 8: Final Conclusions and Perspectives

SUMMARY

The RNA exosome is an essential ribonuclease complex that functions both in the nucleus and cytoplasm. Its dysfunction is associated with many human diseases such as neurodegenerative disorders and cancers (Boczonadi et al., 2014; Di Donato et al., 2016; Fabre and Badens, 2014; Hoskins et al., 2016a; Wan et al., 2012; Weissbach et al., 2015). However, how its dysfunction contributes to the diseases is currently unclear. Therefore, a thorough investigation of the RNA exosome function is of high importance.

In this study, we showed that there are at least two different RNA exosome conformations present *in vivo*, and they have distinct functions (Han and van Hoof, 2016). There was some indirect evidence that suggested different substrate recruitment route to the catalytic subunit, Rrp44, in the RNA exosome. For example, *in vitro* transcribed tRNAi^{Met} directly binds to Rrp44 (Schneider et al., 2007). In addition in CRAC (crosslinking and cDNA analysis), snoRNAs are cross-linked more efficiently to Rrp44 compared to the core subunits experiment, suggesting that they may bind to Rrp44 and bypass the core (Schneider et al., 2012). Furthermore, x-ray crystallography and single particle EM showed that a single strand RNA could be directly recruited to Rrp44 bypassing the central channel of the exosome (Liu et al., 2014; Makino et al., 2013a). However, evidence of the presence of an RNA exosome conformation that directly recruits substrates to Rrp44 in the cell was lacking. Here, we provide the first *in vivo* evidence that the Rrp44^{da} (direct access)-exosome is present. Considering that the Rrp44^{da}-exosome is specifically required for the degradation of hypomodified tRNAi^{Met} and truncated 5S rRNA, which are aberrant RNAs, it is possible that Rrp44^{da}-exosome is involved in nuclear RNA surveillance. In addition, since these substrates are highly

structured, the Rrp44^{da}-exosome would be required for initial trimming of short single stranded 3' end of highly structured substrates. Furthermore, depending on the secondary structure of RNA, both the Rrp44^{da} and Rrp44^{ch}-conformations would be required for degradation. Our initial RNA sequencing analysis suggests that Rrp44^{da}-conformation is important for snoRNA processing, and further identification of specific substrates would provide insight into how the two conformations of the RNA exosome function in cell.

While the RNA exosome alters its conformation to deal with RNA substrates with vastly different characteristics once delivered, cofactors are required to recruit the exosome to specific substrates (Butler and Mitchell, 2011). Multiple nuclear and cytoplasmic cofactors of the exosome have been identified, and thorough *in vitro* analyses of recombinant cofactors have identified how the exosome cofactors interacts with the RNA exosome or other cofactors (Kowalinski et al., 2016; Makino et al., 2015; Schuch et al., 2014; Wasmuth et al., 2014; Zinder et al., 2016). Rrp6 is a nuclear cofactor of the exosome that has both catalytic and structural roles (Butler and Mitchell, 2011). However, the functional significance of the interactions remains to be elucidated. It has been shown that Rrp6 interacts with the exosome via its C-terminal domain, and with Mtr4, an RNA helicase, through its N-terminal domain (Schuch et al., 2014). It appears that Mtr4 interacts with the RNA exosome through Rrp6 as Rrp6 functions as an adaptor protein that mediates the Mtr4-exosome interaction. However, a viable phenotype of a *RRP6* null strain led us to ask whether there are Rrp6-independent interactions between Mtr4 and the RNA exosome. To test this, we thoroughly investigated the Rrp6-Mtr4 and Rrp6-exosome interactions. I show that Rrp6 functions beyond known biochemical and structural interactions, and Mpp6 functions redundantly with Rrp6 to mediate interaction

of the RNA exosome with other cofactors such as Mtr4. Therefore, we propose that the cofactor-exosome interactions are largely redundant, and the redundancy ensures the proper processing and degradation of RNAs that are critical for cellular functions.

In conclusion, this study improved our understanding in the RNA exosome function. I show that the RNA exosome alternates its conformation to deal with RNA substrates with vastly different characteristics. I also found that the exosome cofactors, Rrp6 and Mtr4, function beyond known biochemical and physical functions. Moreover, the exosome-cofactor interactions are largely redundant, and the redundancy is partly mediated by Rrp6 and Mpp6. To completely understand the function of the RNA exosome, there are still numerous questions to be answered. The following discussion will include some future directions for this study that would further improve our knowledge in the function of the exosome.

Two conformations of the RNA exosome

Identification of additional substrates of the Rrp44^{da}-exosome

Northern blot and RNA sequencing analyses showed that the Rrp44^{ch}-exosome globally regulates a variety of transcripts, while the Rrp44^{da}-exosome is required for processing or degradation of specific substrates such as hypomodified tRNA^{iMet}, truncated 5S rRNA, and snoRNAs (Chapter 3 and 4). Technical limitation of northern blot has been overcome by the RNA sequencing analysis, which allows a global insight into the transcriptome. RNA sequencing identified a limited set of mRNAs and CUTs that were affected by *rrp44-da*, and future experiments could focus on what differentiates these substrates from other mRNAs and CUTs. Using RNA sequencing, we also show

that snoRNAs are specific substrates of Rrp44^{da}-exosome. A recent CRAC study also suggested that snoRNAs use the direct access conformation of the RNA exosome (Delan-Forino et al., 2017). While CRAC and RNA sequencing identified snoRNAs as specific substrates of the Rrp44^{da}-exosome, it is still possible that many other substrates are missing in the analyses. The Rrp44^{da}-exosome appears to be important for processing or degradation of small RNA species such as tRNA, 5S rRNA, and snoRNAs, and small RNAs are likely missed in total RNA sequencing analyses due to masking effects by longer transcripts. In addition, transcripts that are not abundant could be lost among the more abundant transcripts. Especially, the CRAC analysis relies on RNA crosslinking to proteins, the concentration of proteins may be limited, U-rich RNAs should be more readily recovered in CRAC and different proteins cross-link with different efficiencies, possibly due to the amino acid residues in close proximity to U residues. Thus, both RNA seq and CRAC may have missed some substrates.

Therefore, it is necessary to overcome the limitations described above to identify specific substrates of Rrp44^{da}-exosome. For this, I propose additional RNA sequencing strategies. In chapter 3, we depleted rRNA by Ribozero and sequenced the remaining RNA. Small RNA sequencing analysis or poly(A) selection would also identify another subset of RNA exosome substrates. In either approach RNA from *rrp44-da* and *rrp41-L* mutant strains and a wild-type strain will be isolated. RNA will then be further purified by oligo(dT) selection or by gel electrophoresis and excising RNA species shorter than 300 nt. Synthetic spike-in set of small RNAs can be used as control for normalization. This analysis would identify known substrates of the Rrp44^{da}-exosome such as snoRNAs, which would validate the approach. In addition, I expect to identify novel substrates of

the Rrp44^{da}-exosome. Moreover, in combination of the previous studies that identified the specific substrates of Rrp44^{da} and Rrp44^{ch}-exosome, it will provide a further insight how the two conformations of the RNA exosome functions in cell.

One potential pitfall of this approach is that the Rrp44^{da}-exosome is redundant with Rrp6, and the redundancy could hinder the identification of specific substrates. Therefore, to remove the redundancy, strains with a catalytically inactive Rrp6 (i.e. *rrp6-D238N* and *rrp6-D238N rrp44-da* strains could be included in the RNA sequencing analyses. Another pitfall is that some of the effects of the *rrp44-da* mutation may not be linked to the effect on conformation. Enzymatic analysis of Rrp44-da indicates it is less active than wild-type Rrp44 (John Zinder and Chris Lima, Pers. Comm.). The Lima lab has identified a mutation in Rrp43 that, like *rrp44-da*, destabilizes the direct access conformation. My unpublished data show that like *rrp44-da* this *rrp43-ΔL1* mutation suppresses the growth defect of *rrp41-L*. Future transcriptomic analysis could also include this *rrp43-ΔL1* mutation. These approaches would allow a near complete identification of Rrp44^{da}-exosome substrates, and it will provide a better understanding in the function of the direct access conformation.

Determine whether the mutations identified in cancers affect the direct access conformation

In the chapter 3, we have suggested that mutations identified in multiple myeloma patients may be associated with the dysfunction of the direct access path of the RNA exosome because only hDis3 (a Rrp44 homolog) is mutated in many cancers. Although there are dozens of the exosome mutations identified in multiple myeloma, they are only

located in Rrp44 (Weissbach et al., 2015). Several identical mutations in yeast Rrp44 yielded slow growth of yeast and showed synthetic growth defect with *rrp6Δ* (Tomecki et al., 2014). In addition, these mutations affected exonuclease activity of Rrp44. Specifically, yeast strains that carry the disease mutations accumulated the full-length 5'ETS (5'External Transcribed Spacer), which is similar to what I showed for the *rrp44-da* mutation exosome. Therefore, the accumulation of the known Rrp44^{da} substrate and the complete segregation of cancer mutations in Rrp44 from the other exosome subunits suggests that those mutations may specifically affect the function of the Rrp44^{da}-exosome. To test this, disease mutations could be introduced into the yeast Rrp44. If the disease mutations are associated with the Rrp44^{da}-exosome, it is expected that mutations only show synthetic phenotype with *rrp6Δ* since the Rrp44^{da}-exosome only functions in the nucleus. In addition, we showed that the Rrp44^{da}-exosome is specifically required for the degradation of hypomodified tRNA^{iMet} and truncated 5S rRNA (Han and van Hoof, 2016) (Chapter 3). To test whether the cancer mutations of Rrp44 affect the Rrp44^{da}-exosome function, the mutant constructs could be introduced into the *trm6-504 gcn2 rrp44Δ* strain, and degradation of hypomodified tRNA^{iMet} and truncated 5S rRNA could be analyzed by growth assay and northern blot as described in chapter 3. Moreover, the genetic interaction of the mutant constructs with *rrp41-L* can be tested because we observed that *rrp44-da* suppressed the growth defect phenotype of *rrp41-L*.

The above described experiments will determine if the mutations identified in multiple myeloma affect the Rrp44^{da}-exosome. It is still unclear whether those mutations identified in multiple myeloma are driving mutations or not. However, if they affect the function of the Rrp44^{da}-exosome, it would provide a novel strategy to treat multiple

myeloma as suggested previously (Tomecki et al., 2014). Since the inhibition of endonuclease activity significantly reduce the growth of *rrp44-da* strain, in which the Rrp44^{da} formation is disrupted, compounds that inhibit the endonuclease activity would specifically reduce the growth of cells carrying the *RRP44* mutations (Han and van Hoof, 2016) (Chapter 3). Alternatively it might be possible to develop drugs that restore the balance between the channel and direct access conformations of the mutated human RNA exosome. Moreover, with identification substrates of the Rrp44^{da}-exosome, this study would provide a further understanding in the function of the RNA exosome and its association with cancers such as multiple myeloma and acute myeloid leukemia.

Identify the suppressors of the growth phenotype of *rrp41-L*

In the chapter 3, we unexpectedly found that the slow growth phenotype caused by disruption of the Rrp44^{ch}-exosome in *rrp41-L* was suppressed by disruption of the Rrp44^{da}-exosome, *rrp44-da*. I speculate that the balance between the two conformations is important for the optimal growth of the RNA exosome. However, none of the RNA processing defects of *rrp41-L* we analyzed are reverted back to normal by introducing *rrp44-da*, although a slight alleviation was seen in RNA sequencing analysis (chapter 4). How the growth phenotype of *rrp41-L* is suppressed by *rrp44-da* is largely enigmatic.

I expect that investigation of this result would further improve our understanding in the RNA exosome function. Since that *rrp41-L* is lethal at 37°C and *rrp44-da* suppressed this lethality, we can use a suppressor screen to identify mutations that allow the growth of *rrp41-L* at 37°C. For this, a *rrp41Δ* strain that carries *rrp41-L* will be incubated at 30°C for over dozens of generations to accumulate spontaneous mutations.

The resulting cells will be plated on solid media followed by incubation at 37°C. Cells that grow in the media would carry mutations that suppresses the phenotype of *rrp41-L*. Viable cells can be backcrossed to a wild-type strain of yeast multiple times to remove background mutations, and the resulting yeast strains will be subjected to whole genome sequencing. I expect that mutations in *RRP44* that disrupt the Rrp44^{da}-exosome would be isolated, which would validate the approach. Identified mutations in selected genes can be individually validated by introducing the mutations into *rrp41-L*. This is expected to find factors that enhance or activate the Rrp44^{da}-exosome function. It is hard to predict what genes would be identified in the screening, but it could be known exosome cofactors or novel factors. Therefore, by identifying suppressors of the growth phenotype of *rrp41-L*, this study would provide further knowledge in the function of the Rrp44^{da}-exosome and its relationship with Rrp44^{ch}-exosome.

Function of Rrp6

Determine how Rrp6 localizes to nucleus

Since Rrp6 is a nuclear cofactor of the RNA exosome, it is expected to be critical for Rrp6 to localize in the nucleus for its proper functions. In the chapter 6, we suggested that three signals, the NLSs of Rrp6, Rrp47, and the RNA exosome, redundantly mediate the nuclear localization of Rrp6. I show that GFP-Rrp6 Δ N Δ C is diffuse in the cytoplasm, indicating that either N- or C-terminal domains are important for the nuclear localization. However, either deletion of the N-terminal domain or deletion of the exosome association region (EAR) in the C-terminus did not affect the Rrp6 localization. In addition, simultaneous deletion of the C-terminal EAR and NLS (GFP-Rrp6 Δ EAR Δ NLS) did not

result in mislocalization of Rrp6. Thus, we concluded that the exosome/cofactor interaction and NLS are redundant in the nuclear localization of Rrp6. Mislocalization of GFP-Rrp6 Δ EAR Δ NLS in *rrp47* Δ and in *rrp47*- Δ NLS supports this conclusion because Rrp47 interacts with the N-terminus of Rrp6 in the nucleus. Therefore, the nuclear localization of Rrp47 and the interaction of Rrp47 with Rrp6 is critical for the localization of GFP-Rrp6 Δ EAR Δ NLS.

The nuclear localization could be differentially regulated by three signals, the interaction with Rrp47/exosome and NLS. To test this, dynamics of Rrp6 localization using mutant Rrp6 proteins in which each signal is deleted could be tested by FRAP (Fluorescence Recovery After Photobleaching) (Jacobson et al., 1976). This experiment would allow us to understand how those three signals regulate the localization of Rrp6.

Determine the *in vivo* function of Rrp6 lasso

A recent structural and biochemical analyses showed that the C-terminal domain of Rrp6 carries multiple functions (Wasmuth and Lima, 2017). It contains the exosome association region (EAR) and a region called “lasso” that enhances the *in vitro* activity of the RNA exosome. However, the *in vivo* function of the lasso is currently unclear. Our genetic analyses in the chapter 6 suggested that the C-terminal domain of Rrp6 has multiple functions since restoring the exosome interaction of Rrp6 Δ C by the C-terminal fusion of the exosome interacting domain of Ski7 (Rrp6 Δ C_Ski7-116-226) did not fully complement the phenotype of *rrp6* Δ C. Physical interaction of the Rrp6 Δ C_Ski7-116-226 with the RNA exosome was fully restored, indicating that the C-terminal domain of Rrp6 has other functions than the exosome interaction. I expect that the functions that are

defective in Rrp6 Δ C_Ski7-116-226 is attributed to the lasso. Those functions are redundant with *mpp6* Δ and the Arch domain of Mtr4 as *rrp6* Δ C_Ski7-116-226 was synthetic lethal with *mpp6* Δ and *mtr4-archless*, respectively. Since we show that the Arch domain of Mtr4 potentially interacts with the RNA exosome, and Mpp6 mediates the exosome-cofactor interactions, it is possible that the lasso is related to the interaction of other cofactors.

First, we could test whether the disrupted function of Rrp6 Δ C_Ski7-116-226 is attributed to lasso of Rrp6. For this, the lasso would be fused to Rrp6 Δ C_Ski7-116-226. *rrp6* Δ C_Ski7-116-226_lasso could be tested for its genetic interaction with *mpp6* Δ or *mtr4-archless*. If the *rrp6* Δ C_Ski7-116-226_lasso is a fully functional *RRP6* allele, it will suggest that the function of lasso is redundant with Mpp6 and the Arch domain of Mtr4. I could further generate a *rrp6* Δ lasso allele to confirm the genetic analyses.

The co-IP experiment in the chapter 6 showed that the interaction of Mpp6 with Rrp6 is reduced when the C-terminal domain of Rrp6 is deleted. It is possible that the lasso is important for the Mpp6 interaction. Therefore, we could also test the physical interaction of Rrp6 Δ lasso by pulling down Mpp6-TAP followed by western blot using anti-Rrp6 antibody. In summary, experiments described above will determine the *in vivo* function of lasso, and it would provide further understanding in the Rrp6 that carries multiple functions in the nucleus.

Determine the function of the N- and C-termini of Rrp6 by RNA sequencing

In the chapter 6, we show that the N- and C-terminal domains of Rrp6 function beyond known structural and biochemical roles. While the genetic analyses provide

valuable information regarding the presence of novel functions of the N- and C-termini of Rrp6, it is still unclear what those functions are. Since *rrp6* Δ *N* is synthetic lethal with *mtr4* Δ *N*, isolating point mutations in the Rrp6 N-terminus that are synthetic lethal with *mtr4* Δ *N* allows identification of important regions in the N-terminal domain of Rrp6. For this, the *rrp6* mutant library that contains various mutations in the N-terminal domain could be generated by error-prone PCR. The library would be introduced into the *mtr4* Δ *N* strain that carries a wild-type *RRP6* allele in a URA3 plasmid. The resulting transformants can be replica plated on control selective media that allow growth of transformants and 5-FOA for a synthetic lethal screen. Plasmids can be isolated from transformants that shows synthetic lethal phenotype with *mtr4* Δ *N* followed by sequencing to identify mutations. To test what function of Rrp6 is disrupted in isolated mutants, growth, 5-FU sensitivity, and cofactor/exosome interaction would be tested followed by functional analysis using northern blot.

Identification of RNA substrates that accumulate in *rrp6* Δ *N* or *rrp6* Δ *C* should provide insight into the function of the deleted regions. I already tested accumulation of several known substrates, but northern blot is technically limited. Therefore, to determine the novel functions of the N- and C-termini of Rrp6 we could conduct RNA sequencing analyses. To isolate known functions of N or C-terminal domains which is the Mtr4 interaction and the exosome interaction, respectively, we could include Rrp6 mutants in which the Mtr4 or the exosome interaction is specifically disrupted. Thus, a wild-type *RRP6*, *rrp6-I14E_R18E* (disruption of the Mtr4 interaction), *rrp6* Δ *C*, *rrp6* Δ *EAR*, and *rrp6* Δ *lasso* should be included in the sequencing analyses. In addition, novel *rrp6* mutants that are isolated by the experiment in the previous paragraph should be included.

The functional redundancy of Rrp6 with Mpp6 also would mask the effect of mutations. Functional redundancy with Mpp6 in mediating the cofactor-exosome interactions can be removed by conditional depletion of *MPP6* using a GAL promoter. As a result, we would expect to find specific substrates accumulation of mutant strains, and it would identify functions of the N- and C-termini of Rrp6. In addition, in combination with the genetic analyses of the *rrp6Δlasso* mutant in the previous aim, we would expect to determine the *in vivo* function of the lasso as well. Moreover, we will be able to determine the redundant function of Rrp6 with Mpp6. Therefore, these experiments would thoroughly elucidate the specific functions of Rrp6, and we also would be able to determine the *in vivo* function of Mpp6, which is normally undetected in *mpp6Δ* due to the redundancy with Rrp6.

Function of Mpp6

Determine whether How Mtr4 interacts with the RNA exosome and Mpp6

The results in chapters 6 and 7 suggest that Mpp6 may redundantly mediate the cofactor and exosome interactions with Rrp6. The synthetic lethal phenotype of *rrp6ΔN*, in which the Mtr4 interaction is disrupted, with *mpp6Δ* and the synthetic growth defect of *rrp6-I14E_R18E*, in which the Mtr4 interaction is specifically disrupted, with *mpp6Δ* suggest

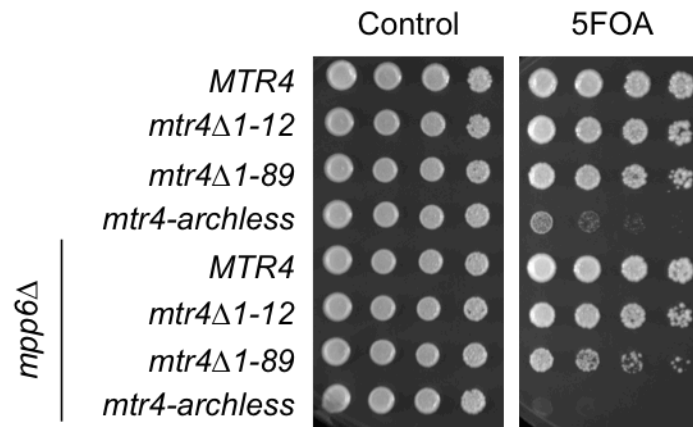


Figure 8.1. Genetic interaction of *mpp6Δ* with *mtr4* mutants.

The *mtr4Δmpp6Δ* strain that carries *MTR4* in a *URA3* plasmid was transformed with *mtr4* mutant plasmid and a wild-type *MPP6* or a vector control. Resulting transformants were serially diluted and spotted on media containing 5FOA.

that Mpp6 interacts with Mtr4. However, a direct interaction of the two proteins has not been shown. A preliminary genetic experiment showed that *mtr4Δ1-89*, in which the Rrp6 interaction is disrupted, shows a growth defect with *mpp6Δ*, and it has a synthetic lethal phenotype with *mtr4-archless* (Fig. 8.1). This result suggests that the Arch domain of Mtr4 is redundant with Mpp6, indicating that the Mtr4 interacts with the RNA exosome independent of Mpp6. It is particularly interesting because Mtr4-archless is still able to interact with Rrp6. The Arch domain of Mtr4 interacts with Nop53 and Utp18 for ribosomal RNA processing and degradation (Thoms et al., 2015). Thus, it is possible that Mpp6 interacts with those Arch-interacting proteins for ribosomal RNA processing. In addition, the synthetic growth phenotype of *mtr4Δ1-89* with *mpp6Δ* suggests that Mpp6 may mediate interaction of Mtr4 with the RNA exosome. Moreover, the fact that *mtr4Δ1-89* is not synthetic lethal with *mpp6Δ* indicates that Mtr4Δ1-89 still is able to interact with the RNA exosome or Rrp6. This interpretation is consistent with our previous prediction that the Arch domain interacts with the exosome independent of Rrp6.

Taken together, data suggest that there are multiple interactions of Mtr4 with Rrp6 or the RNA exosome. It appears that the Mtr4-exosome interaction could be mediated by Rrp6, Mpp6, or the Arch domain of Mtr4. However, the details of the interactions are missing. Therefore, Mtr4-exosome interaction should be thoroughly investigated. For this, Mtr4, Mtr4Δ1-12, Mtr4Δ1-89, and Mtr4-archless proteins can be immunoprecipitated using anti-Mtr4 antibody. Pull-down fractions could be analyzed by western blot to test the interaction of the Mtr4 proteins with Rrp6, Mpp6, and Rrp44, one of the core subunits. Based on our genetic analyses, we expect that Mtr4Δ1-12 and Mtr4Δ1-89 still retain their interactions with Rrp6 or the RNA exosome. This would be

striking since the deleted N-terminus in Mtr4 Δ 1-89 is the only known interaction site of Mtr4 with Rrp6.

In addition, we show that the N-terminal 89 residues of Mtr4 have functions other than the Rrp6 interaction since the deletion N-terminal was synthetic lethal with disruption of the Mtr4 interaction in Rrp6 (Chapter 6). The unknown function could be the interaction with Mpp6, as Mpp6 appears to redundantly mediate the interaction of cofactors with the RNA exosome. A slight growth defect, but not synthetic lethality, of *mtr4 Δ 1-89* with *mpp6 Δ* supports this conclusion that the function of Mpp6 may be the interaction with the N-terminal region of Mtr4. Thus, we expect that Mtr4 Δ 1-12 would still retain its interaction with Mpp6, while Mtr4 Δ 1-89 shows reduced interaction. Therefore, the experiment would provide a thorough understanding of Mtr4 interactions with other exosome cofactors such as Rrp6 and Mpp6, and the RNA exosome core. As we discussed in the chapter 6, the cofactor-exosome interactions appear to be largely redundant, and it would ensure the proper RNA processing and degradation that are critical processes for life.

Identification of regions in Mpp6 that are important for its interaction with the RNA exosome and cofactors

Our data suggest that Mpp6 interacts with multiple proteins including Mtr4, Rrp6, and the RNA exosome (Chapter 6 and 7). This raises the question of how Mpp6 interacts with other proteins. Mpp6 is 186 amino acid residues long and rich in basic residues (23% K and R), suggesting a possible interaction with nucleic acids. It has been shown that the C-terminal region of Mpp6 interacts with Rrp6, but its interaction with other proteins is

largely unclear (Kim et al., 2016b). Therefore, the interaction of Mpp6 with Rrp6, Mtr4, and the RNA exosome should be thoroughly investigate. because Mpp6 lacks recognizable domains but instead may be largely intrinsically disordered a series of N- and C-terminal truncations could be constructed. Genetic interactions of these *mpp6* mutants with *rrp6Δ* and *csl4ΔN* should be tested since *rrp6Δ* and *csl4ΔN* showed synthetic lethality with *mpp6Δ*. It would allow us to determine what regions in Mpp6 are redundant with Rrp6 or the N-terminal domain of Csl4. As a result, we would be able to determine where in Mpp6 Rrp6 or the RNA exosome interact. Subsequent *in vivo* pull-down experiments using TAP-tagged version of Mpp6 mutants would test the physical interactions of Mpp6 with Rrp6 or the RNA exosome. Therefore, the experiments described above should determine how Mpp6 interacts with cofactors and the RNA exosome. Mpp6 is the least understood exosome cofactor, yet is conserved between yeast and humans, and this further characterization will provide valuable insight into how the RNA exosome interacts with its cofactors and functions in the cell.

CONCLUDING REMARK

In this study, we show that there are at least two different conformations of the RNA exosome present *in vivo*, and they have distinct functions. One conformation utilizes the central channel of the RNA exosome to recruit RNA substrates, while the other directly recruits substrates to the catalytic subunit, Rrp44. I show that the RNA exosome alters its conformations to deal with specific substrates. In addition, the balance between the two conformations appears to be critical for the optimal function of the RNA

exosome. This study also thoroughly investigated the interaction of the RNA exosome with its nuclear cofactors and identified novel genetic interactions. The results indicate that the cofactor-exosome interactions are largely redundant, and we speculate that redundancy of the important interactions ensure proper RNA processing or degradation that are critical for normal cellular functions. However, specific interaction sites may be important for specific functions.

Dysfunction of the RNA exosome is associated with many different human diseases such as neurodegenerative disorders, cancers, and syndromic diarrhea. Thus, the thorough understanding of the RNA exosome function is of high importance. This study clearly improved our understanding how the RNA exosome deals with RNA substrates with vastly different characteristics and how it interacts with its cofactors to specifically processes or degrades RNA substrates. Moreover, this study will serve as a foundation stone to further help to improve our understanding in the RNA exosome.

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