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Characterization Of The Rna Binding And Rna Degrading Subunits Of The Eukaryotic Exosome

Borislava Tsanova

Characterization of the RNA binding and RNA degrading subunits of the eukaryotic exosome

by

Borislava B. Tsanova, B.S.

APPROVED: _____
Supervisory Professor, Ambro van Hoof, Ph.D.

_____
Theresa M. Koehler, Ph.D.

_____
Michael C. Lorenz, Ph.D.

_____
Ann-Bin Shyu, Ph.D.

_____
Eric J. Wagner, Ph.D.

APPROVED: _____
Dean, The University of Texas
Graduate School of Biomedical Sciences at Houston

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Borislava B. Tsanova B.S.

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DEDICATIONS

*I dedicate this work to my husband **Maxim Maximov**. I could not have done it without his support.*

*I also dedicate it to my two wonderful daughters **Ivana** and **Andrea** whom I love with all of my heart.*

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Characterization of the RNA binding and RNA degrading subunits of the eukaryotic exosome

Borislava B. Tsanova, B.S.

Supervisory Professor, Ambro van Hoof, Ph.D.

The exosome is an essential complex of ten proteins involved in the processing and degradation of many RNAs in the cell. These include various stable RNAs, mRNAs, and aberrant transcripts both in the nucleus and in the cytoplasm.

In this work I characterize the three members of the exosome “cap”, the RNA binding proteins Rrp4, Rrp40, and Csl4. I determine that in spite of their structural similarity, they each have a unique essential role. Second, I determine that two of the cap proteins Rrp4 and Rrp40 have a role in bridging subunits of the PH ring of the exosome. The third cap protein Csl4 was shown not to have a bridging role. Further, I show that stable interaction with the exosome is not required for the essential role of Csl4.

In addition, I look at the physiological importance of the nuclease domains of the exosome. I show that the exonuclease is the primary activity for the exosome and that in its absence the iron starvation response of the cell is activated.

Despite numerous studies of the exosome, only a few address the physiological relevance of the catalytic activity. This work shows that the exonuclease of the exosome is important for oxidative stress protection and affects glucose metabolism

and provides a basis for future research into the physiological importance of this essential RNase.

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Chapter 1: Introduction, background, and significance

INTRODUCTION AND BACKGROUND

RNA degradation and processing

RNA degradation is used by all cells for applying temporal control of gene expression as well as maintaining its fidelity by eliminating aberrant transcripts. RNAs are degraded either at the end of the RNA life or if they are aberrant. Such aberrant RNAs may result from genetic mutations, such as nonstop mutations or mistakes in processing the RNA, such as hypomodification of tRNAs. In each case, however, the persistence of the aberrant RNA will lead to errors in gene expression, thus the degradation serves a role as a surveillance mechanism to preserve the fidelity of gene expression.

The initial transcription of RNAs results in a primary RNA transcript. The primary RNA transcript is then processed and sometimes edited to produce the final mature RNA transcript. RNA processing refers to the addition or removal of components of the RNA. Processing reactions include shortening of the primary RNA transcript from either the 5' or the 3' end and in the case of mRNAs the addition of a 5' 7-methyl guanosine cap, addition of a 3' poly(A) tail, and RNA splicing to remove introns (Lodish, 2000).

Often these two processes, RNA degradation and RNA processing are carried out by the same enzyme. However the recognition of a transcript intended for degradation from processing is thought to be accomplished by interaction with accessory proteins. Some proteins associate with the ribonuclease, in which case they are usually referred to as cofactors, while other proteins associate with the

RNA and form complexes called ribonucleoproteins (RNPs). It is believed that the combination of these factors and their interactions allows the cell to determine the fate of a particular transcript (Schilders et al., 2006).

Ribonucleases are classified as either endoribonucleases or exoribonucleases. Endoribonucleases cleave the phosphodiester bond within the RNA polynucleotide chain, while exoribonucleases cleave phosphodiester bonds at the end of the chain. The exonucleases can be further subcategorized into either 5'→3' exoribonucleases or 3'→5' exoribonucleases depending on the end from which they start degrading the RNA (from the 5' end or from the 3' end respectively; Arraiano et al., 2013).

Additionally, ribonucleases may not associate with protein complexes, such as the 5'→3' exoribonuclease Xrn1, or they are assembled into larger protein complexes such as the major 3'→5' exoribonuclease, the exosome.

The eukaryotic exosome

The major 3'→5' exoribonuclease in eukaryotes and archaea is the exosome (Chlebowski et al., 2013; Dziembowski et al., 2007). In eukaryotes, the exosome also has an endonuclease domain (Lebreton et al., 2008; Schaeffer et al., 2009; Schneider et al., 2009). The eukaryotic exosomes that have been studied so far are well conserved through evolution (Chlebowski et al., 2013; Decker, 1998). The exosomes of *Saccharomyces cerevisiae* and the human exosome are very similar both in structure and in function (Sloan et al., 2012). They also include homologues

of the same protein subunits. This similarity makes *S. cerevisiae* a good model organism for studying the eukaryotic exosome.

Structure of the exosome

The exosome is a complex of ten proteins, each of which is essential for life. There are several structures of eukaryotic exosomes including crystal structures of human and yeast exosomes (Liu et al., 2006; Makino et al., 2013) and EM reconstitutions of the exosome (Malet et al., 2010; Wang et al., 2007). Additionally, the catalytic subunit Rrp44 was independently crystallized with RNA and in a complex with Rrp41 and Rp45 (Bonneau et al., 2009; Lorentzen et al., 2008).

Based on the published structures listed above, nine of the ten proteins assemble in a cylindrical structure, composed of two rings, and the catalytic subunit is located on one side of this cylinder (Figure 1-1). The first ring is termed the exosome “cap” and it is composed of three RNA binding proteins, Rrp4, Rrp40, and Csl4. Each one of them has an S1 domain which is a known RNA binding domain originally identified in ribosomal protein S1. These S1 domains form a ring on the side of the exosome where RNA enters. The cap of the exosome is thought to mediate the initial interaction with the RNA substrate and/or interact with protein cofactors of the exosome; therefore it may be important in determining substrate specificity.

The middle portion of the exosome is composed of a larger ring called the PH ring, because of the RNase PH domains found in each of its component proteins. However, unlike RNase PH, the eukaryotic PH ring is inactive, lacking catalytic

activity. In archaea the PH ring is where catalysis occurs, but the active sites of eukaryotic exosomes have point mutations rendering them inactive (Dziembowski et al., 2007; Liu et al., 2006). The PH ring is composed of six proteins, Rrp41, Rrp42, Rrp43, Rrp45, Rrp46, and Mtr3. The 3'end of the RNA to be degraded is threaded through the PH ring as it emerges from the cap (the RNA binding ring) into the catalytic subunit Rrp44.

The tenth subunit, Rrp44, is located on the side where the RNA exits the ring (Wang et al., 2007). It has an exonuclease domain which faces the RNA exit site of the exosome and an endonuclease domain which faces away from the PH ring cylinder (Malet et al., 2010). Rrp44 is the only site of RNA degradation in the eukaryotic exosome. For detailed review on the structure and function of Rrp44 see Chapter 5.

The RNA binding proteins of the exosome

Studies of individual eukaryotic RNA binding proteins are limited. For example, RNA binding affinity has been studied only in Rrp40 and the reports are conflicting with one study reporting no RNA binding while another showing RNA binding by Rrp40 (Luz et al., 2007; Oddone et al., 2007). Eukaryotic Csl4 has been studied specifically for its role in the cytoplasmic mRNA degradation by the exosome and a point mutation in its Zn ribbon-like domain (see chapter 4) was found to affect specifically 3'→5' mRNA degradation by the exosome (van Hoof et al., 2000b).

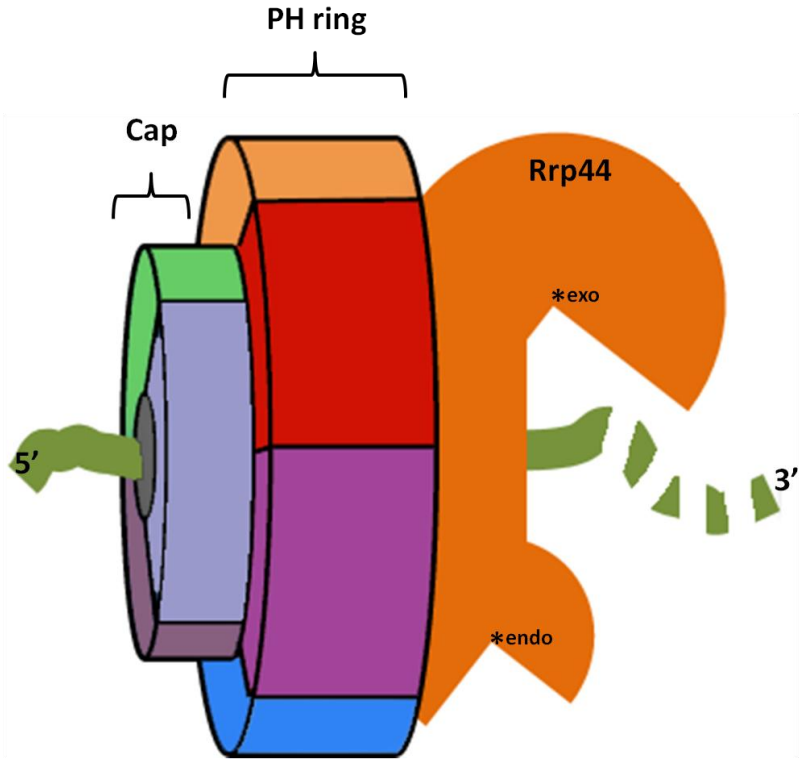


Figure 1-1. Structure of the exosome. Depiction of the structure of the 10 subunit eukaryotic exosome with RNA (in green). Locations of the exonuclease and the endonuclease within Rrp44 are show with asterisks (*).

In archaea, the exosome has two RNA binding proteins, Rrp4 and Csl4 which are homologous to Rrp4 and Csl4 of *S. cerevisiae* respectively. Archaeal Rrp4 is homologous to Rrp40 from eukaryotes, too. Archaea have several forms of the exosome, which differ by the stoichiometry of their RNA binding proteins while the PH ring remains the same. Exosomes with three Csl4 proteins, three Rrp4 proteins, or heterogeneous composition of the cap with mixed Rrp4 and Csl4 stoichiometry have been described (Buttner et al., 2005; Walter et al., 2006). The reason for this variability seems to be that, at least in archaea, the RNA binding proteins confer specificity to the exosome. This specificity is conferred in two ways. First, Rrp4 and Csl4 provide different substrate specificities to the exosome. Rrp4 exosomes prefer A-rich substrates while Csl4 exosomes prefer A-poor substrates (Roppelt et al., 2010). Second, different exosome complexes interact with different cofactors. For example, DnaG only interacts with a Csl4 exosome, but not with an Rrp4 exosome (Hou et al., 2013). The binding of DnaG enhances the interaction of a Csl4 exosome with A-rich substrates which are otherwise preferred by the Rrp4 exosome. Even though there is high conservation of the RNA binding proteins it is unknown if their role in providing substrate specificity and/or protein interactions is also conserved in higher organisms.

Catalytic activity of the exosome

Exosomes from all organisms have exoribonuclease activity. In archaea, the PH ring is the site of RNA degradation (Lorentzen et al., 2005). Eukaryotic exosome

have point mutations in the PH ring subunits, which render them catalytically inactive (Dziembowski et al., 2007; Liu et al., 2006). However, in eukaryotes there is a tenth subunit, Rrp44, which provides the catalytic activity of the exosome. More specifically, Rrp44 has processive hydrolytic 3'→5' exoribonuclease activity instead of the PH ring catalysis found in the archaeal exosome (Liu et al., 2006). Additionally, eukaryotes have evolved endoribonuclease activity, which is also found in Rrp44 but in a separate domain (Lebreton et al., 2008; Schaeffer et al., 2009; Schneider et al., 2009).

Most eukaryotes have one Rrp44 subunit which harbors both the exonuclease and the endonuclease domains. Human cells have subfuncitonalized the ribonucleases of the exosome even further. This subfunctionalization involves both cellular compartmentalization as well as catalytic activity. There are three homologues of Rrp44 in humans: hDIS3, hDIS3L, and hDis3L2. hDIS3 is found predominantly in the nucleus of human cells, while hDIS3L and hDIS3L2 are strictly cytoplasmic. Only the nucleus- localized hDis3 has endonuclease activity, while the other two homologues have only exoribonuclease activity (Astuti et al., 2012; Staals et al., 2010; Tomecki et al., 2010). In *Schizosaccharomyces pombe,* Dis3L2 does not associate with the exosome but still takes part in the cytoplasmic mRNA degradation pathway (Malecki et al., 2013).

Role of the exosome in the nucleus

The exosome was first discovered in *S. cerevisiae* as a nuclear ribosomal RNA processing machine required for the maturation of the 5.8S rRNA (Mitchell et al., 1997). Specifically, it was discovered that in the nucleus the complex plays a role in the maturation of 5.8S rRNA and also of the degradation of the 5' External transcribed spacer (ETS) of rRNA (de la Cruz et al., 1998). During the 5.8S rRNA maturation, the exosome degrades the 7S rRNA down to 5.8S + 30 nucleotides and then the final processing is dependent on Rrp6 (Allmang et al., 1999a). After the initial discovery, many studies have added various roles for the exosome making the exosome one of the most versatile cellular machines affecting most RNAs in the cell.

In addition to rRNA maturation, the exosome also takes part in the processing of snoRNAs, and snRNAs (Allmang et al., 1999a; van Hoof et al., 2000a). The 3' end of snRNAs U1, U4, and U5 are processed by the exosome. In this role the exosome functions together with Rrp6 by completing the final 3' processing steps. The exosome degrades the initial 3' nucleotides and then the final nucleotides are degraded in Rrp6-dependent manner.

snoRNAs can be independently transcribed or intron-derived. The exosome processes several snoRNAs together with its nuclear cofactor Rrp6, but not all snoRNAs require the exosome for 3' maturation. As with the 5.8S rRNA and snRNAs, Rrp6 is only required for the final steps of snoRNA maturation, specifically the trimming of the last 3 nucleotides (Allmang et al., 1999a; van Hoof et al.,

2000a).

In addition to processing, the exosome takes part in surveillance mechanisms, specifically the degradation of hypomodified $tRNA^{Met}$, misprocessed rRNAs, cryptic unstable transcripts (CUTs), and the cotranscriptional surveillance of pre-mRNAs, (Andrulis et al., 2002; Kadaba et al., 2004; Thiebaut et al., 2006).

The essential role of the exosome is also believed to be in the nucleus. Deletion of the cytoplasmic cofactors of the exosome, which are required for all known cytoplasmic roles, is not lethal (Rhee et al., 1989; Sommer and Wickner, 1987; van Hoof et al., 2000b; Widner and Wickner, 1993). Additionally, some of the nuclear exosome cofactors are essential, specifically Mtr4 and all three components of the Nrd1/Nab3/Sen1 complex (Liang et al., 1996; Steinmetz and Brow, 1996; Wilson et al., 1994). It is not known what specific role of the exosome complex is essential because many of the nuclear roles of the exosome are potentially essential.

In the cytoplasm, the exosome takes part in the 3'→5' degradation of normal and aberrant mRNAs (see *Normal mRNA degradation by the exosome in the cytoplasm* in this chapter*).* In addition various exosome roles require different cofactors, thus it seems that the specificity of the degradation/processing targets is not determined by the exosome itself.

Nuclear exosome cofactors

In the nucleus the exosome associates with Rrp6, an exonuclease homologous to RNase D from *Escherichia coli.* Rrp6 localizes to the nucleus in *S. cerevisiae*, but in

human cells and trypanosomes it has been found to be both in the nucleus and in the cytoplasm (Brouwer et al., 2001; Haile et al., 2007). *In vitro*, Rrp6 forms a complex with the exosome of *S. cerevisiae* (Makino et al., 2013). Because Rrp6 also interacts with the exosome *in vivo*, it is inferred that this complex forms in the cell's nucleus (Allmang et al., 1999b). While Rrp6 functions together with the exosome in 5.8S rRNA maturation where it is required for the degradation of the last 30 nucleotides (Briggs et al., 1998), and maturation of snRNAs and snoRNAs (Allmang et al., 1999a; van Hoof et al., 2000a), it also has exosome independent roles. For example, it degrades the last three nucleotides of some snoRNAs in an exosome independent manner (Allmang et al., 1999a; van Hoof et al., 2000a). Rrp6 has also been found to be required independently of the exosome for cell proliferation and error-free mitosis in *Drosophila melanogaster* cells (Graham et al., 2009b)

Additional nuclear cofactors of the exosome include Mtr4 and the TRAMP complex. Mtr4 is an RNA helicase which functions together with the exosome for its processing functions, specifically for processing of 5.8S rRNA, snoRNA, and snRNAs (Allmang et al., 1999a; de la Cruz et al., 1998; van Hoof et al., 2000a). For the degradation function of the exosome, Mtr4 is assembled in a complex, the TRAMP complex, with an RNA binding protein and a poly(A) polymerase, either Air1 or Air2 and either Trf4 or Trf5 respectively (Grzechnik and Kufel, 2008; LaCava et al., 2005; Rougemaille et al., 2007; Vanacova et al., 2005). Trf4 and Trf5 add short poly(A) tails to RNAs and these poly(A) tails are thought to promote

degradation by the exosome and it appears as each of them has different target RNAs (San Paolo et al., 2009).

Another complex with which the exosome is known to associate in the nucleus is the Nrd1/Nab3/Sen1 complex. Together with the exosome it functions in the processing of snRNAs and snoRNAs and degradation of misprocessed mRNAs and cryptic unstable transcripts (CUTS;Conrad et al., 2000; Thiebaut et al., 2006; Vasiljeva and Buratowski, 2006). Additionally the Nrd1/Nab3/Sen1 complex was shown to be involved, together with Rrp6 and the TRAMP complex, in the 3' end processing of the *CTH2* mRNA, which codes for a protein functioning in iron response (Ciais et al., 2008). Nrd1 and Nab3 have also been implicated in regulating the cellular response to nutrient availability (Darby et al., 2012)

As described, the exosome functions in processing or degradation of virtually all types of RNAs in the nucleus. For its various roles the exosome associates with many proteins and/or protein complexes. Most of these nuclear cofactors of the exosome have exosome-independent roles. Additionally, combinations of cofactors have been identified functioning together for some roles of the exosome. Such highly complex organization is indicative of the complicated nature of RNA recognition and degradation in the nucleus, of which the exosome is a major player.

Role of the exosome in the cytoplasm

In the cytoplasm the exosome plays a role in three general processes. First, it takes part in one of the two pathways for degradation of normal mRNAs. Second, it degrades aberrant mRNAs. Third, it has an antiviral role. For a depiction of normal an aberrant mRNA degradation see Figure 2.

Cytoplasmic cofactors of the exosome

For all of its known cytoplasmic roles the exosome requires Ski7 and the Ski complex. The Ski complex is composed of Ski2, Ski3, and two copies of Ski8 and has been proposed to interact with the cap of the exosome through Ski7 (Halbach et al., 2013). Ski7 is believed to function as a scaffold protein because it was found to interact with both the exosome and the Ski complex (Araki et al., 2001). A point mutation in Csl4, *csl4G253E* (*published* as *ski4-1)* that disturbs the exosome's role in the cytoplasm also does not coimmunoprecipitate with Ski7 nor the Ski complex suggesting that the interaction with the Ski complex is required for the cytoplasmic exosome function (van Hoof et al., 2000b).

Normal mRNA degradation by the exosome in the cytoplasm

In *S. cerevisiae* most mRNAs are deadenylated by the Ccr4-Not complex before being degraded (Decker and Parker, 1993; Tucker et al., 2002; Tucker et al., 2001). The deadenylation is a rate limiting step for the normal mRNA degradation. After

deadenylation, the mRNA can be degraded either from the 5' end or from the 3' end (Decker and Parker, 1993; Muhlrad et al., 1995). For a depiction of cytoplasmic mRNA degradation see Figure 1-2. When mRNAs are degraded from the 5' end, the 7-methyl cap is removed by the decapping enzyme Dcp1/Dcp2 and then the mRNA is degraded by Xrn1 (Beelman et al., 1996; Dunckley and Parker, 1999; Muhlrad et al., 1994; Steiger et al., 2003). From the 3' end mRNAs are degraded by the exosome, and the cap is then recycled by Dcs1 (Jacobs Anderson and Parker, 1998; Liu et al., 2002; Wang and Kiledjian, 2001). These pathways function in a redundant manner because each of them is not essential on its own but blocking both is synthetic lethal (Jacobs Anderson and Parker, 1998). It is likely however that during normal cellular processes, both pathways take place simultaneously. Studies suggest that the 3'→5' pathway, which as stated above is the one in which the exosome is involved, is predominant in humans (Wang and Kiledjian, 2001), while the 5'→3' pathway is predominant in *S. cerevisiae* (Jacobs Anderson and Parker, 1998).

Aberrant mRNA degradation by the exosome in the cytoplasm

The cytoplasmic exosome has been found to take part in mRNA surveillance for aberrant mRNAs, specifically mRNAs that lack a poly(A) tail or a stop codon (van Hoof et al., 2002).

The nonstop mRNA degradation pathway is conserved from yeast to humans and unlike regular mRNA degradation, it is deadenylation independent (Frischmeyer et

al., 2002). Because translation is required for nonstop mRNA decay and because ribosomes have been found to stall at the 3' end of nonstop mRNAs, it is believed that the exosome is recruited to the nonstop mRNA after, at least, the first round of translation (Figure 1-2 B; Ito-Harashima et al., 2007; van Hoof et al., 2002). Ski7, as well as each of the Ski complex proteins, Ski2, Ski3, and Ski8, were also found to be required for nonstop mRNA decay (van Hoof et al., 2002). Interestingly, the exoribonuclease and the endoribonuclease of the exosome were redundant for degrading nonstop mRNAs (Schaeffer and van Hoof, 2011).

Because there are no stop codons, the 3' UTR and the poly(A) tail would also be translated resulting in a poly(lysine) tag for the aberrant protein which in turn would be targeted for degradation by the 26S proteasome. A genetic screen for suppressors of nonstop mRNA decay identified several mutations involving the proteasome, thus supporting this model (Wilson et al., 2007).

Antiviral role of the exosome

The cytoplasmic exosome also has antiviral role. *S. cerevisiae* commonly contains an endogenous L-A virus. Additionally, some strains contain M satellite RNA which codes for a toxin (killer toxin) that is secreted from the cell and kills neighboring cells that are not infected. The expression of the M satellite is dependent on RNA-dependent RNA polymerase which is encoded by the L-A virus (Wickner et al., 1991). The cytoplasmic exosome is implicated in surveillance of the M satellite because all of the *ski* mutants were identified in *S. cerevisiae* in a screen for

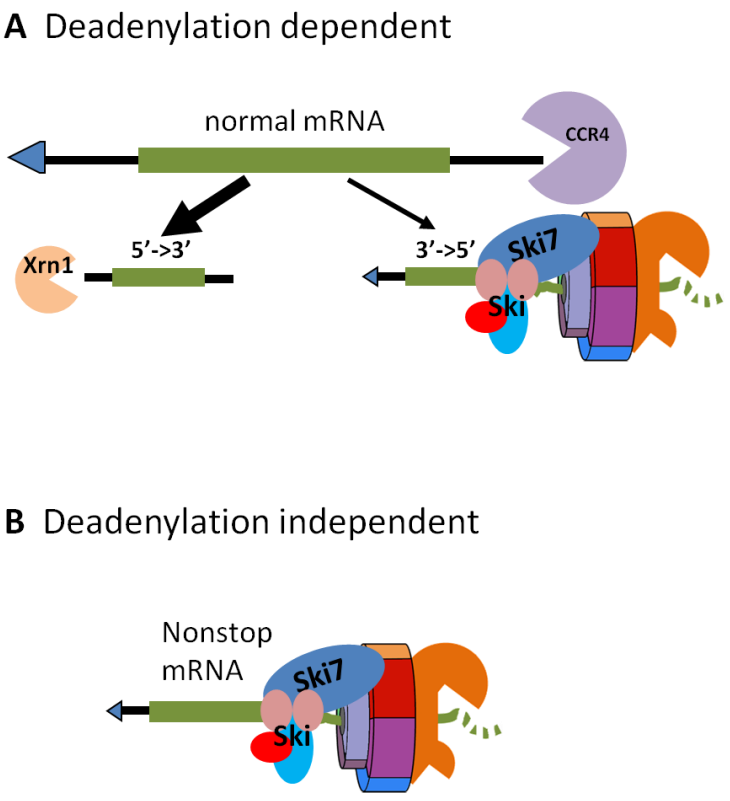


Figure 1-2. Cytoplasmic mRNA degradation by the exosome. A. The exosome in the normal mRNA degradation pathway. This pathway is deadenylation dependent. mRNAs are first deadenylated by the Ccr4-Not complex and then degraded either 5'→3' by Xrn1 (after decapping by Dcp2, not shown here) or 3'→5' by the exosome. Ski = ski complex. B. Degradation of aberrant mRNAs by the exosome as exemplified by a nonstop mRNA. Deadenylation is not needed and these mRNAs are degraded directly by the exosome which is facilitated by the ski complex and Ski7.

increased secretion of the killer toxin (Ridley et al., 1984; Toh et al., 1978). Two of the proteins, Ski4 and Ski6, later were determined to be the exosome components Csl4 and Rrp41 respectively (Mitchell et al., 1997; van Hoof et al., 2000b) and several of these encode cytoplasmic cofactors of the exosome such as Ski7 and the Ski complex proteins Ski2, Ski3, and Ski8 (Brown et al., 2000). The exact mechanism of exosome involvement is not known, but it seems that the secretion of the killer toxin is affected by lowering the viral RNA levels presumably by degrading it.

SIGNIFICANCE OF THIS STUDY

Exosome-dependent processing and degradation of RNAs affects virtually all cellular processes

Degradation of RNA is an essential process, important for normal cellular function at many levels. RNA surveillance, maturation and gene expression control involve RNA degradation. Therefore, it is of high importance that the processes by which RNA is degraded are understood. Fundamental understanding of these processes includes both the basic task of characterizing the proteins that make up the RNA degrading complexes as well as understanding the global effect these RNase enzymes have on gene expression.

This study characterizes the cap proteins of the exosome, which is the major 3'→5' exoribonuclease in eukaryotes and archaea. These three proteins are at the site of RNA entry and have several RNA binding domains. This study shows that not only do they have a functional role in the exosome, but they likely also have a structural role. It adds to our understanding of how the complex is held together and gives us a possible explanation of why three so similar proteins have unique roles.

Further, this research addresses the global effect of the exonuclease on the cell. It shows that the ultimate effect on gene expression is not as extensive as would be expected from the number and variety of substrates of the exosome. Identifying the major cellular processes that are affected by the exosome is significant because it adds to our understanding of global exosome regulation as well as opens the way to new research in the area of functional relevance in addition to the more technical study of individual substrates.

Importance to translational research

The exosome is important in human related research for four general reasons. First, mutations of exosome components themselves cause disease in humans. Specifically related to this study, mutations in Rrp40 cause severe neurological problems in infants (Wan et al., 2012). For detailed description of Rrp40 mutations related to health see Chapter 3. In addition, a mutation in two of the cytoplasmic co-factors of the exosome, Ski2 and Ski3, cause Syndromic diarrhea, a GI disorder (Fabre et al., 2012). Finally, mutations in one of the human homologues of Rrp44,

Dis3L2 cause Perlman syndrome which is a genetic disorder characterized by susceptibility to a childhood associated nephroblastoma (Astuti et al., 2012). Second, mutations that target the mutated transcript to the exosome, such as nonstop mutations, cause disease in humans such as hemorrhages, muscular dystrophy and Diamond-Blackfan anemia (Ameri et al., 2007; Cacciottolo et al., 2011; Klauer and van Hoof, 2012; Willig et al., 1999). Third, the exosome is a target of the immune system in the case of the autoimmune disease polymyocitis/scleroderma (Allmang et al., 1999b). Fourth, the exosome's activity is also targeted for cancer treatment by the drug 5FU (Fang et al., 2004; Silverstein et al., 2011).

The exosome has antiviral role in *S. cerevisiae*. Because it is such a conserved and versatile RNase it is also possible that it has antiviral role in human cells. Therefore a study of the east exosome's activity will be beneficial for future work in understanding how cells detect and neutralize infection by RNA viruses.

Understanding how the exosome works, may help develop new therapeutic options for people that have exosome-related diseases. Moreover, because the exosome structure and function are so well conserved from yeast to human, *S. cerevisiae* is a well-suited model organism for its study. The knowledge gained from the *S. cerevisiae* exosome may benefit the research when applied to the human organism.

Chapter 2: Materials and methods

Yeast strains, media and growth conditions

Strains

All yeast strains used in this study are haploid and are listed in Table 2-1.

Media

All of the *S. cerevisiae* strains used in this work were grown, unless otherwise specified, in YPD (yeast peptone dextrose) medium composed of 1% yeast extract, 2% peptone, 2% dextrose or in synthetic complete (SC) drop-out powder obtained from Sunrise Science Products, lacking the specified nutrient for plasmid selection. YPD medium with 5.74 mM of 5-fluoroorotic acid (5FOA) was used to select for strains that have lost a plasmid with a *URA3* marker for plasmid shuffle assay (see below).

Growth conditions

Unless otherwise noted all cultures were started overnight in 5 ml YPD and then transferred the next morning to the specified medium and grown at 30^{o}C.

For assays requiring RNA or protein isolation, cell cultures were grown to OD_{600}=0.6, centrifuged, the supernatant was removed and the cells were washed with H_2O, collected and frozen at -80^{o}C.

Table 2-1 Strains used		
Strain name	**Genotype**	**Reference**
yAV1104	*MATa; leu2-Δ0; lys2-Δ0; ura3-Δ0; his3-Δ1; rrp4Δ::NEO* [*RRP4, URA3*]	(Schaeffer et al., 2009)
yAV1107	*MATa; leu2-Δ0; LYS2; MET15; ura3-Δ0; his3-Δ1; rrp40Δ::NEO* [*RRP40, URA3*]	(Schaeffer et al., 2009)
yAV1047	*MATa; leu2-Δ0; LYS2; met15Δ0; ura3; his3-Δ1; csl4Δ::NEO* [*CSL4, URA3*]	(Schaeffer et al., 2009)
yAV40	*MATα; trp1-; leu2-3; lys2-201; ura3-52; HIS4; CUP1; rrp4-1; ade1-100*	This study
yAV245	*MATa; trp1; LEU2; lys2; ura3-52; HIS4; CUP1; mtr14-1, ade1; aro-; HIS3; HIS1*	T2639 from A. Tartakoff
yAV229	*MATa; TRP1; leu2-3; LYS2; ura3-52; his4-519; CUP1; ade1-100; rrp4::GAL10-protA-RRP4*	(Mitchell et al., 1997)
yAV253	*MATa; trp1; leu2Δ1; LYS2; ura3-52; HIS4; CUP1; GAL::rrp40; his3Δ200; gal2; galΔ108*	(Mitchell et al., 1997)
yAV255	*MATa; trp1; leu2-Δ1; LYS2; ura3; HIS4; CUP1; GAL::csl4; his3Δ200; gal2; galΔ108*	(Mitchell et al., 1997)
yAV1128	*leu2-Δ0; ura3-Δ0; his3-Δ1; rrp4Δ::HYG; rro40Δ::NEO; MET15* [*RRP4, URA3*] [*RRP40, URA3*]	This study
yAV340	*MATa; trp1; leu2; lys2; ura3; HIS4; CUP1::LEU2PM; dcp1-2:;TRP1; ski4-1*	(van Hoof et al., 2000b)
yAV1117	*MATa, his3Δ1, leu2Δ0, ura3Δ0, met15Δ0, RRP43-myc::HIS3*	(Schaeffer et al., 2009)
yAV1129	*MATα; leu2-Δ0; ura3-Δ0; his3-Δ1; rrp44Δ::NEO* [*RRP44, LEU2*]	This study
yAV1131	*MATα; leu2-Δ0; ura3-Δ0; his3-Δ1; rrp44Δ::NEO* [*RRP44 D551N, LEU2*]	This study
yAV1284	*MATa; TRP1; leu2-; lys2; ura3-; HIS4; CUP1; his3; rrp44Δ::NAT* [*RRP44, LEU2*]	This study
yAV1135	*MATα; leu2-Δ0; ura3-Δ0; his3-Δ1; rrp44Δ::NEO* [*RRP44 D171A, LEU2*]	This study

Oxidative stress media

Oxidative stress was achieved by adding 2.0 μM H_2O_2 or 50 μM of menadione to the medium in which the strain were grown. Both liquid and solid media were supplemented with the same concentrations. For some experiments 1.0 μM H_2O_2 was used, because *rrp44-exo*$^-$ stopped growing at 2.0 μM H_2O_2.

Iron supplementation and depletion

When iron was added to medium, it was added as 100 μM FAS (ferrous ammonium sulfate). To chelate iron, 100 μM of the iron chelator bathophenanthroline disulfonate (BPS) was added to the medium.

Respiratory growth media

YEP + 2% glycerol or YEP + 2% ethanol media were used to stimulate respiratory growth conditions.

Yeast transformation

Transformation was done as described (Schiestl and Gietz, 1989). Briefly, yeast cells were incubated with 1μg of plasmid in 300 μL of PLATE (40%v/v PEG3350, 0.1M LiAc, 10 mM TrisHcl, 1 mM EDTA) solution and 10μL heat denatured ssDNA. Incubation was done at room temperature overnight. Then the cells were centrifuged, the PLATE solution removed and the yeast cells were plated on selective medium.

Table 2-2. Plasmids used

Name	Description	Marker	Vector backbone	Insert : origin	Vector: origin
pAV188	His3 nonstop reporter	*URA3*	CEN	(van Hoof et al., 2002)	
pAV302	Csl4 3' UTR	*LEU2*	CEN	PCR with oAV366 and oAV118	pRS415
pAV303	Csl4 1-126	*LEU2*	CEN	PCR with oAV365 and oAV372	pAV302
pAV304	Csl4 1-251	*LEU2*	CEN	PCR with oAV365 and oAV371	pAV302
pAV305	Csl4	*LEU2*	CEN	PCR with oAV365 and oAV370	pAV302
pAV335	Csl4 111-292	*LEU2*	CEN	PCR with oAV405 and oAV370	pAV305
pAV421	Rrp4 3'UTR in pRS415	*LEU2*	CEN	PCR with oAV424 & oAV425	pRS415
pAV425	Rrp4 full length	*LEU2*	CEN	PCR with oAV426 & oAV429	pAV421
pAV441	Rrp4 1-105	*LEU2*	CEN	PCR with oAV426 & oAV500	pAV421
pAV442	Rrp4 1-190	*LEU2*	CEN	PCR with oAV426 and oAV501	pAV421
pAV443	Rrp4	*URA3*	CEN	Digest of pAV426 with XbaI and XhoI	pRS416
pAV444	Gal_Csl4 in pRS425	*LEU2*	2μ	pAV345 digestion with PvuII	pRS425
pAV445	Gal_Rrp4 in pRS425	*LEU2*	2μ	pAV346 digestion with PvuII	pRS425
pAV446	Gal_Rrp40 in pRS425	*LEU2*	2μ	pAV 347 digestion with PvuII	pRS425
pAV447	Rrp40 1-65	*URA3*	CEN	PCR of Rrp40 (1-65) with oAV 432 and oAV506	pRS436
pAV448	Rrp40 1-140	*URA3*	CEN	PCR of Rrp40 (1-140) with oAV432 and oAV507	pAV436
pAV449	Rrp40	*URA3*	CEN	PCR of Rrp40 (1-240) with oAV 432 and oAV508	pAV436
pAV459	Rrp40 1-65	*LEU2*	CEN	pAV447 digestion with XbaI and HindIII	pRS415
pAV460	Rrp40 1-140	*LEU2*	CEN	pAV448 digestion	pRS415
pAV461	Rrp40	*LEU2*	CEN	pAV449 - digestion	pS415
pAV466	Rrp40 65-240	*LEU2*	CEN	PCR with oAV531 and oAV431	pAV465
pAV467	Rrp40 140-240	*LEU2*	CEN	PCR with oAV532 and oAV431	pAV465
pAV469	Rrp40 65-240	*URA3*	CEN	pAV466	pRS416
pAV470	Rrp40 140 -240	*URA3*	CEN	pAV467	pRS416
pAV471	Rrp4 105-359	*LEU2*	CEN	PCR with oAV528 and oAV425	pAV464
pAV472	Rrp4 190 -359	*LEU2*	CEN	PCR with oAV529 and oAV425	pAV464
pAV476	TAP tag	*LEU2*	CEN	PCR with oAV556 and oAV557	pRS415
pAV477	5' UTR of Rrp 4 + 3' TAP tag	*LEU2*	CEN	PCR with oAV556 and oAV557	pAV464
pAV478	5' UTR of Rrp 40 + 3' TAP tag	*LEU2*	CEN	PCR with oAV556 and oAV557	pAV464

Table 2-2. Plasmids used (continued)

Name	Description	Marker	Vector backbone	Insert : origin	Vector: origin
pAV487	Rrp4 1-105-TAP	*LEU2*	CEN	PCR with oAV533 and oAV558	pAV476
pAV488	Rrp4 1-190-TAP	*LEU2*	CEN	PCR with oAV533 and oAV559	pAV476
pAV489	Rrp4 105-359-TAP	*LEU2*	CEN	PCR with oAV528 and oAV560	pAV477
pAV490	Rrp4 190-359-TAP	*LEU2*	CEN	PCR with oAV529 and oAV560	pAV477
pAV491	Rrp4-TAP	*LEU2*	CEN	PCR with oAV533 and oAV560	pAV476
pAV492	Rrp40 1-65-TAP	*LEU2*	CEN	PCR with oAV534 and oAV561	pAV476
pAV493	Rrp40 1-140-TAP	*LEU2*	CEN	PCR with oAV534 and oAV562	pAV476
pAV494	Rrp40 65-240-TAP	*LEU2*	CEN	PCR with oAV531 and oAV563	pAV478
pAV495	Rrp40 140-240-TAP	*LEU2*	CEN	PCR with oAV532 and oAV563	pAV478
pAV496	Rrp40-TAP	*LEU2*	CEN	PCR with oAV534 and oAV563	pAV476
pAV532	Rrp4 S152A	*LEU2*	CEN	Mutagenesis with oAV595 and oAV596	pAV426
pAV533	Rrp4 S152E	*LEU2*	CEN	Mutagenesis with oAV597 and oAV598	pAV426
pAV534	Csl4 S94A	*LEU2*	CEN	Mutagenesis with oAV599 and oAV600	pAV305
pAV535	Csl4 S94E	*LEU2*	CEN	Mutagenesis with oAV601 and oAV602	pAV305
pAV541	Rrp4 1-105	*HIS3*	CEN	Digestion of pAV441	pRS413
pAV542	Rrp4 1-190	*HIS3*	CEN	Digestion of pAV442	pRS413
pAV543	Rrp4-TAP	*URA3*	CEN	Digestion of pAV491 with SacI and XhoI	pRS416
pAV544	Csl4 W272A	*LEU2*	CEN	Mutagenesis with oAV617 and oAV618	pAV305
pAV545	Csl4Y268A,T270A,W272A	*LEU2*	CEN	Mutagenesis with oAV619 and oAV620	pAV305
pAV550	Rrp4 1-105 + Rrp40 65-240 Chimera	*LEU2*	CEN	PCR from pAV461 with oAV607 and oAV609	pAV487
pAV551	Rrp4 1-190 + Rrp40 140-240 Chimera	*LEU2*	CEN	PCR from pAV461 with oAV608 and oAV609	pAV488
pAV552	Rrp40 1-65+ Rrp4 105-359 Chimera	*LEU2*	CEN	PCR from pAV426 with oAV604 and oAV606	pAV492
pAV553	Rrp40 1-140 + Rrp4 190-359 Chimera	*LEU2*	CEN	PCR from pAV426 with oAV605 and oAV606	pAV493
pAV554	Csl4 - V210A, R211A	*LEU2*	CEN	Mutagenesis with oAV629 and oAV630	pAV305
pAV555	Csl4 - D214A, R215A	*LEU2*	CEN	Mutagenesis with oAV631 and oAV632	pAV305
pAV556	Csl4 - D216A, R217A	*LEU2*	CEN	Mutagenesis with oAV633 and oAV634	pAV305
pAV557	Csl4 - S236A, L237A	*LEU2*	CEN	Mutagenesis with oAV635 and oAV636	pAV305
pAV558	Csl4 110-292 W272A	*LEU2*	CEN	Mutagenesis with oAV617 andoAV618	pAV 335
pAV559	Csl4 110-292 Y268A,T270A,W272A	*LEU2*	CEN	Mutagenesis with oAV619 andoAV620	pAV335
pAV560	Csl4 110-292 V210A, R211A	*LEU2*	CEN	Mutagenesis with oAV629 and oAV630	pAV 335
pAV561	Csl4 110-292 D214A, R215A	*LEU2*	CEN	Mutagenesis with oAV631 and oAV632	pAV335

Table 2-2. Plasmids used (continued)

Name	Description	Marker	Vector backbone	Insert : origin	Vector: origin
pAV562	Csl4 110-292 D216A, R217A	*LEU2*	CEN	Mutagenesis with oAV633 and oAV634	pAV335
pAV563	Csl4 110-292 S236A, L237A	*LEU2*	CEN	Mutagenesis with oAV635 and oAV636	pAV335
pAV569	Csl4 110-250	*LEU2*	CEN	PCR of pAV305 with oAV405(SphI) and oAV371(BamHI)	pAV305
pAV582	Csl4 S1(110-250) - TAP	*LEU2*	CEN	PCR from pAV569 with oAV365 and oAV669	pAV476
pAV583	Csl4 Zn(248-291) - TAP	*LEU2*	CEN	PCR from pAV336 with oAV365 and oAV661	pAV476
pAV584	Csl4-TAP	*LEU2*	CEN	PCR from pAV305 with oAV365 and oAV661	pAV476
pAV640	Rrp40 1-65+ Rrp4 105-359	*HIS3*	CEN	Digestion of pAV552	pR413
pAV641	Rrp40 1-140 + Rrp4 190-359 Chimera	*HIS3*	CEN	Digestion of pAV553	pRS413
pAV642	Rrp40 (1-65)+Rrp4 (105-190) + Rrp40 (140-240) Chimera	*HIS3*	CEN	Digestion of pAV565	pRS413
pAV643	Rrp40	*HIS3*	CEN	Digestion of pAV461	pRS413
pAV646	Csl4 1-126-TAP	*LEU2*	CEN	PCR from pAV305 with oAV365 and oAV699	pAV476
pAV647	Csl4 1-250-TAP	*LEU2*	CEN	PCR from pAV305 with oAV365 and oAV668	pAV476
pAV648	Csl4 111-292-TAP	*LEU2*	CEN	PCR from pAV305 with oAV365 and oAV61	pAV476
pAV649	Csl4 111-292 D251A, L252A	*LEU2*	CEN	Mutagenesis with oAV700 and oAV701	pAV335
pAV650	Csl4 111-292 G253A	*LEU2*	CEN	Mutagenesis with oAV702 and oAV703	pAV335
pAV651	Csl4 111-292 V254A,V255A	*LEU2*	CEN	Mutagenesis with oAV704 and oAV705	pAV335
pAV652	Csl4 111-292 M267A	*LEU2*	CEN	Mutagenesis with oAV706 and oAV707	pAV335
pAV653	Csl4 111-292 M275A, P278A	*LEU2*	CEN	Mutagenesis with oAV708 and oAV709	pAV335
pAV654	Csl4 111-292 E284A, R286A, K287A	*LEU2*	CEN	Mutagenesis with oAV710 and oAV711	pAV335
pAV655	Csl4 111-292 K290A	*LEU2*	CEN	Mutagenesis with oAV712 and oAV713	pAV335
pAV666	Csl4 1-126-FLAG	*LEU2*	CEN	PCR of pAV303 with oAV365(XbaI) and oAV723(BamHI)	pAV302
pAV667	Csl4 1-251-FLAG	*LEU2*	CEN	PCR of pAV304 with oAV365(XbaI) and oAV724(BamHI)	pAV302
pAV668	Csl4 111-292-FLAG	*LEU2*	CEN	PCR of pAV335 with oAV365(XbaI) and oAV725(BamHI)	pAV302
pAV669	Csl4 247-292-FLAG	*LEU2*	CEN	PCR of pAV336 with oAV365(XbaI) and oAV725(BamHI)	pAV302
pAV670	Csl4-FLAG	*LEU2*	CEN	PCR of pAV305 with oAV365(XbaI) and oAV725(BamHI)	pAV302
pAV679	Csl4 1-126 S94E	*LEU2*	CEN	Mutagenesis of pAV666 with oAV601 and oAV602	pAV666

Table 2-2. Plasmids used (continued)					
pAV680	Csl4 111-292-TAP V210A,R211A	*LEU2*	CEN	Mutagenesis of pAV648 with oAV629 and oAV630	pAV648
pAV681	Csl4 111-292-TAP D214A, R215A	*LEU2*	CEN	Mutagenesis of pAV648 with oAV631 and oAV632	pAV648
pAV682	Csl4 111-292_TAP D216A, R217A	*LEU2*	CEN	Mutagenesis of pAV648 with oAV633 and oAV634	pAV648
pAV683	Csl4 111-292-TAP S236A, L237A	*LEU2*	CEN	Mutagenesis of pAV648 with oAV635 and oAV636	pAV648
pAV684	Csl4 111-292-TAP D251A, L252A	*LEU2*	CEN	Mutagenesis of pAV648 with oAV700 and oAV701	pAV648
pAV685	Csl4 111-292-TAP G253A	*LEU2*	CEN	Mutagenesis of pAV648 with oAV702 and oAV703	pAV648
pAV686	Csl4 111-292-TAP V254A, V255A	*LEU2*	CEN	Mutagenesis of pAV648 with oAV704 and oAV705	pAV648
pAV687	Csl4 111-292-TAP M267A	*LEU2*	CEN	Mutagenesis of pAV648 with oAV706 and oAV707	pAV648
pAV688	Csl4 111-292-TAP Y268A,T270A,W272A	*LEU2*	CEN	Mutagenesis of pAV648 with oAV619 and oAV620	pAV648
pAV689	Csl4 111-292-TAP Y268A	*LEU2*	CEN	Mutagenesis of pAV648 with oAV657 and oAV658	pAV648
pAV690	Csl4 111-292-TAP T270A	*LEU2*	CEN	Mutagenesis of pAV648 with oAV659 and oAV660	pAV648
pAV691	Csl4 111-292-TAP W272A	*LEU2*	CEN	Mutagenesis of pAV648 with oAV617 and oAV618	pAV648
pAV692	Csl4 111-292-TAP M275A, P278A	*LEU2*	CEN	Mutagenesis of pAV648 with oAV708 and oAV709	pAV648
pAV693	Csl4 111-292-TAP E284A, R286A, K287A	*LEU2*	CEN	Mutagenesis of pAV648 with oAV710 and oAV711	pAV648
pAV694	Csl4 111-292-TAP K290A	*LEU2*	CEN	Mutagenesis of pAV648 with oAV712 and oAV713	pAV648
pAV786	cyc1_LacZ	*URA3*	2μ	(Marx and Lidstrom, 2001)	
pAV804	cyc1_aft1BS_LacZ	*URA3*	2μ	Oligonucleotides cloning with oAV886 and oAV88	pAV786
pAV843	Aft1-GFP	*URA3*	2μ	(Crisp et al., 2003)	
pRS413	empty vector	*HIS3*	CEN	(Mumberg et al., 1995)	
pRS415	empty vector	*LEU2*	CEN		
pRS416	empty vector	*URA3*	CEN		
pRS425	empty vector	*LEU2*	2μ		

Plasmids used in this study

Plasmids used in this study are listed in Table 2-2. All plasmids, unless otherwise noted, have an endogenous promoter and 3' UTR to ensure expression at normal endogenous level. Additionally each plasmid has a marker for selection. The markers used in this work were *URA3*, *LEU2*, or *HIS3*.

C-terminal TAP tag cloning

All of the proteins that were tagged with a TAP tag were tagged at their C-terminus by removing the stop codon of the sequence to be tagged and adding a G followed by a *Pst*I site (G CTGCAG). That positions the TAP tag in frame and results in adding 2 alanine residues and a glycine residue between the tagged protein and the TAP tag. For a depiction of the TAP-tagging see Figure 2.1.

Site-directed mutagenesis

Site directed mutagenesis was performed using Quick Change Protocol (Stratagene) with the indicated PCR primers and PfuUltra DNA polymerase (Stratagene). PCR settings were as suggested by the manufacturer as follows: initial denaturation - 95^{o}C for 30 seconds; 18 cycles of: 95^{o}C for 30 seconds, 55^{o}C for 1 minute, 68^{o}C – for 1 min per kb of plasmid; one addition cycle of 68^{o}C for 1 min per kB of plasmid length. The PCR was then treated with 1μl *Dpn*I (NEB) for 1 hour at 37^{o}C. 2 μl of the *Dpn*I treated reaction were transformed in chemically competent *E. coli* cells, plated on LB+0.5 mg/ml Ampicillin, and grown at 37^{o}C overnight. Colonies were then picked, grown on LB + Amp and screened by

restriction digestion (each primer has a restriction site that does not change the protein coding sequence for screening) and then the sequence was confirmed by DNA sequencing.

Growth assays

Liquid growth assays were started with yeast cells at OD_{600}=0.1 from an overnight culture in YPD, or required selective medium, at 30^{o}C.

Plate growth assays were done as follows. 5x serial dilutions, starting with OD_{600}=0.6 were done in a 96-well plate and then were spotted on selective plates and grown at the indicated temperature. Unless otherwise indicated, all pictures were taken either on day 3 or on day 4 after plating.

Plasmid shuffle assay

The purpose of this assay is to replace an essential protein with a mutant version of the same protein. A haploid yeast strain with a knock out of the particular gene in the genome and a plasmid encoded wild-type version with a URA3 marker was transformed with a mutant gene on a plasmid with the LEU2 marker. Transformants were selected on SC-Leu-Ura medium, and then growth assays, as described, were performed on 5FOA. 5FOA selects for cells that have lost the *URA3* marker effectively shuffling the LEU2 vector for the URA3 vector (Boeke et al., 1984). Growth on 5FOA indicates that the LEU2 born mutant can substitute for the essential role of the wild-type gene.

Table 2-3. Primers used

Name	Sequence
oAV365	CGCGATCTAGAGGAATTCGAATATGATAGACATCC
oAV366	GGTATCGATAAGCTTCGGTGGTATTGTAGG
oAV370	CGCGAGGATCCTTAAAAAGGTTTGGCACATTTGCGCTT
oAV371	CGCGAGGATCCTTAGTCATTCCTTGCAGTGGTCAAA
oAV372	CGCGAGGATCCTTAATTAGCATATTTGTTGGTTTTACGAC
oAV405	CGCTGGCATGCCTTCCTGGGACAGAAAAGGGTCGTAAAACC
oAV424	CGCGAGGATCCCAAAAGCCGCTGTTTACACATTTATAAAC
oAV425	CGCGACTCGAGCTGCTACCTTTGCCAGTGGTAAACC
oAV426	CGCGATCTAGAGGAATACTGGCAGTGCGATTTTCATGC
oAV429	CGCGAGGATCCTATTAGTTGCCGTTACCTCTCA
oAV430	CGCGAGGATCCATGGTATAATTAAAATATATAAACACATAGAT
oAV431	CGCGAAGCTTCGTATTAGATATGTGCACTATAGTTGTCG
oAV432	CGCGATCTAGAGCGTTATACTGCGTTATACTGCG
oAV500	CGCGAGGATCCTATTAGGCATAACGACCTTTCAATGGAATTACCG
oAV501	CGCGAGGATCCTATTACTTTAGAGACCTTGTATGTAAAGAGGC
oAV506	CGCGAGGATCCTAGTTTAAATGTATCTCTTACTAGAATAGTCT
oAV507	CGCGAGGATCCTAGTTTAAGTTGAGTCAAAACATTCTATTTCGGC
oAV508	CGCGAGGATCCTAGTTTACTCCTCCTTGACCGTAAGTAT
oAV527	CGCGATCTAGACGCTTGTGTGACTACGCAAAGATTTAG
oAV528	CGCGATCTAGAAACAGATATGGCCCCAGAAACTGGTGATCACGTCGTAGGG
oAV529	CGCGATCTAGAAACAGATATGAAGTACGGGAAGTTAAGAAACGGG
oAV530	CGCGATCTAGACTCTTGGTTAGTCGAAATGCTGG
oAV531	CGCGATCTAGACATACAAGATGATTCCATCTGTAAACGATTTTGTAATCG
oAV532	CGCGATCTAGACATACAAGATGACTACAGGACGCGATGCTGGTTTCGG
oAV533	CGCGAGAGCTCGGAATACTGGCAGTGCGATTTT
oAV534	CGCGAGAGCTCGCGTTATACTGCGTTATACTG
oAV556	CGCCTGCAGGTCGACGGATCCCCGGGTTAATTAATCCA
oAV557	CGCAAGCTTTGCCGGTAGAGGTGTGGTCAATAAGAGCGAC
oAV558	CGCCTGCAGCGGCATAACGACCTTTCAATGGAATTACCGATAGCA
oAV559	CGCCTGCAGCCTTTAGAGACCTTGTATGTAAAGAGGCACT
oAV560	CGCCTGCAGCGTTGCCGTTACCTCTCATTTTTTCGGCGGT
oAV561	CGCCTGCAGCAATGTATCTCTTACTAGAATAGTCTATATA
oAV562	CGCCTGCAGCAGTTGAGTCAAAACATTCTATTTCGGCTTC
oAV563	CGCCTGCAGCCTCCTCCTTGACCGTAAGTATTTCTTTAAA
oAV595	CCGGGTGGAATTCTAAGAAGAAAAGCTGAAAGTG
oAV596	CACTTTCCTCTTTTCTTCTTAGAATTCCACCCGG
oAV597	CCGGGTGGAATTCTAAGAAGAAAAGAGGAAAGTG
oAV598	CACTTTCAGCTTTTCTTCTTAGAATTCCACCCGG
oAV599	GGAAGAAAAATCAGTCGACGCAGCACCTAATGATGTCACAAG

Table 2-3. Primers used (continued)

Name	Sequence
oAV600	CTTGTGACATCATTAGGTGCTGCGTCGACTGATTTTTCTTCC
oAV601	GGAAGAAAAATCAGTCGACGCAGAACCTAATGATGTCACAAG
oAV602	CTTGTGACATCATTAGGTTCTGCGTCGACTGATTTTTCTTCC
oAV604	CGCGACTGCAGCTGCCCCAGAAACTGGTGATCACGTCGTAGG
oAV605	CGCGACTGCAGCTAAGTACGGGAAGTTAAGAAACGGGATG
oAV606	CGCGACTCGAGCTGCTACCTTTGCCAGTGGTAAACC
oAV607	CGCGACTGCAGCTATTCCATCTGTAAACGATTTTGTAATCGG
oAV608	CGCGACTGCAGCTACTACAGGACGCGATGCTGGTTTCGG
oAV609	CGCGCTCGAGCGTATTAGATATGTGCACTATAGTTGTCG
oAV612	CGCGAGGGCCCGCCACCATGTCCGAAGTTATCACAATTACCAAGCGG
oAV613	CGCGAGGGCCCGCCACCATGTCTACGTTCATATTCCCTGGTGATAGC
oAV614	CGCGAGGGCCCGCCACCATGGCATGCAATTTTCAGTTTCCAGAAATAGC
oAV617	GGTGGATTAATGTATGCTACAGACGCGCAAATGATGACTTCACCG
oAV618	CGGTGAAGTCATCATTTGCGCGTCTGTAGCATACATTAATCCACC
oAV619	GGTGGATTAATGGCTGCTGCAGACGCGCAAATGATGACTTCACCG
oAV620	CGGTGAAGTCATCATTTGCGCGTCTGCAGCAGCCATTAATCCACC
oAV621	CGCGACTCGAGGCCACCATGTCTACGTTCATATTCCCTGGTGATAGC
oAV629	GGTATAATCCGATCGCAGGATGCAGCGTCTACAGACAGAGAT
oAV630	ATCTCTGTCTGTAGACGCTGCATCCTGCGATCGGATTATACC
oAV631	GGTCTCAGGATGTACGGTCTACAGCCGCCGATCGTGTGAAAGTG
oAV632	CACTTTCACACGATCGGCGGCTGTAGACCGTACATCCTGAGACC
oAV633	GGATGTACGGTCTACCGATCGAGCTGCTGTGAAAGTGATAGAATG
oAV634	CATTCTATCACTTTCACAGCAGCTCGATCGGTAGACCGTACATCC
oAV635	GGGCGCAAGTTTTAGCAGCCGGCGACGGA ACCAACTACTATTTG
oAV636	CAAATAGTAGTTGGTTCCGTCGCCGGCTGCTAAAACTTGCGCCC
oAV653	CGGGAGATATTGTTCGAGCTCAAGTTTTAGCATTAGGTGACGG
oAV654	CCGTCACCTAATGCTAAAACTTGAGCTCGAACAATATCTCCCG
oAV655	CGGGAGATATTGTTCGAGCTCAAGTTTTATCAGCAGGTGACGG
oAV656	CCGTCACCTGCTGATAAAACTTGAGCTCGAACAATATCTCCCG
oAV657	GGTGGATTGATGGCTGCTACAGACTGGCAAATGATGACTAGTCCG
oAV658	CGGACTAGTCATCATTTGCCAGTCTGTAGCAGCCATCAATCCACC
oAV659	GGTGGATTGATGTATGCTGCAGACTGGCAAATGATGACTTCACCG
oAV660	CGGTGAAGTCATCATTTGCCAGTCTGCAGCATACATCAATCCACC
oAV661	CGCCTGCAGCAAAAGGTTTGGCACATTTGCGCTTTTCTGTAGCGCC
oAV668	CGCTCTAGAATGCTTCCTGGGACAGAAAAGGGTCGTAAAACCAACAAATATGCTAAT
oAV669	CGCTGCAGCATTCCTTGCAGTGGTCAAATAGTAGTTGGTTCCGTCACC
oAV670	CGCTCTAGAATGGACCTTGGGGTCGTGTTCGCCAGAGCTGCTAATGGTGCTGGTGG
oAV699	CGCCTGCAGC ATTATTAGCATATTTGTTGGTTTTACGACCC
oAV700	CCACTGCAAGGAATGCCGCTGGGGTCGTGTTCGCCCGAGCTGCT
oAV701	AGCAGCTCGGGCGAACACGACCCCAGCGGCATTCCTTGCAGTGG

Table 2-3. Primers used (continued)

Name	Sequence
oAV702	GGAATGACCTTGCGGTCGTGTTCGCCCGAGCTGCT
oAV703	AGCAGCTCGGGCGAACACGACCGCAAGGTCATTCC
oAV704	GGAATGACCTTGGGGCCGCGTTCGCCCGAGCTGCT
oAV705	AGCAGCTCGGGCGAACGCGGCCCCAAGGTCATTCC
oAV706	GGTGCCGGCGGATTGGCGTATGCTACAGACTGGC
oAV707	GCCAGTCTGTAGCATACGCCAATCCGCCGGCACC
oAV708	GCTACAGACTGGCAAATGGCGACTTCTGCAGTTACAGGCGC
oAV709	GCGCCTGTAACTGCAGAAGTCGCCATTTGCCAGTCTGTAGC
oAV710	CCGGTTACAGGCGCTACAGCCAAGGCGGCATGTGCCAAACC
oAV711	GGTTTGGCACATGCCGCCTTGGCTGTAGCGCCTGTAACCGG
oAV712	CGCTACAGAAAAGCGCAAATGTGCCGCACCTTTTTAAGGATCC
oAV713	GGATCCTTAAAAAGGTGCGGCACATTTGCGCTTTTCTGTAGCG
oAV723	GCGGGATCCTCATTTGTCATCGTCGTCCTTGTAGTCATTATTAGCATATTTGTTGGTTTTACGACCC
oAV724	GCGGGATCCTCATTTGTCATCGTCGTCCTTGTAGTCATTCCTTGCAGTGGTCAAATAGTAGTTGGTTCCG TCACC
oAV725	GCGGGATCCTCATTTGTCATCGTCGTCCTTGTAGTCAAAAGGTTTGGCACATTTGCGCTTTTCTGTAGC
oAV746	CGCTACAGAAAAGCGCAAATGTGCCGCACCTTTTGCTGCAGG
oAV747	CCTGCAGCAAAAGGTGCGGCACATTTGCGCTTTTCTGTAGCG
oAV748	CGC GCTGCAGGT TCTACGTTCATATTCCCTGGTGATAGCTTTCCTGTAG
oAV751	CCAGAAATAGCTTATCCAGGTAAGCTTGCATGCCCACAGTACGG
oAV752	CCGTACTGTGGGCATGCAAGCTTACCTGGATAAGCTATTTCTGG
oAV753	GTAAAAAATATTGCGGTTGCAGTGCTTCCTGGTACCGAAAAGGGT
oAV754	ACCCTTTTCGGTACCAGGAAGCACTGCAACCGCAATATTTTTTAC
oAV886	GATCTCTTCAAAAGTGCACCCATTTGCAGGTGCTCC
oAV887	TCGAGGAGCACCTGCAAATGGGTGCACTTTTGAAGA

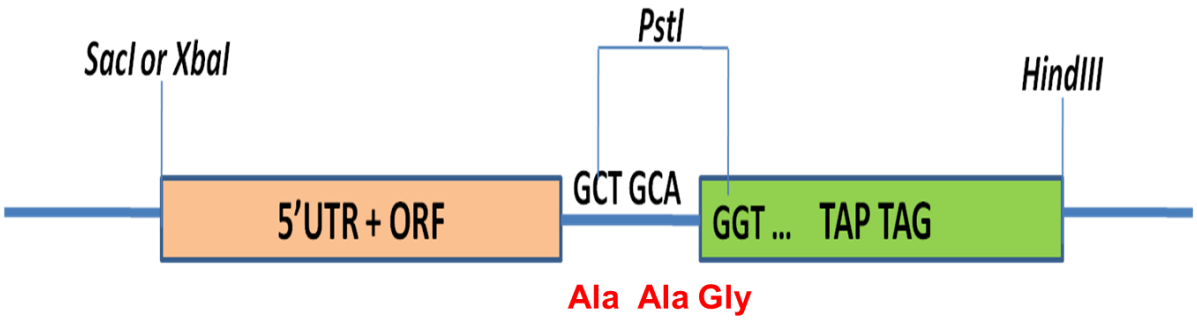


Figure 2.1 Depiction of the TAP tag cloning. Protein sequence, either full-length or truncated, was fused at the C-terminus with the TAP tag in the manner depicted above in order to place both sequences in frame. The blue line depicts the nucleotide sequence and the resulting amino acid sequence between the TAP tag and the protein of interest is depicted on the bottom in red.

Cytoplasmic mRNA degradation assay

Cytoplasmic mRNA degradation assays were performed essentially as described (van Hoof et al., 2000b). Briefly, to test if a protein is involved in the 3→5' cytoplasmic mRNA degradation pathway, a strain deleted for the particular protein was supplemented with a plasmid encoding that same protein or a mutant. The strain also had a *dcp1-2* temperature-sensitive allele of DCP1. Transformants were grown on selective medium at room temperature. Next, growth assays were performed, as described above, at room temperature, 30^{o}C and 37^{o}C. Since the *dcp1-2* allele is temperature-sensitive, at non-permissive temperature this strain does not have a functional 5'→3' decay pathway and can only survive if the 3'→5' pathway is functional. The functionality of the 3'→5' would be provided for by the plasmid-encoded protein.

Nonstop mRNA degradation assay

This assay was completed as described (van Hoof et al., 2002). Briefly, a strain with a deletion of one gene on the chromosome, complemented with a mutant allele of that gene on a plasmid, was transformed with a vector carrying a mutant *his3* gene which lacks a stop codon, *his3-nonstop.* After selecting for transformants, growth assays were performed as described above, on SC-His medium. Strains that are proficient in nonstop mRNA degradation rapidly degrade the *his3-nonstop* mRNA which leads to failure to grow on SC-His medium. Strains that are deficient in nonstop mRNA degradation do not recognize the *his3-nonstop* as aberrant mRNA and do not degrade it, which allows for the production of histidine and the strain

grows. The positive control used for this assay was a *ski7Δ* strain because *ski7Δ* does not have nonstop mRNA decay.

Protein isolation

Total protein was isolated form *S. cerevisiae* using the alkaline lysis method. Briefly, cells were grown to OD_{600} = 0.8 and 4 OD units of cells were removed, centrifuged, the supernatant was removed and the cell pellet was suspended in 150 ml of lysis solution (1.85 M NaOH with 1% β-mercaptoethanol) and incubated on ice for 10 minutes. 75 ml TCA were added to precipitate the protein and the precipitated protein was pelleted using centrifugation. The protein pellet was then washed with 1 ml acetone, resuspended in 100 ml of 2x loading buffer and heated at 65^{o}C for 10 minutes to dissolve. 10 μl of sample was used for the Western blotting.

Western blotting

Proteins were resolved on 10% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane using electrophoresis (Towbin et al., 1979). The membrane was blocked in 5% milk in TBST (Tris-Buffered Saline with 1ml/L Tween® 20), and then treated with primary antibody for 1 hour, washed three times in TBST, treated with secondary antibody HRP conjugated for 1 hour, washed again three times in TBST, and the proteins were detected using ECL Plus Western Blotting Detection Reagents (GE Healthcare) per manufacturer's protocol.

Co-immunoprecipitation (Co-IPs)

Co-IPs were completed as described (Schaeffer et al., 2012). Briefly, cells were grown to OD_{600} 0.8, centrifuged, and the cells were collected and washed with ddH_2O. The cell pellet was resuspended in 200 μl of cold IP50 buffer (50 mM TRIS-HCl pH7.5, 50 mM NaCl, 2 mM $MgCl_2$, 0.1% Triton) + 0.5M EDTA, 1 μM β-mercaptoethanol, 0.1 mM PMSF, and 1 "Mini-complete protease inhibitor tablet EDTA free"(Roche) per 10 ml of buffer. The above sample was vortexed with glass beads for 7 min at 4°C, centrifuged, the supernatant was collected and total protein was quantified in spectrophotometer using Biorad protein assay Dye reagent. 40 mg of total protein was used for the Western blotting.

RNA isolation

RNA was isolated by the "Hot phenol" method essentially as described (He et al., 2008). Briefly, cells were grown to OD_{600}=0.6 and collected by centrifugation and removal of the supernatant. 500 μl of RNA buffer A (0.5 M NaoAc, 0.01M EDTA, 1% DEPC 1% SDS) was added to the cell pellet followed by 500μl of RNA Phenol/BufferA 1:1 ratio, heated to 65°C. The mixture was heated to 65°C for 4 minutes, centrifuged, upper aqueous layer mixed with 500 μl of RNA/BufferA was heated to 65°C again for 4 min. and the previous step repeated. Next, the upper aqueous layer was mixed with 500μl of Buffer A +TE/Chloroform at room temperature (Phenol: Chloroform is 1:1 ratio) then centrifuged and upper layer precipitated by the addition of 1 ml of 100% cold ethanol and incubating at -80°C for

at least one hour. Ethanol was removed and replaced with 70% ethanol and then the 70% ehtanol was replaced with DEPC treated water. RNA pellet was resuspended by 5 freeze/thaw cycles and absorbance was measure at 260nm.

RNA gel electrophoresis

Polyacrylamide gels (11.25 ml of 40% 19:1 acrylamide:bisacrylamide, 7.5 ml of 10x TBE, 30 ml of DEPC treated H_2O, 34.5g urea, 100μl TEMED, 100μl 40% APS) were run to detect RNA fragments smaller that 500nts such as 5.8S rRNA. 5' ETS, U4, tRNAs, etc. as indicated in the text. The electrophoresis was run in TBE buffer. After resolving the RNA by size, it was transferred onto a nylon membrane using electroblotting in 0.5x TBE buffer for a total 180 volt hours and then the membrane was subjected to UV crosslinking.

1.3% agarose gels (72 ml ddH_2O, 18 ml formaldehyde, 1.3% agarose, 5μL 10 mg/ml ethidium bromide 10 ml 10x MOPS buffer,) were run for detection of iron response mRNAs FIT3 and TIS11. MOPS buffer is 41.8 g MOPS (3-(N-morpholino)propanesulfonic acid), 16.6 ml NaAc, 20 ml 0.5 M EDTA in 1L H_2O, pH 7.0. Agarose gels were run in 1x MOPS buffer and transferred overnight via capillary action onto nylon membranes and then the membrane was subjected to UV crosslinking.

Northern blotting

The sequences of all oligos used as probes for northern blotting are listed in Table 2.3. All probes were ^{32}P 5' α-phosphate - labeled.

Northern blot analysis was performed as described (Meaux and Van Hoof, 2006). Briefly, nylon membranes onto which RNA was transferred, either from polyacrylamide or from agarose gels (see above), were washed with prewash buffer (0.1x SSC, 0.1% SDS) at 65^{o}C for 1 hr, then prehybridized in prehybridization buffer (10x Denhardt's solution, 6x SSC, 0.1% SDS) at 42^{o}C for 1 hr and probed overnight in the same buffer with added radioactively labeled probe at 42^{o}C. Membranes were washed in wash buffer (6xSSC,0.1% SDS) 3 times (for 5 min, 5 min, and 20 min) and signals were detected by exposing phosphor screens for empirically determined amount of time and visualized using a STORM840 PhosphorImager (Amersham). A probe for the RNA component of the Signal Recognition Particle was used as a loading control for both agarose and polyacrylamide gels.

β-galactosidase assay

β-galactosidase assays were completed per manufacturer's instructions (Promega). Briefly, cells were grown to OD_{600}=0.6. 50 μl of cell culture was mixed with 50 μl of Beta-Glo reagent (Promega). The mixture was incubated for 30 min. at room temperature in 96-well plates and light output and OD_{600} were measured using Biotech Synergy MX plate reader. Light output was normalized to OD for each well.

ROS detection assay

ROS detection assays were completed after optimization as suggested by the manufacturer (Life technologies). Overnight cultures were diluted to OD_{600}=0.2, then after growing for 1.5 hr were either treated with H_2O_2 (for the cells that were oxidatively stressed) or left untreated. After 1hr treatment 1 ml of culture was centrifuged and the medium was removed. The cells were washed in phosphate buffered saline (PBS), resuspended in 1 ml PBS and 25 μM 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) was added. Fluorescence was detected at 525 nm with excitation at 495 nm and Relative Light Units were normalized to OD in each sample and then all samples were normalized to wild-type.

Glucose utilization assay

To measure percentage of glucose in the medium, cells were grown in liquid YPD culture as described for 24 hr. OD_{600} was measured every two hours and at the same time 1 ml of culture was removed and frozen for analysis. Glucose was measured using Glucose (HK) Assay Kit (Sigma) used per manufacturer's instructions.

Ethanol output assay

Ethanol was measured using EnzyChom™ Ethanol Assay Kit (BioAssay Systems) per manufacturer's instructions.

Table 2-4. Oligonucleotides used for Northern blotting		
Name	**Target**	**Sequence**
oAV224	SRP	GTCTAGCCGCGAGGAAGG
oAV777	5.8S rRNA	TTTGCGTGCGTTCTTCATC
oRP756	*U4*	
oAV872	*TIS11*	GGGAGTTTCCTGCACTTGGC
oAV873	*FIT2*	CGACGGCTTGAGTGACGGTC
oAV973	Rnase P	GTTCGCCACTAATGACGTCC
oAV976	precursor to tRNA Arg-Asp	AGAAACAAAGCACTCACGAT
oAV978	tRNA Glu	CCGAAGCGGGGAGTCGAACCC
oAV979	tRNA Asp	CCGCGACGGGGAATTGAACCC
oAV981	*CAF20*	CGTCGTCTTCGTCTTCACTTTCC
oAV982	*REV1*	CTTTCCCACGTTTGGAAGCC
oAV983	YMR304C-A	GCTTTTAGGTAATGCCGC
oAV1025	*ATM1*	GCCCGAAGACCCAACTATTGCGG
oAV1137	*CYB2*	CGGTAGAACCGTACGCGCGG
oAV1138	*CYT1*	GCTGGGGCGTGCAATCCGTG
oAV1141	*RIP1*	CCTGTACGTGGATTTGCTAGCCAGC
oAV1142	*COR1*	GCCAAAGACCTCTTGAACTGG
oAV1151	5' ETS	CGAACGACAAGCCTACTG

Chapter 3: Characterization of Rrp4 and Rrp40 RNA binding proteins of the exosome

INTRODUCTION

Currently, it is unknown how the exosome recognizes substrate RNAs. Recognition is likely to occur at the site of RNA entry where the three cap proteins of the exosome are located. Because of their location and because each of the cap proteins contains a known nucleic acid binding domain, S1, the cap proteins are likely candidates to bind RNA and/or exosome cofactors. Therefore, they may be important for providing specificity to the exosome.

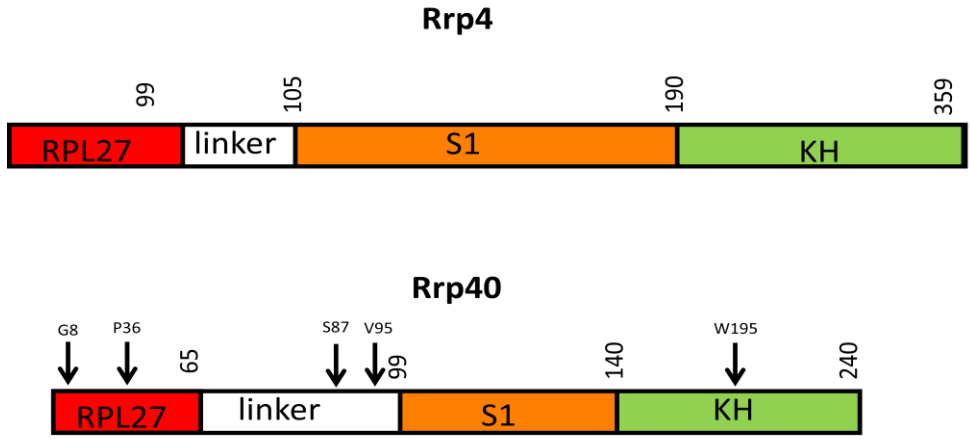
Mutations in the human ortholog of Rrp40, hRrp40, have been associated with various human diseases, suggesting the importance of this particular protein to human physiology, particularly in neurological conditions. For example, mutations in hRrp40 V80F and D132A are associated with early onset spasticity, distal amyotrophy, and cerebellar atrophy (Zanni et al., 2013). Additionally, mutations G31A, A139P, D132A, and W238A have been associated with Pontocerebellar hypoplasia type 1 (Biancheri et al., 2013; Rudnik-Schoneborn et al., 2013; Schwabova et al., 2013; Wan et al., 2012). Therefore. characterizing the cap proteins of the exosome is important for understanding basic cellular RNA metabolism, but also may have implications for the improvement of human health.

Two of the cap proteins, Rrp4 and Rrp40 share almost identical domain organization. Each has an N-terminal RPL27-like domain connected to the middle S1 domain by a linking sequence, and a KH (K homology) domain (Figure 3-1).

The N-terminal domains of Rrp4 and Rrp40 are similar to a domain found in ribosomal protein Rpl27, which is involved in protein-protein interactions, thus they are referred to from now on as RPL27-like domains. Other than this similarity, nothing much is known about these domains. With regards to their interactions with the PH ring of the exosome, the RPL27-like domain of Rrp4 interacts with Rrp41, while the RPL27-like domain of Rrp40 interacts with Rrp46 (Liu et al., 2006).

The S1 domains are well known RNA binding domains, first identified in the ribosomal protein S1 (Subramanian et al., 1981). These domains are found in many RNA binding proteins and are highly conserved from prokaryotes to eukaryotes. All three major exoribonucleases of *E. coli,* RNase II, RNase R, and PNPase, contain S1 domains. In eukaryotes, in addition to all three cap proteins of the exosome, S1 domains are found in the initiation factor 2α (eIF2α) and in proteins important for releasing mRNAs from the spliceosome such as Prp22 and dead box protein 8. It has been shown that S1 domains have specific target sequences along the RNA (Boni et al., 1991). However, there does not seem to be any major difference from one S1 domain to another with respect to binding of particular RNAs. The S1 domain of RNase II was successfully swapped with the S1 domains of either RNase R or PNPase, even though there was some loss of activity (Amblar et al., 2007). Thus it seems that the S1 domains were functioning best when in the context of the protein with which they evolved. Further, the same study showed that neither the degradation properties, nor the ability to deal with secondary structures were related to the S1 domains, but the S1 domains were capable of inducing multimerization.

A



B

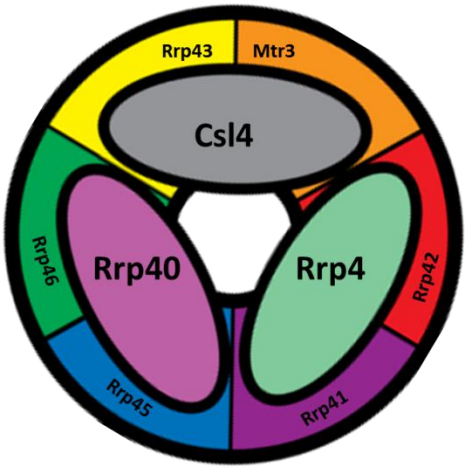


Figure 3-1 Domain organization of Rrp4 and Rrp40. A. Depiction of domain organization of the Rrp4 and Rrp40 protein sequences. The numbers indicate residue number. Arrows above Rrp40 point to residues corresponding to disease-causing mutations in human Rrp40 (hRrp40). G8=hRrp40 G31, P36=hRrp40 V80, S87=D132, V95=hRrp40 A139, W195=hRrp40 W238. **B**. A view of the exosome from the cap protein side. Shown is the location of the cap proteins in relation to the PH ring proteins.

Finally, both proteins have a KH domain at their C-terminal end. KH domains are nucleic acid binding domains, fist identified in the human heterogeneous nuclear ribonucleoprotein (hnRNP) K (Baber et al., 1999; Siomi et al., 1993). KH domains are often found in repeats and can function either independently or cooperatively with the other KH domains (Garcia-Mayoral et al., 2007). Like the S1 domains, they are also conserved though evolution and are found in bacterial proteins, such as PNPase of *E. coli* and also in numerous eukaryotic proteins. The best known example is Fragile mental retardation 1(FMR1) a mutation in which causes fragile X syndrome (Gibson et al., 1993). The KH domains are a rare case in which there are two folds, type I and type II, that result from the highly similar sequences of the KH motifs (Grishin, 2001). Interestingly, while Rrp4 and Rrp40 share similar structure along their other domains, there are significant differences in their KH domain topologies (Liu et al., 2006). The effect of these differences is not known.

Both Rrp4 and Rrp40 are essential proteins. However, given their high sequence homology, it would be unusual that the domains are needed for the essential function of the exosome versus having redundant roles. There are three possible functions of Rrp4 and Rrp40. First, they may play a structural role in the assembly of the exosome complex. When the human exosome structure was solved the authors were unable to assemble the PH ring without including the three cap proteins which strongly suggested that at least one of the cap proteins was needed for the exosome to assemble (Liu et al., 2006). Second, they may bind substrate RNA destined for degradation or processing by the exosome. This is based on the fact that each of these proteins contains two RNA binding domains. Third, Rrp4 and

Rrp40 may provide a platform for binding other protein cofactors of the exosome. And finally, these three possible functions of the cap proteins are not mutually exclusive. Therefore the purpose of this study is to determine the role of Rrp4 and Rrp40 in the exosome.

RESULTS

All of the domains of Rrp4 are essential

Even though both Rrp4 and Rrp40 are essential, it is not know which of their domains are responsible for their essential role. To determine which domain of Rrp4 is responsible for the essential function, several truncations of Rrp4 were constructed: rrp4RPL27-like, rrp4RPL27-like +S1, rrp4S1+KH, and rrp4KH which had the RPL27-like domain, the RPL27-like + S1 domain, the S1+KH domain, and only the KH domain respectively. Each of the truncations was inserted into a vector with the *LEU2* marker and the truncations were between the native 5' and 3' UTRs of Rrp4. Two different methods were chosen to test the ability of these constructs to substitute for the essential function of Rrp4: introducing the constructs in strains in which Rrp4 was deleted and introducing them into a strain in which Rrp4 could be shut-off via a galactose-regulated promoter.

First, a strain with *rrp4Δ* on the chromosome, complemented by a vector with wild-type *RRP4* and an *URA3* marker, was transformed with the *LEU2* plasmids with the truncations and transformants were selected on SC-Leu-Ura. Next, a plasmid shuffle assay was carried out by growing the transformants on 5FOA to select for strains that have lost the *URA3* plasmids (for more details on the plasmid shuffle assay refer to Chapter 2 Materials and Methods). If any truncation was able to substitute for the essential function of Rrp4 then the strain in which it is transformed would be able to grow on 5FOA. None of the constructs grew on 5FOA indicating that they were unable to substitute for the essential role of Rrp4 (Figure 3-2 A).

To confirm these results in an independent way, the same constructs were used to transform a strain in which Rrp4 is under the control of the *GAL* promoter (*GAL_RRP4*) allowing for shutting off the expression when glucose is added to the medium. Transformants with truncations that were grown on medium with glucose had a growth rate similar to the negative control (an empty vector), indicating that they could not substitute for the essential role of Rrp4 (Figure 3-2 B). The little growth seen for these constructs as well as for the negative control is probably due to minimal expression from the *GAL* promoter allowing for low level of Rrp4. The only construct that was able to complement the Rrp4 shut-off was the full-length Rrp4, indicating that the truncations were not able to substitute for the essential role of Rrp4.

To confirm that the truncated proteins were expressed, the truncations were tagged with a TAP tag (Rigaut et al., 1999) at their C-terminal end. The TAP tag has a Protein A moiety as a part of it, therefore expression was confirmed by a Western blot probed with anti-Protein A antibody (Figure 3-2 C). Each truncation was expressed to a level comparable to wild-type Rrp4 indicating that the truncations were not viable for reasons other than lack of protein expression. A growth assay with these TAP-tagged truncations was also conducted in *rrp4Δ* strain to verify that the TAP tag did not alter the phenotype (Figure 3-2D). The full length TAP-tagged Rrp4 grew as well as the untagged Rrp4 indicating that the TAP tag did not cause alteration in the growth phenotype. Therefore the truncation and TAP tag addition did not affect the expression and the essential role of Rrp4.

Given all of the above data, it was concluded that all of the domains of Rrp4 are essential for life, as none of the truncations could substitute for the essential role of the full-length Rrp4.

All of the domains of Rrp40 are essential

To determine the essential domains of Rrp40, similar sets of experiments as for Rrp4 were carried out. Truncations of Rrp40 were created- Rrp40RPL27-like, Rrp40RPL27-like +S1, Rrp40S1+KH, and Rrp40KH. In the case of Rrp40, in addition to the knock-out, and *GAL*-regulated Rrp40, a temperature sensitive allele of Rrp40 was also used.

First, a plasmid shuffle assay with the Rrp40 truncations introduced into an *rrp40Δ* strain was conducted in the same way as for the *rrp4Δ* strain. None of the constructs grew on 5FOA indicating that they were unable to substitute for the essential role of Rrp40 (Figure 3-3 A).

Second, a strain with *GAL* promoter regulated Rrp40 was transformed with vectors bearing each of the truncations and was grown on medium supplemented either with glucose or galactose. Figure 3-3B shows that only the full length Rrp40 was able to substitute for the lack of Rrp40.

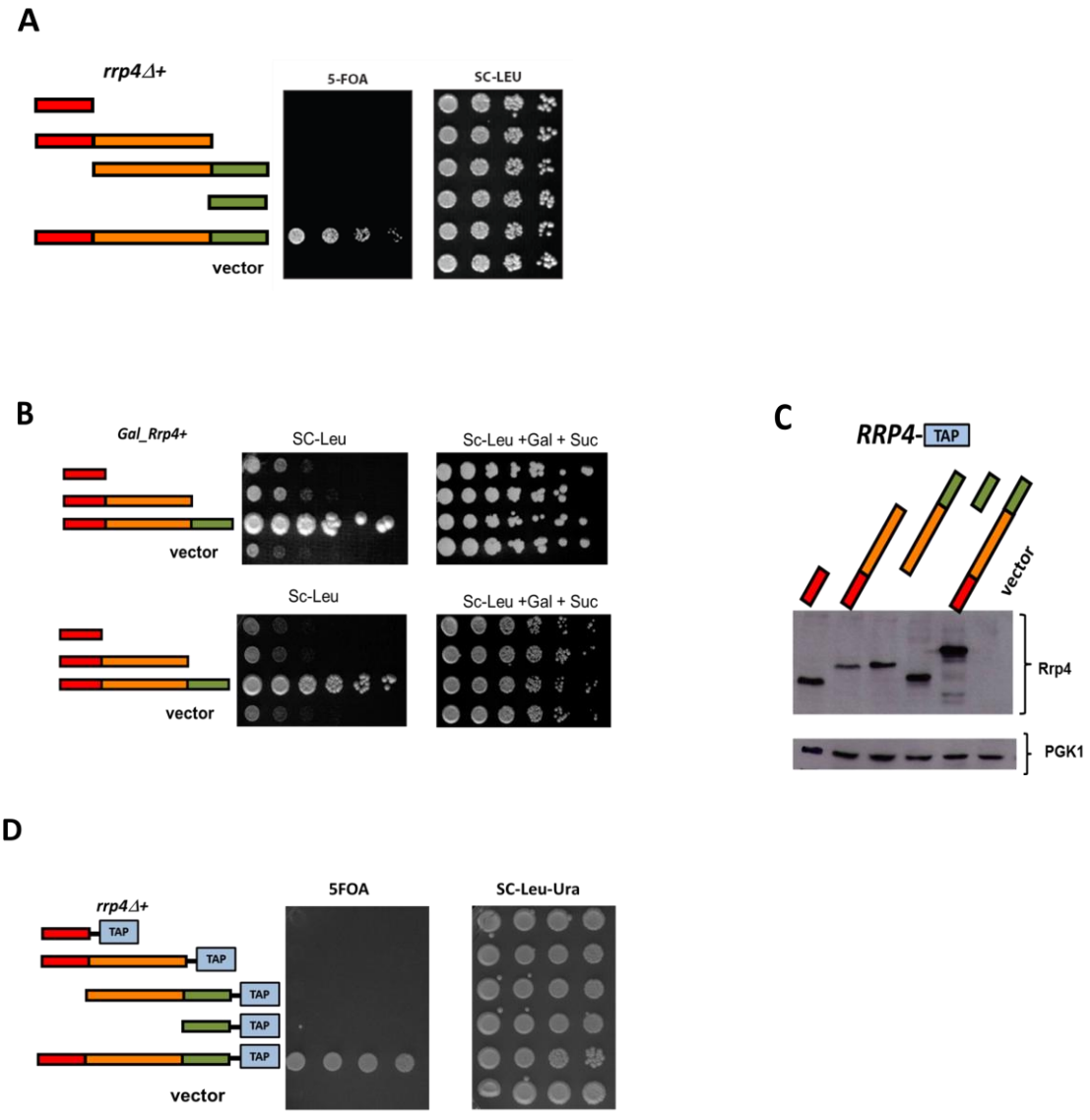


Figure 3-2. All of the domains of Rrp4 are essential. A. Plasmid shuffle assay of the depicted truncations of Rrp4 in an *rrp4Δ* strain (Schaeffer et al., 2009). B. The depicted truncations were transformed into a strain in which Rrpp4 is under the control of the *GAL* promoter (*GAL*_Rrp4) and grown either without galactose (SC-Leu) or with galactose and sucrose (SC-Leu+Gal+Suc). C. Western blot analysis of the depicted truncations of Rrp4. D. Plasmid shuffle assay of the depicted truncations of Rrp4, TAP-tagged, in an *rrp4Δ* strain.

Third, a strain bearing a temperature sensitive allele of Rrp40, *mtr14-1,* (Smith et al., 2011*)* was transformed with each of the vectors encoding the Rrp40 truncations. After selecting for transformants at room temperature, they were transferred to either non-permissive temperature at 36^{o}C or grown at the permissive temperature of 23^{o}C. As with the previous two assays, only full length Rrp40 was able to substitute for the essential role of Rrp40 (Figure 3-3D).

To determine if each truncation was expressed, the truncations were tagged with a TAP tag at their C-terminus. A Western blot was conducted (Figure 3-3C) and confirmed the expression of only the full-length Rrp40 and the truncation with only KH domain. There was also a very low level expression of the Rrp40RPL27-like +S1 truncation, but no visible bands for Rrp40RPL27-like or Rrp40S1+KH. The reason why some of the Rrp40 truncations were not expressed is not known, but is possible that factors related to proper protein folding may play a role. These data suggest that all of the domains of Rrp44 are needed for exosome function, but in the case of the non-expressed truncation a stability issue due to improper folding cannot be ruled out.

The cap proteins have unique essential roles.

Because Rrp4 and Rrp40 are so similar in their domain organization, it is possible that one of them can substitute for another. To test if each of them has a unique role, each was overexpressed into a deletion strain of the other. Because the

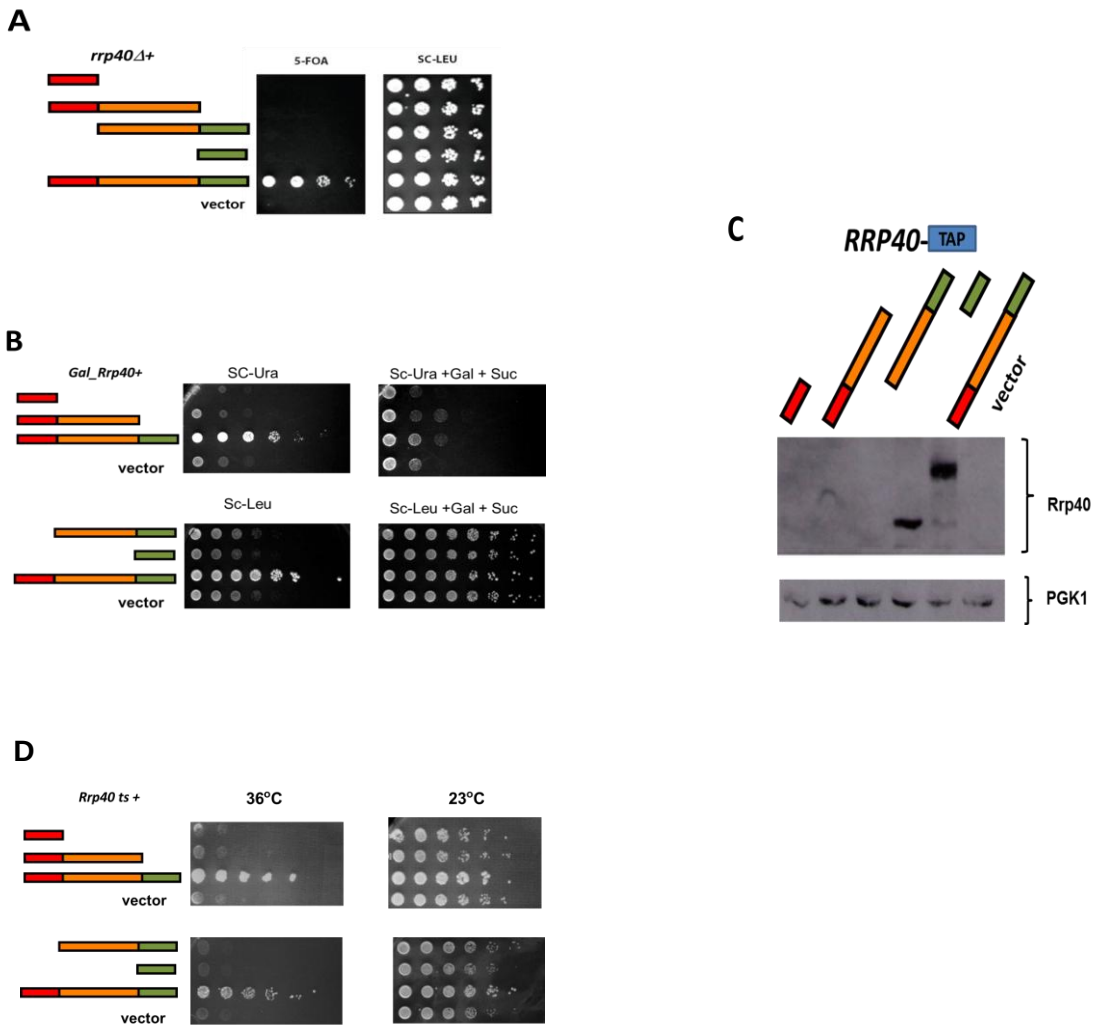


Figure 3-3. All of the domains of Rrp40 are essential. A. Plasmid shuffle assay of the depicted truncations of Rrp40 in an *rrp4Δ* strain (Schaeffer et al., 2009). B. The depicted truncations were transformed into a strain in which Rrp40 is under the control of the *GAL* promoter (Gal_Rrp4) and grown without galactose (SC-Leu) and with galactose and sucrose (SC-Leu+Gal+Suc). C. Western blot analysis of the depicted truncations of Rrp40 D. The depicted truncations were transformed into a strain with temperature sensitive Rrp40 (*Rrp40 ts*) and grown at permissive (23^{o}C) and non-permissive (36^{o}C) temperature.

deletions strains already have the rest of the exosome genes on their chromosomes, the additional genes were introduced behind the strong and inducible *GAL* promoter and in a 2μ vector, in order to overexpress the protein. 2μ vectors are high copy vectors used for high expression (Futcher and Cox, 1984). There are two reasons for designing the assay this way. First, it is possible that deletion of one protein, either Rrp4 or Rrp40, is stoichiometrically unfavorable and even if the other protein can substitute for the essential function, there isn't enough of it available. Such situation would lower the total number of exosome complexes in the cell to a minimum of half of the original. If however, the other protein is overexpressed, it may be able to substitute for the essential function of the other. Second, increasing concentration of proteins by overexpression increases their chance of interacting with lower affinities. Therefore, even if Rrp40 has weaker interaction with the exosome at the place of Rrp4 (and vice versa), overexpressing it will increase the chance for interaction. Additionally, since the third cap protein Csl4, also has a similar domain, it was also included in the assay. The transformants were grown on 5FOA + galactose. 5FOA was used to select for transformants that have lost the plasmid with the *URA3* marker (wild-type gene) and galactose was used to induce expression from the vector. None of the proteins were able to substitute for any of the other two cap proteins (Figure 3-4). In each strain, the only transformants that grew were the ones transformed with the corresponding protein for positive control.

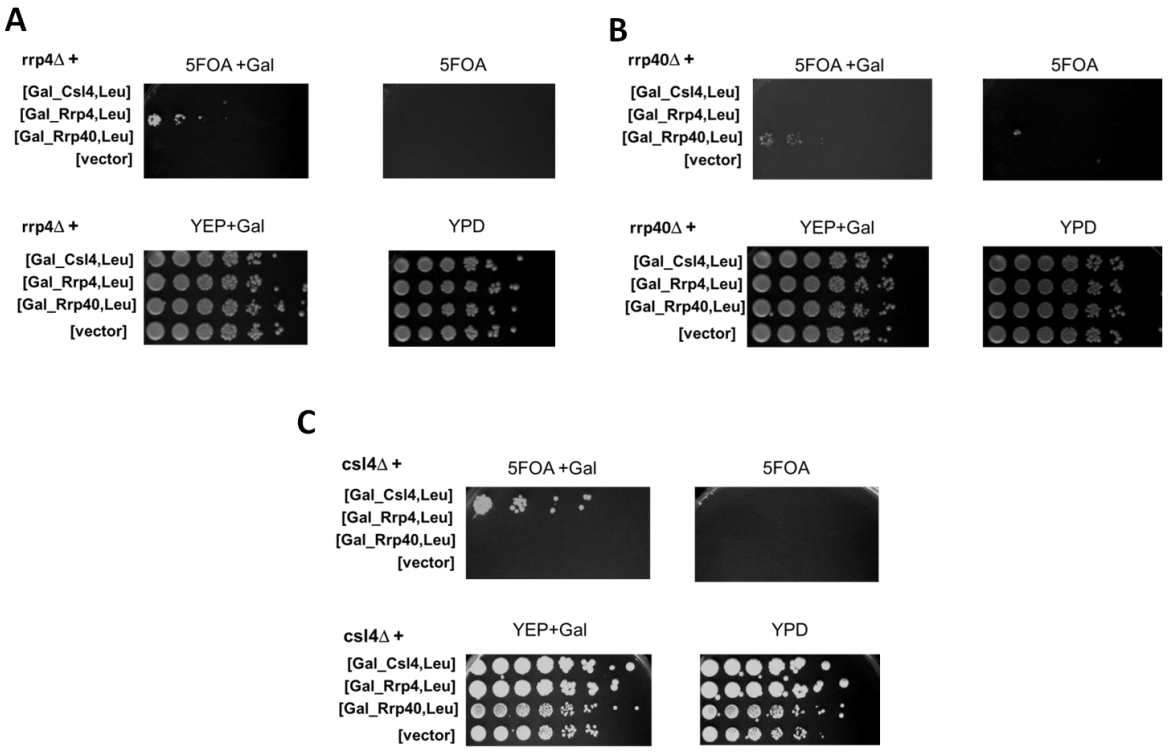


Figure 3-4. Overexpression of each cap protein in a deletion strain of the other cap proteins A plasmid shuffle assay of A. *rrp4Δ* B. *rrp40Δ* and C. *csl4Δ* strain transformed with the indicated vectors in which each of the cap proteins was under the control of the *GAL* promoter.

The above data indicate that Rrp4, Rrp40, and also Csl4, have unique roles that cannot be substituted by overexpressing another one of the cap proteins.

The homologous domains of Rrp4 and Rrp40 are not interchangeable

As mentioned earlier, there is a possibility that the essential role of Rrp4 and Rrp40 is structural, functional, or both. Since the data so far indicated that each of the domains of Rrp4 and Rrp40 is needed for exosome function, either one of these possibilities could not be ruled out. The structural role for either Rrp4 or Rrp40 would be to bridge two dimers of the exosome PH-ring subunits. One way to check for a structural role is to express the proteins as separate domains, *in trans*. However, not all truncations of Rrp40 were stable, therefore it is impossible to express Rrp40 *in trans*. Conversely, all truncations of Rrp4 were expressed at levels comparable to wild type.

To verify that at least one of the roles for Rrp4 is structural, an *in trans* expression assay was conducted. Briefly, *rrp4Δ* strain with a wild type Rrp4 on an *URA3* vector was transformed with the following truncations: one clone was transformed with rrp4RPL27-like on *LEU2* vector and rrp4S1+KH on *HIS3* vector; another clone was transformed with rrp4RPL27-like +S1 on a *LEU2* vector + rrp4KH on a *HIS3* vector. The transformants were then transferred to 5FOA plates to select for los of the *URA3* (with wild-type Rrp4) vector. Neither of the two combinations of Rrp4 domains expressed *in trans* was able to complement an *rrp4Δ,* only the full length Rrp4 was able to complement it (Figure 3-5 A) suggesting that Rrp4 may play a

structural role in the exosome. This is consistent with the idea that Rrp4 is important for the exosome structure by bridging two dimers of the exosome PH-ring.

Since some of the truncations of Rrp40 were not stable on their own, we decided to make various chimeras of Rrp4 and Rrp40. The purpose of this experiment was to test whether the particular location of the domains was important for growth. Six chimeric proteins were made in which various domains were combined (for depiction see figure:3-5 B and C) rrp4 RPL27-like _rrp40S1+KH, rrp4 RPL27-like +S1_rrp40KH, rrp40RPL27-like _rrp4S1+KH, rrp40RPL27-like +S1_rrp4KH, rrp4RPL27-like _rrp40S1_rrp4KH, and rrp40RPL27-like _rrp4S1_Rrp40KH. Each of these chimeras was introduced into strains with a deletion of either RRP4 (*rrp4Δ*) or RRP40 (*rrp40Δ*). In *rrp4Δ*, only the positive control, a plasmid bearing a full length Rrp4, was able to complement a deletion (Figure 3-5 B) and in *rrp40Δ* only the positive control, a full length Rp40, was able to complement a deletion (Figure 3-5 C).

Transforming chimeras in single deletion strains of either *rrp4Δ,* or *rrp40Δ* has the possibility of changing the stoichiometry of the possible exosome complex because the resulting strain never has one of each domains. For example an *rrp4Δ* strain transformed with a chimera rrp4 RPL27-like +S1_rrp40KH will have two KH domains from Rrp40 and no KH domain from Rrp4. To correct for that a domain switch experiment was also conducted in double deletion *rrp4Δrrp40Δ* mutants. Neither the RPL27-like, nor the S1, nor the KH domain switch was able to

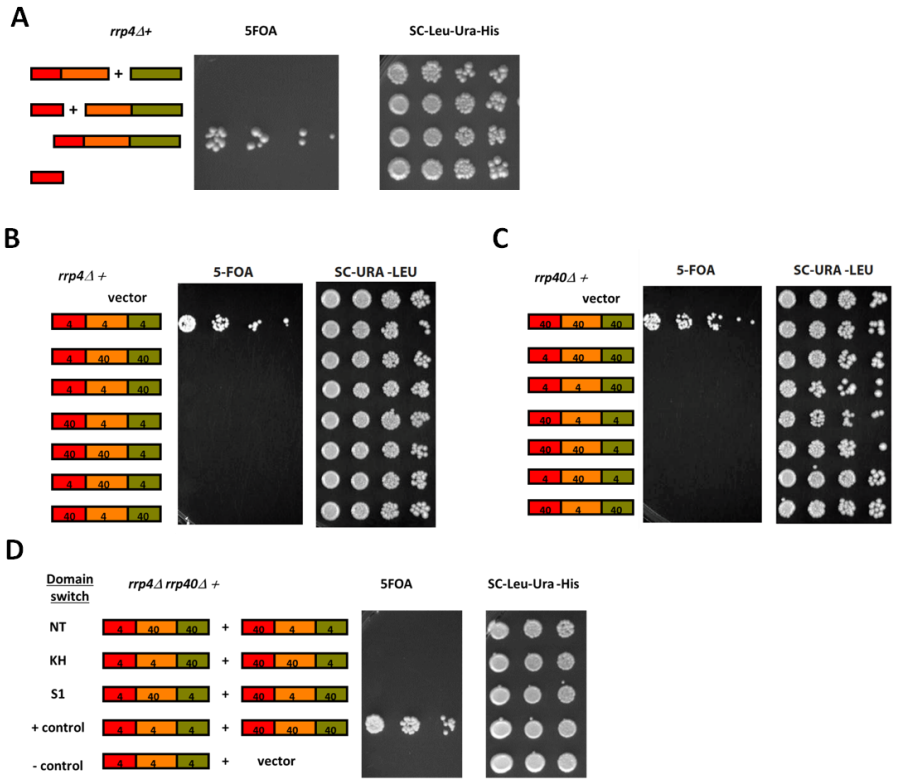


Figure 3-5 Rrp4 and Rrp40 have a structural role in the exosome A. Plasmid shuffle assay of the expression of Rrp4 *in trans(Schaeffer et al., 2009)*. One of the truncations is on a plasmid bearing a *LEU2* marker, and the other on a plasmid bearing a *HIS3* marker. B. Plasmid shuffle assay of the expression of a truncation Rrp4/40 protein in an *rrp4Δ* strain. The numbers on the pictures indicate from which proteins (4=Rrp4, 40=Rrp40) does the indicated domain come (Schaeffer et al., 2009). C. Plasmid shuffle assay of the expression of a truncation Rrp4/40 protein in an *rrp40Δ* strain (Schaeffer et al., 2009). D Plasmid shuffle assay of the expression of Rrp4 and Rrp40 in which a domain has been switched. Indicated domains are noted on the left (NT=N terminal domain, S1 domain, or KH domain) in a double deletion strain *rrp4Δrrp40Δ.*

complement the double deletion of Rp4 and Rrp40. The inability to switch domains also suggests that there is a structural role for Rrp4 and Rrp40.

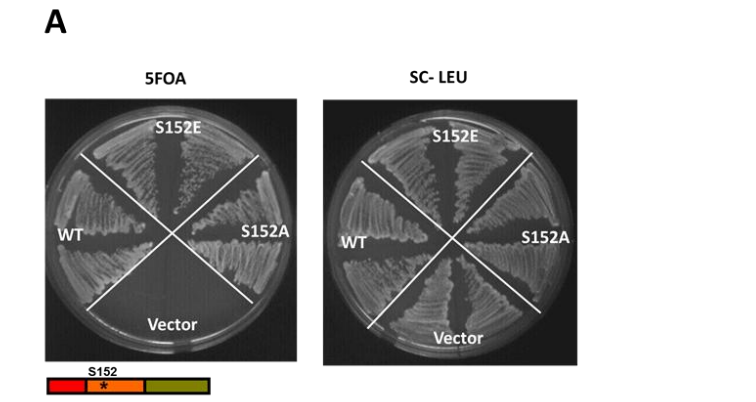
A conserved phosphorylation is not important for the essential role of Rrp4 or for the nonstop mRNA surveilance by the exosome.

A conclusion that Rrp4 and Rrp40 have structural roles, does not rule out the possibility that they also have functional roles. Both proteins have RNA binding domains suggesting that they bind RNA that is to be degraded by the exosome. To further look into a possible functional role, a search of two phosphorylation databases (PhospoPep and PhosphoGRID) was performed for possible posttranslational modifications. There was no evidence that Rrp40 was posttranslationally modified, however Rrp4 did have several predicted phosphorylation sites, specifically S28, S46, S152, S256, and S268. Only one of these phosphorylation sites, S152, was also found to be conserved in human and murine Rrp4 homologues and is located in the S1 domain close to where RNA enters the central channel of the exosome (Synowsky et al., 2006). This suggested that this particular modification was important for function. In order to test if this modification was important for viability residue 152 was mutated to alanine to mimic the phosphor-free state and to glutamic acid to mimic the phosphorylated state. Both mutants grew at wild-type level indicating that neither mutation affected the essential role of Rrp4 (Figure 3-6 A). These mutations were also tested by a nonstop assay to determine if they affected the cytoplasmic surveillance role of the

exosome. Briefly, an *rrp4Δ* strain yAV1104 with the Rrp4 phosphorylation mutants on a plasmid was transformed with a plasmid with a *HIS3* gene in which the stop codon is mutated (*his3-nonstop*) pAV188. The transformants were then grown with and without supplemented histidine in the medium. If the *his3-nonstop* mRNA is recognized as aberrant by the exosome, then it is quickly degraded. Thus, that strain will not grow on medium lacking histidine. If, however, there is a problem with cytoplasmic nonstop mRNA degradation, then the *his3-nonstop* mRNA will not be degraded, and a protein will be translated which will then allow for growth on medium lacking histidine. Figure 3-6 B shows that there is no growth of either point mutant indicating that there is no effect on the cytoplasmic mRNA degradation of nonstop mRNA by the exosome. Taken together, these data indicate that this conserved site of Rrp4 is not important for the essential role of Rrp4 or for the nonstop mRNA surveilance of the exosome.

Excess Rrp4 is stable

Some studies have suggested that exosome subunits localize to different sites in the cell. Specifically, by using indirect immunofluorescence with antibodies to endogenous exosome subunits, it was found that in *Drosophila melanogaster* S2 cells, various exosome components localize differently (Graham et al., 2006). The authors concluded that there may be differently assembled exosome complexes. However, a careful analysis of their data shows that Rrp4 was enriched in foci that were markedly different from the foci seen for Rrp40 and Rrp42 (a PH ring subunit



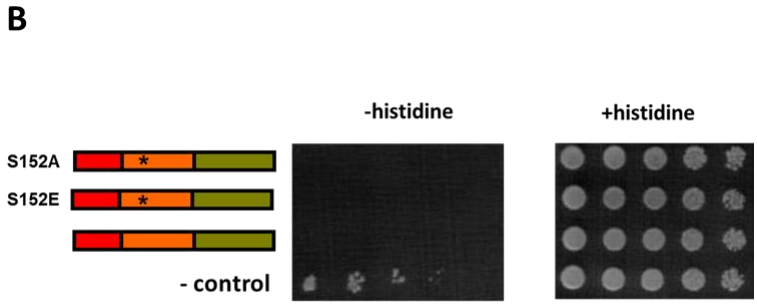


Figure 3-6. The posttranslational phosphorylation of Rrp4 is not important for viability or nonstop mRNA degradation. A. Plasmid shuffle assay of point mutants of Rrp4 S152E and S152A. Two independently derived clones of each mutant were plated. The location of S152 along the Rrp4 protein sequence is indicated with an asterisk on the picture below. B. Non-stop assay (see material and methods) of the same mutants in an *rrp4Δ* strain.

of the exosome) suggesting that Rrp4 may have a role outside of the exosome, since it does not localize with a PH ring subunit. Presumably, if such a role exists, Rrp4 would be stable on its own, not as a part of the exosome complex.

To test if Rrp4 is stable when not associated with the exosome, several experiments were completed. First, a wild-type strain was transformed with a plasmid encoding full-length, TAP- tagged Rrp4 with a *URA3* marker (Rrp4_TAP,URA) and a high copy plasmid with *GAL* regulated full-length Rrp4-ProteinA with an *LEU2* marker(GAL_Rrp4-PtnA, *LEU2*). The purpose of the experiment was to overexpress Rrp4 via the plasmid with the *GAL* promoter, and to verify the level of the total Rrp4 by a Western blot with an anti-Protein A antibody that detects the Protein A in the high copy vector and the TAP tag. If Rrp4 is not stable when not associated with exosome, it would be expected that the levels of the antibody detectable (i.e. TAP-tagged + Protein A tagged) Rrp4 would be the same compared to a strain in which there is no *GAL*_Rrp4-PtnA. However, the levels of Rrp4 increased (Figure 3-7 A). The increase seen in the Western blot is due to a Protein A moiety that is cloned in frame with the Gal_RRP4 gene, resulting in the expression of a Protein A-tagged Rrp4. These data suggest that Rrp4 is stable out of the exosome.

A second experiment to address the stability of Rrp4 when not in the exosome complex was completed. A vector with a Tap-tagged Rrp4 with a *LEU2* marker (Rrp4_TAP, *LEU2*), was introduced either in wild-type *S. cerevisiae* strain or in an *rrp4Δ* strain, complemented with a wild-type RRP4 allele on a vector with a *URA3* marker (Rrp4, *URA3*). The strains were then grown on 5FOA to select for the loss of

the *URA3* vector in the *rrp4Δ* strain. Therefore after plasmid shuffle both strains have the Rrp4_TAP vector, but the wild-type strain has a chromosomally originating wild-type Rrp4 while the *rrp4Δ* strain doesn't. As seen in Figure 3-7 B, both strains have the same amount of Rrp4_TAP even though the wild-type strain has the wild-type Rrp4 on the chromosome. These data also suggest that Rrp4 is stable out of the exosome complex.

A third experiment was completed for an additional confirmation of the stability of Rrp4 out of the exosome complex. This experiment utilizes temperature sensitive mutants of components of the cap, the PH ring, and the catalytic subunit. The mutants were a temperature-sensitive mutant of Mtr3, *mtr3-1*, a temperature-sensitive mutant of Rrp40, *mtr14-1*, and a temperature sensitive mutant of Rrp44, *mtr17-1*. These mutations were discovered as a part of a screen for mutants defective in nuclear mRNA export (Kadowaki et al., 1994). Each one of these strains and a wild-type strain were transformed with a vector with Rrp4_TAP and incubated at permissive (23^{o}C) and non-permissive (37^{o}C) temperature and then the amount of Rrp4_TAP was determined and compared. As seen from the results in Figure 3-7 C, there was no change in the amount of Rrp4_TAP in any of the strains at both temperatures. The results of this assay taken together with the first two experiments suggest that when Rrp4 is in excess, it is stable.

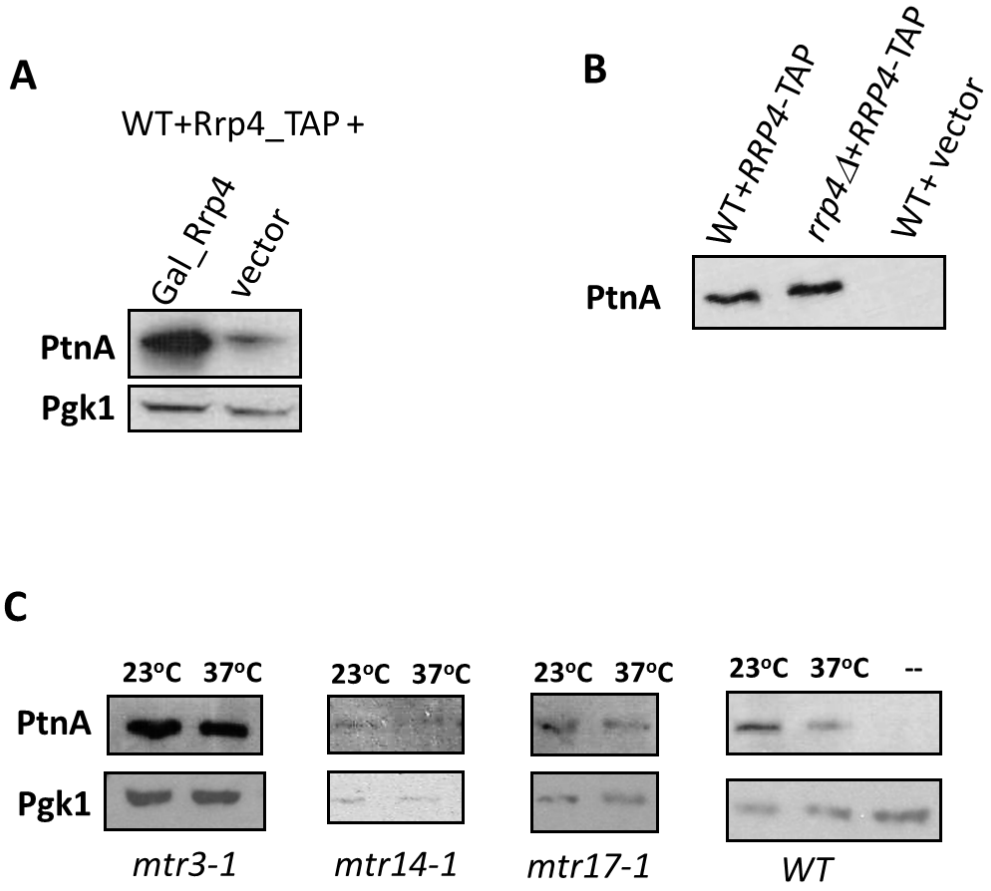


Figure 3-7 Rrp4 is stable out of the exosome complex. Western blots probed with either anti-ProteinA antibody (PtnA) or anti-Pgk1 for loading control. A. Wild-type *S. cerevisiae* strain, transformed with a plasmid with Rrp4_TAP and either *GAL*_Rrp4-PtnA or empty vector. B. Rrp4_TAP was introduced to either WT (ln1) or *rrp4Δ* (ln2) strain. Ln3 is a negative control WT+ vector. C. Temperature sensitive strains (listed below the Western blots) were transformed with an Rrp4_TAP and grown at the indicated temperatures. – (very right lane) is WT strain transformed with an empty vector control, grown at 23°C.

DISCUSSION

The data presented in this chapter begin to uncover the possible roles of the two RNA binding proteins Rrp4 and Rrp40. I have determined that the essential function of Rrp4 and Rrp40 does not lie only in one domain, but rather expression of the entire proteins is necessary for life. Additionally, Rrp4 and Rrp40 have unique roles that cannot be substituted by the other one, despite having such a similar domain organization. However, the data about the unique domain roles may not be valid because after this study was concluded, a new crystal structure of the exosome was published where the domain organization of Rrp4 and Rrp40 was changed (Makino et al., 2013). In particular there is a large difference of the boundaries of the S1 domain of Rrp40 which may have affected the results of the S1 domain switch (Figure 3-8).

One line of evidence presented here points to a structural role for both Rrp4 and Rrp40. First, both the RPL27-like and the KH domains of Rrp4 and Rrp40 are essential. Second, the RPL27-like domain of Rrp4, which interacts with Rrp41, and the RPL27-like domain of Rrp40, which interacts with Rrp46 are not interchangeable. The KH domains of both proteins are also not interchangeable. The KH domain of Rrp4 interacts with Rrp42, and the KH domain of Rrp40 interacts with Rrp43. Third, at least for Rrp4 I have established that the RPL27-like and the KH domains need to be in the same polypeptide. These data, taken together with data from other studies, specifically that the cap proteins were needed for the assembly of the exosome complex (Liu et al., 2006), indicate that Rrp4 and Rrp40 are needed to provide bridging contacts for the underlying PH ring proteins. After

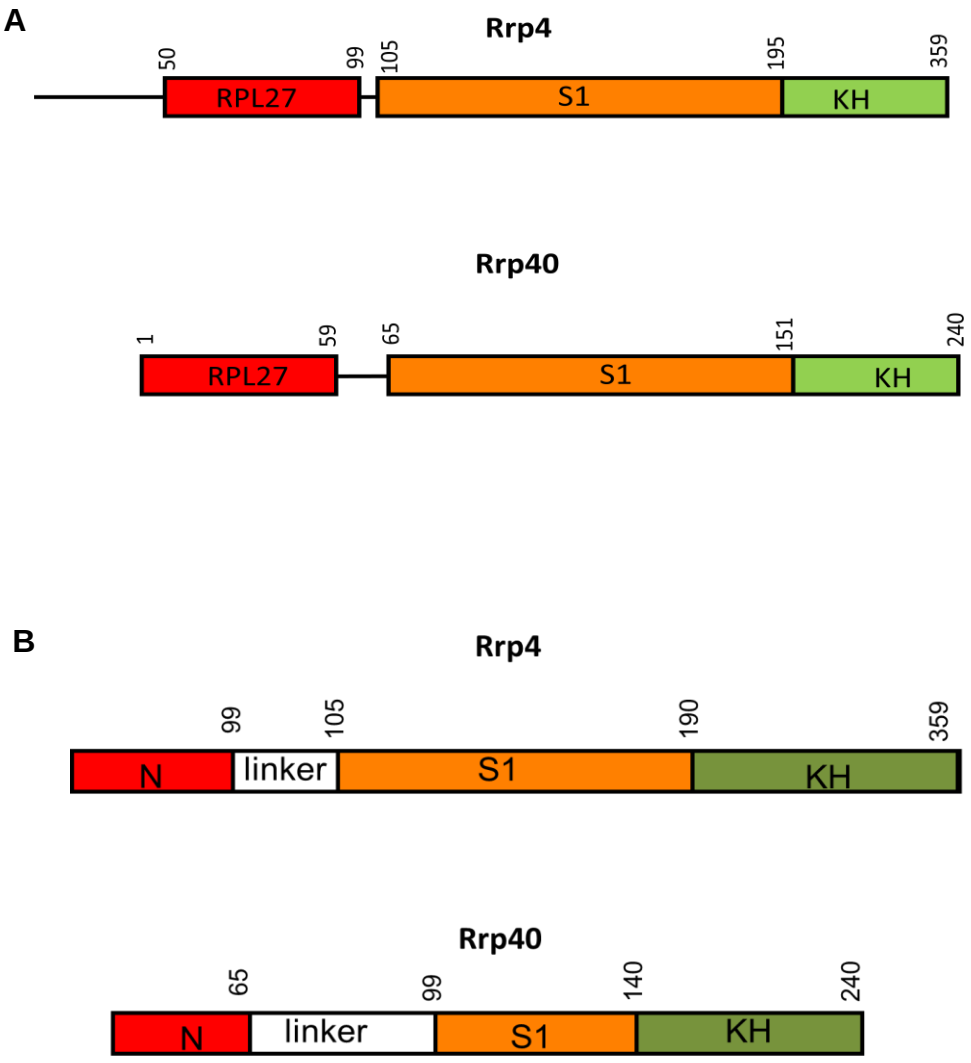


Figure 3-8. Revised domain organization of Rrp4 and Rrp40. A. Domain organization of Rrp4 and Rrp40 according to the crystal structure published by (Makino et al., 2013). B. Domain organization of Rrp4 and Rrp40 used to create the truncations used in this study.

this study was concluded, a study was published of reconstitution of the exosome, where the authors show that in vitro only Rrp40 was needed for the assembly of the PH ring of the exosome (Malet et al., 2010). While these data support my conclusion about the structural role of Rrp40, it does not support a model for a structural role for Rrp4. However, since this study was carried out *in vitro*, it is still possible that Rrp4 has a structural role, but that the exosome exists *in vivo* in two or more conformations. Another possible explanation is that Rrp4 may be added late during exosome assembly or it may be transiently associated with the exosome. Such unstable association may also explain the stability of Rrp4 out of the exosome complex, which is discussed below.

The presence of multiple RNA binding domains at the site of RNA entry into the exosome, points to a functional role for Rrp4 and Rrp40, specifically, binding of exosome's substrates. Point mutations of three basic residues in a loop in the S1 domain of Rrp4 were lethal suggesting that the essential role of Rrp4 is as a part of the exosome because the S1 domain binds RNA destined for degradation (Malet et al., 2010).

Additionally, Rrp4 seems also to have a functional role outside of the exosome complex. This conclusion is based on two separate pieces of data. First, Rrp4 has been shown to localize to distinct foci in *D. melanogaster* (Graham et al., 2006). Second, my data show that Rrp4 it not degraded when in excess. What this functional role is, is currently unknown. However, other exosome components have also been found to have a role outside of the complex. For example, Rrp46 was found to assemble in dimers, in addition to its assembly in the exosome (Yang et

al., 2010). Therefore it is possible that Rrp4 also has exosome independent roles. Future studies may address this possibility. The phosphorylation of Rrp4 suggest that there may be an additional regulation needed, for some other role of Rrp4, but that this possible role is non-essential given the fact that the phosphorylation itself was not essential. The phosphorylation site however was next to the essential basic residues that were found to be essential. It would be interesting to determine what if any regulation the phosphorylation of Rrp4 exerts on the proteins and what is the role of Rrp4 outside of the exosome.

Chapter 4. Characterization of the exosome cap protein Csl4

INTRODUCTION

Csl4 is one of the cap proteins of the exosome, together with Rrp4 and Rrp40. Csl4 was first identified in a screen for Cep1 synthetic lethality (Baker et al., 1998). Prior to that, a separate screen for viral super killer mutants identified eight so-called Ski proteins (Toh et al., 1978). It was later discovered that Csl4 is homologous to one of the genes identified in the superkiller screen, Ski4, and that it is a component of the exosome (Allmang et al., 1999b; van Hoof et al., 2000b).

The exosome associates with different proteins in the nucleus and in the cytoplasm and it is believed that these associations determine the specificity of substrate degradation or processing. However, the mechanism of these cofactor associations remains unknown. Csl4 is the only protein of the exosome complex which is currently known to have a mutation specifically disturbing cytoplasmic mRNA degradation, both for normal and nonstop transcripts. Therefore, Csl4 is a likely candidate to mediate the different roles of the exosome in the nucleus and in the cytoplasm.

Csl4 consists of three domains. The N-terminal domain is structurally related to ribosomal protein L27. In the middle of the protein there is an S1 domain which is an RNA-binding domain. The C-terminal domain is a Zn ribbon-like domain, which is structurally similar to Zn ribbons, but it lacks the cysteine residues characteristic of Zn ribbons. Therefore it does not bind Zn (Figure 4-1). For more detailed information on the RPL27-like and S1 domains in general, please refer to Chapter 3. In the Zn-ribbon domain of the archaeal homolog of Csl4 the cysteine residues

are present and thus archaeal Csl4 is likely to bind a Zn atom (Buttner et al., 2005). In *S. cerevisiae* Cslp4 also has a linker region between the RPL27-like domain and the S1 domain, which is absent from its human homolog. A point mutant *csl4-1,* in the promoter of Csl4, reduces expression of Csl4 and has been found to be defective in rRNA processing while the cytoplasmic mRNA degradation function of the exosome was not affected (van Hoof et al., 2000b). Another mutant, *ski4-1,* has a point mutation G253E located in the Zn ribbon-like domain and is defective in mRNA degradation but has normal rRNA processing phenotype (van Hoof et al., 2000b). These two mutants in effect separate the role of the exosome in the nucleus from its role in the cytoplasm.

Two general possibilities were considered when aiming to identify what was the essential role of Csl4. First, Csl4 could have a structural role in stabilizing the exosome complex. When the crystal structure of the human exosome complex was solved, the authors reported unsuccessfully trying to assemble just the PH ring without the cap proteins Csl4, Rrp4, and Rrp40. They were only able to assemble the entire complex once the cap proteins were added (Liu et al., 2006). This suggested that the cap proteins, one of which is Csl4, could play a structural role in the assembly of the complex. It was not further investigated whether all of them or only particular ones were needed for the exosome assembly. A second possible role for Csl4 is that it could have a functional role on the exosome complex. No enzymatic activity has been identified for Csl4, however it contains potential nucleic acid binding domains (S1 and Zn-like) and a possible protein-binding domain (RPL27-like), and it is located at the site of the proposed entry of substrate into the

exosome (Wang et al., 2007). It is known that the exosome functions with various protein cofactors but the locations of interactions between these cofactors and the exosome are unknown. Therefore is it possible that Csl4, being located at the proposed entry site of the substrate, mediates entry of the substrate into the exosome central channel by interacting with other protein factors, binding substrates, or both. This chapter outlines the experiments that were carried out in order to identify and distinguish these possible role of Csl4.

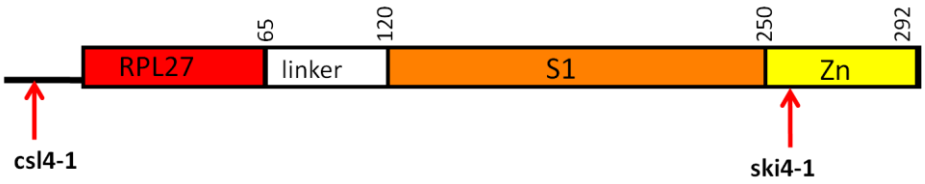


Figure 4-1 Domain organization of Csl4. Csl4 has three domains; the RPL27-like domain is the N-terminal domain, followed by a linker region which is not present in human homologues, an S1 domain and the a Zn ribbon-like domain. Numbers indicate residues. Point mutants csl4-1 is located in the promoter as depicted, and ski4-1 is located in the Zn ribbon-like domain.

RESULTS

Csl4 does not stabilize the exosome by bridging contacts

To determine which domain of Csl4 is essential, C-terminal truncations of Csl4 were created and used in a plasmid-shuffle assay (for details of the assay see materials and methods). Briefly, a *csl4Δ* strain was transformed with plasmids with either wild-type Csl4 or truncated Csl4. The truncations used were Csl4 1-120 (RPL27-like+linker), Csl4 1-250(RPL27-like +linker+ S1 domains), Csl4 65-292(linker + S1+Zn ribbon-like domains), and 120-292(S1 + Zn ribbon-like domains). Transformants were selected on SC-Leu-Ura. Next, a plasmid shuffle assay was performed on 5FOA to determine if any of the truncations could substitute for the essential role of Csl4. It was found that the RPL27-like+linker domain was sufficient for viability even after removing the Zn ribbon-like domain and the S1+ the Zn ribbon-like domains (Figure 4-2A). However, based on Csl4's buried surface from the crystal structure of the human exosome, the RPL27-like domain should only interact with one of the PH ring subunits, Mtr3 (Liu et al., 2006). Therefore a truncation that only interacts with one subunit cannot bridge the underlying proteins. These data suggested that the essential role of Csl4 was not in stabilizing the exosome by bridging contacts between the PH subunits. In confirmation of these results, a paper published in 2007 also indicated that the yeast exosome can be assembled without Csl4 *in vitro* (Wang et al., 2007).

The RPL27-like domain and the S1+ Zn ribbon-like domains are redundant for the essential function of Csl4

To further confirm which domains were essential for the role of Csl4, N-terminal truncations of Csl4 were also created and used in a plasmid shuffle assay. Surprisingly, the RPL27-like domain was not essential and viability was conferred as long as the S1+ Zn ribbon-like domains were expressed *in cis* (Figure 4-2 A). The Zn ribbon-like truncation did not grow (not shown). To clarify whether the S1 or the Zn ribbon -like domain contributed to the essential role in the Csl4-S1+Zn-ribbon-like truncation, the S1 domain was expressed by itself and was found not to be sufficient for life (Figure 4-2 B). Therefore, the S1+Zn ribbon-like domains were also sufficient for viability.

Expression of the truncations was checked by Western blot using TAP-tagged truncations and all but the Zn ribbon-like domains were found to be expressed (Figure 4-2 C). Because the Western blot was done using TAP-tagged truncations of Csl4, but the initial growth assay was done using untagged truncations, it was also verified by a growth assay that the addition of the TAP tag did not alter the phenotype of the viable mutants (Figure 4-2 D). The above data indicate that the RPL27-like domain and the S1+ Zn ribbon-like domains are redundant for the essential function of Csl4. This is consistent with a view that if an exosome-interacting domain of Csl4 is expressed the essential function of Csl4 can be carried out.

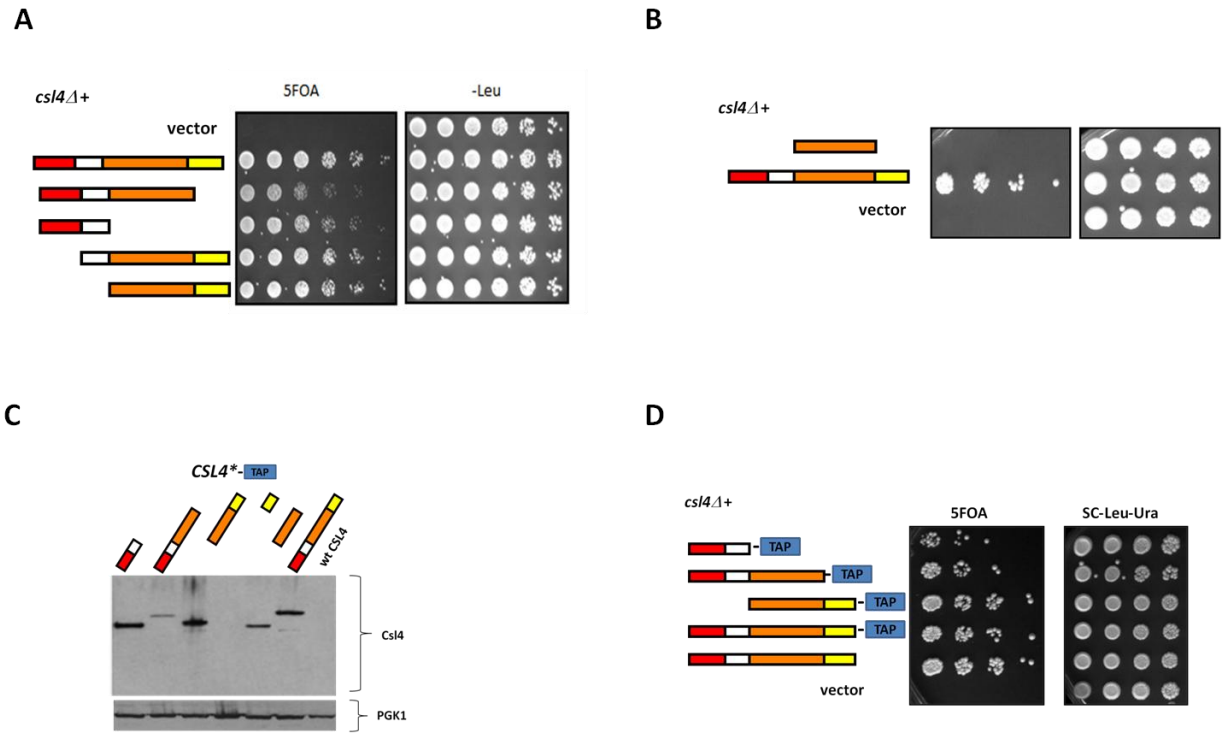


Figure 4-2. The RPL27-like domain and the S1+ Zn ribbon-like domains are redundant for the essential function of Csl4. A and B Plasmid shuffle assay. *csl4Δ* strain was transformed with plasmids with the indicated truncations and LEU2 markers. C. A Western blot of the depicted TAP-tagged truncations of Csl4. PGK1- loading control. D. Plasmid shuffle assay. *csl4Δ* strain was transformed with plasmids with the indicated truncations with a C-terminal TAP tag and LEU2 markers.

Because it was determined that Csl4 does not stabilize the exosome by bridging interactions, to further characterize its role (both essential and non-essential), several possibilities were addressed using the viable truncations of Csl4. First, the nuclear function of the exosome was tested; second, the cytoplasmic function of the exosome was tested; third, posttranslational modifications were tested; fourth, conserved surface residues were mutated.

The ZN ribbon-like domain is required for the known functions of the cytoplasmic exosome.

To correlate the growth data with known biological roles of the exosome, the domains of Csl4 that were found to sustain viability, were tested for the known nuclear roles of the exosome. Specifically, I tested the 7S pre-rRNA processing and 5' ETS degradation, which are known to be affected in exosome depletions (Allmang et al., 1999a). The processing of the 7S pre-rRNA was slightly affected in the truncation bearing only the RPL27-like domain, but unaffected in other viable truncations. There was no effect on the final 5.8S rRNA processing and on the degradation of the 5' ETS (Figure 4-3). This indicated that viable Csl4 truncations behaved almost as wild type in rRNA degradation and processing.

The same domains were also tested for known cytoplasmic exosome roles, specifically cytoplasmic mRNA degradation and nonstop mRNA degradation.

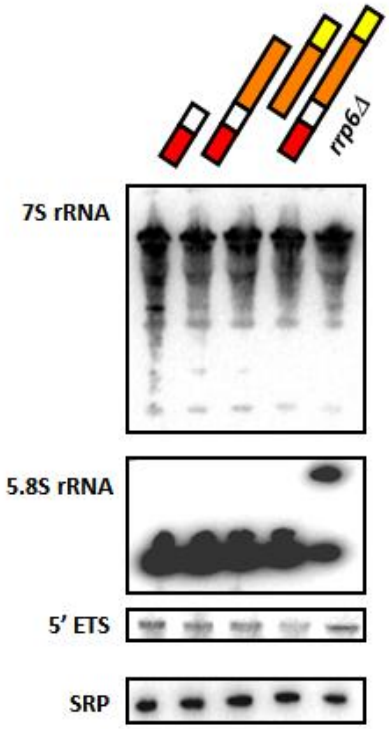


Figure 4-3 Viable truncation of Csl4 behave as wild type for rRNA processing and degradation. Northern blot analysis of *csl4Δ* strain transformed with the indicated truncations. The RNA for which it was probed are listed on the left of the pictures. SRP (the RNA moiety of the signal recognition particle) is used as a loading control.

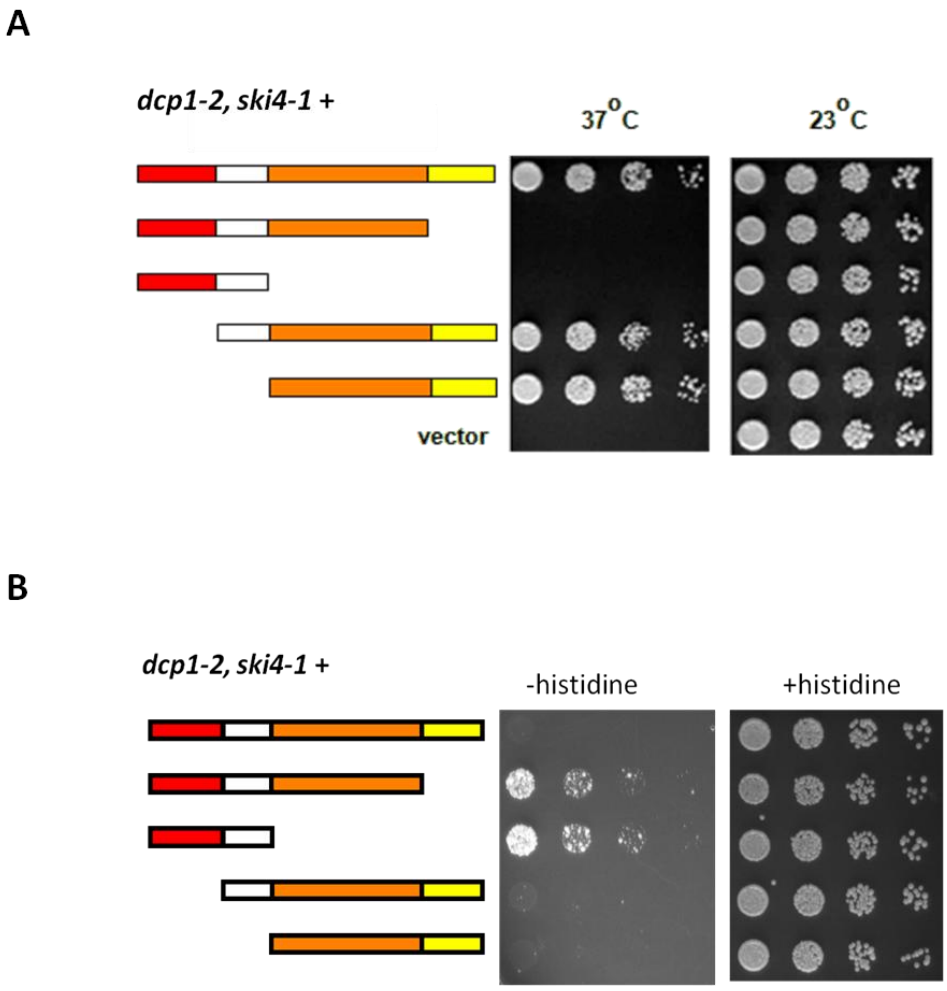


Figure 4-4 Cytoplasmic mRNA degradation and nonstop mRNA surveillance by the viable Csl4 truncations. A. Cytoplasmic mRNA degradation assay of the depicted truncations B. nonstop assay of the depicted truncations.

Briefly, to completely abolish cytoplasmic mRNA degradation, which is essential, both 5'→3' and 3'→5' degradation pathways must be blocked. A strain with a temperature sensitive mutation *dcp1-2* and a mutation *ski4-1* was used. The *ski4-1* mutation blocks the 3'→5' mRNA degradation pathway. The *dcp1-2* mutation conditionally blocks the 5'→3' mRNA degradation pathway when transferred to non-permissive temperature. For description of the assay, see Chapter 2 "Materials and methods". The *dcp1-2 ski4-1* mutant was transformed with vectors encoding viable truncations of Csl4 and then grown at permissive and non-permissive temperatures. At the non-permissive temperature both pathways for mRNA degradation are blocked and this strain can only survive if a truncation of Csl4 can substitute for the mRNA degradation role of wild-type Csl4. As seen in Figure 4-4 A, truncations lacking the Zn ribbon-like domain did not survive, while other truncations did, indicating that the Zn ribbon-like domain is required for mRNA degradation by the exosome. Additionally, the truncations were tested for nonstop mRNA surveillance by the exosome. A *csl4Δ* strain supplemented by truncations of Csl4 were transformed with a vector carrying a HIS3 gene lacking a stop codon (*his3-nonstop*) and grown with and without histidine. Only the truncations lacking the Zn ribbon-like domain grew on medium lacking histidine, indicating that the Zn ribbon - like domain is required for nonstop mRNA degradation by the exosome. Therefore, the Zn ribbon-like domain of Csl4 is required for all known cytoplasmic roles of the exosome.

Posttranslational phosphorylaiton at S94 is not important for the essential role Csl4.

To continue looking into the role of Csl4, a search of PhospoPep and PhosphoGRID phosphorylation databases was performed for possible phosphorylation sites. It was found that Csl4 could potentially be phosphorylated in the linker between the RPL27-like and the S1 domain, at residue S94 (Synowsky et al., 2006).To test if the phosphorylation was required for viability, residue 94 was mutated to alanine and to glutamic acid to mimic the phosphor-free and the phosphorylated state. Both mutants were able to substitute for the essential role of Csl4, indicating that this particular potential posttranslational modification was not required for viability (Figure 4-5).

The Zn ribbon-like domain is essential in the absence of the RPL27-like domain

While expressing the S1 and Zn ribbon-like domains together was sufficient for viability, expressing either one by itself was not. To determine which domain, S1 or Zn ribbon-like, carried the essential function, 23 conserved amino acids were mutated in either the S1 or the Zn ribbon-like domain. All of the mutations were created in a truncation of Csl4 which lacks the RPL27-like domain because the RPL27-like domain is viable on its own as determined previously. Therefore its presence on its own confers viability. All of the amino acids were changed to alanine. There were four double mutants in the S1 domain: V210A R211A, D214A

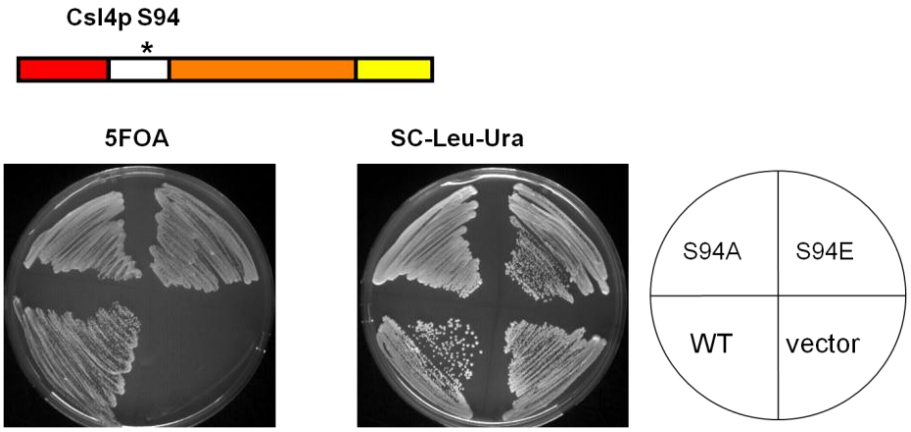


Figure 4-5. The posttranslational phosphorylation of Csl4 is not important for the essential role Csl4. Plasmid shuffle assay of point mutants of Csl4 S194A and S94E. The location of S94 along the Csl4 sequence protein sequence is indicated with an asterisk on the picture above.

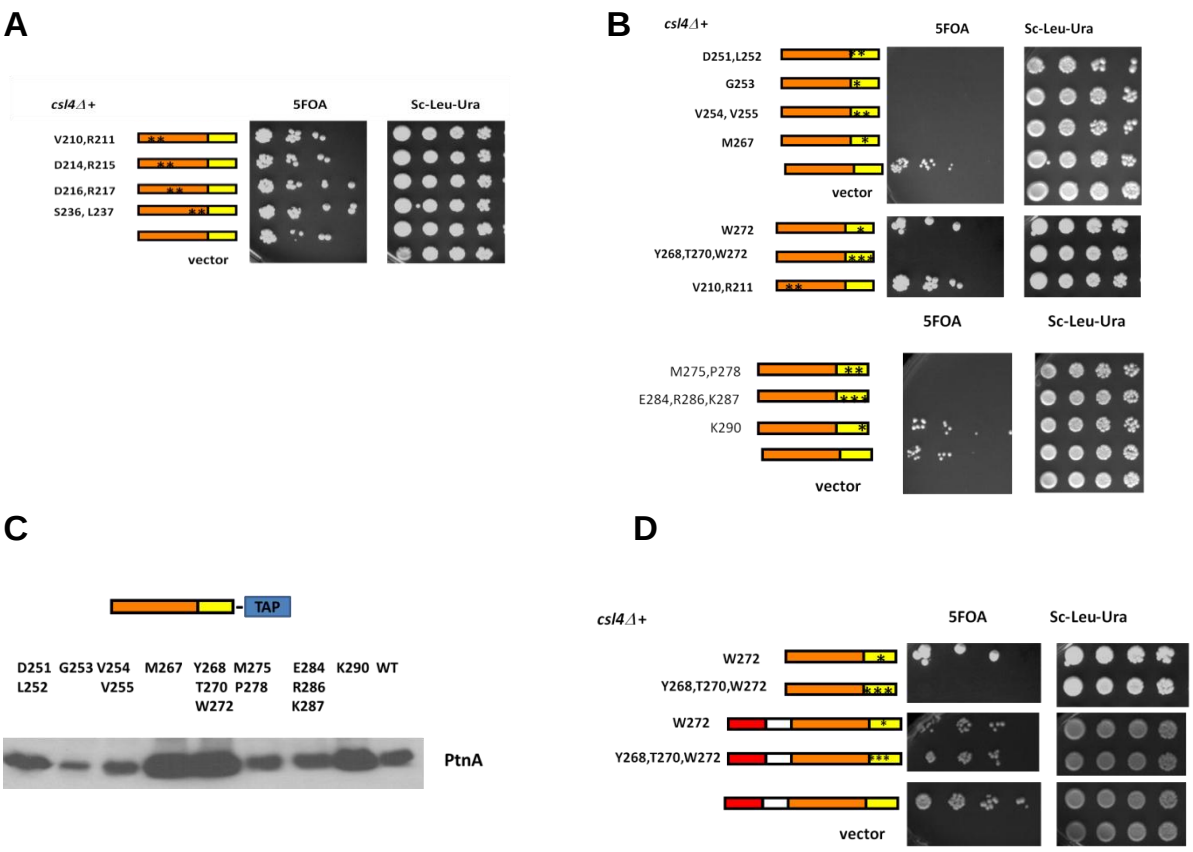


Figure 4-6 The Zn ribbon-like domain is essential in the absence of the RPL27-like domain. Plasmid shuffle assays of the indicated truncations. Strain, cls4Δ , is indicated in the left upper corner, the mutations are indicated at the left of each picture, medium indicated above the picture of each growth assay A. Point mutants of residues located in the S1 domain in truncations lacking the RPL 27 domain. B. Point mutants of residues located in the Zn ribbon-like domain. D. Point mutants of residues located in the Zn ribbon-like domain in truncations with or without the RPL27-like domain C. A Western blot of TAP-tagged truncation of Csl4 lacking the RPL 27 domain with the indicated point mutations above each blot.

R215A, D216A R217A, and S236A L237A. All of these mutants were viable as determined by a plasmid shuffle assay (Figure 4-6 A) suggesting that the S1 domain was not essential for viability. To check if the Zn ribbon-like domain was essential for viability, nine mutants were created in the Zn ribbon-like domain: D251A L252A, G253A, V254A V255A, M267A, W272A, Y268A T270A W272A, M275A P278A, E284A R286A K287A, and K290A. With the exception of W272A and K290A, all of the rest of the mutations were lethal (Figure 4-6 B). To confirm that the lack of growth was due specifically to the point mutations, and not due to lack of expression, the expression of these mutants, except W272A which was viable, was confirmed by a Western blot (Figure 4-6 C). Addition of the RPL27-like domain to the Y268A T270A W272A mutant, restored viability suggesting that the RPL27-like domain and the Zn-ribbon domain may have a functional redundancy for the essential role of Csl4 (Figure 4-6 D). Overall, these data indicate that in Csl4 the Zn ribbon-like domain is essential in the absence of the RPL27-like domain.

The essential role of the Zn ribbon-like domain in the absence of the RPL27-like domain overlaps with its role in mRNA degradation and nonstop mRNA decay.

To determine if the essential role of the Zn ribbon-like domain in the absence of the RPL27-like domain was specific to the nuclear exosome, the cytoplasmic exosome function was also tested. Each of the nonviable truncations was transformed into a double mutant *dcp1-2 ski4-1* and after selection for

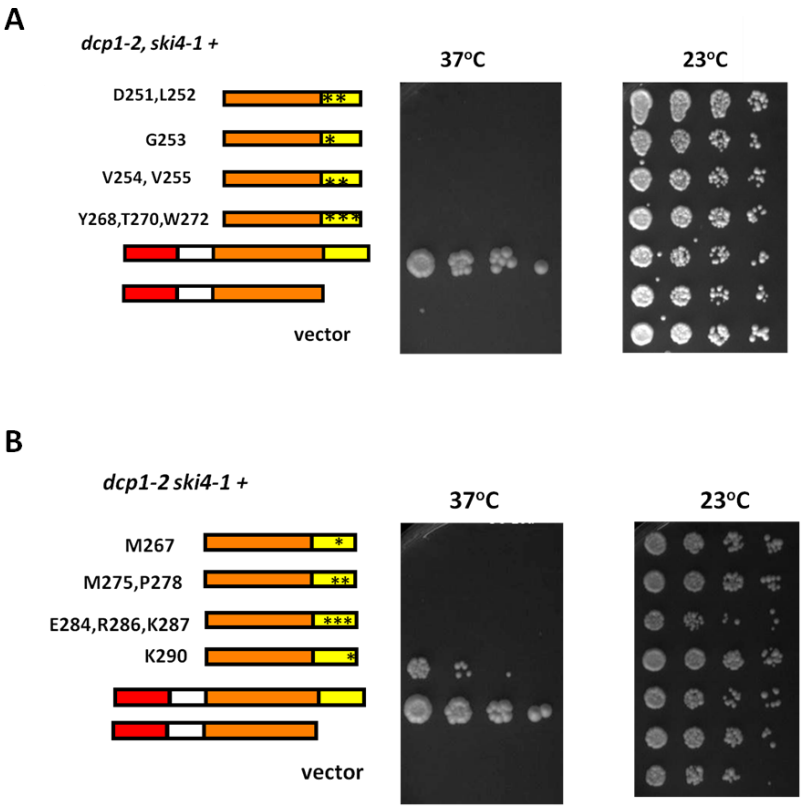


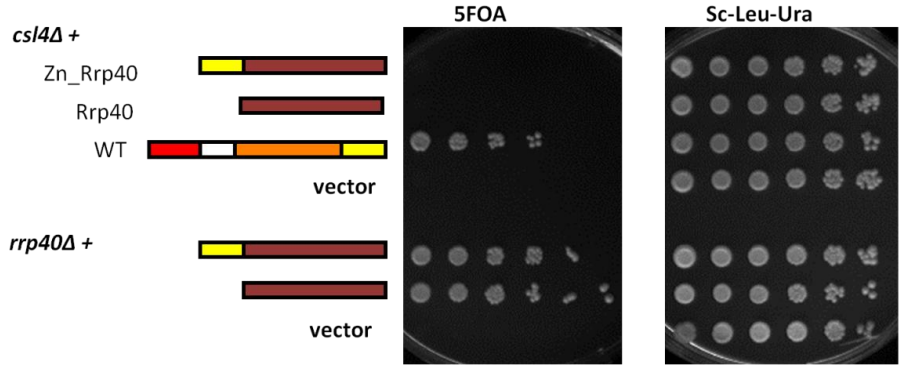
Figure 4-7. Point mutations in the Zn ribbon-like domain are defective in mRNA degradation in the absence of RPL 27 domain. A and B. mRNA degradation growth assays of the indicated point mutations in the S1+Zn ribbon-like truncation of Csl4.

transformants, grown at permissive and non-permissive temperatures. Each of the mutants that was not able to substitute for the essential role of Csl4 was also not able to substitute for mRNA decay function (Figure 4-7 A and B). In contrast, the mutant that was viable, K290A, was also able to substitute for the mRNA decay functions. These results suggest that the essential role that the Zn ribbon-like domain carries out in the absence of the RPL27-like domain, overlaps with its cytoplasmic roles in mRNA degradation and nonstop mRNA decay.

Localizing the Zn ribbon-like domain to the exosome is not sufficient for the essential role of Csl4

One possible explanation of the data presented above is that the Zn ribbon-like domain has an important function and that the S1 domain may allow it to carry out this function by recruiting the Zn ribbon-like domain to the exosome. To test this hypothesis, the Zn ribbon-like domain of Csl4 was fused to the N-terminus of Rrp40 (Zn-Rrp40). The reason for designing the chimera in this manner, versus fusing it to the C-terminus, was that the N-terminus of Rrp40 is the domain at which Rrp40 interacts with the Zn ribbon-like domain of Csl4.Therefore this chimera is expected to localize the Zn ribbon-like domain to the site where it is usually positioned on the exosome. A vector with the Zn-Rrp40 chimera was transformed into a *cl4Δ* strain

A



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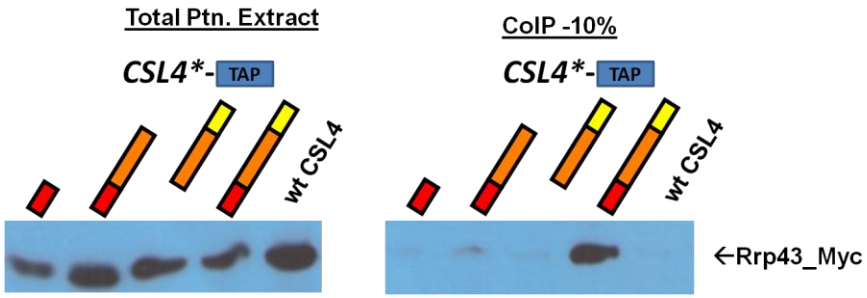

Figure 4-8 Csl4's essential role may not be as a part of the exosome complex. A. Plasmid shuffle assay of the indicated chimeras in *csl4Δ* and *rrp40Δ* strains. B. A Western blot of Co-immunoprecipitation of Myc-tagged Rrp43 with TAP-tagged Csl4 truncations.

and also into an *rrp40Δ* strain and after selecting for transformants, both stains with appropriate controls were grown on 5FOA for a plasmid shuffle assay. The Zn-Rrp40 chimera was able to complement an *rrp40Δ* strain indicating that the protein is expressed (Figure 4-8 A bottom 3 lanes). However, the Zn-Rrp40 chimera was not able to complement a *csl4Δ* strain, indicating that localizing the Zn domain to the exosome was not sufficient for viability (Figure 4-8 A).

Multiple domains contribute to Csl4 association with the exosome

Multiple domains were found to be sufficient for viability; however it was not clear if each of them was required for localization to the exosome. Therefore, an experiment was carried out to test which domain of Csl4 was required for localization to the exosome. TAP-tagged Csl4 truncations were transformed into a strain with a Myc-tagged Rrp43. Co-immunoprecipitation was performed with Protein A beads and then a Western blot was performed on the total protein extract and the bound fraction. As seen in Figure 4-8 B, Rrp43 only interacted with the full length Csl4, but not with any of the other otherwise viable truncations, suggesting that these truncations were not interacting with the core exosome. This indicates that more than one domain is necessary for the association of Csl4 with the exosome.

DISCUSSION

The data presented in this chapter uncover at least partially the possible roles Csl4 may play in the exosome. Given the fact that the other two exosome proteins, Rrp4 and Rrp40 were determined to have structural roles, it is interesting that Csl4 does not seem to bridge PH ring subunits of the exosome. This is despite the fact that two of its three domains are very similar and it is also located at the same side of the exosome at the RNA entry. Other studies have found that Csl4 is not as tightly associated with the exosome as Rrp4 and Rrp40, further supporting the idea that it does not stabilize the exosome by bridging interactions, but instead has a functional role (Synowsky et al., 2006). The potential phosphorylation site found on Csl4 at residue S94 suggests that there may be some regulation, however no effect on cell viability was discovered suggesting that regardless of the regulation (if any) this modification does not involve the essential role of Csl4. After the conclusion of this study a new crystal structure of the exosome was published(Makino et al., 2013). Therefore the domain organization of Csl4 was revised (Figure 4-9). However, the changes to Csl4 were very slight and considering how the truncation were made (see Chapter 2, Table 2-2) should not have any effect on the conclusions based on the experiments in this chapter.

In addition to its essential and likely nuclear role, Csl4 also has a cytoplasmic role and the Zn ribbon-like domain was found to be required for it. A mutant *ski4-1*, which has a point mutation in the Zn ribbon-like domain of Csl4, cannot do cytoplasmic mRNA degradation (van Hoof et al., 2000b). However how it exerts its effect was not known. This point mutation is only four amino acids away from the S1

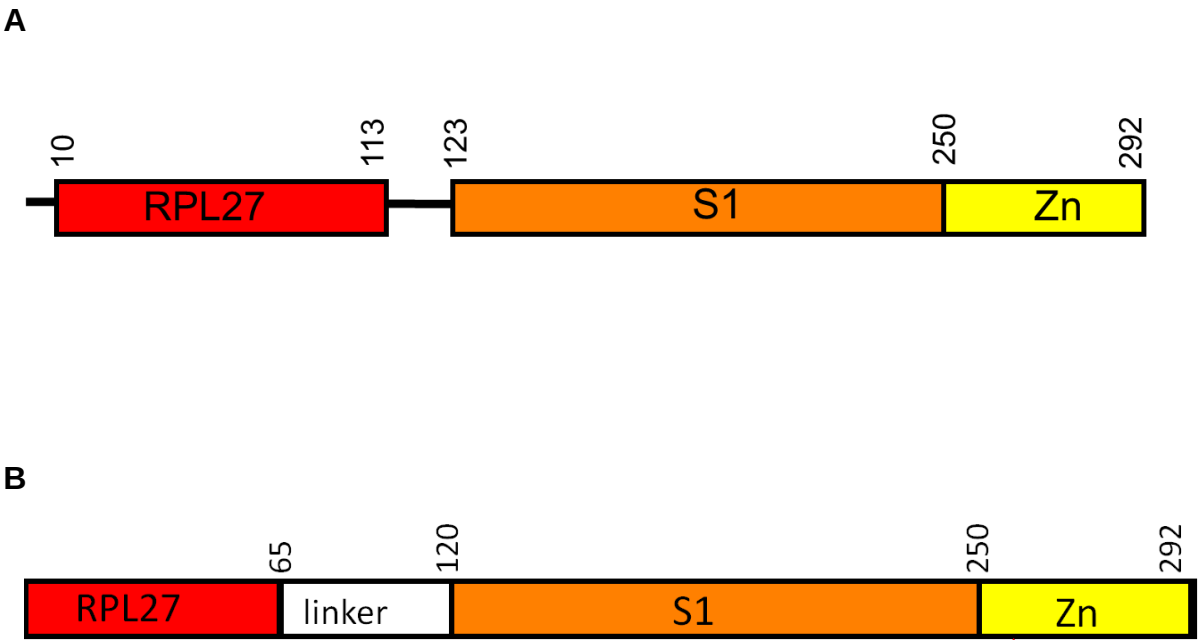

Figure 4-9. Revised domain organization of Csl4. A. Domain organization of Csl4 according to the crystal structure published by (Makino et al., 2013). B. Domain orgnizaiton of Csl4 used to create the truncations used in this study.

domain; therefore it was possible that alteration in the S1 domain, not the Zn ribbon-like domain was responsible for this effect. The data presented here indicate that it is the Zn ribbon-like domain that is required for cytoplasmic mRNA degradation and also for nonstop mRNA decay because a truncation lacking the Zn ribbon-like domain, but containing the entire S1 domain did affect the cytoplasmic role.

A very intriguing finding is that while Csl4 is an essential protein, it does not contain a single essential domain. Viability is conferred as long as either the RPL27-like domain or the S1+Zn domain are expressed. This suggests that whatever the essential function of Csl4 is, these two distinct parts of the protein must be able to perform it. Each of these two parts of Csl4 interacts independently with the exosome. It is therefore possible that a protein cofactor or RNA interact independently with separate moieties on Csl4 and when only one or the other is expressed there is still residual interaction capable of carrying out the essential function of Csl4 (Figure 4-10).

Looking to further narrow down which domain of the S1+Zn truncation actually carries out the essential role of this truncation, it was found that in fact it was the Zn ribbon-like domain. This conclusion was based on the fact that mutations in conserved residues along the Zn ribbon-like domain were inviable, while point mutations along conserved residues in the S1 domain did not affect viability. These same mutations also affected the non-essential cytoplasmic roles of Csl4 in normal mRNA decay and nonstop mRNA degradation.

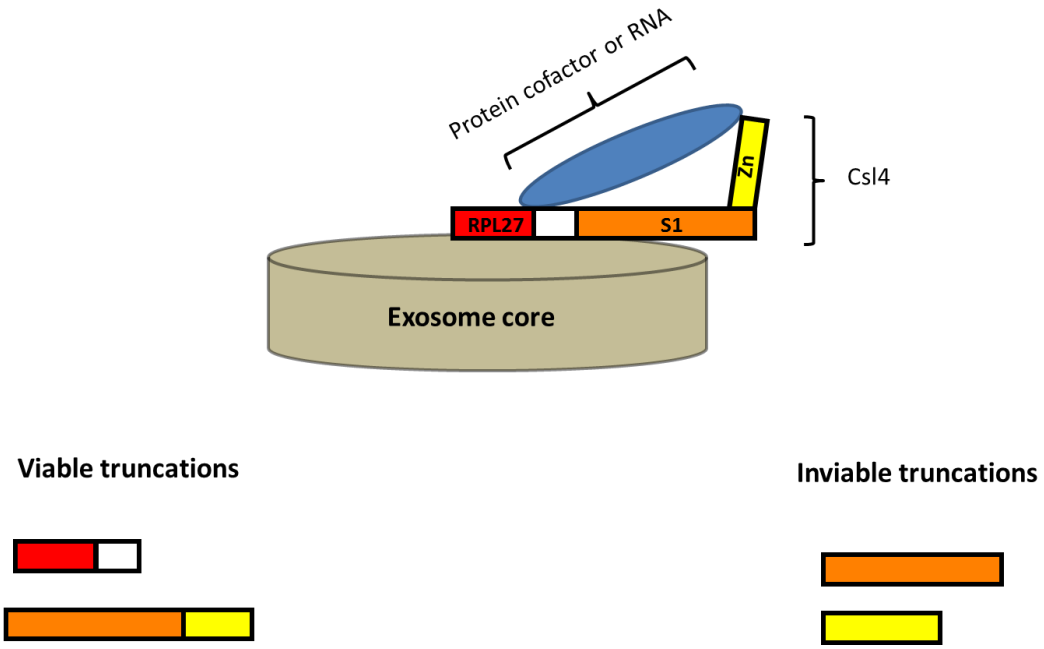

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Figure 4-10. Model for interaction of Csl4 with a protein co-factor or RNA. A protein cofactor or RNA that are required for the essential role of Csl4 interacts with both the RPL27-like and the Zn ribbon-like domain and interaction with the exosome is also required for viability. On the bottom left is a depiction of the truncations that were sufficient for growth and on the bottom right is a depiction of the truncations that were not sufficient for growth.

One interpretation of these data is that the point mutations of conserved residues affect the structure of the Zn ribbon-domain rendering it non-functional. Another possibility is that these residues are important for contact and stable interactions with a protein cofactor or RNA substrate which are weakened even further in the absence of the RPL27-like domain. Finally a combination of these interpretations may be what is actually going on and some mutations may affect structure while others may affect binding. Regardless of that, it is the Zn ribbon-like domain that overlaps with the RPL27-like domain for the essential role of Csl4. However, the Zn ribbon-like domain had to be expressed together with the S1 domain. The S1 domain is where the S1+Zn interact with the exosome. That is consistent with a model in which RNA or protein cofactor(s) bind two separate ends of Csl4 and need to be brought to the exosome, and in this case the S1 domain may be fulfilling that role.

Csl4 may participate in a mixed RNA-protein interaction in addition to binding exclusively RNA or proteins. For example archaeal Csl4 is required for interaction of the exosome with DnaG in order to improve interaction with A-rich RNAs (Hou et al., 2013). Csl4 is capable of binding both RNA and protein, Rrp6, on different surfaces (Makino et al., 2013). Therefore it is possible that there is more than one essential role for Csl4 and it binds RNA for one and proteins for a different essential role.

Chapter 5: The exoribonuclease of Rrp44 affects iron response

Disclaimer: This chapter includes experiments completed by other people. The microarray and the calculations of mRNA levels were completed in the laboratory of Dr. Allan Jacobson, University of Massachusetts. The PARE analysis was completed in the laboratory of Dr. Pamela Green at the University of Delaware.

INTRODUCTION

Structural organization of Rrp44, the catalytic subunit of the RNA exosome

Rrp44 is the only catalytically active subunit of the exosome (Dziembowski et al., 2007; Liu et al., 2006). It has a 3'→5' exonuclease activity and an endonuclease activity (Dziembowski et al., 2007; Greimann and Lima, 2008; Lebreton et al., 2008; Schaeffer et al., 2009; Schneider et al., 2009). All known exosome activities are affected in an exonuclease point mutant, but not in an endonuclease point mutant. These include nuclear roles such as processing of 5.8S rRNA and snoRNAs and degradation of the 5' ETS of rRNA (Allmang et al., 1999a; Mitchell et al., 1996; van Hoof et al., 2000a). In addition, cytoplasmic degradation of normal mRNAs also seems to be affected only in the exoribonuclease mutant (Schaeffer and van Hoof, 2011). In the case of cytoplasmic nonstop mRNA degradation the exonuclease and the endonuclease domains are redundant, but still, there is no known substrate that is specific only to the endonuclease (Schaeffer and van Hoof, 2011)

Rrp44 has 5 domains. From the N to the C terminus the domain organization is as follows: there is a PIN domain which harbors the endonuclease activity, two cold shock (CSD) domains CSD1 and CSD2, an RNB domain which has the 3'→5' exoribonuclease activity, and an S1 domain which is an RNA binding domain (Figure 5-1). In addition, at the very N-terminus of the protein there is a conserved region of 33 amino acids followed by a CR3 region which consists of three conserved cysteine residues (Cairrao et al., 2005; Schaeffer et al., 2012). Each of the domains of Rrp44 is found both in eukaryotic and prokaryotic organisms and

with the exception of the PIN domain, the rest of the domains are shared between Rrp44 and its bacterial homologues RNase II and RNase R (Amblar et al., 2006; Vincent and Deutscher, 2009). Point mutants are available that completely inactivate the catalytic activities of the exosome. A point mutation D171A completely abolished the endonuclease activity (Schaeffer et al., 2009), while a point mutant D551N eliminates the 3'→5' exoribonuclease activity (Dziembowski et al., 2007).

Rrp44 contacts the exosome at the site opposite of the RNA binding proteins (see Chapter 1, Figure 1-1). RNA that is threaded through the PH ring reaches the RNB domain of Rrp44 where it is hydrolytically degraded in the 3'→5' direction. The exonuclease faces the central channel and thus any substrate that emerges from it, suggesting that RNA that goes through the channel is destined for exoribonuclease degradation. It is not clear however why the endonuclease faces away from the central channel (Chapter1, Figure 1-1). Studies suggest that the RNA is delivered to the endonuclease through the central channel as well, since mutations in the central channel were shown to affect the activity not only of the exonuclease, but also of the endonuclease (Drazkowska et al., 2013; Wasmuth and Lima, 2012)

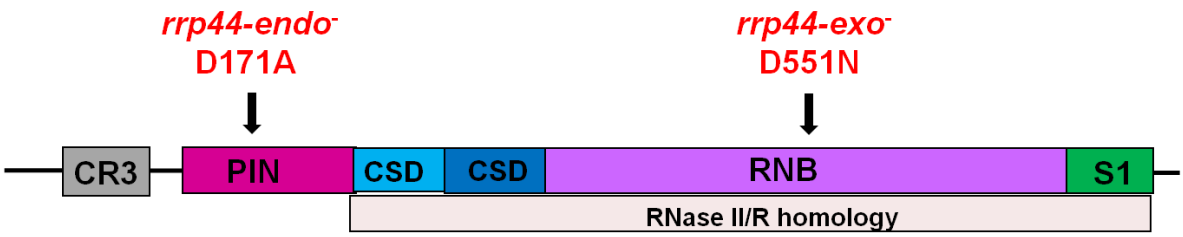


Figure 5-1. Domain organization of Rrp44. The domains of Rrp44 are depicted. Locations of the nuclease point mutants are shown above the protein and the part of Rrp44 that is homologous to RNase II and RNase R is shown below the protein.

While Rrp44 is the only catalytic subunit of the eukaryotic exosome, it may not always be associated with the exosome. For example, there are three proteins that are homologues of Rrp44 in humans, and they localize to different cellular compartments (Tomecki et al., 2010). In *S. cerevisiae* and in *Drosophila melanogaster* Rrp44 is not as tightly associated with the exosome as the rest of the subunits (Allmang et al., 1999b; Graham et al., 2009a). An Rrp44 mutant that has the N-terminus deleted does not associate with the exosome (Schneider et al., 2009). Studies in *Arabidopsis thaliana* and in trypanosomes did not find that Rrp44 associates with the exosome, however it is still possible that *in vivo* Rrp44 does in fact associate with the exosome in these organisms (Chekanova et al., 2002; Estevez et al., 2001). Finally, microarray studies on knock downs of Rrp44, Rrp6, and core exosome subunits, showed that the substrates of each differed suggesting again exosome independent roles for these proteins (Kiss and Andrulis, 2010). Regardless of whether it does or it doesn't have exosome independent functions, Rrp44 does provide the catalytic activity of the exosome thus it is important for all of the exosome's functions.

Functional studies of Rrp44 and the exosome

Most studies of the exosome and Rrp44 have concentrated on substrate specificities and structure, thus functional studies are limited. However there are several studies that associate the exosome and Rrp44 with particular cellular roles. Rrp44 has been found to alter circadian rhythm in *Neurospora crassa* (Guo et al., 2009). Yeast Rrp44 mutant has also been found to have a defect in microtubules

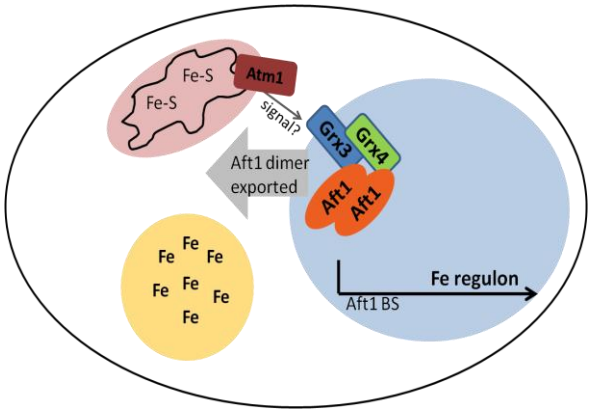
(Smith et al., 2011). Finally, a study showed that Rrp44 affects chromosome segregation and mitotic progression in *Schizosaccharomyces pombe* by affecting kinetochore formation and kinetochore-microtubule interactions (Murakami et al., 2007).

Iron response in S. cerevisiae

Iron balance is critically important for the cell, because iron is on the one hand an essential element required for the production of all proteins with an iron-sulfur cluster, but on the other hand too much free iron is toxic. Therefore the intake of iron is tightly regulated (Figure5-2).

It is not known how low iron is sensed, however an inhibitory signal is known to come from the mitochondria and to be dependent on the mitochondrial transporter Atm1 (Rutherford et al., 2005). This in turn downregulates the glutathione reductases Grx3 and Grx4 which promote export from the nucleus of the major iron response regulator Aft1 by forming a complex with Fra1/2 and stimulating Aft1's dimerization (Li et al., 2009; Pujol-Carrion et al., 2006). When Aft1 dimerizes, it is expelled form the nucleus and thus cannot activate transcription of the iron response genes (Ojeda et al., 2006; Yamaguchi-Iwai et al., 2002).

Iron replete conditions:



Iron deplete conditions:

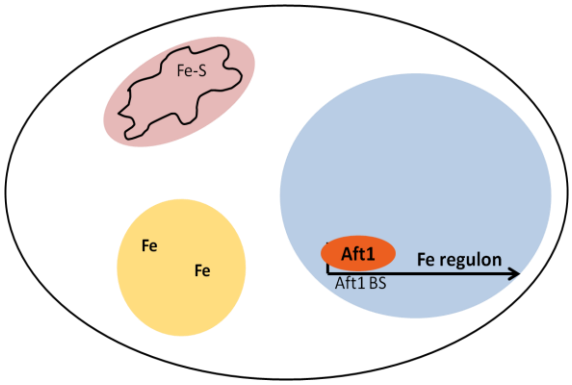


Figure 5-2 Activation of iron response in *S. cerevisiae*. During iron replete conditions an unknown inhibitory signal is translocated from the mitochondrial transporter Atm1. This stimulates Grx3 and Grx4 to induce Aft1 dimerization and subsequent export out of the nucleus. During iron replete conditions there is no signal form the mitochondria, thus no Grx3/4 induced Aft1 dimerization and the monomeric Aft1 remains in the nucleus and binds to Aft1 binding site (Aft1 BS) in the promoters of genes of the iron regulon. Blue circle – nucleus, yellow circle – vacuole, light purple oval – mitochondrion.

When Grx3 and Grx4 are not available to bind Aft1 and facilitate its export into the cytoplasm, the monomeric Aft1 remains in the nucleus and binds to promoters with a specific Aft1 binding site to activate transcription. The genes activated by Aft1 are collectively called the iron regulon.

The iron regulon includes three different classes of genes, which are further regulated depending on the cellular responses. Activation of Aft1 leads first to upregulation of the iron intake mechanisms; second, the cell switches, wherever possible, to iron independent pathways; third, iron stored in the vacuole is released. These events occur in the order listed here and if one is sufficient to overcome the iron deficiency then the next does not take place (Philpott and Protchenko, 2008).

To determine the primary activity of the exosome I have looked at the exonuclease and endonuclease activities independently. I used rrp44-*exo*$^-$ and *rrp44-endo*$^-$ mutants to look at the effects they have on known exosome substrates, growth rate, and gene expression. Additionally, in collaboration with Pamela Green's lab from University of Delaware, I tried to identify specific substrates of the endonuclease. Data presented in this chapter show that the primary activity of the exosome is the exoribonuclease and that a major consequence of inactivating the exoribonuclease is the activation of iron response.

RESULTS

The primary activity of Rrp44 is in the exoribonuclease

Since the exosome has both an exonuclease and an endoribonuclease, it is unclear which activity if important for which roles of the exosome. To test which activity is important for growth, point mutants of either the exonuclease, D551N (*rrp44-exo*$^{-}$*),* or the endonuclease, D171A (*rrp44-endo*$^{-}$), were transformed into an *rrp44*Δ strain and were grown for twenty four hours in liquid YPD medium. Growth was only affected in the exonuclease but not in the endonuclease mutant indicating that the exonuclease is important for growth under standard lab conditions (Figure 5-3).

Additionally, a deep sequencing of internally cleaved RNA fragments was concluded with the help of Pamela Greens' lab at the University of Delaware, searching for products specific to the endonuclease (Figure 5-4). Parallel analysis of RNA ends (PARE) was used to construct libraries of cleavage products of wild type and *rrp44-endo*$^{-}$ mutant (German et al., 2009). Briefly, RNA was isolated from wild type and *rrp44-endo*$^{-}$ and polyadenylated RNA was purified. Next, an adapter specific to a 5' phosphate and with a *Mme*I restriction enzyme binding site was ligated to the 5' of the RNA. The adapter ligation was followed by reverse transcription (Figure 5-4) and then a second DNA strand was synthesized. The resulting dsDNA was then cleaved with *Mme*I. *Mme*I one cleaves 20 bp away from the *Mme*I site therefore the resulting 20 bp fragments captured the site of the internal cleavage and 13 bp downstream.

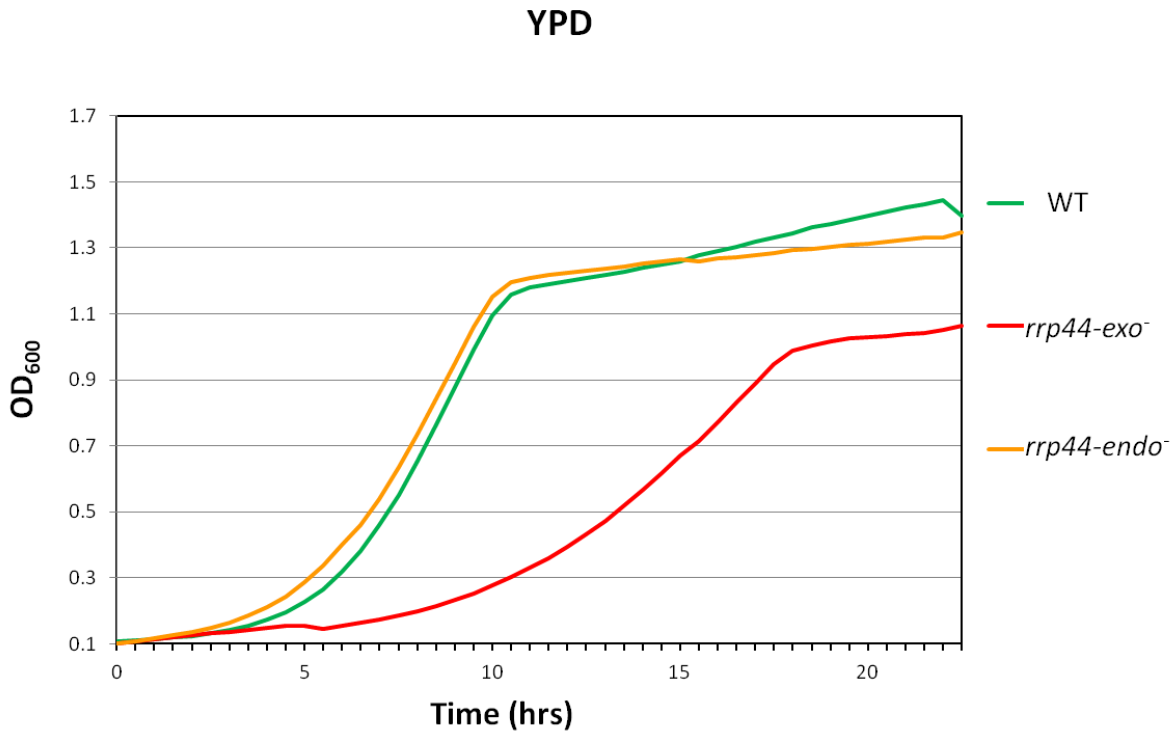


Figure 5-3 Growth is affected only in the exonuclease but not in the endonuclease mutant. A growth curve representing average of three independent clones for 24 hrs growth in liquid YPD medium. Green is wild-type, red - *rrp44-exo*$^{-}$, and orange - *rrp44-endo*$^{-}$

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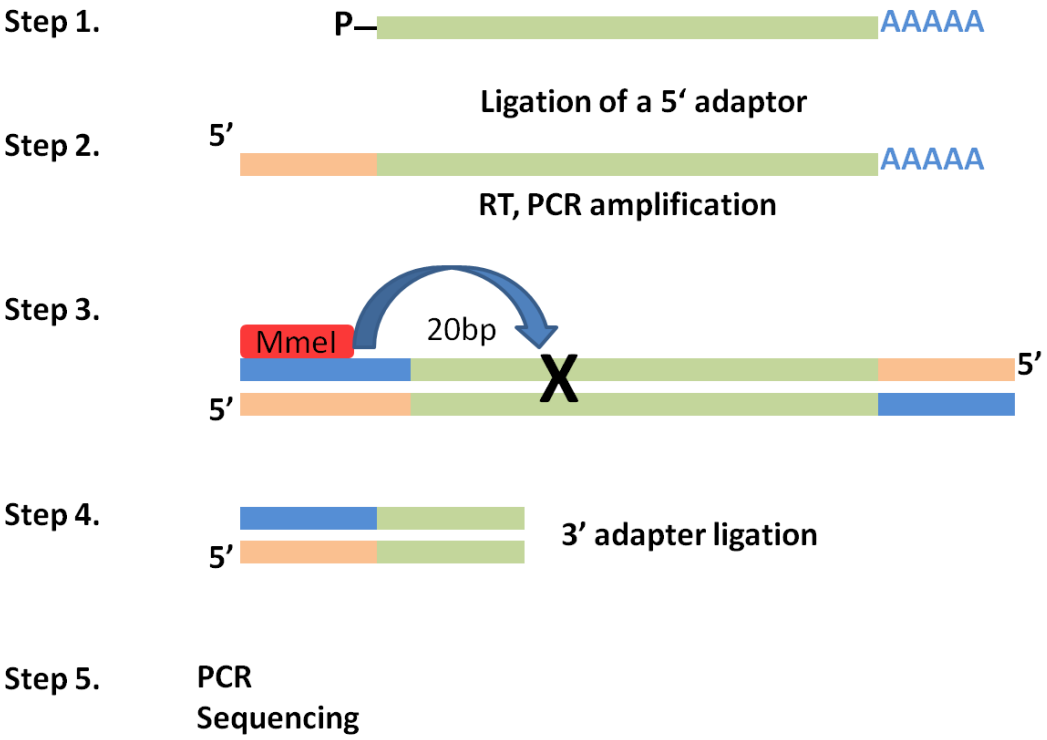


Figure 5-4. Selection of internally cleaved products for deep sequencing. Step 1- polyadenylated RNA is isolated. Cleavage products are expected to have a 5’ phosphate to which an adaptor with MmeI recognition site is ligated. Step 2- the RNA is reverse transcribed and PCR amplified for a second strand synthesis; Step3- Digestion with MmeI which cuts 20bp away from its recognition site; Step 4- A 3’ adaptor is ligated to the 20bp signature reads; Step 5- The sequences are PCR amplified and sequenced.

This figure is based on (German et al., 2009)

Two wild-type libraries and one *rrp44-endo*$^{-}$ library were constructed. The sequencing generated 1 282 573 20 bp unique reads that matched to the genome. Fragments smaller than 20 bp were not detected. All identified mRNAs had less than 5x increase in *rrp44-endo*$^{-}$ compared to wild type suggesting that the degradation of any particular mRNA is not controlled by the endonuclease. Thirteen candidate genes were identified based on PARE signatures that were present in the wild-type libraries but not present in the *rrp44-endo*$^{-}$ mutant. Of these the best three candidates were CAF20, REV1, and a dubious ORF YMR304C-A (Figure 5-5 A). Northern blots for REV1, and YMR304C-A were unable to detect these RNAs. The levels of the CAF20 mRNA showed no difference between wild-type and *rrp44-endo*$^{-}$ on a Northern blot (Figure 5-5 B). Two tRNA genes were also a part of the gene set of the thirteen candidates – tRNA-Asp (tRNA(GUC)L1) and tRNA-Glu (tRNA(CUC)D). Northern blots of these two tRNAs also showed no differences in the levels of these transcripts between wild-type and *rrp44-endo*$^{-}$ strains (Figure 5-5 C).The tRNA Asp identified in the PARE analysis is initially synthesized as a dicistronic transcript together with tRNA Arg and then the transcript is cleaved by RNase P which also cleaves the 5' ends of other tRNA transcripts (Engelke et al., 1985; Schmidt et al., 1980). Therefore Northern blots were also probed for these two transcripts. Neither tRNA-Arg-Asp nor RNase P showed any difference in transcript levels between wild type and *rrp44-endo*$^{-}$*.* Based on these analyses it was concluded that no specific substrates for the endonuclease activity of Rrp44 were identified using the PARE analysis method.

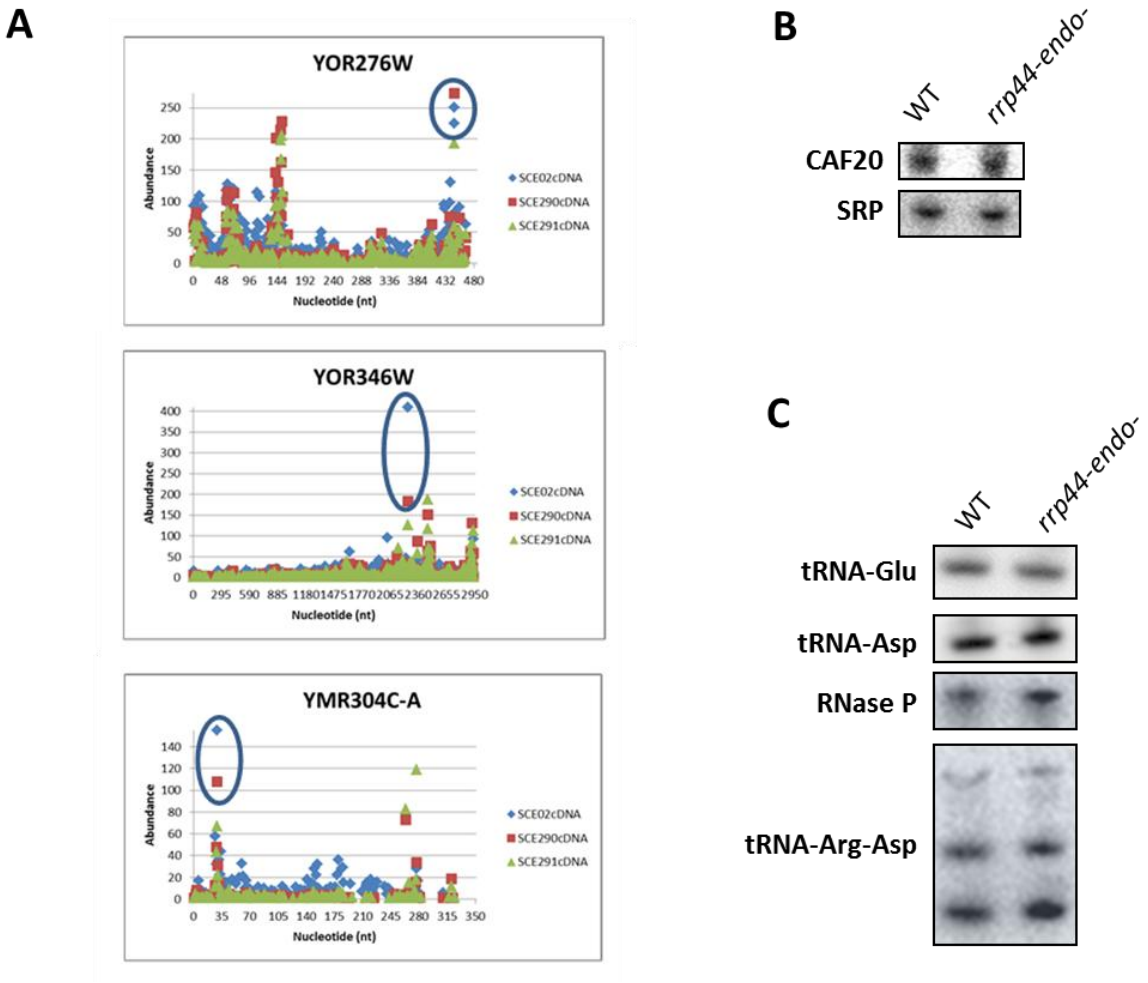


Figure 5-5 PARE analysis did not identify specific substrates for the endonuclease of Rrp44. A. Plots of the three best candidate genes. YOR276W= CAF20, YOR346W=REV. SCE291 (green) is the library from the *rrp44-endo*$^{-}$ strain. SCE02 (blue) and SCE290 (red) are libraries form the wild-type strain. B. Northern blot analysis of CAF20 mRNA in wild-type (WT) and *rrp44-endo*$^{-}$ strains. C. Northern blot analyses of tRNAGlu, tRNAAsp, RNase P, and tRNA-Arg-Asp (polycistronic) transcripts.

A microarray analysis of poly(A) RNA from *rrp44-exo*$^{-}$ and *rrp44-endo*$^{-}$ also did not find any effects on gene expression for the endoribonuclease (for details on the microarray see the sections below).

Therefore, considering that the endonuclease does not affect known exosome roles and growth phenotype, that the deep sequencing of cleavage products failed to identify specific endonuclease products, and a microarray failed to identify effects on gene expression, it was concluded that the primary activity of Rrp44 is carried out by the exonuclease.

Iron response is activated in *rrp44exo*$^{-}$ mutant

To characterize the effects the endoribonuclease or the exoribonuclease activity of Rrp44 have on gene expression, poly(A) RNA was isolated from wild-type, *rrp44-exo*$^{-}$, and *rrp44-endo*$^{-}$ strains and hybridized to a Affymetrix *S. cerevisiae* / *S. pombe* tiling microarray. Hybridization and initial analysis were done by the Allan Jacobson's Lab at the University of Massachusetts Medical School. The RNA was isolated in quadruplicate and mRNAs that were upregulated at least two-fold were selected for further analysis. No mRNAs were affected in *the rrp44-endo*$^{-}$ mutant, however 84 mRNAs were upregulated and 63 were downregulated in the *rrp44-exo*$^{-}$ (Table 5-1 and Table 5-2)

To assess if there was enrichment of any particular cellular processes, a gene ontology (GO) analysis was used. The main GO terms that were identified were all

related to iron uptake. Altogether, 10 of the 84 mRNAs (12%) were directly implicated in iron uptake. To verify the microarray results, a Northern blot was completed with *FIT2* and *TIS11* probes (Figure 5-6). In addition, RNA was isolated from an independently created *rrp44-exo*$^-$ mutant and the same RNAs was analyzed by a Northern blot. Increase in *FIT2* and *TIS11* mRNAs was noted in both the original and the independently created strain, thus it was concluded that the upregulation of iron response is a major physiological response in *rrp44-exo*$^-$.

Iron response in the *rrp44-exo*$^-$ is activated by activation of Aft1

Since a mutant that is defective specifically in RNA degradation (*rrp44-exo*$^-$) was used for the microarray, there were two possibilities for the increased abundance of the iron regulon mRNAs. First, the exonuclease could be degrading these mRNAs which would lead to their increase in an exonuclease mutant. Second, the increase may be secondary to some other effect caused by the exonuclease which leads to activation of the transcription factor responsible for activating transcription of these mRNAs. Aft1 is the transcription factor that is the major factor responsible for regulating iron response. Aft1 has been shown to bind to a ANTGCACCC elements in promoters of Aft1-regulated genes and stimulate transcription (Yamaguchi-Iwai et al., 1996).

Table 5-1 Microarray data – genes upregulated at least twofold in all four samples				
Gene	Fold elevation	Aft1 BS	Synonym	Description
	9.61		YDR042C	ORF, Uncharacterized
	8.47		YKL183C-A	ORF, Uncharacterized
FIT2	7.73	yes	YOR382W	siderophore-iron transporter
	6.78		YHR022C-A	ORF, Uncharacterized
	5.93		YHR214C-E	ORF, Uncharacterized
	5.73		YGR121W-A	ORF, Uncharacterized
FIT3	5.51	yes	YOR383C	Siderophore-iron transporter
	5.50		YAR075W	Nonfunctional protein
TIS11	5.47	yes	YLR136C	mRNA-binding protein expressed during iron starvation
ECM12	5.25		YHR021W-A	ORF, Uncharacterized
PRM6	5.07		YML047C	Pheromone-regulated protein
	4.74		YML007C-A	ORF, Uncharacterized
SIT1	4.73	yes	YEL065W	Transporter of siderophore-iron chelates
	4.26		YBR298C-A	ORF, Uncharacterized
	4.13		YAL064W	Protein of unknown function
	4.09		YOR394C-A	ORF, Uncharacterized
FIG1	3.98		YBR040W	Integral membrane protein required for efficient mating
	3.98		YOL155W-A	ORF, Uncharacterized
	3.89		YMR001C-A	ORF, Uncharacterized
AGA2	3.88		YGL032C	Adhesion subunit of a-agglutinin of a-cells
DIP5	3.88		YPL265W	Dicarboxylic amino acid permease
GRE1	3.87		YPL223C	Stress induced hydrophillin
	3.86		YOL097W-A	ORF, Uncharacterized
	3.82		YHR214C-D	ORF, Uncharacterized
ARN1	3.79	yes	YHL040C	Transporter of siderophore-iron chelates
	3.72		YLL066W-B	ORF, Uncharacterized
HMX1	3.69	yes	YLR205C	ER localized, heme-binding peroxidase
NRD1	3.67		YNL251C	nuclear RNA-binding protein
	3.59		YBR296C-A	ORF, Uncharacterized
	3.57		YGR204C-A	ORF, Uncharacterized
SET6	3.46		YPL165C	Protein of unknown function
ARP10	3.39		YDR106W	Component of the dynactin complex
	3.37		YOL162W	ORF, Uncharacterized
PDH1	3.29		YPR002W	Mitochondrial protein that participates in respiration, induced by diauxic shift
	3.29		YOR032W-A	ORF, Uncharacterized
	3.24		YNL277W-A	ORF, Uncharacterized

Table 5-1 (continued) Microarray data – genes upregulated at least twofold in all four samples

Gene	Fold elevation	Aft 1 BS	Synonym	Description
PRM5	3.21		YIL117C	Pheromone-regulated protein
	3.17		YCR108C	ORF, Uncharacterized
HUG1	3.16		YML058W-A	Protein involved in the Mec1p-mediated checkpoint pathway
VMR1	3.13		YHL035C	Vacuolar membrane protein
	3.11		YGR035C	ORF, Uncharacterized
MAL33	3.09		YBR297W	MAL-activator protein
ARN2	3.09	yes	YHL047C	Transporter of siderophore-iron chelates
FRE2	3.07	yes	YKL220C	Ferric and cupric reductase
PRM2	3.05		YIL037C	Pheromone-regulated protein
CUE2	3.05		YKL090W	Protein of unknown function
TAD2	3.02		YJL035C	Subunit of tRNA-specific adenosine-34 deaminase
IMD1 ; IMD2	3.00		YAR073W	Nonfunctional protein with homology to IMP dehydrogenase
HES1	2.98		YOR237W	Protein implicated in the regulation of ergosterol biosynthesis
TIR4	2.96		YOR009W	Cell wall mannoprotein
ALP1	2.94		YNL270C	Basic amino acid transporter
NCA3	2.92		YJL116C	Protein involved in mitochondrial organization
	2.92		YOL163W	ORF, Uncharacterized
COS12	2.87		YGL263W	Protein of unknown function
MIG2	2.87		YGL209W	Zinc finger transcriptional repressor
	2.81		YER078W-A	ORF, Uncharacterized
CIN1	2.77		YOR349W	Tubulin folding factor D
RKM5	2.76		YLR137W	Protein lysine methyltransferase
FUS2	2.74		YMR232W	Cytoplasmic protein localized to the shmoo tip
FYV5	2.72		YCL058C	Protein involved in regulation of the mating pathway
MND1	2.71		YGL183C	Protein required for recombination and meiotic nuclear division
	2.69		YLR108C	Protein on unknown function
AYT1	2.68		YLL063C	Acetyltransferase
FLO1	2.67		YAR050W	Lectin-like protein involved in flocculation
ZPS1	2.67		YOL154W	Putative GPI-anchored protein
	2.65		YOL114C	ORF, Uncharacterized
FET3	2.60	yes	YMR058W	Ferro-O2-oxidoreductase
ARG82	2.57		YDR173C	Protein involved in regulation of arginine-responsive and Mcm1p-dependent genes
	2.56		YNL024C	ORF, Uncharacterized

Table 5-1 (continued) Microarray data – genes upregulated at least twofold in all four samples				
Gene	**Fold elevation**	**Aft 1 BS**	**Synonym**	**Description**
	2.52		YLR412C-A	ORF, Uncharacterized
	2.48		YJL136W-A	ORF, Uncharacterized
MIG3	2.46		YER028C	Transcriptional regulator; role in catabolite repression and ethanol response
ENB1	2.45	yes	YOL158C	Endosomal ferric enterobactin transporter
RRN3	2.44		YKL125W	Protein required for transcription of rDNA by RNA polymerase I
	2.44		YJL027C	ORF, Uncharacterized
	2.40		YGR146C-A	ORF, Uncharacterized
CCC2	2.39		YDR270W	Cu(+2)-transporting P-type ATPase
DAN3	2.30		YBR301W	Cell wall mannoprotein
	2.30		YIL089W	Protein of unknown function
MTC3	2.27		YGL226W	Protein of unknown function
PCK1	2.26		YKR097W	Phosphoenolpyruvate carboxykinase
AIM4	2.12		YBR194W	Protein proposed to be associated with the nuclear pore complex

Table 5-2 Microarray data- genes downregulated at least twofold in all four samples

Gene	Fold elevation	Synonym	Description
ARO10	0.05	YDR380W	Phenylpyruvate decarboxylase
GYP5	0.06	YPL249C	GTPase-activating protein (GAP) for yeast Rab family members, involved in ER to Golgi trafficking
ARO9	0.16	YHR137W	Aromatic aminotransferase
INH1	0.17	YDL181W	Protein that inhibits ATP hydrolysis by the F1F0-ATP synthase
GRX4	0.18	YER174C	Monothiol glutaredoxin
MGA1	0.20	YGR249W	Protein similar to heat shock transcription factor
PHM6	0.22	YDR281C	Protein of unknown function
HFM1	0.25	YGL251C	Meiosis specific DNA helicase
YFH7	0.25	YFR007W	Putative kinase
SPO1	0.26	YNL012W	Meiosis-specific protein
LIN1	0.26	YHR156C	Non-essential component of U5 snRNP
HXT7 ; HXT6	0.26	YDR342C	High-affinity glucose transporter
ISF1	0.27	YMR081C	Serine-rich, hydrophilic protein
PHO89	0.27	YBR296C	Na+/Pi cotransporter, active in early growth phase
NRT1	0.27	YOR071C	High-affinity nicotinamide riboside transporter
SPL2	0.28	YHR136C	Protein with similarity to cyclin-dependent kinase inhibitors
	0.28	YNL040W	ORF, Uncharacterized
GMC1	0.29	YDR506C	Protein involved in meiotic progression
PHO5	0.30	YBR093C	Repressible acid phosphatase
FMP48	0.30	YGR052W	ORF, Uncharacterized
CYB2	0.31	YML054C	Cytochrome b2
LAP4	0.31	YKL103C	Vacuolar aminopeptidase
TIR1	0.31	YER011W	Cell wall mannoprotein , expression is downregulated at acidic pH
COX4	0.31	YGL187C	Subunit IV of cytochrome c oxidase
	0.31	YKL151C	NADHX dehydratase
DDR48	0.33	YMR173W	DNA damage-responsive protein
TAX4	0.34	YJL083W	Protein involved in regulation of phosphatidylinositol 4,5-bisphosphate concentrations
TPO4	0.34	YOR273C	Polyamine transport protein
	0.34	YPL113C	Glyoxylate reductase
AGP1	0.34	YCL025C	Low-affinity amino acid permease,, involved in uptake of asparagine, glutamine, and other amino acids
	0.34	YNL134C	Protein of unknown function
EDS1	0.34	YBR033W	ORF, Uncharacterized
TIR3	0.35	YIL011W	Cell wall mannoprotein
	0.35	YHR202W	ORF, Uncharacterized

Table 5-2 (continued) Microarray data- genes downregulated at least twofold in all four samples			
Gene	**Gene**	**Gene**	**Gene**
CYT1	0.36	YOR065W	Cytochrome c1
HSP30	0.36	YCR021C	Hydrophobic plasma membrane protein
GDH1	0.37	YOR375C	NADP(+)-dependent glutamate dehydrogenase
CYC1	0.38	YJR048W	Cytochrome c, isoform 1
ARG1	0.38	YOL058W	Arginosuccinate synthetase
GGC1	0.38	YDL198C	Mitochondrial GTP/GDP transporter
CUE5	0.38	YOR042W	Protein containing a CUE domain that binds ubiquitin
ALD5	0.38	YER073W	Mitochondrial aldehyde dehydrogenase
RTC3	0.38	YHR087W	Protein involved in RNA metabolism
ARG3	0.39	YJL088W	Ornithine carbamoyltransferase
VTC3	0.39	YPL019C	Vacuolar membrane protein
GPH1	0.39	YPR160W	Glycogen phosphorylase required for the mobilization of glycogen
GAD1	0.40	YMR250W	Glutamate decarboxylase
CSR2	0.40	YPR030W	Nuclear protein with a potential regulatory role in utilization of galactose and nonfermentable carbon sources
PHO11 ; PHO12	0.40	YAR071W	One of three repressible acid phosphatases, a glycoprotein that is transported to the cell surface
	0.40	YPL014W	ORF, Uncharacterized
GSY1	0.41	YFR015C	Glycogen synthase
WSC4	0.41	YHL028W	ER membrane protein
RIP1	0.42	YEL024W	Ubiquinol-cytochrome-c reductase, a Rieske iron-sulfur protein of the mitochondrial cytochrome bc1 complex
ARG5,6	0.42	YER069W	Protein that is processed in the mitochondrion to yield acetylglutamate kinase and N-acetyl-gamma-glutamyl-phosphate reductase, which catalyze the 2nd and 3rd steps in arginine biosynthesis
	0.42	YJR129C	ORF, Uncharacterized
TFB6	0.42	YOR352W	Subunit of TFIIH complex
CPA2	0.43	YJR109C	Large subunit of carbamoyl phosphate synthetase
RIP1	0.43	AFFX-r2-Sc-RIP1-M	Ubiquinol-cytochrome-c reductase, a Rieske iron-sulfur protein
MNN1	0.44	YER001W	Integral membrane glycoprotein of the Golgi complex
HXT4	0.44	YHR092C	High-affinity glucose transporter of the major facilitator superfamily
COR1	0.45	YBL045C	Core subunit of the ubiquinol-cytochrome c reductase complex (bc1 complex)
MEP1	0.45	YGR121C	Ammonium permease
SMF1	0.46	YOL122C	Divalent metal ion transporter

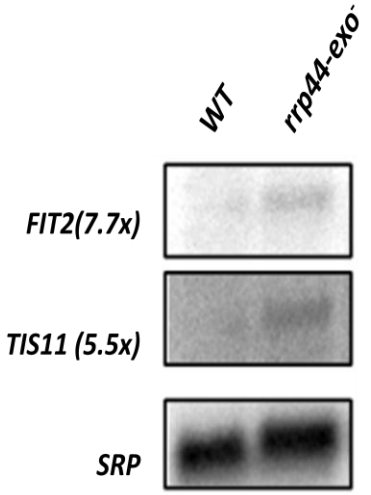
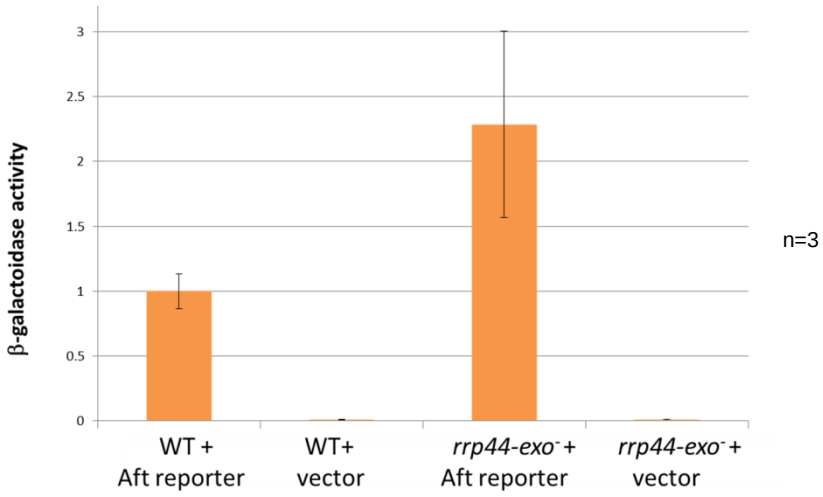


Figure 5-6 Verification of microarray results. A Northern blot with probes for FIT2 and TIS11 in wild-type (WT) and rrp44-exo-. The numbers in parenthesis indicated the fold elevation in the microarray. SRP is the RNA part of the signal recognition particle, used for loading control.

Each of the iron response genes that were upregulated in the microarray had a binding site for Aft1 in its promoter region. These data suggested that *rrp44-exo*$^-$ may cause Aft1 activation, which in turn would increase the transcription of the iron response mRNAs.

To test whether Aft1 was activated, two assays were conducted. First, an indirect *lacZ* based assay was completed. Briefly, a *lacZ* reporter gene with a minimal *CYC1* promoter and an Aft1 binding site was constructed. As a control, the same vector, but without the Aft1 site was used. The reporter plasmids were transformed into wild-type and *rrp44-exo*$^-$ strains and a beta-galactosidase activity assay was carried out. Beta-galactosidase levels were increased 2.1 fold in the *rrp44-exo*$^-$ strain compared to the wild-type strain (Figure 5-7 A). In addition, when comparing only the levels of the control reporter, without the Aft1 binding site, in wild type versus *rrp44-exo*$^-$, these levels were not significantly different (Figure 5-7 B). This indicates that *rrp44-exo*$^-$ does not affect the level of the reporter itself. To directly observe Aft1 localization a construct with GFP-tagged Aft1 was transformed into an *rrp44-exo*$^-$ strain and in wild-type strain. Aft1 was observed to be localized in the nucleus in 44% of the cells, while only in 15% of the wild-type cells, which is consistent with it being activated in the *rrp44-exo*$^-$ mutant (Figure 5-8).

A



B

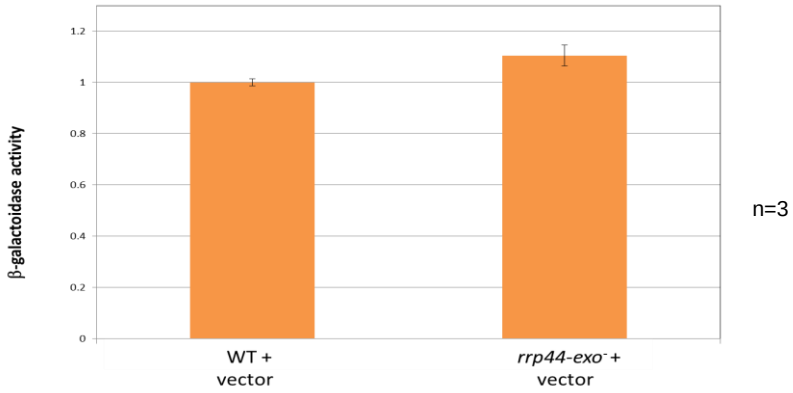


Figure 5-7 Aft1 is activated in *rrp44-exo⁻*. A. Beta-galactosidase activity of wild-type and rrp44-exo- transformed with a reporter with either Aft1 binding site or without (vector). The data is averaged of 3 experiments and normalized to wild-type. B. A close-up views of the wild-type versus rrp44-exo- transformed with only the reporter. This is the same data as above, but normalized to the wild-type + vector strain.

Taken together these data suggest that the increased levels of iron regulon mRNAs was not directly caused by the exonuclease by means of these mRNAs not being degraded, but due to activation of Aft1. Therefore the exonuclease indirectly affects the activation of iron response in *S. cerevisiae*.

The iron response activation in *rrp44-exo*$^{-}$ is not due to aberrant ATM1 expression

The inhibitory signal for the activation of iron response is known to be dependent of the mitochondrial transporter Atm1.To test whether Atm1's expression was affected in the *rrp44-exo*$^{-}$ mutant, a Northern blot was completed with probe for *ATM1*. *ATM1* was not affected in the microarray and as seen in Figure 5-9 there were no differences in the expression of *ATM1* in wild type and the *rrp44-exo*$^{-}$ mutant. This suggests that the improper iron activation is not due to an aberrant mitochondrial signaling event. One of the regulators of Aft1, Grx4, was one of the downregulated mRNAs in the microarray; however numerous attempts to detect *GRX4* by Northern blots or RT PCR failed, thus the microarray down regulation could not be verified.

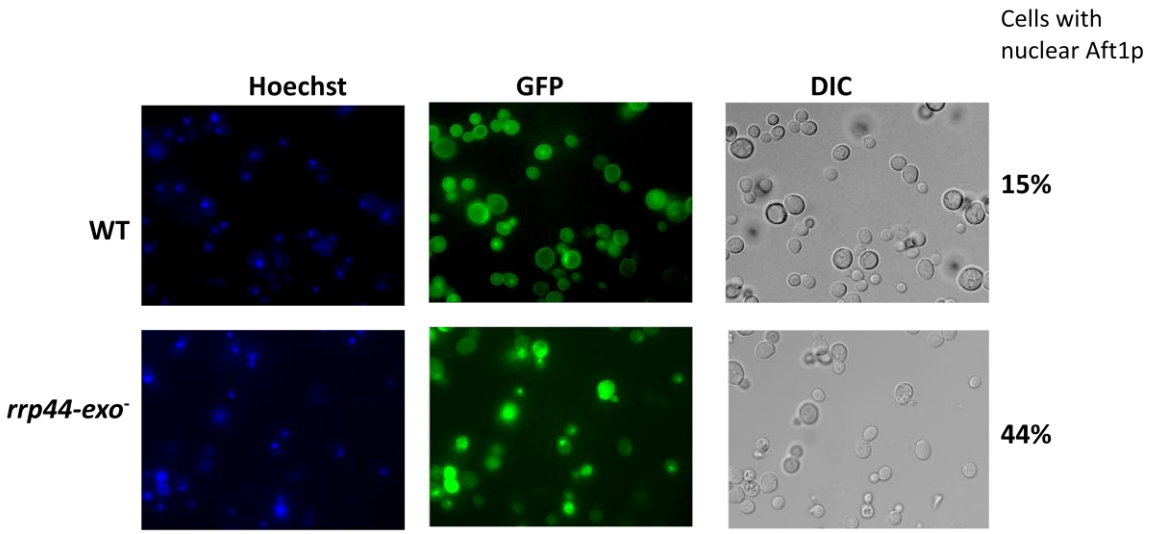


Figure 5-8. Aft1 localizes to the nucleus in higher percentage of *rrp44-exo*$^-$ cells than in wild-type cells. Fluorescent microscopy of wild-type and *rrp44-exo*$^-$ cells transformed with Aft1-GFP reporter.

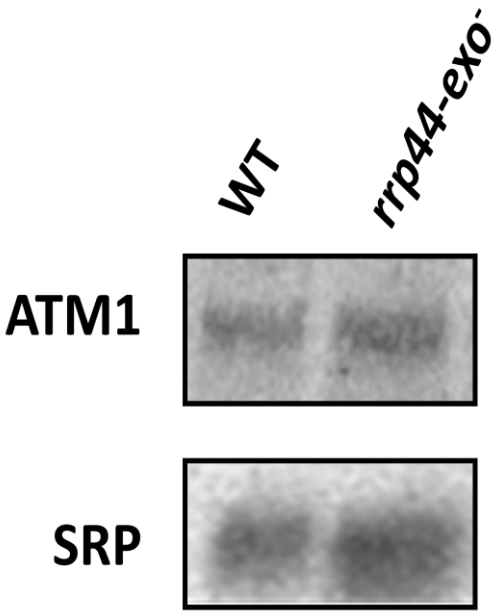


Figure 5-9. The levels of *ATM1* are the same in wild type and rrp44-exo⁻. Northern blot with probes for *ATM1* and *SRP* (loading control) in wild-type (WT) and *rrp44-exo⁻*.

DISCUSSION

This chapter examines the importance of the different ribonuclease domains of Rrp44 and their effect on gene expression. I have shown that the primary activity of Rrp44 is in the exoribonuclease domain. In addition I have determined that a major consequence of the exonuclease inactivation is the indirect activation of iron response.

Interestingly, I was unable to find any specific substrates for the endonuclease and there was no effect of gene expression in the *rrp44-endo*$^{-}$ mutant as determined by the microarray analysis. These data are consistent with the data that *rrp44-endo*$^{-}$ also does not have a growth phenotype and with other studies that also were unable to find substrates for the endonuclease (Schneider et al., 2012). However, knockouts of the endonuclease and exonuclease of Rrp44 are synthetically lethal. Therefore for the essential role of Rrrp44, the endonuclease overlaps with the exonuclease. The data presented here suggest that all of the endonuclease substrates are also substrates for the exonuclease, while the opposite is not true. It is possible that for the most critical substrates, the ones that cause the synthetic lethality phenotype, the exosome has developed a back-up system by using two separate active sites to ensure their degradation or processing. It would be interesting to find out what the effect on gene expression is in a knock-out of both enzymatic activities on Rrp44. However, since a double mutant is inviable such an experiment inherently has certain limitations. For example, it can only be done shortly after knocking out both enzymatic activities possibly not giving it enough

time for major changes to occur. Yet, it would be interesting to determine if new substrates, not affected in the single mutant, emerge from such a screen.

Activation of iron response seems to be a major consequence of the inactivation of the exonuclease. Therefore, in the wild-type strain, the exonuclease actually functions as a negative regulator of iron response. Iron response is a very complicated process which includes various control points. For example, if iron is low, iron importers are upregulated and iron is imported into the cell. However, if the influx of iron is still not sufficient, then a reorganization of metabolic processes take place to include enzymes that are iron independent and if the influx of iron is sufficient then this metabolic reorganization does not occur. Yet another check point exists, in the case when the influx of iron and the metabolic reorganization are still not sufficient, the iron stored in the cellular vacuole is released. Therefore even when the major regulator of iron response Aft1 is activated, there are more checkpoints downstream of it to ensure the correct response is carried out. The exonuclease of Rrp44 seems to function on the initial response to iron deprivation, because neither genes for metabolic reorganization, nor vacuolar genes involved in iron response were affected in *rrp44-exo*$^{-}$.

Two of the three total functional studies of Rrp44 have found a connection between microtubules and Rrp44 (Murakami et al., 2007; Smith et al., 2011). Here, I find that the major transcription factor of iron response is activated in *rrp44-exo*$^{-}$. Interestingly, Aft1 was recently found to interact with kinetochore proteins (Hamza

and Baetz, 2012). It would be interesting to find out if a connection between these two phenotypes, Aft1 activation and kinetochore-microtubule defects, exists.

It was surprising to find out that the effect on gene expression was relatively modest, with 84 upregulated and 63 downregulated mRNAs. Since the exosome is an exoribonuclease, the downregulated mRNAs were certainly indirectly affected and in addition I determined that for at least the 10 iron response mRNAs, the effect was also indirect. The fact that there is so much indirect effect can be explained by the fact that the essential role of Rrp44 is in the nucleus, while in the cytoplasm the role of the exosome in mRNA degradation overlaps with that of the enzymes from the 5'→3' mRNA degradation pathway. Therefore, any effect on gene expression that is exosome specific is more likely to be affected by the nuclear exosome, thus most likely to be indirect.

Another cytoplasmic role for the exosome is the degradation of aberrant nonstop mRNAs. This role is independent of the 5→3' degradation pathway, thus it is exosome specific. However, it is not likely that the mRNAs seen in the microarray are the results of lack of nonstop mRNA degradation, because for that role the exonuclease and the endonuclease were shown to be redundant (Schaeffer and van Hoof, 2011). In the strains used for the microarray, *rrp44-exo*$^{-}$ and *rrp44-endo*$^{-}$, there is always either exonuclease or endonuclease activity of Rrp44 which would rapidly degrade such mRNAs therefore they would not be detected by the microarray.

Again, as with the lack of identified substrates for the endonuclease, one possible explanation for the modest effect on gene expression is the possible overlap of

degradation or processing roles between the exonuclease and the endonuclease for the most critically important substrates. The nucleus is the location where the exosome's role is essential. In human cells only the Rrp44 homologue that localizes to the nucleus has retained an endonuclease activity (Tomecki et al., 2010). This is consistent with the idea that the two activities may overlap for the most critical substrates.

Chapter 6: The exoribonuclease of Rrp44 affects glucose fermentation and production of reactive oxygen species

INTRODUCTION

Iron metabolism and oxidative stress in S. cerevisiae

Iron is an essential element needed for the activity of many enzymes. Due to the high reduction potential of the Fe3+/Fe2+ pair, iron is a part of several proteins in the electron transport chain (ETC). In the ETC iron is found as a part of iron-sulfur proteins where it is a part of iron-sulfur clusters. It is also found in cytochromes, where it is contained in their heme groups. The purpose of the ETC is to build proton gradient to be used as energy for the ATP synthase (for a detailed review of the ECT in yeast, refer to de Vries and Marres, 1987; Schagger, 2002). Oxidation of NADH and $FADH_2$ however requires the initial transfer of protons together with electrons. As H_2 atoms are transported across the membrane they are oxidized and H^+ is pumped across the membrane, while electrons are shuttled via the rest of the ETC to the terminal electron acceptor, in this case oxygen (Becker et al., 2003). Iron in the iron-sulfur clusters receives electrons one at a time, as the protons are pumped through the membrane (Becker, Kleinsmith et al. 2003). Therefore iron is a critical element for the establishment of proton gradient in the mitochondrial intermembrane space and consequently for the cellular ability to produce ATP (Figure 6-1).

However, as essential as iron is for respiration, it is also important to be kept in balance. If iron is in excess and is not sequestered either in the vacuole or as a part of a protein, free iron can cause the formation of reactive oxygen species (ROS). ROS are constantly produced during normal cellular processes and are also

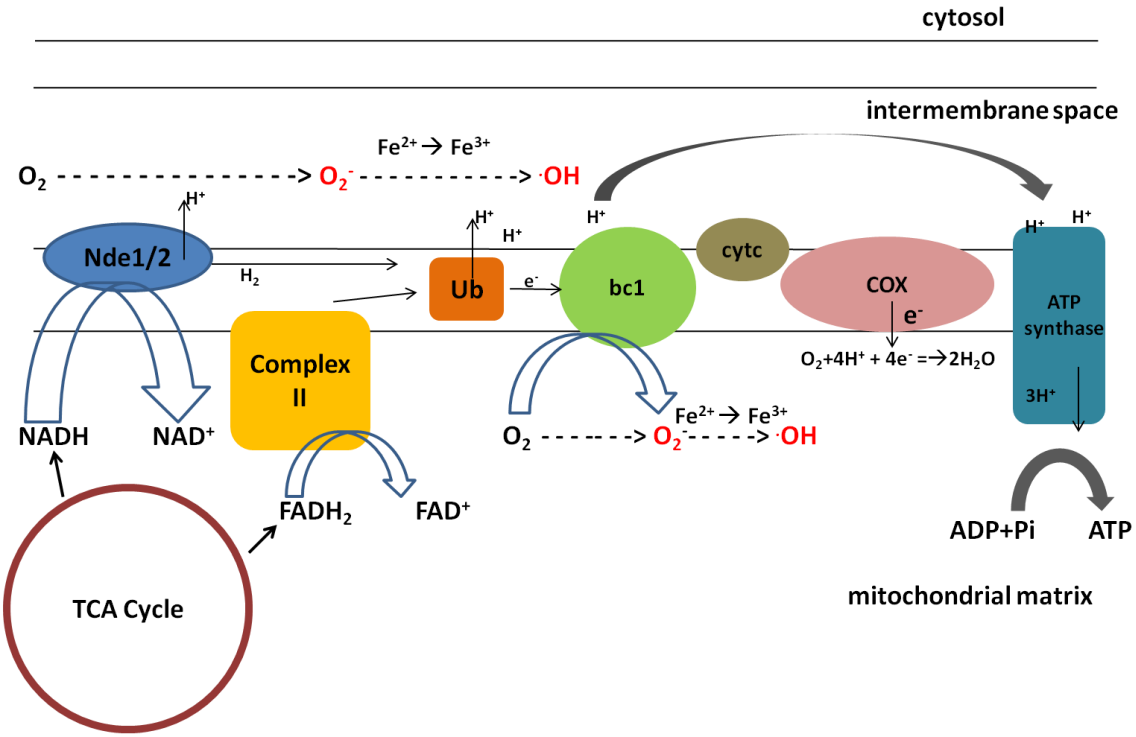


Figure6-1. The electron transport chain (ETC) of S. cerevisiae. *S. cerevisiae* does not have complex I, instead NADH from the TCA cycle is oxidized by the NADH oxidases Nde1 and Nde2. FADH2 from the TCA cycle is oxidized by Complex II. The NADH oxidases and complex II each transfer electrons to Ubiquinone(Ub), a mobile electron carrier, which in turn transfers them to the bc1 complex (also called complex III). From the bc1 complex, electrons are transferred to another shuttle molecule. Cytochrome C. Cytochrome C shuttles the electrons to Cytochrome C oxidase (COX) also called complex 4, which in turn transfers them to the terminal electron acceptor oxygen. As electrons are transferred, protons are pumped through the inner mitochondrial membrane into the intermembrane space and the proton gradient that is created is used by the ATP synthase to make ATP from ADP and inorganic phosphate. The two places where electrons leak from the ETC are at the Nde1/2 and the bc1 complex. If excess free iron is available, reactive oxygen species (ROS) are formed.

constantly detoxified. Damage caused by ROS is also constantly repaired, and in the event of oxidative stress, the cell responds by a series of events collectively called the oxidative stress response.

Location and timing of production of ROS in *S. cerevisiae*

The mitochondrial membrane is the main location of ROS production. This is due to electrons escaping the ETC during respiration and reacting with other molecules (Murphy, 2009). However, *S. cerevisiae*, when grown on glucose (or any fermentable sugar) as a carbon source, grows preferentially by fermentation, thereby shutting off respiration and producing ethanol which is excreted outside of the cell. When glucose is no longer available, *S. cerevisiae* switches to respiration and oxidizes the remaining non-fermentable carbon source. The process of switching to respiration is accompanied by a major change in transcription and slowing down of growth rate and it is referred to as a diauxic shift. (Figure 6-2) (Zaman et al., 2008). Respiratory metabolism requires the use of the ETC, thus more ROS are produced, but also more oxidative stress responses are upregulated to neutralize the effect of these ROS.

Detoxification of ROS

Detoxification of ROS is mainly accomplished enzymatically via detoxifying enzymes. The major ROS detoxifying enzymes are superoxide dismutases (SODs), catalases, and peroxidases (Herrero et al., 2008). These enzymes are upregulated upon oxidative stress to detoxify the produced ROS.

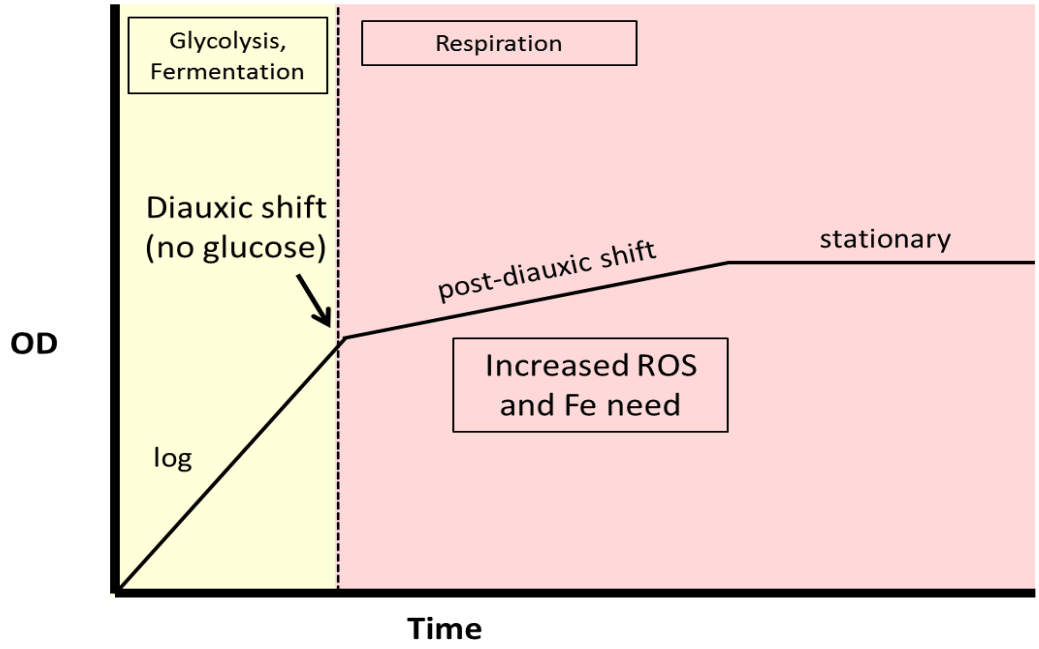


Figure 6-2. Depiction of the two growth phases of *S. cerevisiae* when using glucose as carbon source. During the first phase (yellow shaded area) *S. cerevisiae* grows by fermenting the glucose in the medium and excreting ethanol. As glucose is used up a metabolic change, referred to as the diauxic shift occurs. Growth rate slows and *S. cerevisiae* reorganizes it metabolism to utilize the ethanol in the medium by respiration (pink shaded area).

Oxidative damage repair

Oxidative stress causes damage in virtually all cellular molecules, including DNA, RNA, proteins, and lipids. Sulfhydryl groups of proteins are especially vulnerable to oxidative damage. *S. cerevisiae* has two major systems to repair protein damage from oxidation, the thioredoxin (TRX) and the glutaredoxin (GRX) systems, as well as other reductases. Both the TRX and the GRX systems use small molecules which contain cysteine. When a protein is oxidized, the thioredoxins or the glutaredoxins reduce it by oxidizing their own cysteines. Both systems are complemented by reductases which in turn reduce the thioredoxins or the glutaredoxins to recycle them (Grant, 2001).

Regulation of oxidative stress response

Oxidative stress responses are regulated at the transcriptional level by the transcription factors Yap1 and Skn7 (Brown et al., 1993; Hirata et al., 1994; Krems et al., 1996). Yap1 is involved in transcriptional control of all thioredoxin and glutathione dependent system as well as the pentose phosphate pathway (PPP). The PPP's activation is needed in order to provide reducing equivalents for both systems. Skn7 is only required for activation of some but not all proteins of the TRX system.

Chapter 5 showed that iron response is activated indirectly in the *rrp44-exo*$^{-}$ mutant. Experiments presented in this chapter were conducted to investigate the cause of this activation. Data presented in this chapter shows that ROS levels are

elevated in *rrp44-exo*$^{-}$ mutant and that the ROS elevation is functionally related to iron response activation. Further, I show that the exoribonuclease mutation causes aberrant glucose fermentation.

RESULTS

The exonuclease is required for protection from hydrogen peroxide induced oxidative stress

As excess iron can lead to increased production of ROS, the sensitivity of the *rrp44-exo*$^-$ mutant to oxidative stress was tested. *rrp44-exo*$^-$ was grown both in liquid YPD medium supplemented with 2 μM hydrogen peroxide (H_2O_2; Figure 6-3 A) or on solid YPD medium (Figure 6-3B) supplemented with 2 μM H_2O_2. In both cases the *rrp44-exo*$^-$ mutant did not grow while the wild-type control grew, indicating that *rrp44-exo*$^-$ is hypersensitive to oxidative stress.

To test if the sensitivity was specific to hydrogen peroxide or if other oxidative stressor could cause similar sensitivity, *rrp44-exo*$^-$ was grown on medium supplemented with 50 μM menadione which is another oxidative stress agent. The difference between menadione and H_2O_2 is that menadione does not cause an increase of H_2O_2 but an increase of superoxide (Goldberg and Stern, 1976). Growth on medium with menadione was not affected compared to growth on YPD (Figure 6-4A) indicating that the hypersensitivity was specific to H_2O_2.

To test if the knockout of the exonuclease caused sensitivity to other general stressors, *rrp44-exo*$^-$ strain was also grown at high temperature, 37^{o}C and low temperature, 15^{o}C (Figure 6-4 B). There was no effect on growth compared to growth on YPD at 30^{o}C. These data indicated that *rrp44-exo*$^-$ was not generally sensitive to stress. Taken together, I concluded that *rrp44-exo*$^-$ is hypersensitive to hydrogen peroxide induced oxidative stress.

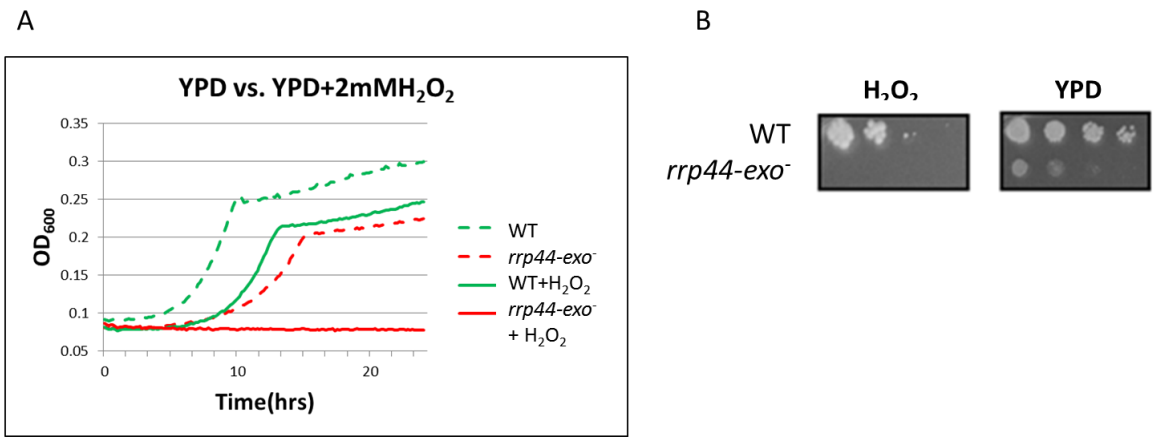


Figure 6-3. *rrp44-exo*$^-$ is hypersensitive to hydrogen peroxide. A. *rrp44-exo*$^-$ and wild-type (WT) *S. cerevisiae* strain were grown in liquid YPD versus YPD+2 μM H_2O_2 cultures. Dashed lines represent growth in YPD; solid lines represent growth in YPD+2μMH_2O_2. Green - wild-type; red - *rrp44-exo*$^-$. B. Growth assay on wild-type and *rrp44-exo*$^-$ on solid medium with and without 2 μM H_2O_2.

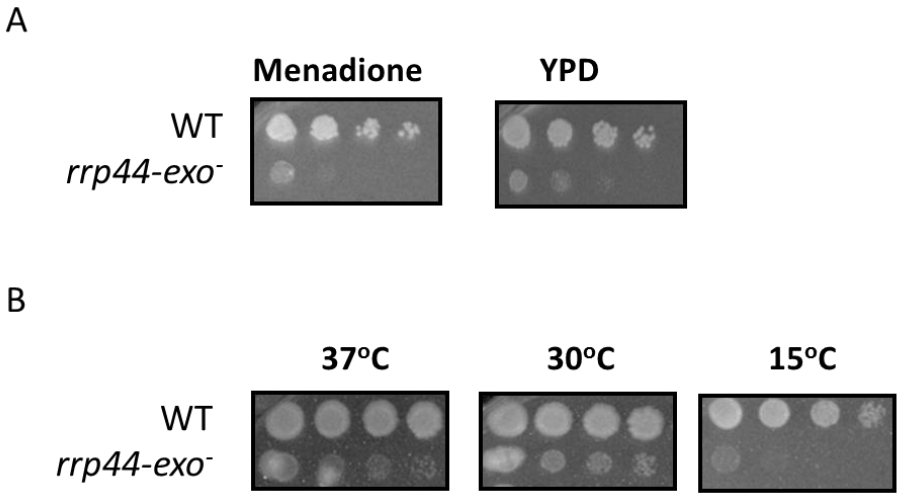


Figure 6-4. *rrp44-exo*$^-$ is not hypersensitive to menadione or temperature stress. A. Plate growth assay of wild-type and *rrp44-exo*$^-$ on YPD+ 50 μM menadione and on YPD. B. Growth assay on YPD at the indicated temperatures.

The exonuclease mutant has increased intracellular levels of ROS

Either higher production of ROS or the inability to detoxify normal ROS levels can lead to the observed H_2O_2 hypersensitivity phenotype. To test if *rrp44-exo*$^-$ has increased ROS levels, an ROS detection assay was carried out. Briefly, wild-type and *rrp44-exo*$^-$ cells were grown in YPD and in YPD + 1 μM H_2O_2. Next, H_2DCFDA (2',7'-dichlorodihydrofluorescein diacetate) reagent was added and fluorescence, indirectly indicating ROS levels, was measured in a plate reader (for details of the assay see Chapter 2). During growth in YPD medium *rrp44-exo*$^-$ had 2x elevated ROS levels relative to wild-type and in YPD+1 μM H_2O_2 the *rrp44-exo*$^-$ had 2.3x elevated ROS levels over the wild-type strain (Figure6-5).

As mentioned earlier, either increased production or defective detoxification can lead to increased levels of ROS. However, the microarray data did not point to any enzymes normally involved in ROS detoxification. These data, and the results from the ROS detection assay above, suggest that the exonuclease mutant has increased production of ROS.

Altered iron response and sensitivity to oxidative stress in the *rrp44-exo*$^-$ mutant are functionally connected.

Many factors affect if iron response is activated. It is possible that the iron response activation in *rrp44-exo*$^-$ is related to the ROS production or it is possible that the activated iron response is independent of the ROS production. To test that, I added iron to oxidatively stressed cells and monitored growth rate.

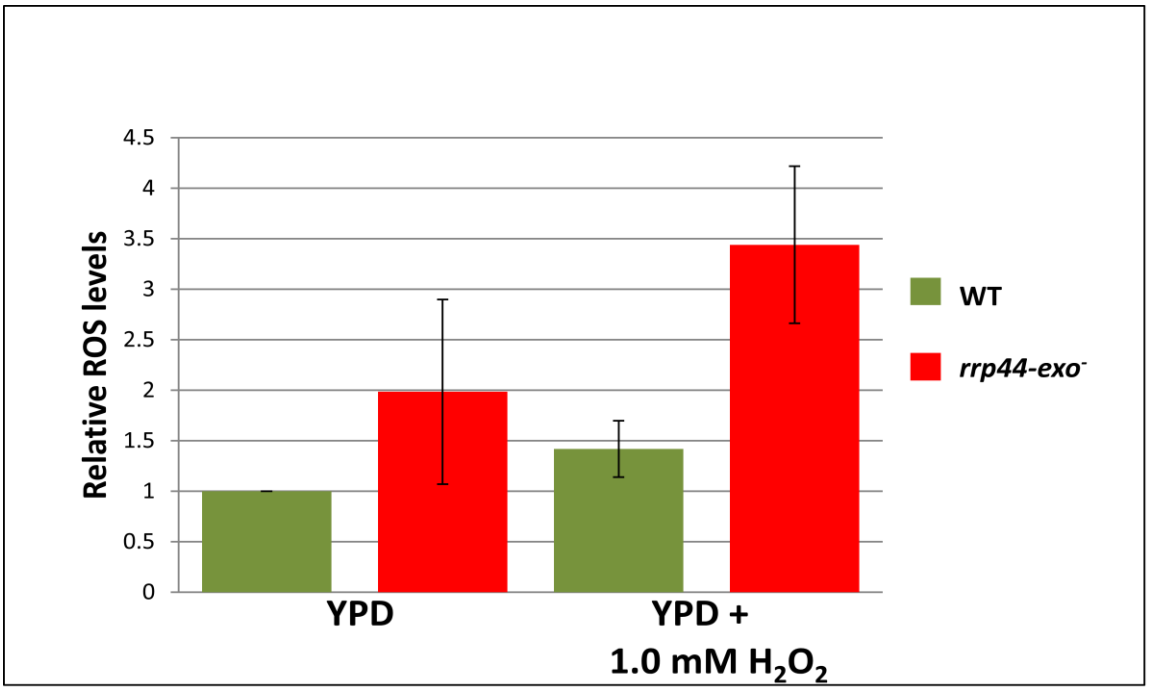


Figure 6-5. *rrp44-exo*$^-$ has increased ROS levels. Relative ROS levels were measured in wild-type(green) and *rrp44-exo*$^-$ (red) with and without H_2O_2 treatment as indicated. n=3

If growth rate is improved, that would indicate that the iron response and oxidative stress are functionally related in *rrp4-exo*$^-$ because it would mean that iron response itself is not misregulated, but it rather a consequence of oxidative stress. If however there was increased sensitivity or no change, then this would indicate that these two processes are affected by the exonuclease independently of each other.

Wild-type and *rrp44-exo*$^-$ cells were stressed with 1 μM H_2O_2 for one hour and then 100 μM Ferrous Ammonium Sulfate (FAS) was added to the medium as a source of Fe^{3+}. Figure 6-6A shows that addition of FAS improved the growth rate of *rrp44-exo*$^-$ but did not affect the wild-type strain. This improvement in growth was specific to oxidatively stressed cells as addition of FAS to cells grown in YPD without H_2O_2 did not have any effect on growth rate (Figure 6-6B).

Growth on solid medium supplemented with 1000x Fe and with low iron was not affected in *rrp44-exo*$^-$ compared to growth on SC (Figure 6-6C). In this case SC medium was chosen over YPD because SC medium has defined amounts of nutrient including iron, therefore making it possible for exact iron calculations to be conducted for the 1000x medium.

To further support the hypothesis the *rrp44-exo*$^-$ has higher requirements for iron, instead of a misregulated iron response, a Northern blot was conducted with a probe against *FIT2*. Both wild type and *rrp44-exo*$^-$ were grown either on YPD, or on increasing concentrations of H_2O_2 – 0.5 μM H_2O_2 and 1.0 μM H_2O_2. As shown earlier, *rrp44-exo*$^-$ upregulates the iron regulon without additional oxidative stress. However both wild type and *rrp44-exo*$^-$ upregulate iron response upon oxidative

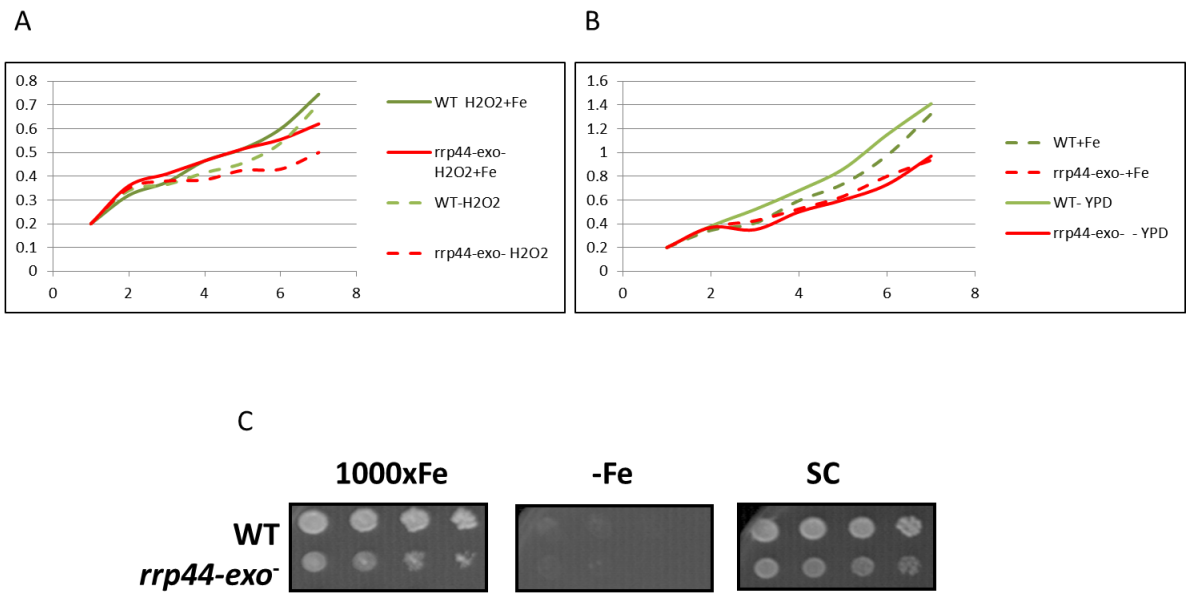


Figure 6-6. Iron response and oxidative stress are functionally related in *rrp44-exo*$^-$.

A Growth of wild type and *rrp44-exo*$^-$ in YPD stressed with H_2O_2 for one hour (dashed lines) or stressed with H_2O_2 for one hour followed by supplementation by 100 μM Fe (solid lines). B. Growth of wild-type and *rrp44-exo*$^-$ - in YPD (dashed lines) or YPD+ 100 μM Fe (solid lines). C. Growth assay of wild type and *rrp44-exo*$^-$ on synthetic complete (SC) medium with addition of 1000xFe or without iron.

stress indicating the upregulation of iron response is a normal physiological response of the cells during stress with H_2O_2 (Figure 6-7).

The exonuclease of the exosome affects the cellular growth during fermentative conditions, but not during respiratory conditions

In wild-type cells, oxidative stress is produced mostly during respiration. However *S. cerevisiae* grown in log phase on YPD medium grows by fermentation until all of the glucose is used up and then it switches to respiration, during which it uses up the ethanol that has been excreted in the medium during the fermentative growth phase (Zaman et al., 2008). To compare growth rates during respiratory conditions, wild-type strain and *rrp44-exo*$^-$ mutant were grown in YEP + 2% glycerol medium. This medium has glycerol instead of glucose as a sole carbon source. Since glycerol in a non-fermentable carbon source, growth on this medium forces *S. cerevisiae* to grow by respiration. Interestingly, during respiratory growth on YEP+ glycerol, wild-type cells and *rrp44-exo*$^-$ growth was the same (Figure 6-8A). The results were specific to respiratory growth, not only to glycerol, because wild-type and *rrp44-exo*$^-$ grew with the same phenotype on medium supplemented with ethanol (YEP+ 2% ethanol) also. The similar growth rates were also confirmed with growth in liquid YEP+ glycerol medium (Figure 6-8B). Based on the above data I concluded that *rrp44-exo*$^-$ affects only fermentative growth rate but not the respiratory growth.

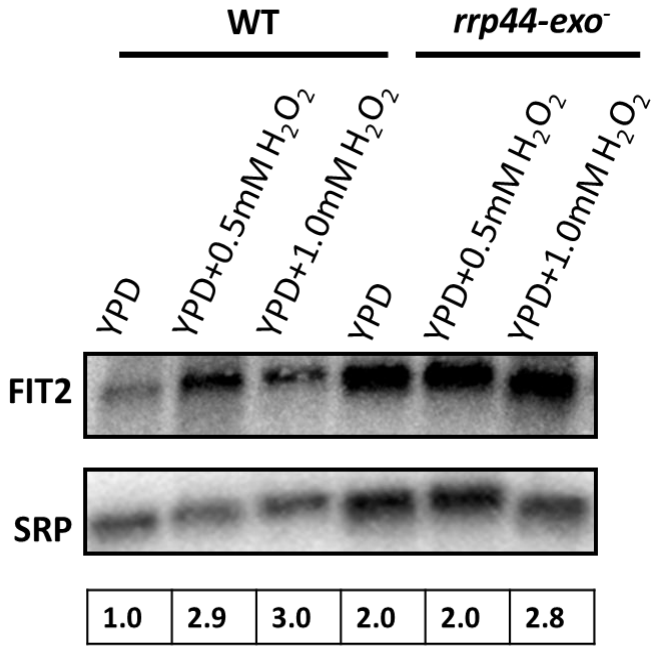


Figure 6-7. Activation of iron response after oxidative stress. Northern blots probed for *FIT2* of wild-type and *rrp44-exo*$^-$ treated with increasing amounts of H_2O_2, as indicated.

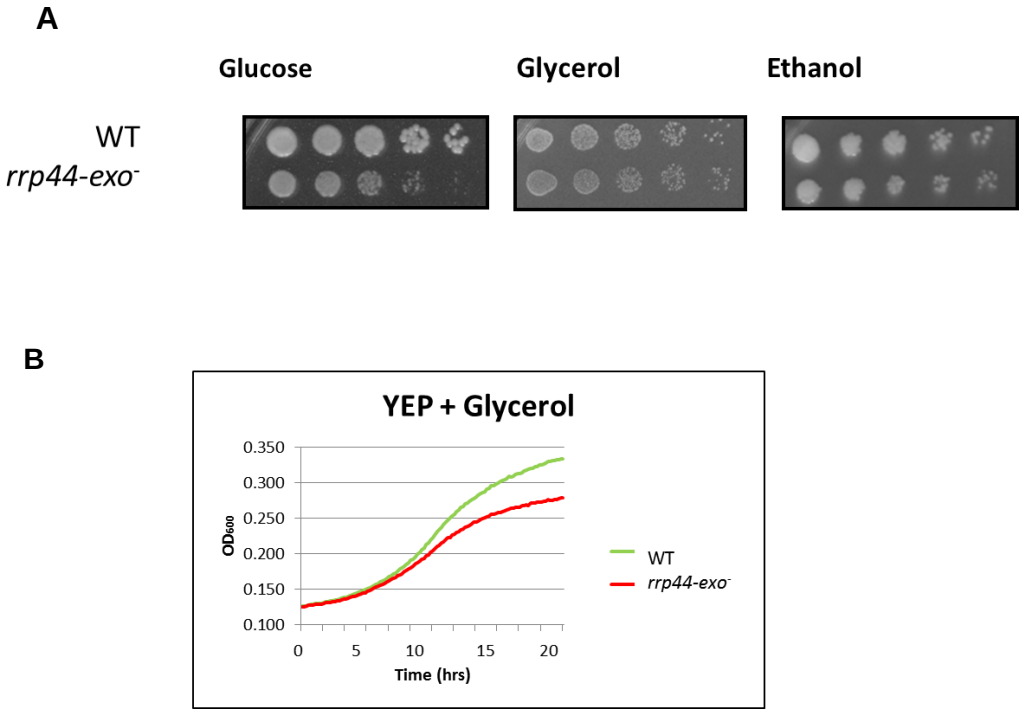


Figure 6-8. *rrp44-exo*$^-$ does not have severe growth defect compared to wild-type during respiratory growth. A. Wild type and *rrp44-exo*$^-$ growth assay on YEP medium supplemented with 2% glucose, 2% glycerol, or 2% ethanol. B. Growth assay of wild type and *rrp44-exo*$^-$ in liquid YEP+ glycerol medium.

Slow growth phenotype of rrp44-exo- correlates with increased ROS levels

If the increased ROS causes the slow growth phenotype, then it is expected that there will be no increase in ROS compared to wild type under conditions when the *rrp44-exo*$^-$ grows with the same rate as wild type. The only such known condition is respiratory growth. To check the ROS levels during respiratory growth, the ROS detection assay with H_2DCFDA was repeated after growing the cells in YEP + glycerol. The ROS levels in wild-type and *rrp44-exo*$^-$ cells had no significant difference (Figure 6-9). This indicated that ROS increase is a possible explanation for the slow growth phenotype of the *rrp44-exo*$^-$ mutant.

RNA processing and degradation defects of *rrp44-exo*$^-$ are independent of its slow growth phenotype and do not correlate with increase of ROS

Another possible explanation of the slow-growth phenotype is that it is caused by defects in processing and degradation of stable RNAs such as rRNA and snRNA that are known substrates for the exonuclease. To test if the defects in the transcripts were still present during respiratory growth, Northern blots were done with RNA isolated from cells grown in YEP + glycerol and probes against 5.8S rRNA, 5'ETS, and U4 snRNA. None of the defects present in YPD growth disappeared upon growth in YEP + glycerol (Figure 6-10). These data indicate that processing and degradation defects of *rrp44-exo*$^-$ are independent of its slow growth phenotype and does not correlate with increase of ROS.

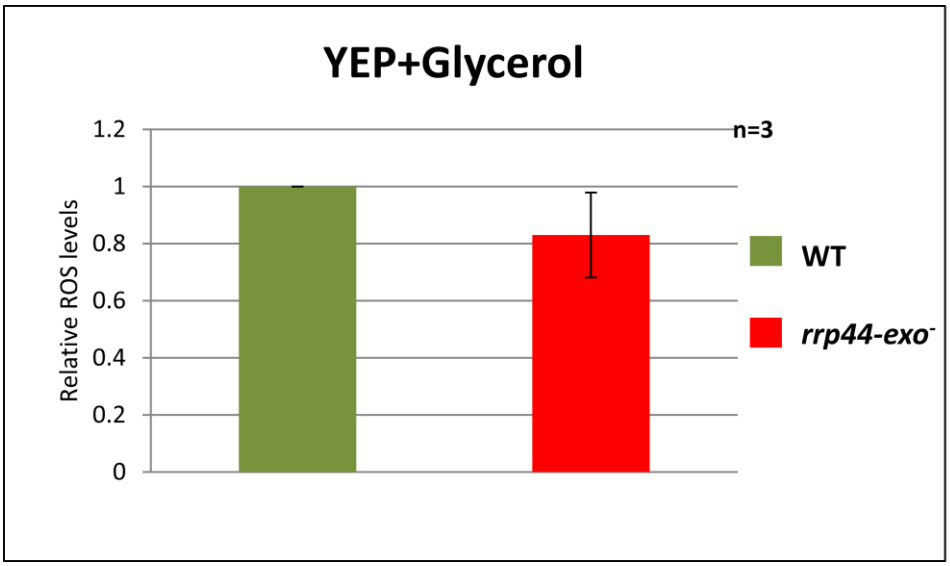


Figure 6-9. **During respiratory growth *rrp44-exo*$^-$ and wild type have the same ROS levels**. Relative ROS levels were measured in wild type (green) and *rrp44-exo*$^-$ (red) grown in YEP+ 2% glycerol medium.

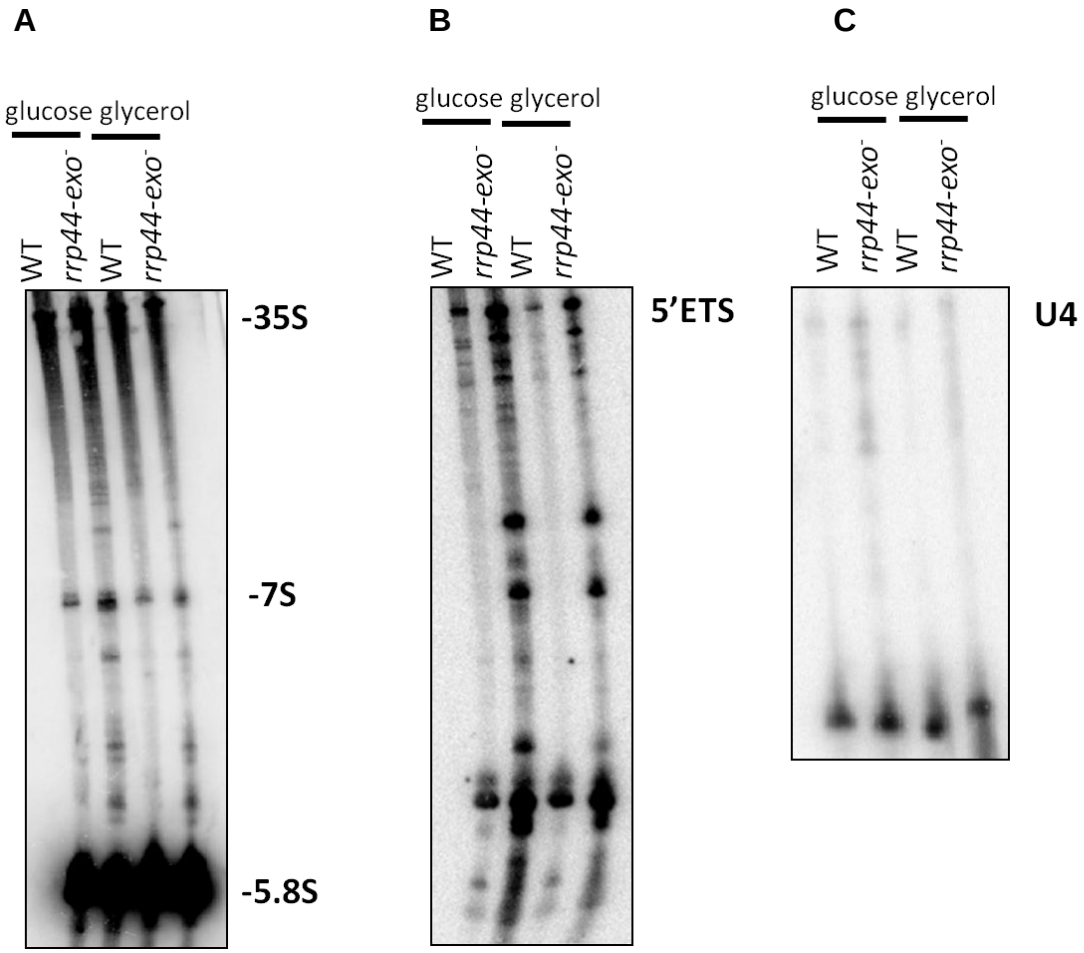


Figure 6-10. **Processing and degradation defects of *rrp44-exo*$^-$ are independent of its slow growth**. Northern blot analyses of wild type and *rrp44-exo*$^-$ grown either by fermentation (glucose) or respiration (glycerol). The blots were probed for A. 5.8S rRNA B. 5'ETS, and C. U4 snRNA.

***rrp44-exo*⁻ causes aberrant glucose metabolism**

The fact that *rrp44-exo*⁻ grows at a different rate compared to wild type during respiratory and fermentative conditions suggests that there is an aberrancy occurring during fermentative growth. One possibility is that *rrp44-exo*⁻ prematurely activates the ETC, going into respiration before all of the glucose is used up. Another possibility is that *rrp44-exo*⁻ produces different metabolites during glucose fermentation than the wild type.

To test if the ETC was prematurely activated, Northern blots with probes against components of the bc1 complex (complex III) were conducted. The bc1 complex has also been postulated to be the site of most oxidative stress production (Drose and Brandt, 2008). None of the components of the bc1 complex were upregulated (Figure 6-11) and one, Rip1, which is an iron sulfur protein, had lower levels, both in fermentative (with glucose as a carbon source) and respiratory (with glycerol as a carbon source) conditions. This indicates that the *rrp44-exo*⁻ does not upregulate ETC genes.

To further test if there is aberrancy with the glucose metabolism, the glucose intake, the ethanol output, and growth were measured over twenty four hour period of growth in YPD. Both wild-type cells and *rrp44-exo*⁻ used the same amount of glucose when corrected for growth rate, however *rrp44-exo*⁻ had lower ethanol output (Figure 6-12). Glucose metabolism in *S. cerevisiae* produces ethanol, but it

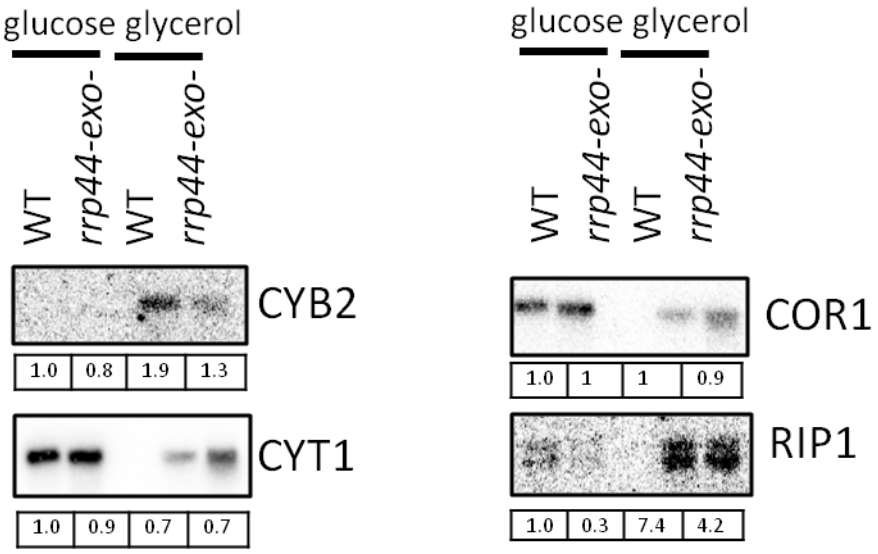
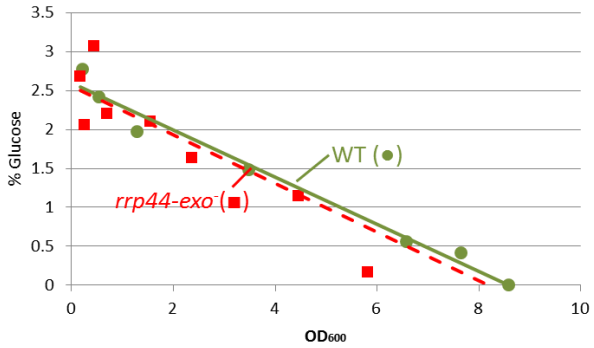


Figure 6-11. **Levels of ETC transcripts are not upregulated in *rrp44-exo*$^-$.** Northern blot analyses of wild type and *rrp44-exo*$^-$ grown either by fermentation (glucose) or respiration (glycerol). The probe is indicated on the right of each blot. Relative levels compared to SRP are shown below each blot.

A



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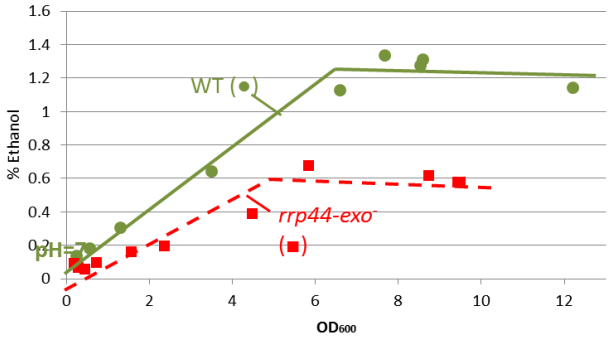

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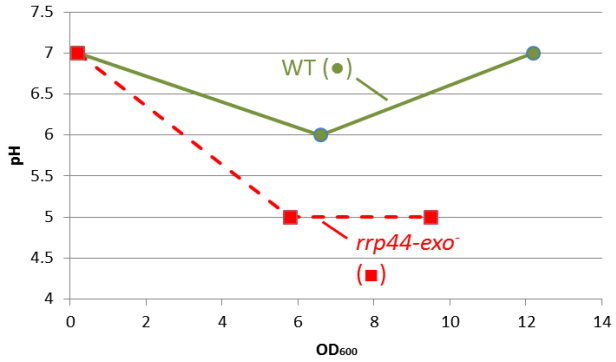

Figure 6-12. **Glucose metabolism is aberrant in *rrp44-exo⁻*.** Wild-type and *rrp44-exo⁻* were grown in YPD over 24hr period in triplicates. A. Percentage of glucose in the medium. B. Percentage of ethanol in the medium. C. pH of the medium in which wild type and *rrp44-exo⁻* strains were grown was measured at the indicated OD_{600},

also produces lactic acid and acetic acid. The production of lactic or acetic acid will lower the pH of the medium. To test if either of these two metabolites may be produced, the pH of the medium was measured. Interestingly, it was found that the pH of the *rrp44-exo*$^-$ is 2 points lower, at 5, than the starting pH of 7 indicating acidification of the medium. While the medium of the wild type strain i salso initially acidified, it is recovered and goes back to neutral pH=7 again during the respiratory growth. All of the above data indicate that glucose metabolism is aberrant in *rrp44-exo*$^-$ with lower production of ethanol and higher production of acidic metabolites, possibly lactate, acetate, or both.

DISCUSSION

The data presented in this chapter show the physiological importance of the exonuclease of Rrp44 during major cellular processes such as generation of oxidative stress and glucose metabolism.

The fact that addition of iron to oxidatively stressed cells improves the growth rate in the *rrp44-exo*$^{-}$ mutant, shows that the observed activation of iron response is not in fact a misregulation of the iron response itself. Instead, the iron response activation is a physiological consequence of another currently unknown aberrancy resulting from the lack of the exonuclease activity. Increase of ROS causes upregulation of iron response and the hypersensitivity phenotype is only observed during a condition under which the exonuclease mutant has higher ROS levels than wild-type cells.

Since initially the reason for examining ROS sensitivity was the possibility of too much iron causing increase in ROS, it seems counterintuitive that the opposite would also be true and too much ROS would cause increase in iron response. However, it should be considered that iron can exist in two different forms in the cell – free iron or bound iron and this could make a difference in the way oxidative stress and iron response could be related. Iron is rarely found in free form. It is usually sequestered in the vacuole or it is bound as a part of a protein prosthetic group. Too much free iron would cause oxidative stress and would be resulting in lowering of iron import and activating vacuolar sequestration of iron. However, too much oxidative stress also damages the bound iron, resulting in increased needs for iron due to damage in iron-sulfur clusters (Imlay, 2003). Therefore simply looking

at the fact that iron response is activated and having shown that it is related to oxidative stress rather than misregulated, it can reasonably be suggested that it is the bound iron that is affected and not that the cell has too much free iron. That conclusion is supported by the fact that only the iron sulfur protein in the bc1 complex of the ETC was affected and it had a lower than wild-type level. Additionally, no vacuolar genes related to iron storage were affected in the exonuclease mutant, further supporting this hypothesis because the vacuole is the site for iron storage in *S. cerevisiae*.

I have established that the increased ROS is related to the iron activation. The next question that needs to be answered is what causes the increased ROS in the *rrp44-exo*$^-$ mutant, if it is not activation of the ETC. A major possibility is that the aberrant glucose metabolism itself causes the ROS generation. Since the *rrp44-exo*$^-$ strain had less ethanol, and had lowered pH in the medium, it was concluded that either lactic acid or acetic acid production maybe increased in this mutant. Both lactic acid and acetic acid are known to induce oxidative stress in *S. cerevisiae* (Abbott et al., 2009; Semchyshyn et al., 2011). Furthermore lactate is known to react with ferric ion to form a complex which then, via the Fenton reaction, reacts with H_2O_2 to enhance the OH production (Ali et al., 2000). Finally, a microarray examining the role of acid stress on gene expression, found that iron response genes, regulated by Aft1, were upregulated upon stress with either lactic acid or acetic acid (Kawahata et al., 2006). Therefore it is possible that altered glucose metabolism, resulting in higher production of either lactic or acetic acid, is the reason for the increase of ROS and iron response in the *rrp44-exo*$^-$ strain.

The pH of the medium, however, was lower even after the diauxic shift, yet ROS production was at wild-type levels. An explanation of this phenomenon could be that after the diauxic shift, enzymes that detoxify ROS are upregulated and lower the ROS levels. In fact it has been shown that overexpression of catalase reduced oxidative stress induced by lactic acid (Abbott et al., 2009).

It would be interesting if in fact all of the physiological responses examined in this and the previous chapter stem from the aberrant fermentation that the *rrp44-exo*$^{-}$ carries out. Further experiments to distinguish the exact type and ratio of fermentative processes that are carried out will help further narrow down what exactly is affected in the pathway, Regardless of the results, the fact that RNA metabolism affects glucose metabolism is intriguing by itself and finding the exact regulatory RNA will be very exciting.

Chapter 7: Conclusions and perspective

The RNA exosome is a major exoribonuclease in eukaryotes and archaea, which also has endonuclease activity in eukaryotes. The work presented in this dissertation examines some important aspects of the RNA metabolism by the exosome. First, I have begun characterizing the cap proteins of the RNA exosome. These proteins, Rrp4, Rrp40, and Csl4, because of their location at the entry site of the substrate, are possibly important in stabilizing the substrate and/or providing specificity to the exosome. Specificity may be provided either directly by binding to particular RNAs or indirectly, by interacting with other exosome co-factors or RNA bound proteins which in turn provide specificity. Second, I have examined the physiological relevance of the exonuclease for cellular processes. Therefore, both of these aspects of my research, the characterization of the structure/function relationships of the proteins at the site of RNA entry and the physiological effect of the exosome, will serve as important basis for future research into the role of the exosome.

CONCLUSIONS AND FUTURE DIRECTIONS IN THE STUDY OF THE EXOSOME CAP PROTEINS

Rrp4 and Rrp40 have a structural role in the exosome

Rrp4 and Rrp40 are essential proteins and, as presented in Chapter 3, all of their three domains need to be expressed for viability. As similar as these two proteins are structurally, I have shown that they each have unique functions that cannot be

substituted by the other one. Additionally, chimeras of the two proteins do not substitute for each other indicating that the precise location of the proteins, not only their close proximity to the exosome entrance site, is important. Finally, *in cis* expression of Rrp4 domains also does not substitute for the essential role of Rrp4. All of these data strongly suggest that Rrp4 and Rrp40 have a structural role in the assembly of the exosome complex. Interestingly, an *in vitro* study showed assembly of the exosome with any two of the cap proteins, and also an exosome with only Rrp40 (Malet et al., 2010). The Malet et al. study implies that only Rrp40 is absolutely required for a stable assembly of the exosome which contradicts the data shown in Chapter 3 that suggests that Rrp4 also has a role in bridging PH subunits. However it is possible that if the exosome exists in more than one conformation, Rrp4 may be needed for only one. Such a scenario may be missed by *in vitro* studies, because confirmations may be driven be specific biochemical or genetic factors that are not available in the context of the *in vitro* studies. In fact, such a scenario may be missed also in the context of *in vivo* studies as they are usually carried out under specific standard conditions that do not vary. As I have shown in Chapter 6, the exonuclease mutant has different growth phenotype, ROS amount, and iron response activation depending on the carbon source. These differences were missed in traditional studies done with glucose and during logarithmic growth phase. A comprehensive study, compiling data from various exosome studies, has pointed out that published TAP-tagged purifications of the exosome differ in the composition of the proteins that elute with one or another exosome subunit suggesting that there may be different exosome complexes (Kiss et al., 2012). It is

also known that at least in archaea, the exosome exists in different confirmations, specifically of the cap proteins including homotrimers of either Rrp4 or Csl4, and heterotrimer composed of both Rrp4 and Csl4 (Lorentzen et al., 2007; Roppelt et al., 2010; Witharana et al., 2012). Therefore it is possible that a confirmation of the exosome, driven by some other, unknown conditions, does in fact require Rrp4 for its structural integrity.

In addition, at least for Rrp4, my data imply that it has a functional role outside of the exosome complex. This role is currently unknown and I have suggested experiments (see below) to address this proposed role. It is also possible that Rrp4 has a non-essential role, given the fact that it is post-translationally modified by phosphorylation, but this phosphorylation is not required for viability. The most likely possibility is that this phosphorylation has a regulatory role. To determine a possible role for the phosphorylation, I have suggested a series of experiments designed to comprehensively look at the effects of the potential regulation by the phosphorylation.

Csl4 does not stabilize the exosome by bridging PH ring proteins

Unlike Rrp4 and Rrp40, I have determined that the third cap protein Csl4 does not have role in bridging subunits of the PH ring of the exosome, thus it follows that it has only a functional role. Therefore the essential role of Csl4 may be to bind RNA or protein cofactors. At this time either of these possibilities is likely.

Whether the proposed RNA or protein binding takes place initially at the entrance site of the exosome central channel is debatable. Based on the research I have presented in this work, I cannot currently rule out that RNA or proteins are bound by Csl4 away from the exosome complex. The fact that viable Csl4 truncations lose most of their interactions with the exosome suggests that stable interactions are not required for viability. Further experiments need to address this possibility by repeating the experiment in a strain in which *CSL4* is deleted and the truncations are the only Csl4 available in these cells, as described in the conclusion section of Chapter 4.

Following are several experimental approaches that will help answer important questions regarding the cap proteins of the exosome. First, what are the roles of the individual domains of the cap proteins? Second, what are the functions of the cap proteins? Third, what, if any, are the roles of the cap proteins exclusive of the exosome complex? And finally, what are the roles of the phosphorylation of Rrp4 and Csl4?

Future directions: Determining the roles of individual domains of the cap proteins

To determine the role of specific domain interactions, the TAP tagged truncations of the individual Rrp4, Rrp40, and Csl4 protein can be used. Briefly, plasmids with truncations and full length Rrp4, Rrp40, and Csl4 sequences would be transformed individually into a wild-type *S. cerevisiae* strain. After growth to log phase cells will

be harvested, and total protein will be isolated. The total proteins will be run through a IgG sepharose column to pull-down the TAP-tagged protein along with any protein binding partners. The bound fraction will then be analyzed by mass spectroscopy to identify all the binding partners. Comparing of the mass spectroscopy data of the binding partners of the full-length protein and the truncation will indicate which proteins (if any) are missing from the fractions bound to truncations. To determine if a domain does in fact interact with a given protein, the individual domain and its proposed protein binding partner should be expressed and purified from *E. coli* and *in vitro* binding assay should be carried out to confirm that the proposed interaction is only with that domain and it is direct. In addition to the technical challenges, one caveat of this experiment is that if the role of a domain is to bind RNA, this will be missed because this approach only tests for protein-protein interaction. If no change in protein-protein interaction is discovered, a possibility that this particular domain binds RNA should be considered. However, a role of a domain may be to both interact with proteins and RNA at different surfaces, therefore the interpretation of the results should be carefully done, to avoid overreaching conclusions should a binding partner for a domain is discovered. To avoid such errors, an assay for RNA binding can also be done. Since the cap proteins are believed to bind RNA non-specifically, the use of complicated assays such as RIP-seq (RNA immunoprecipitation followed by high-throughput sequencing) may not be warranted as the amount of data from these high throughput assays may be overwhelming to allow for straightforward interpretation. *In-vitro* assays for exosome activity have been done with generic RNA substrates, thus such substrates can be used in gel-

shift assays together with the various Rrp4, Rrp40, and Csl4 truncations. Thus, both of these experiments, the truncation pull-down and the RNA binding assay should add to the characterization of the roles of the individual domains of the cap proteins. One exception will be the RPL27-like domain and the S1 domains of Rrp40. For these two domains individual distinction will not be possible using this method as they are not expressed individually, but a point mutagenesis approach may be more suitable.

Future directions: Determining the functions of the cap proteins

I have shown that the cap proteins have a functional role. However what that functional role is not clear. It is possible that the functional role is the same as for the exosome complex, or there is a possibility that individual proteins contribute to different functional roles. To determine the functional role of the cap proteins, microarray analyses with poly(A) RNA can be completed. Because each of these proteins is an essential protein, they cannot be deleted. However they can be at least temporary depleted. One widely used method, and one that has also been used with the exosome proteins is depletion by the use of the *GAL* promoter (Allmang et al., 1999a). However, in Chapter 6 of this dissertation I have shown that glucose metabolism is affected in the exosome mutant. I have not specifically examined growth on galactose, but given the aberrancies seen when glucose is used as a carbon source, it is my opinion that a sugar regulated promoter should be avoided when completing experiments involving the exosome. The best option

would be to use a promoter, such as tetracycline regulatable promoters that are independent of sugar type and availability and are also commercially available (Gari et al., 1997).

To look at the role of the cap proteins the Tet-off system can be used with the wild-type TAP-tagged sequence of Rrp4, Rrp40, or Csl4 and transformed into the corresponding deletion strain. The depletion, after tetracycline addition, will be monitored by a Western blot with antibody against the TAP tag and simultaneously cells would be harvested, and total RNA will be extracted to be used for microarray analysis.

Several conclusions can be drawn for such data, depending on the final results. First, the microarray results of cap protein depletion may show completely overlapping results with the microarray results of the *rrp44-exo*$^{-}$ presented in this dissertation. Such results, even though unlikely, would suggest that both the cap protein and Rrp44's functional roles are as a part of the exosome complex. Second, the microarray results of cap protein depletion may have some, but not all overlapping results with the microarray results from the *rrp44-exo*$^{-}$ and not have additional RNA changes. Such results will also suggest that the cap protein's functional role is as a part of the exosome complex, because a role outside of the exosome complex would involve different, not overlapping substrates. Third, in addition to overlapping results, the cap protein depletion microarray may present additional changes in RNA not detected in the *rrp44-exo*$^{-}$ microarray, which would suggest that the particular cap protein has a role outside of the exosome complex. Fourth, because the exosome proteins are essential, depletion of one of them may

lead to changes not directly associated with the exosome, but provoked by other mechanisms related to the cell dying and this should be kept in mind while investigating the results from the microarray.

Future directions: Determining the cap proteins roles outside of the exosome complex

Several lines of evidence suggest that the cap proteins may have roles outside of the exosome. First, cap proteins have been visualized in distinct cellular foci (Graham et al., 2006). Second, I have shown in this dissertation that at least Rrp4 is stable out of the exosome complex. This stability assay can be extended to Rrp40 as well. To examine possible roles outside of the exosome complex, point mutations leading to dissociation with the exosome can be created by analyzing the interacting surfaces between the cap protein and the exosome subunits of the PH ring. A coimmunoprecipitation assay if the mutant proteins with the exosome should be done to confirm that the mutants do not bind the exosome. To determine if the essential role of the cap protein is outside of the exosome complex, plasmid shuffle assay with the mutated cap protein can be used with a strain which has tetracycline-regulatable wild-type cap protein. This experiment will be suitable specifically for Csl4, since Rrp40 is known to have a structural role, and my data suggest that Rrp4 also may have a structural role. If the essential role of Csl4 is out of the exosome complex, upon depletion with tetracycline, there will be no effect on growth of this mutant compared to a mutant in which wild-type Csl4 has been introduced.

Alternatively, if the essential role of Csl4 is as a part of the exosome complex, then upon depletion of the wild-type Csl4, the growth will slow down and the strain will ultimately stop growing.

To look for non-essential roles of the cap proteins that are not as a part of the exosome complex, TAP-tagged mutants that do not interact with the exosome, as described above, can be introduced into wild-type strain of *S. cerevisiae* and pull-down assays can be completed followed by mass spectrometry analyses to look for interacting proteins. The protein binding profile of these mutants can then be compared to mass spectrometry profile of wild-type cap protein to determine which, if any, protein interactions are specific to the mutant proteins.

Future directions: Determining the role of posttranslational modifications of Rrp4 and Csl4

The presence of posttranslational modifications in Rrp4 and Csl4 suggest that these two proteins are regulated. I have shown that such regulation is not related to the essential function of these proteins, but nevertheless it would be interesting to know why these proteins are posttranslationally modified. Posttranslational modifications could be used by the cell to regulate protein localization, abundance, or interactions with other proteins. To determine what is the role of the phosphorylation of Rrp4 and Csl4, the phosphomutants of Rrp4 and Csl4 that I created for this work (see Chapters 3 and 4) can be used in variety of assays. First, to look for protein binding partners on a global scale, pull-down assays followed by

mass spectrometry can be done with these mutants and compared to wild-type. Second, to look for changes in gene expression, microarrays can be completed with these mutants and compared to wild-type strains. Third, to look for changes in abundance, the phosphomutant protein levels can be compared to wild-type corresponding protein by Western blots. Forth, to look for localization changes, the mutants can be tagged with GFP and their cellular localization can be observed by florescent microscopy. Altogether, completing this set of experiments should generate data which will determine the role of the posttranslational phosphorylation of these two proteins.

CONCLUSIONS AND FUTURE DIRECTIONS IN THE STUDY OF THE EXONUCLEASE FUNCTIONAL IMPORTANCE

The exonuclease is the primary activity of the exosome

The catalytic subunit of the Rrp44 has two RNase activities. First, it has an exonuclease activity and second it has an endonuclease activity. Interestingly, using a microarray analysis and an analysis of internally cut RNAs I have not been able to identify any substrates for the endonuclease. Yet the endonuclease is able to substitute for the essential role of Rrp44, even though a strain having only endonuclease activity grows slower. In contrast, a strain having only exonuclease activity does not have any apparent growth defect. Together with the data that known substrates of the exosome are only affected in exonuclease mutant, this

indicates that the exonuclease is the main activity of Rrp44. One explanation of the lack of specific substrates coupled with the ability to provide viability would be that the endonuclease's role may be to provide supporting function to the exonuclease. For example if the endonuclease cleaves substrates that have stronger secondary structures to speed up their degradation, it may not have specific substrates unique to it. Rather, in the absence of the exonuclease, the endonuclease would cleave all essential substrates and if that slows down the process, may result in the slower growth phenotype. Another possibility is that just for the essential substrates the exosome has developed a second degradation site, to ensure that they are processed and/or degraded.

Future directions: Determining the essential substrates of the exosome

The modest effect in gene expression was surprising given the wide range of substrates of the exosome. One possibility is that many substrates are affected by both activities and knocking only one out does not result in appreciable mRNA changes. An experiment that can be carried out in order to determine the effect on gene expression of Rrp44 is to attempt to complete a microarray analysis or high-throughput sequencing using a double mutant *rrp44-exo*$^{-}$ *rrp44-endo*$^{-}$. Briefly, a *rrp44-exo*$^{-}$ *rrp44-endo*$^{-}$ strain carrying a Tet-regulatable *rrp44-exo*$^{-}$ on a plasmid, could be grown in liquid YPD medium at OD_{600}=0.6 and then the expression from the Tet Promoter will be shut off by addition of Tetracycline to the medium. This will result in a complete knock-out of the ribonucleic activities of Rrp44. Such mutant is

inviable and will stop growing eventually, however depletion will be possible. The duration of the depletion will have to be empirically determined. Cells can then be collected, RNA extracted and sent either for microarray hybridization or high-throughput sequencing. For a control a knockout of only the exonuclease can be used. Any RNA the level of which is changed in the double mutant will be considered to be resulting from overlapping functions of the exonuclease and the endonuclease and will further be investigated. A possible difficulty of this experiment is that the time during which both activities will be knocked-out may not be sufficient to determine any appreciable changes especially in non-coding stable RNAs. mRNAs half-life in *S. cerevisiae* vary greatly with 91% of the transcripts have a half-life between 12 and 29 minutes (Munchel et al., 2011) but stable RNAs persist throughout the life of the cell and appreciable depletion could be seen upon cell division. Exosome mutants start accumulating defects in 7S rRNA and 5'ETS 8hrs after a Tetracycline shut off of the Tet-off promoter, and the experiment was carried out for 22 hours (Dziembowski et al., 2007).

The results from the experiment with this double mutant will point to why the exosome is essential because they will give the changes in gene expression. The results from a deep sequencing experiment with this mutant will be more complicated to interpret because the exosome affects may different RNAs. However, the deep sequencing approach is more useful if the specific substrates of the exosome are to be identified.

Another approach that can be taken to identify essential roles of the exosome is to carry out a genetic complementation screen. A library of *S. cerevisiae* ORFs can be

transformed into an rpp44-*exo*$^-$ *rrp44-endo*$^-$ mutant to screen for loss of synthetic lethality. However this approach may only be effective if the synthetic lethality is due to one or a few genes. If the synthetic lethality results from a global effect on gene expression then this genetic test will not be useful.

Model of how *rrp44-exo*$^-$ affect physiological responses of the cell

In chapter 6 I showed that the *rrp44-exo*$^-$ mutant affects glucose metabolism and that during glucose growth, *rrp44-exo*$^-$ outputs less ethanol than wild-type strain while simultaneously acidifying the medium more than the wild-type strain. The slow growth on glucose correlated with higher ROS production. Additionally I showed that iron response was activated during oxidative stress and that addition of iron improved the growth of an oxidatively stressed *rrp44-exo*$^-$ mutant. Based on comprehensive analysis of the above data, I have developed a model of how *rrp44-exo*$^-$ affects iron response in YPD medium (Figure 7-1). Knowing that lactic acid causes oxidative stress, I postulate that the *rrp44-exo*$^-$ mutant produces more acid (either lactic or acetic acid) during glucose metabolism, which in turn leads to the production of increased amount of ROS and therefore to activation of iron response (Figure 7-1). Both acetic and lactic acid induce oxidative stress in *S. cerevisiae* (Abbott et al., 2009; Semchyshyn et al., 2011), therefore either one will be capable of inducing such physiological response. Additionally, both acetic and lactic acid induce iron response genes similarly to the ones induced in the *rrp44-exo*$^-$ mutant (Kawahata et al., 2006).

Future directions: Testing the model of how the *rrp44-exo$^-$ mutant* affects physiological responses of the cell

There are several experiments that could be carried out in order to test my model. First, there are two possibilities for the lower pH in the medium of *rrp44-exo$^-$* : one is increase in lactic acid; the other is increase in acetic acid. To be able to further narrow down what element of glucose metabolism is affected in *rrp44-exo$^-$* it is necessary to determine which one of these acids causes the increased acidity of the medium. Kits able to measure either one of these acids are commercially available and can be used for that purpose. To test the possibility that acidification causes the slow growth of rrp44-*exo$^-$* an experiment to neutralize the acid in the medium could be carried out. Buffering the medium has been shown to reverse the growth impairments caused by lactic acid and acetic acid toxicity in *S. cerevisiae* (Thomas et al., 2002). Wild-type and *rrp44-exo$^-$* strains could be grown in YPD medium which is monitored for pH change and the pH adjusted by KOH and HCl addition as needed. Improvement in growth rate as compared to wild-type will indicate that the slower growth phenotype is caused by the addition of acid. Conversely, no change in growth phenotype will indicate that slower growth is independent of acid production.

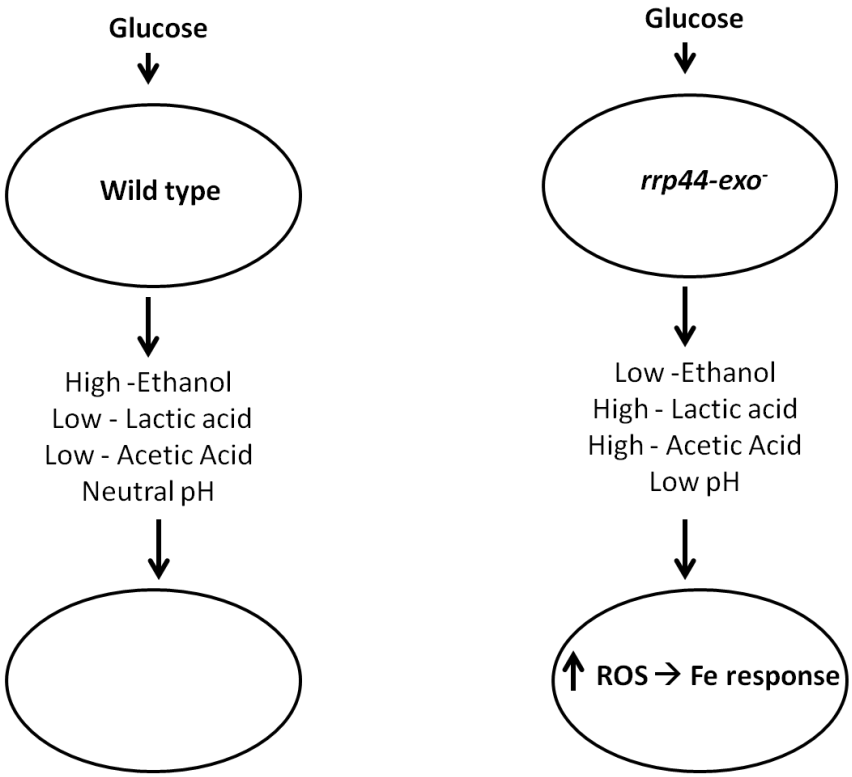

Figure 7-1 A model of how *rrp44-exo*$^{-}$ affect physiological responses of the cell. Left - Wild-type cells during growth with glucose as a carbon source grow preferentially by ethanol fermentation, putting out in the medium mostly ethanol and some lactic and acetic acid. Right – *rrp44-exo*$^{-}$ mutant during growth on glucose as a carbon source have lowered output of ethanol and increased output of either lactic or acetic acid which leads to increase ROS production which in turn leads to activation of the iron response.

Lactic and acetic acid are known to cause an increase in oxidative stress. To test if they are the specific cause for the oxidative stress production in *rrp44-exo*$^-$,wild-type and *rrp44-exo*$^-$ strains could be grown as their `medium is regularly exchanged coupled with ROS measurement and northern blots to probe for iron response mRNAs. In addition, medium form wild-type and *rrp44-exo*$^-$ mutant can be taken and used to supplement a wild-type strain and to measure changes in growth rate, ROS production and iron response mRNAs. In addition, an experiment in which increasing amounts of lactic acid and/or acetic acid are added to the media could be carried out to confirm increase of ROS and iron response activation. Results from these experiments will identify if the source of the physiological responses seen in *rrp44-exo*$^-$ is an excreted product in the medium from aberrant fermentation.

Final remarks

The RNA exosome in a major exoribonuclease involved in numerous cellular processes. This dissertation contributes to the understanding of the importance of this RNA degrading machinery by first characterizing the roles of the three cap proteins in the exosome and second by beginning to elucidate the physiological importance of the exonuclease of the exosome. Lastly, this work provides a testable model to serve as a foundation for future experiments to elucidate the physiological importance of the main catalytic activity of the RNA exosome.

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VITA

Borislava Borissova Tsanova was born on November 13th, 1974 in Sofia, Bulgaria, the daughter of Theodora Moneva and Boris Tsanov. After graduating from high school, she immigrated to the United States where she enrolled in Houston Community College and in 1996 graduated with a diploma of Vocational Nursing. After several years Borislava decided to enroll back in college and in 2006 she graduated with a B.S. degree in Applied Microbiology from University of Houston – Downtown. In August 2006 Borislava joined UT - Graduate School of Biomedical Sciences in Houston and in May 2007 joined the laboratory of Dr. Ambro van Hoof.

Permanent address: 10312 Brinwood Drive, Houston, TX77043