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The Role of the Arched Helicases in Exosome-Mediated Function

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THE ROLE OF THE ARCHED HELICASES IN EXOSOME-MEDIATED FUNCTION

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RNA processing and degradation are two important functions that control gene expression and promote RNA fidelity in the cell. A major ribonuclease complex, called the exosome, is involved in both of these processes. The exosome is composed of ten essential proteins with only one catalytically active subunit, called Rrp44. While the same ten essential subunits make up both the nuclear and cytoplasmic exosome, there are nuclear and cytoplasmic exosome cofactors that promote specific exosome functions in each of the cell compartments. To date, it is unclear how the exosome distinguishes between RNA substrates. We hypothesize that compartment specific cofactors may promote the substrate specificity of the exosome.

In this work, I characterize several cofactors of the exosome, both nuclear and cytoplasmic. First, I describe the arch domain, which is a unique domain in a nuclear and a cytoplasmic cofactor of the exosome. Specifically, I show that the arch domain of nuclear exosome cofactor, Mtr4, is required for specific exosome-mediated activities and overlaps functionally with the exosome-associated exonuclease, Rrp6.

Further, I show that the arch domain of Ski2 is required for the degradation of normal and aberrant mRNAs.

Additionally, this work describes in detail the Mtr4 domains involved in the physical association with other RNA processing proteins. Further, I characterize the minimal Mtr4-binding region in a third exosome cofactor, Trf5.

Understanding how exosome cofactors synergistically promote exosome function will provide us a better understanding of how the exosome complex precisely regulates its catalytic activities. As described here, cofactors play a major role in determining the substrate specificity of the nuclear and cytoplasmic exosome. Moreover, specific accessory domains, which are not involved in the catalytic function of the cofactor, are required for substrate targeting of the eukaryotic RNA exosome.

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Chapter 1: Introduction, Background and Significance

INTRODUCTION AND BACKGROUND

Post-transcriptional modifications are essential for RNA function

RNAs are macromolecules that are important for all cellular functions. There are a variety of RNA types, which vary in length, shape, and ultimately function. After transcription, RNAs are in a premature state (pre-RNA), which renders them nonfunctional. Thus, each RNA must undergo specific modifications and cleavages to achieve its mature form. This intricate and customized process requires many different enzymes and regulatory RNAs. During this process RNA intermediates transiently associate with proteins forming ribonucleoproteins (RNPs). RNPs modulate the transport, cleavage, translation (if mRNA), and degradation of the RNA. Many of these RNA binding proteins belong to multi-protein complexes involved in the processing of the RNA. RNPs play a crucial role in determining the fate of the RNA (Hafidh *et al.*, 2011).

The RNA metabolic pathway is very intricate, therefore mistakes can be made along the RNA maturation process. Thus, many of the RNA modifications and cleavages have redundant or back-up pathways. Additionally, the cell has dedicated surveillance pathways to maintain the fidelity of these essential processes. Furthermore, aberrant RNAs may arise from mutations in the DNA template. Specific mutations encoded in the RNA may then produce truncated proteins or misfolded noncoding RNAs (ncRNA). To avoid these mistakes, aberrant RNAs are efficiently identified and targeted for degradation by ribonucleases (Doma & Parker, 2007).

RNA cleavage also has an important role in the regulation of gene expression. In addition to cleavage for maturation and surveillance purposes, mRNAs are also degraded in order to control overall gene expression. The primary sequence of the mRNA (5' untranslated region and 3' untranslated region, 5' UTR and 3'UTR respectively), as well as the RNA binding proteins that decorate it (especially at the poly(A) tail) seem to dictate the half-life of the mRNA (Luchin *et al.*, 2012, D'Orso *et al.*, 2003, Dodson & Shapiro, 2002, Zhang *et al.*, 2002, Kiss *et al.*, 2012, Rajagopalan & Malter, 1997).

In eukaryotes, the RNA exosome, multi-protein complex, plays three major roles in RNA biogenesis: RNA maturation (3' end cleavage), normal mRNA degradation, and quality control of RNA processing in the nucleus and cytoplasm.

Functions of the RNA exosome

The RNA exosome is involved in two important steps in the RNA maturation process. First, the exosome precisely trims premature transcripts to remove unnecessary bases and allow for the formation of the mature RNA. Second, the exosome identifies RNAs that need to be degraded as a means of regulating the gene expression. The exosome has specific substrates in the nucleus and in the cytoplasm.

Nuclear function of the exosome

In the nucleus, the exosome is involved in the defined trimming of ribosomal RNAs (rRNAs). Specifically, the exosome digests the 3' extended region of the 5.8S rRNA and 7S pre-rRNA. Through its exonuclease activity, the exosome processes the

7S pre-rRNA into the 5.8S+30 pre-rRNA, which is then cleaved by the cofactor Rrp6 (discussed in more detail in page 13).

The exosome is also involved in the trimming of both types of small nucleolar RNA (snoRNAs) precursors, C/D and H/ACA, into their mature form (Allmang *et al.*, 1999a, van Hoof *et al.*, 2000b, Lykke-Andersen *et al.*, 2009). Intron-derived snoRNAs U18, U24, snR44 require the exosome to precisely trim the 3' end of the pre-snoRNA. Exosome depletions (through transcriptional down-regulation) show long polyadenylated snoRNAs of heterogenous lengths (150-300 nts). Independently transcribed snR40 and snR33 also accumulate the extended poly(A) snoRNA species. Certain snoRNAs that are transcribed from a polycistronic transcript require the exosome for their 3' end cleavage during maturation including snR72, snR73, and U14 (van Hoof et al., 2000b, Allmang et al., 1999a). Although the exosome processes all the different types of snoRNAs, it only contributes to the cleavage of specific snoRNAs in each of the different categories. Examples of specific snoRNAs tested that do not require the exosome are intron-derived snR39, and independently-transcribed snR10 and U3 snoRNAs (Allmang et al., 1999a, van Hoof et al., 2000b). Interestingly, snR190, which is co-transcribed with U14 (described above), does not require the exosome (Allmang et al., 1999a, van Hoof et al., 2000b) (Please see snoRNA biogenesis section for more detail). Rrp6, the nuclear cofactor of the exosome, is also involved in the processing of the extended polyadenylated snoRNAs. Similarly to the 5.8S processing pathway, the exosome trims the longer snoRNA precursor and hands it off to Rrp6, which cleaves the final three nucleotides.

Small nuclear RNAs (snRNAs) also require cleavage of the primary transcript by the exosome. snRNAs U1, U4, and U5 are endonucleolytically cleaved by Rnt1 at their 3' ends. The exposed 3' end then becomes accessible to the exosome and Rrp6 to precisely remove specific extra bases from the snRNA precursor (van Hoof et al., 2000b, Allmang et al., 1999a).

In addition to cleaving stable RNAs during their maturation process, the exosome degrades processing by-products and unstable cryptic RNAs. First, the exosome degrades one of the degradation by-products of the 35S pre-rRNA cleavage. The 5' External Transcribed Spacer (5'ETS) is the 5' most region of the 35S precursor, and is first cleaved by an endonuclease (see rRNA biogenesis section for more detail)(Elela *et al.*, 1996). The cleaved product becomes a substrate for exosome degradation (Allmang *et al.*, 1999a, Zanchin & Goldfarb, 1999). Further, the uncharacterized Cryptic Unstable Transcripts (CUTs), and antisense RNAs derived from promoter regions (Promoter-Associated Transcripts (PROMPTS)), are all exclusively degraded by the nuclear exosome (Wyers *et al.*, 2005, Preker *et al.*, 2011, Davis & Ares, 2006, Xu *et al.*, 2009, Neil *et al.*, 2009). Additionally, small un-annotated transcripts (SUTs) have been shown to accumulate in yeast strains depleted of the nuclear exosome, as well as nuclear cofactors of the exosome. SUTs, however, can also be substrates of the 5' to 3' decay pathway in the cytoplasm (Xu et al., 2009, Marquardt *et al.*, 2011).

The exosome also targets normal transcript precursors (i.e pre-rRNAs) and prevents their accumulation during biogenesis (Allmang *et al.*, 2000). The nuclear exosome plays an important role in surveillance by the RNA processing pathway.

Aberrant forms of both coding and noncoding RNAs are identified and targeted for degradation by the exosome (Bousquet-Antonelli *et al.*, 2000, Zanchin & Goldfarb, 1999). Temperature sensitive mutation of the subunits of the exososome and nuclear cofactor show accumulation of hypomodified tRNAs, unspliced mRNAs, and misprocessed rRNAs (Cole *et al.*, 2009, Kadaba *et al.*, 2004, Wang *et al.*, 2008, Bousquet-Antonelli et al., 2000, Allmang et al., 2000, Hilleren *et al.*, 2001). For example, the exosome is involved in the degradation of rRNA intermediates 23S and 21S, which are 5' extended versions of the mature 18S rRNA (Allmang et al., 2000).

Cytoplasmic functions of the exosome

In the cytoplasm, the exosome is involved in degradation of mRNAs for both regulation of gene expression and as a surveillance mechanism. First, the cytoplasmic exosome functions in one of the two redundant pathways of normal mRNA degradation (Figure 1.2). The rate-liming step in normal mRNA degradation involves the deadenylation of the mRNA. The exposed 3' end of the mRNA can be exonucleolytically cleaved by the exosome. The mRNA cap is then removed by the scavenging decapping enzyme Dcs1 (Schaeffer & van Hoof, 2011, Jacobs Anderson & Parker, 1998). Every mRNA has a specific half-life, and through RNA decay, the exosome plays an important role in regulating gene expression in the cell.

Second, the exosome targets aberrant mRNAs for degradation in the cytoplasm. Transcripts that contain premature stop codons (nonsense), those that lack in-frame stop codons (nonstop), those that encode long translational pauses (no-go), and those that lack a poly(A) tail are all targeted for degradation by the

cytoplasmic exosome (Doma & Parker, 2006, van Hoof *et al.*, 2002, Lejeune *et al.*, 2003, Meaux & Van Hoof, 2006). In the nonstop mRNA decay pathway, the exosome functions independently of deadenylation and presumably identifies the stalled ribosome associated at the end of the nonstop mRNA (Figure 1.3)(van Hoof et al., 2002, Klauer & van Hoof, 2012a). The exosome degrades the transcript in a 3' to 5' direction and can use either of its ribonucleolytic activities to do so (Schaeffer & van Hoof, 2011).

The structure and composition of the eukaryotic RNA exosome

The RNA exosome is a complex of ten essential proteins that localizes to the nucleus and the cytoplasm (Mitchell *et al.*, 1997, Allmang *et al.*, 1999b). Although the exosome associates with a variety of compartment-specific proteins, in *Saccharomyces cerevisiae* only Rrp4, Rrp40, Rrp41, Rrp42, Rrp43, Rrp44, Rrp45, Rrp46, Mtr3, and Csl4 are invariable and form the core of the exosome complex.

Rrp41, 42, 43, 45, 46 and Mtr3 have homology to the *E. coli* RNase PH enzyme, however, they all lack the enzymatic activities of their bacterial counterpart. These catalytically inactive exosome subunits assemble into a hexameric ring that forms a channel whereby RNA can enter (PH ring) (See Figure 1.1)(Liu *et al.*, 2006, Bonneau., 2009, Malet *et al.*, 2010, Tsanova & van Hoof, 2010, Lorentzen *et al.*, 2007, Januszyk & Lima, 2010).

Figure 1.1

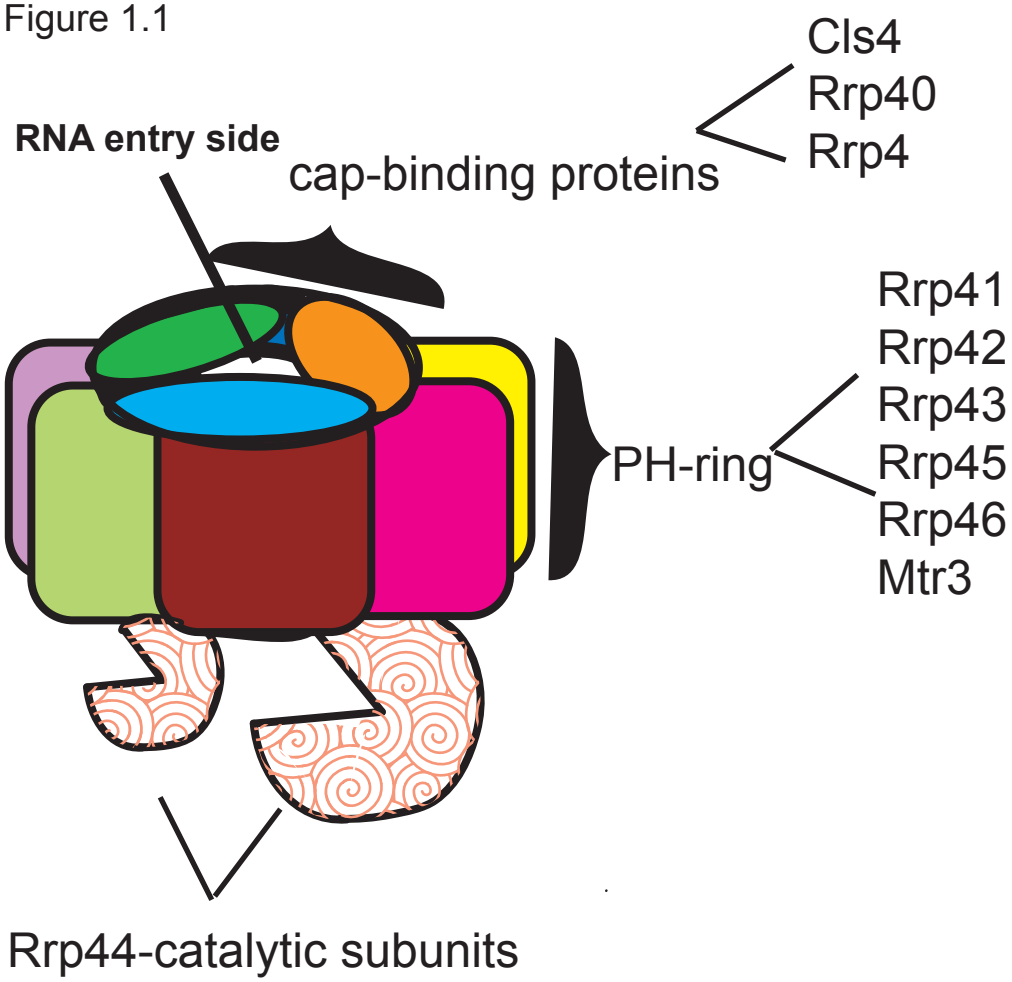

Figure 1.1: Cartoon depiction of the composition and structural features of the eukaryotic RNA exosome.

Figure 1.2

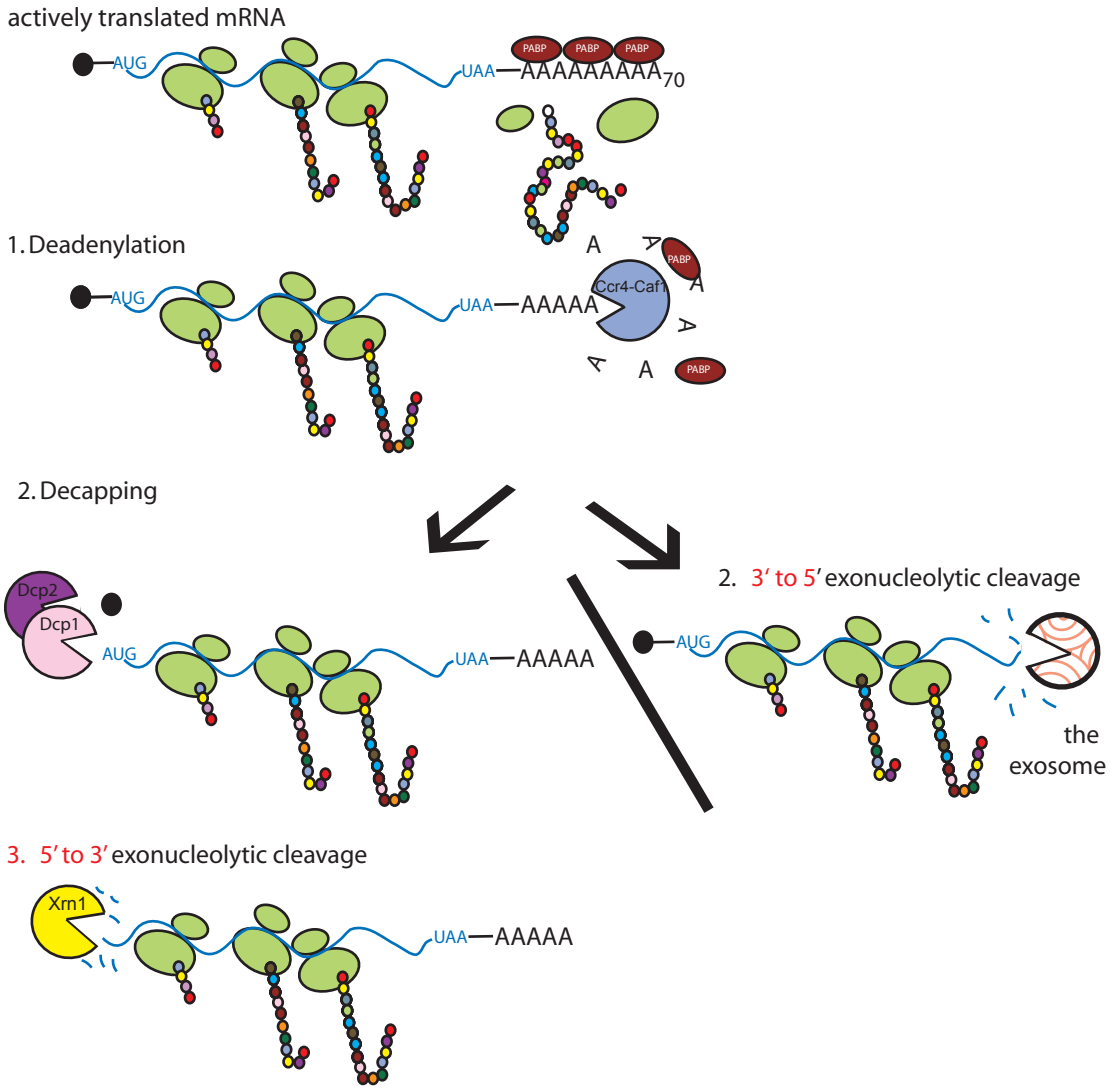

Figure 1.2: Schematic representation of the two normal mRNA decay pathways.
The rate-liming step for normal mRNA degradation involves the deadenylation of the transcript.The transcript then becomes vulnerable for decay from either the 5' or the 3' end. From the 5' end, the mRNA becomes decapped and degraded exonucleolytically by Xrn1. Conversely, the transcript becomes degraded from the 3' end by the RNA exosome. PAPB-poly(A) binding protein, Dcp1/2 are the decapping enzymes, Xrn1 is the 5' to 3'exoribonuclease, Ccr4 is the deadenylase.

Figure 1.3

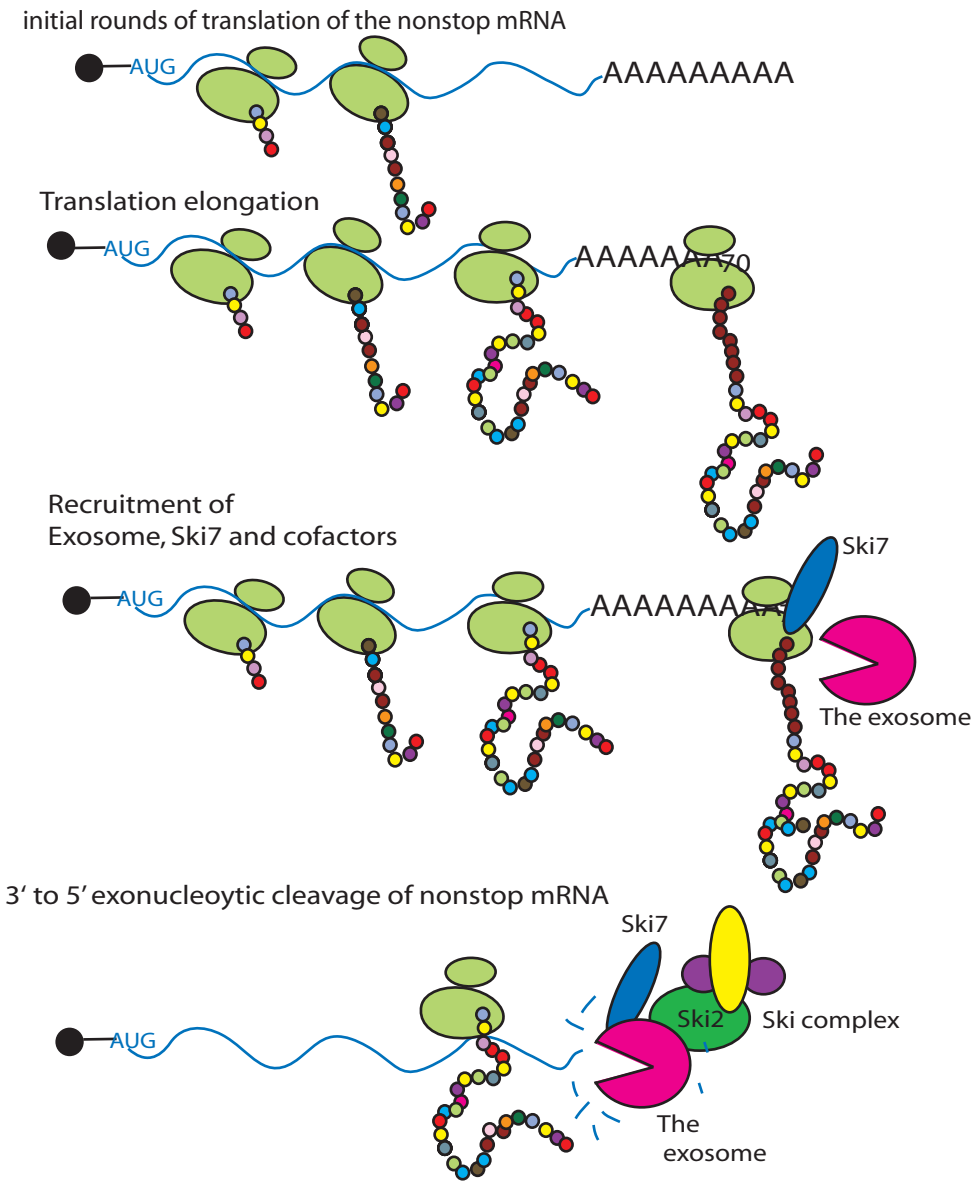

Figure 1.3: Nonstop mRNA decay.
Transcripts lacking termination codons are targeted for nonstop mRNA decay. Unlike normal mRNA decay it does not require deadenylation of the mRNA. The current model involves the ribosome translating to the end of the the poly(A) tail. The exosome, Ski7, and the Ski complex identify the stalled ribosome and promote the 3' to 5' degradation of the transcript.

Csl4, Rrp4, and Rrp40, the cap subunits of the exosome, associate with the RNA entry side of the PH ring (Figure 1.1). The cap proteins of the exosome are putative RNA binding proteins and have homology to the S1/KH family of proteins. Experimental evidence indicates that individually these RNA binding proteins have low intrinsic affinity for RNA (Oddone *et al.*, 2007, Luz *et al.*, 2007). However, combining two of the cap subunits increases their RNA affinity, presumably through cooperative binding (Oddone et al., 2007). It is still unclear whether the cap subunits of the exosome or the PH-ring proteins contribute to RNA binding and substrate specificity of the exosome. Recent experimental evidence suggests that the catalytically inactive core has a role in modulating the nuclease activities of the exosome (Wasmuth & Lima, 2012).

Rrp44 interacts with the opposite side of the PH ring when compared to the cap subunits of the exosome. Rrp44, related to the RNase II family of enzymes, is the only catalytically active protein of the core exosome complex, and it provides the exosome with its exonuclease activity (Dziembowski *et al.*, 2007). Interestingly, Rrp44 also contains an active endonuclease domain (Lebreton *et al.*, 2008, Schaeffer *et al.*, 2009, Schneider *et al.*, 2009). The exonuclease domain, which is at the C-terminus of the protein, faces the putative RNA exit site from the channel of the PH Ring (Figure 1.1). The endonuclease faces away from the channel and the exo-domain, and it is exposed to solvent (Bonneau., 2009). Either the endo- or the exonuclease domains are needed for viability, indicating that they may act redundantly or synergistically (Lebreton et al., 2008, Schaeffer et al., 2009, Schneider et al., 2009)

Cofactors of the exosome

Exosome substrates vary in length, shape, and sequence, therefore, it is unlikely that the exosome identifies these substrates through *cis*-acting elements. It has long been hypothesized that additional proteins that interact with the RNAs determine whether a transcript becomes an exosome target (Chlebowski *et al.*, 2010). The exosome associates with a variety of nuclear and cytoplasmic proteins that promote different exosome activities. *In vitro*, some cofactors enhance the catalytic activities of the exosome core. Below, I describe a few that are pertinent to this dissertation.

Rrp6 is an exoribonuclease that associates with the nuclear exosome

RRP6 (**r**ibosomal **R**NA **p**rocessing) was initially identified as a suppressor of the polyadenylation defect in the *pap1-1* mutation which disrupts the canonical poly(A) polymerase (Briggs et al., 1998). Rrp6 has homology to RNase D in *Escherichia coli,* and is restricted to the nucleus of yeast (Allmang et al., 1999b). Unlike the rest of the exosome core, an *rrp6*Δ is viable, and Rrp6 works synergistically with the exosome in the processing and degradation of substrates in the nucleus (Briggs et al., 1998).

Rrp6 processing and degradation functions

Like the core exosome, Rrp6 has specific processing and degradation functions. An *rrp6*Δ mutant causes accumulation of the rRNA precursor 5.8S+30, which is initially trimmed by the core exosome (Briggs et al., 1998). Further, Rrp6,

together with the exosome, cleaves extended polyadenylated snoRNA precursors (Allmang et al., 1999a, van Hoof et al., 2000b). Independently of the core exosome, Rrp6 removes the last three nucleotides of certain mature snoRNAs (Allmang et al., 1999a, van Hoof et al., 2000b). Additionally, Rrp6 plays a role in degradation of the CUTs and SUTs (Wyers et al., 2005, Xu et al., 2009).

As part of its surveillance role, Rrp6 targets polyadenylated forms of nuclear RNAs (Burkard & Butler, 2000). It is unclear whether these transcripts tagged through polyadenylation correspond to precursors or aberrant forms of the RNA, nevertheless these RNA species accumulate in an *rrp6*Δ strain. In the case of the polyadenylated RNAs, both the exosome and Rrp6 require a second complex of proteins called the TRAMP (**Tr**f4/5, **A**ir1/2, Mtr4 **P**olyadenylation) complex (The TRAMP complex will be discussed in detail in page 15). Rrp6 also plays a role, yet-to-be characterized, in the transition between pre-mRNA processing and commitment to nuclear export (Libri *et al.*, 2002, Rougemaille *et al.*, 2007, Saguez *et al.*, 2008).

Rrp6 has several overlapping functions with the exosome, however specific RNA maturation reactions, such as 5.8S+30 and snoRNA processing, can only be performed by Rrp6 (Kiss & Andrulis, 2010, Gudipati *et al.*, 2012). Mutations that disrupt the physical interaction between Rrp6 and the core exosome do not affect the processing functions of Rrp6 indicating that Rrp6 may perform these independently of the core exosome (Callahan & Butler, 2008).

Mtr4, the nuclear cofactor of the exosome, is essential for life

MTR4, named after **m**RNA **tr**ansport-defective mutant **4**, was initially isolated as a temperature sensitive mutant strain that accumulated poly(A)+ mRNAs in the nucleus (Kadowaki *et al.*, 1994). The mutation *mtr4-1* caused accumulation of discrete foci of poly(A)+ mRNAs in the nucleus of yeast cells. Additionally, when shifted to the non-permissive temperature the mutant *mtr4-1* strain showed modest rRNA defects compared to other *mtr_* mutants isolated (Kadowaki et al., 1994). The rRNA processing intermediates that accumulated in the *mtr4* mutant resembled those of exosome mutants making Mtr4, with its putative helicase activity, an attractive candidate in promoting nuclear exosome function. When the reconstituted exosome complex was incubated with Mtr4 (and other TRAMP subunits), the exosome's catalytic activity was enhanced *in vitro* (LaCava et al., 2005).

Mtr4 is a 120kDa protein and is part of the DExH/D group of helicases (Liang *et al.*, 1996). Mtr4 exhibits triphosphatase activity, which drives the helicase capabilities of Mtr4 (Bernstein *et al.*, 2008). Mtr4 is able to binds single stranded RNA, and unwind duplex RNA in the presence of ATP (Bernstein et al., 2008). The catalytic activities of Mtr4 (helicase and ATPase) are required for cell viability (Bernstein *et al.*, 2006).

Mtr4 localizes to the nucleus where it interacts with the core of the exosome and Rrp6. Mtr4 can promote the processing functions of the exosome. Specifically, Mtr4 is required for the exosome-mediated processing of 5.8S rRNA, snRNA, and snoRNAs (de la Cruz *et al.*, 1998a, Allmang et al., 1999a, van Hoof et al., 2000b).

Further, Mtr4 plays a role in the degradation functions of the exosome, however in this case it assembles into a complex of proteins called the TRAMP complex.

The TRAMP complex

In addition to Mtr4, TRAMP is composed of one noncanonical poly(A) polymerase, either Trf4 or Trf5, and one RNA binding protein, either Air1 or Air2. TRAMP is required for the surveillance functions of the nuclear exosome, namely the targeting and degradation of aberrant forms of tRNAs, mRNAs, snoRNAs, snRNAs, and rRNAs (Rougemaille et al., 2007, Grzechnik & Kufel, 2008, LaCava et al., 2005, San Paolo *et al.*, 2009). Additionally, TRAMP promotes the decay of noncoding uncharacterized RNAs, such as CUTs and SUTs (Wyers et al., 2005, Xu et al., 2009).

Trf4 and Trf5 are composed of a conserved catalytic domain, a central domain, and uncharacterized N- and C-terminal regions. The catalytic domain is similar to other Pol β family of polymerases, and the central region contains a short nucleotide recognition motif (Martin & Keller, 2007). There are no obvious RNA Recognition Motifs (RRM), therefore the RNA binding capabilities may be provided by the Air1/2 TRAMP subunit.

Trf4 and Trf5 (DNA **T**opoisomerase **r**elated **f**uction) add poly(A) tails to exosome substrates, these tails are shorter than the poly(A) tails added to normal mRNAs by Pap1 (Edmonds, 2002, Minvielle-Sebastia & Keller, 1999). Both Mtr4 and the Air proteins are required for proper polyadenylation activity of Trf4 or Trf5. Specifically, at least one of the Air proteins must be present in order for polyadenylation of an RNA substrate to occur (Wyers et al., 2005, LaCava et al.,

2005, Vanacova *et al.*, 2005). Further, Mtr4 is crucial in controlling the polymerase activity of Trf4 by establishing the length of the poly(A) tail added to the substrate (Jia *et al.*, 2011).

In addition to promoting TRAMP function, Trf4 and Trf5 have also been implicated in regulating expression of specific DNA substrates, among these are rDNAs, Ty retrotransposons, and introns (San Paolo et al., 2009, Houseley *et al.*, 2007). Trf4 and Trf5, independent of the other TRAMP subunits, also are implicated in gene silencing, chromatid condensation, and telomeric length (Buhler *et al.*, 2007, San Paolo et al., 2009, Wang *et al.*, 2000). Interestingly, microarray analyses show that Trf4 and Trf5 targets rarely overlap, indicating that there are specific substrates for each of these polymerases (San Paolo et al., 2009).

Air1 and Air2 (**A**rginine methyltransferase-**i**nteracting **R**ING) are zinc-knuckle proteins with five zinc-knuckle (ZnK) motifs. Air2 binds RNA through its second, third, and fourth ZnK (Holub *et al.*, 2012), and it is predicted that Air1 uses the same regions for binding RNA. Znk4 and Znk5 are required for binding to Trf4/5 and disruptions in these regions of Air1/2 block polyadenylation of the substrate by Trf4/5 *in vivo* (Fasken *et al.*, 2011, Holub et al., 2012).

The crystal structure of Trf4-Air2 core was recently solved. The structure contains the catalytic and central domains of Trf4 and the majority of the Znk4 and Znk5 of Air2 (Hamill *et al.*, 2010). Like the *in vivo* results, the structure showed that Znk4 and Znk5 of Air2 interact with the central domain of Trf4 *in vitro.* The N- and C-terminal region of Trf4/5 have yet to be characterized.

It is still unclear what the essential function(s) of the TRAMP complex is. At the present time, it is known that Mtr4 is essential, *trf4Δ/trf5Δ* strains are inviable and *air1Δ/air2Δ* mutants are very slow growing (LaCava et al., 2005, Inoue *et al.*, 2000, San Paolo et al., 2009). Interestingly, a catalytically inactive Trf4 fully complements the *trf4Δ/trf5Δ* strains, indicating that the essential function of Trf4 and Trf5 may not require its polymerase activity and TRAMP assembly may not be essential for life (Wyers et al., 2005).

Rrp6 and the TRAMP Complex

The first clue regarding the functional link between Rrp6 and the TRAMP complex is the accumulation of polyadenylated snRNAs, snoRNAs, and pre-rRNAs in *rrp6*Δ cells (Allmang et al., 1999a, van Hoof et al., 2000b, Kuai *et al.*, 2004). Additionally, aberrant tRNAs are polyadenylated in exosome mutants (Kadaba et al., 2004). Specifically, accumulation of hypomodified tRNAs was suppressed by a mutation in the *TRF4* gene. Once the TRAMP complex was characterized, it became evident that the non-canonical poly(A) polymerases were contributing to the polyadenylation of aberrant substrates (Kadaba et al., 2004, LaCava et al., 2005, Vanacova et al., 2005, Wyers et al., 2005). Furthermore, Rrp6 plays a major role in modulating the poly(A) tail length of aberrant/precursor RNAs in the nucleus in a Trf4-dependent manner (Schmid *et al.*, 2012). Additional RNA substrates that are targeted by TRAMP and cleaved by Rrp6 include the RNA processing intermediates and cryptic transcripts cause by bi-directional transcription (Xu et al., 2009, Neil et al., 2009). Biochemical evidence indicates that the enhanced catalytic activity of the

exosome observed *in vitro* when incubated with the TRAMP complex is mainly attributable to Rrp6 (Callahan & Butler, 2009). However, it is still unclear whether TRAMP-tagged substrates are primarily cleaved by Rrp6, the exosome, or both equally.

Ski2, the cytoplasmic cofactor of the exosome, is involved in normal and aberrant mRNA decay

Ski2 (**S**uper **ki**ller) was first identified as a mutant that allows the overexpression of the yeast killer virus (L-A). *Ski*-mutant strains cause a super-killer phenotype against uninfected cells (Ridley *et al.*, 1984). Further, Ski2 is also implicated in degradation of normal and aberrant mRNAs (Jacobs Anderson & Parker, 1998, van Hoof et al., 2002). All of these RNA degradation functions were also found to require the cytoplasmic form of the exosome (Jacobs Anderson & Parker, 1998).

Mtr4, the helicase cofactor of the nuclear exosome, and Ski2 are paralogs. It is predicted that Ski2, similarly to Mtr4, has helicase activity. This catalytic activity has not been confirmed. However, mutations that disrupt the DEVH (helicase) motif also abolish normal mRNA decay (Brown *et al.*, 2000). Mutants and deletions of *SKI2* are viable and have no obvious phenotype, other than increased killing capabilities when infected with the L-A killer virus (Martegani *et al.*, 1997).

Ski2 is a 146 kDa protein that localizes to the cytoplasm and forms a complex with Ski3 and Ski8 (Brown *et al.*, 2000). Specifically, Ski3 forms the scaffold by which Ski2 and a dimer of Ski8 physically interact (Wang *et al.*, 2005, Synowsky &

Heck, 2008). The N-terminus of Ski2 mediates binding to Ski3. A fourth protein required for the cytoplasmic function of the exosome is the putative GTPase Ski7 (Araki *et al.*, 2001). Ski7 and Ski2 interaction is only possible when the entire Ski complex is assembled (Wang et al., 2005). Unlike its nuclear counterpart Mtr4, Ski2 does not seem to function outside of the Ski complex. The Ski complex is required for all known functions of the cytoplasmic exosome, which include normal, nonstop, nonsense, and no-go mRNA decay (Schaeffer, 2010).

Mtr4 and Ski2 are DExD/H-box helicases that belong to the Ski2-like category of RNA helicases

DNA and RNA helicases are classified into different families depending on the sequence of the specific helicase motifs. Ski2 and Mtr4 are DExD/H-Box helicases, which belong to the largest superfamily of helicases, superfamily II (SF II). Other SF II helicases include the *E. coli'* s DNA helicase RecQ, *Sacharomyces cerevisiae's* eIF4A, and the replication protein NS3 of the Hepatitis C virus (Dumont *et al.*, 2006, Wu, 2012, Andreou & Klostermeier, 2012). DExD/H-box helicases are able to dissociate RNA structures and RNA-binding proteins by rearranging intra- and intermolecular interactions. This remodeling of the RNA molecules is driven by the energy obtained from the DExD/H-box protein-mediated ATP hydrolysis (Bernstein et al., 2006). RNA rearrangements are necessary because they allow the molecule to interact transiently with important proteins and nucleic acids during the RNA maturation process. DExD/H helicases contain eight sequence motifs required for

ATP-binding, hydrolysis, and possibly for interactions with the RNA (see figure 1.4a for more details) (Bernstein et al., 2006, Rocak & Linder, 2004).

Within the DExD/H helicase family, these exosome cofactors are further classified into the Ski2-like (DEVH) helicases, and they all contain the same DEVH sequence in their ATP hydrolysis motif II (see motif depiction in figure 1.4a) Currently, there are four members of the Ski2-like RNA helicase group: Mtr4, Ski2, splicing factor Brr2, and Slh1(Johnson & Jackson, 2012). Additionally, the archaeal DNA helicase Hel308 is also categorized as a Ski2-like helicase (Buttner *et al.*, 2007).

Structures of Mtr4 and Ski2

Hel308, an archaeal DNA helicase, was the first Ski2-like helicase crystal structure to be published. The crystal structure of Hel308 revealed a five-domain organization (Richards *et al.*, 2008). Two RecA (motor domains), domain 3 (winged helix) and 4 (ratchet helix/C-terminal domain) form a ring structure creating a channel where the DNA enters (Richards et al., 2008). Hel308 also contains a fifth accessory domain, which associates with the central channel of the core (Figure 1.4b).

The crystal structures of Mtr4 and Ski2 were solved, and similarly to Hel308, they each formed a ring with four of their domains. The ring structure is termed the 'helicase core' (figure 1.4b and figure 3.1 for more detail) (Halbach *et al.*, 2012, Jackson *et al.*, 2010, Weir *et al.*, 2010). Unlike Hel308, the accessory domain that blocked the exit side of the channel was absent in the Mtr4 and Ski2 structures.

Figure 1.4

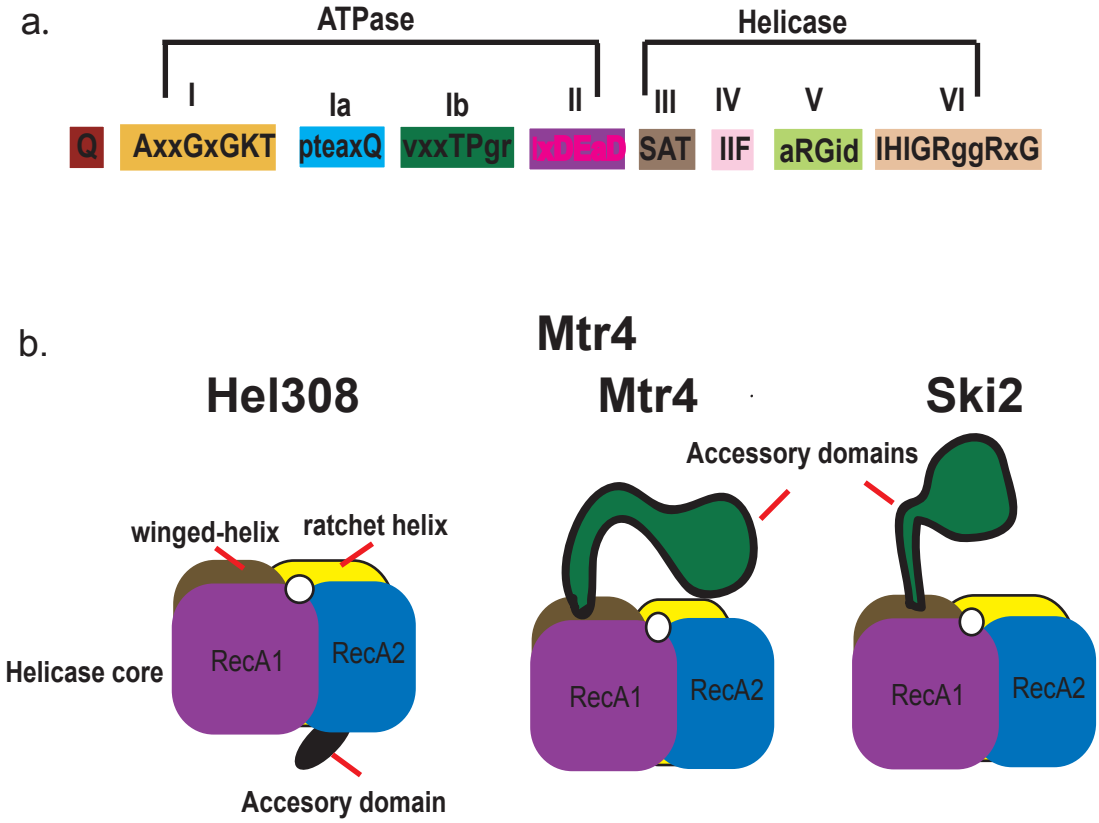

Figure 1.4 and 1.5: Schematic of the conserved motifs and structural features in DExD/H helicases.
a. Important and highly conserved motifs found in RecA1 and RecA2 domains of DExD/H helicases. Capital letters indicate highly conserved residues, while lower case letter indicate lesser conserved residues. Motif I (Walker A motif) is essential for hydrolyzing ATP. Motif II (Walker B),which is used to classify these helicases, is crucial for ATP binding. Motif III plays an important role in bridging the ATPase and RNA binding regions of RNA helicases, however it is not essential for helicase activity. Motif VI is required for RNA unwinding and remodeling functions. **b.** Structural comparison between three members of the Ski2-like family of helicases. All 3 helicases contain the 'helicase core' domains, which assemble into a ring structure. Additional accesory domains provide functional specificity.

Instead, an additional accessory domain of unknown function is present, the arch domain. The arch domain splits the winged helix domain in half and rises above the helicase core of each of these proteins.

Differences between the structure of the arch domain of Mtr4 and the arch domain of Ski2

In general, the arch domain is a conserved feature for both Mtr4 and Ski2, however, there is low identity in the sequence of the arch domain between the two exosome cofactors. Nevertheless, certain structural features are maintained between the two helicases. Some of the specific structural similarities are the size and position of the arch. The arch domain of Mtr4 and Ski2 is 256-260 amino acids in length, and it is inserted in the middle of the winged helix domain (Johnson & Jackson, 2012). Further, both arch domains ascend above the helicase core as two anti-parallel coils leading to a globular fold, termed the fist. Additionally, both domains contain important proline residues that cause the 90-120° bend, and creates the arch fold (Jackson et al., 2010, Johnson & Jackson, 2012).

In addition to the low sequence conservation, there are noteworthy differences between the arch domain of Mtr4 and Ski2. First, the globular domain of Mtr4, termed the 'fist', contains a specific β-barrel fold with a KOW motif. This KOW motif is present in proteins that bind ribosomal RNAs (Kyrpides *et al.*, 1996). Although the arch domain of Ski2 forms the β-barrel fold, there is no KOW motif (Halbach et al., 2012). The lack of a KOW motif in Ski2 is not surprising because the cytoplasmic exosome does not degrade mature and ribosome-associated rRNAs.

Second, both arch domains have been shown to bind RNA, however the level of substrate specificity differs between Mtr4 and Ski2. Namely, the arch domain of Mtr4 preferentially binds aberrant forms of tRNA, while the arch domain of Ski2 promiscuously binds both single and double stranded (structured) RNA (Weir et al., 2010, Halbach et al., 2012). Third, the overall charge of the globular/fist domain of Mtr4 and Ski2 is significantly different. The fist domain of Ski2 is more positively charged. The difference in these important residues may contribute to Ski2's increased RNA binding capabilities.

rRNA biogenesis

Ribosomal RNAs are transcribed as one single polycistronic transcript. The 35S transcript undergoes endonucleolytic and exonucleolytic cleavages to create the mature 5.8S, 18S, and 25S rRNAs. The schematic on Figure 1.5 shows a series endonucleolytic cleavage that separate main sections of the transcript, followed by exonucleolytic cleavage that trims the 5' or 3' end of pre-rRNAs. As described previously, the exosome is primarily involved in degrading the 5'ETS, a processing by-product. Additionally, the exosome cleaves the 3' end of 5.8S pre-rRNA (Figure 1.5). Further, the exosome targets the pre-rRNA for degradation to prevent the assembly of precursor RNAs into the ribosome.

snoRNA biogenesis

Small nucleolar RNAs (snoRNAs) can be divided into two major groups, Box C/D snoRNAs and H/ACA snoRNAs. Box C/D and H/ACA snoRNAs are named for

their two short sequence elements, which are required for their synthesis and stability. Most Box C/D snoRNAs are involved in the backbone modification of rRNAs, namely methylation. Conversely, H/ACA snoRNAs are involved in the pseudouridylation of rRNAs. However, some Box C/D and H/ACA snoRNAs are also involved in pre-rRNA cleavage and assembly without the use of base modification.

Specific snoRNAs are transcribed from either a monocistronic or polycistronic transcript. Further, some snoRNAs are synthesized from the intron lariat released during mRNA splicing. Figure 1.6 depicts the model for the synthesis of each type of snoRNAs. Specifically, the exosome is involved in the trimming of the 3' end of the snoRNAs. Like with the rRNAs, the exosome identifies pre-snoRNAs and targets them for degradation.

Figure 1.5

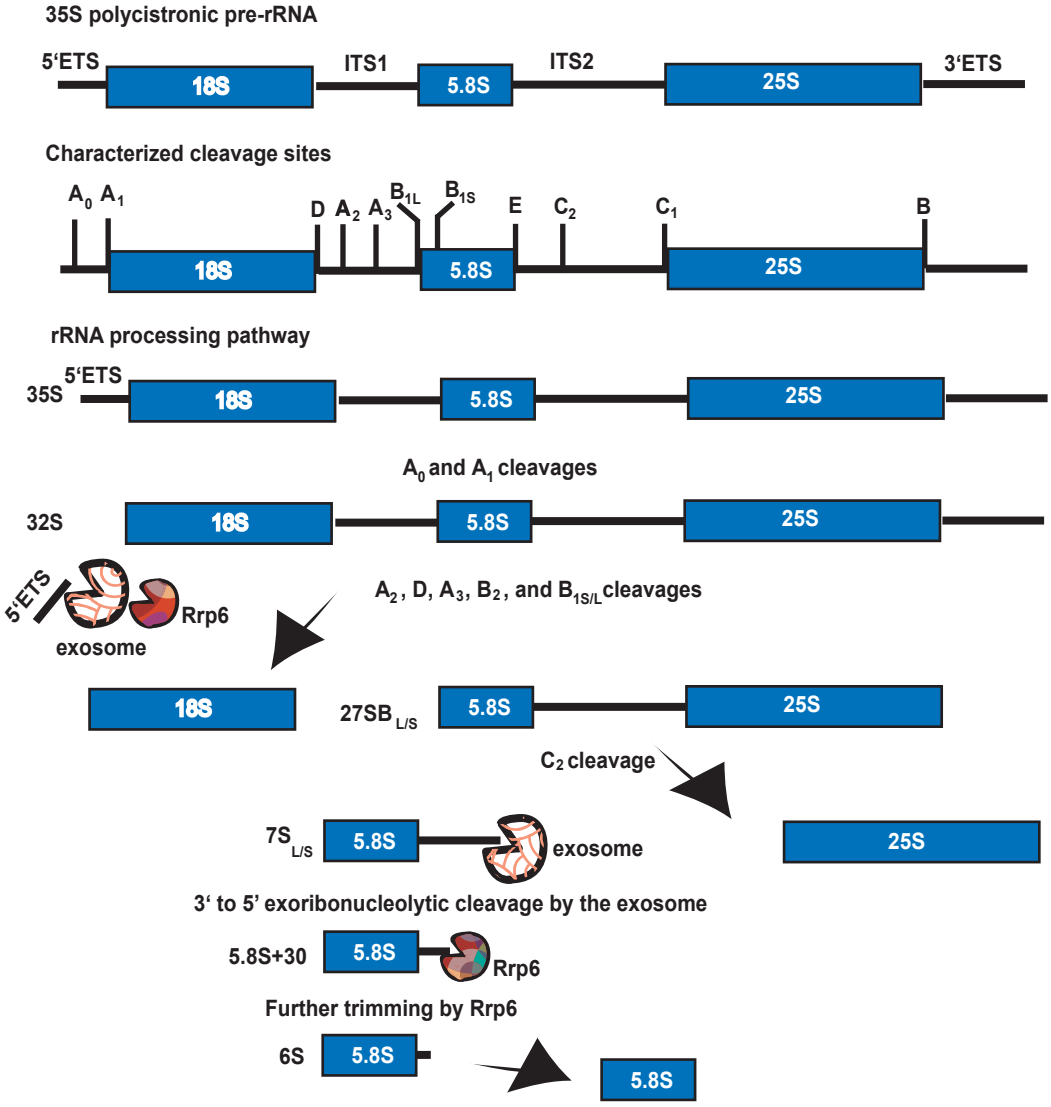

Figure 1.5: Functions of the exosome in the rRNA processing pathway.
rRNA is transcribed as a polycistronic transcript that contains the sequence of the mature 25S, 18S, and 5.8S rRNA. Through a series of endo- and exonulceolytic cleavages, extended forms of the mature rRNA are released and further trimmed into a form that can be assembled into the ribosome. Specifically, the exosome is involved in the degradation of the 5' ETS and the trimming of the 7S and 5.8+30 pre-rRNAs. 5'ETS-5' External Transcribed Spacer, 3'ETS-3' External Transcribed Spacer, ITS1/2-Internal Transcribed Spacer 1/2.

Figure 1.6

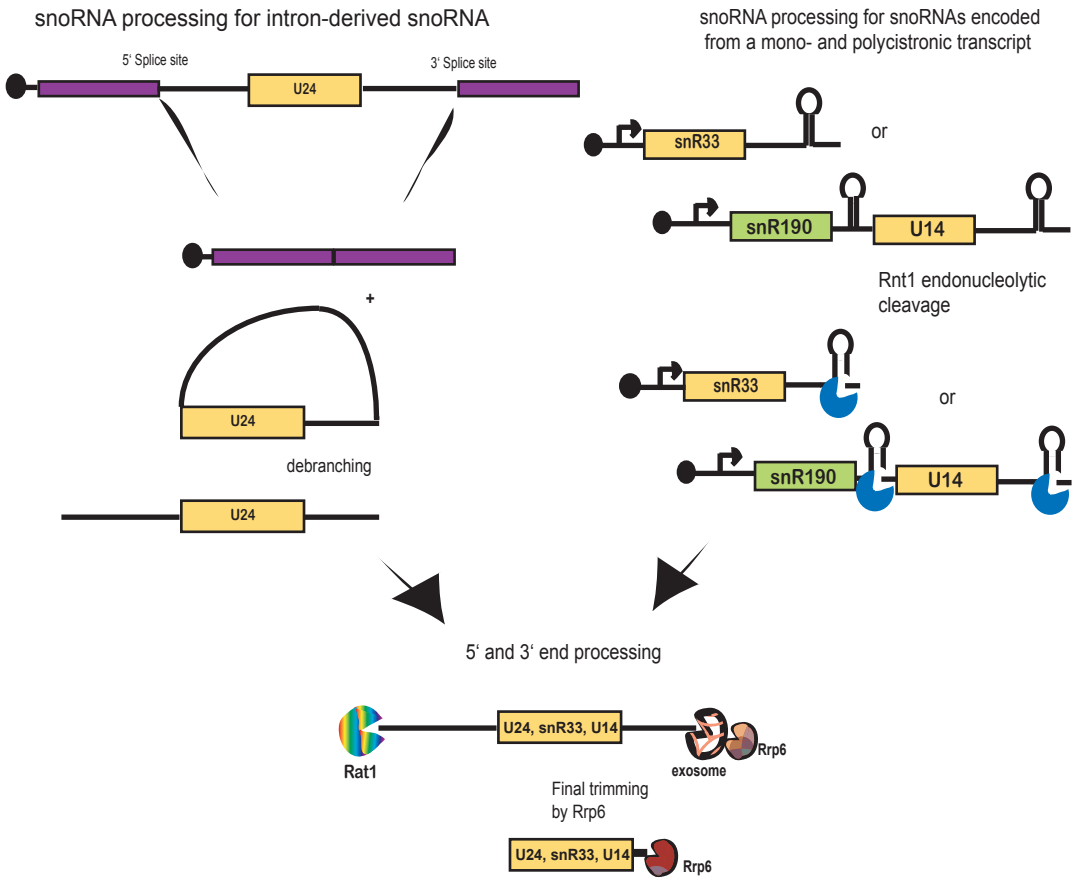

Figure 1.6 snoRNA processing of three different types of snoRNAs

snoRNAs can be expressed from their own promoter, from a polycistronic transcript, or derived from an intron lariat after mRNA splicing. Endonucleases and the debraching enzyme expose the pre-snoRNA at its 5' and 3' end. Exoribonucleases Rat1 and the exosome process the snoRNAs to their mature forms. Like in the 5.8S rRNA biogenesis pathway, Rrp6 trims the last few nucleotides of the mature snoRNA.

SIGNIFICANCE

RNA processing and degradation affect essential cellular functions

Understanding how the exosome targets its substrates will be very important to understand how the cell controls its gene expression. While some RNAs encode proteins, noncoding RNAs are involved in other essential cellular functions such as translation, splicing, base modifications and cleavage reactions. Therefore, any small disruptions in RNA metabolism can lead to a major defect in most, if not all, cellular functions.

RNA degradation and processing is a widely conserved function among all three kingdoms of life. Prokaryotic and archaeal organisms utilize their respective versions of exosome-like complexes (degradosome and archaeal exosome). Discovering the basic principles of how RNA substrates are identified and targeted by the exosome in *S. cerevisiae* will be very applicable to other organisms in the bacterial and archaeal kingdoms.

Modulating exosome functions as means of precisely controlling gene expression

If one can identify what protein features of Mtr4 or Ski2 promote the exosome-mediated cleavage of specific RNAs, then it will be possible to control the stability of specific RNAs and thereby a specific cellular function. RNA is a better candidate for gene control because it is a transient form of information and may be much easier to target than the complex and stable DNA. This control of the exosome's specific functions can be utilized as an additional genetic tool to knock-down certain cellular

functions by targeting a specific type of cellular RNA in organisms that lack or are unable to use knock-outs or RNAi pathways.

Medical Relevance

The eukaryotic exosome is a relevant target for cancer therapies

In addition to controlling gene expression for experimental purposes, the exosome could be used as a target for cancer therapy. For example, rapidly dividing cells such as cancer cells require large amounts of ribosomal RNA. Blocking the production of ribosomal RNAs will stop only the actively proliferating cells from dividing without affecting other types of post-mitotic cells, and consequently lowering the side effects in the rest of the body. Interestingly, Rrp6 has been found to be one of the many targets of the cancer drug 5-fluorouracil (5-FU), a pyrimidine analog used to shrink solid tumors (Hoskins & Scott Butler, 2007, Silverstein *et al.*). 5-FU was initially developed as a uracil analog which would incorporate itself into the DNA/RNA of actively diving cell and create cytotoxic effects that lead to cell death. A more detailed mechanism of how 5-FU affects the exosome has not been elucidated.

The exosome can also be down-regulated to treat genetic diseases

There are other important health implications in controlling RNA stability. Genetic diseases are characterized by mutations in the DNA, which are then passed on to the RNA molecule. Aberrant RNAs are so rapidly targeted for degradation by the exosome that little to no protein is produced. Diseases such as Duchenne's

muscular dystrophy, hemophilia and cystic fibrosis are caused by mutations that prevent the accumulation of important proteins and manifest with life-threatening symptoms (Ameri *et al.*, 2007, Cacciottolo *et al.*, 2011, Finkel, 2010). In this case, down-regulating the RNA degradation functions would promote the stability and translation of the mutated RNA into the proteins these cells are lacking, reducing or eliminating the life-threatening symptoms. Although the proteins encoded by these mutated mRNAs are considered aberrant, allowing them to accumulate during crucial developmental stages could prevent the patient's death.

Additionally, it has been shown that exosome mutations cause a variety of diseases. Recently, it was discovered that mutations in one of the exosome subunits, Rrp40, lead to pontocerebral hypoplasia type 1, a delay in the development of the brain (Wan *et al.*, 2012). Further, mutations in the catalytic subunit of the exosome have been identified in patients with Perlam syndrome, an overgrowth neonatal syndrome (Astuti *et al.*, 2012). Moreover, mutations in the cytoplasmic cofactor, Ski2, cause Syndromic diarrhea, a rare hereditary bowel disorder (Fabre A, 2012). As more diseases are identified, it will become increasingly important to identify the mechanism of function for the exosome in order to treat, diagnose, and prevent the onset of symptoms in these patients.

Chapter 2: Materials and Methods

MATERIALS AND METHODS

Strains, oligonucleotides, and plasmids

Strains, oligonucleotides and plasmids used are described in tables 2.1, 2.2, and 2.3. Unless otherwise specified, all of the experiments were performed in the BY4741 yeast strain background. For the experiments where *MTR4* was overexpressed in the *rrp6Δ* strain, the W303 was utilized. W303-derived *rrp6*Δ strain has a temperature sensitive phenotype at 37°, whereas the BY-derived *rrp6*Δ grows normally at all temperatures. The difference in the phenotypes is not due to lower Rrp6 or Mtr4 protein levels in the W303 parental strain.

Yeast complementation assay

MTR4 and *mtr4-archless* plasmids were introduced into the *mtr4Δ* strain and the *rrp6Δ/mtr4Δ strain* through standard plasmid shuffle technique as described in Jackson et al. 2010. Briefly, isogenic *mtr4Δ* and *mtr4Δ*/*rrp6Δ* strains that contained an *MTR4*, *URA3* plasmid were transformed with expression vectors that encoded either *MTR4* or *mtr4-archless* with a *LEU2* selectable marker. These double transformants were then serially diluted and spotted onto 5-fluoroorotic acid (5-FOA)-containing media to select for cells that had lost the *MTR4*, *URA3* plasmid.

Growth suppression assay

W303 and the isogenic *rrp6Δ* strain Y765 were obtained from Michael Rosbash and transformed with 2μ plasmids encoding Mtr4 and Mtr4-archless

(pAV716 and pAV717 respectively) with the *URA3* selectable marker. Transformants were selected on plates lacking uracil (SC-URA). Single colonies were picked and grown overnight at 30°C. The liquid cultures were then serially diluted 1:5 and spotted onto SC-URA plates. Plates were then placed at 22°C, 30°C, or 37°C.

RNA isolation

Single clones of each of the strains transformed with the *MTR4* and *mtr4-archless* plasmid were streaked onto 5-FOA plates. A patch of cells was grown overnight at 30°C in yeast peptone and dextrose liquid media (YPD). For the overexpression experiment, cells were inoculated and grown in SC-URA plates and liquid media. Cultures were diluted to an A_{600} of 0.2 in 40 mLs of media and allowed to double twice. Cells were obtained through centrifugation and frozen when they reached an A_{600} of 0.8. Total RNA was isolated by standard phenol-chloroform precipitation (Caponigro *et al.*, 1993). Briefly, 150 µL of LET (25 mM Tris pH 8.0, 100 mM LiCl, 20 mM EDTA) was added to the frozen cell pellets. Phenol and phenol-chloroform solutions were equilibrated with LET prior to the RNA isolation experiment. 150 µL of phenol and 250 µL of acid washed beads were added to the solution containing the LET resuspended cells and mixed by vortexing for five minutes. 250 µL of pure chloroform and 250 µL of DEPC-treated water were added to the fragmented cell suspension. To separate the different cell components, the samples were centrifuged at maximum speed for two minutes. The clear aqueous phase was removed and added to a new eppendorf tube containing 400 µL of phenol-chloroform. Next, the samples were centrifuged for two minutes and the

aqueous phase was removed and added to a third tube containing 1 mL of 95% ethanol and 40 µL of 3M NaAc. The RNA was precipitated at -80C° for 30 minutes and centrifuged at maximum speed for 30 minutes. The aqueous layer was removed and the RNA pellet was air-dried, resuspended in 100 µL of DEPC-treated water and quantified using the GE NanoVue Spectrophotometer.

Northern blot analysis

Samples containing 10 µg of RNA were loaded onto a denaturing urea-polyacrylamide gel and subjected to eletrophoresis for 4 hours. RNA-containing polyacrylamide gels were transferred to a nylon membrane using 0.5X TBE at 4°C. 5' hydroxyl nucleotides were labeled with gamma-^{32}P ATP using T4 polynucleotide kinase (PNK). The RNA-containing membrane was hybridized with 5' labeled nucleotides for specific RNA defects (oligonucleotide table 2.3). As a loading control, the RNA subunit of the signal recognition particle (oAV224) was used.

Western blot analysis

Cells were obtained as explained in Northern blot analysis, except only 20mL of liquid media was used to grow the cells. 5-FOA cured strains were grown in YPD and strains overexpressing Mtr4/Mtr4-archless were grown in SC-URA liquid media. Protein was obtained using the glass bead method (Jazwinski, 1990). Briefly, frozen pellets were washed once with 500µL of IP50 (50mM Tris HCl pH 7.5, 50mM KCl, 2mM $MgCl_2$, 0.1% Triton X with an EDTA-free protease inhibitor tablet (Roche), PMSF, and 0.7µL of BME), and supernatant was removed. Further, 300µL of IP50

and 300μL of glass beads were added to the washed pellet and vortexed at 4°C for 2 minutes, incubated on ice for 1 minute (repeated 5 times). Cell suspensions were centrifuged at 7,000 revolutions per minute for 7 minutes at 4°C, supernatant was saved and 6X protein loading buffer was added. Proteins were resolved using 12% SDS PAGE and transferred to a nitrocellulose membrane. The blot was analyzed using antibodies against Mtr4 (de la Cruz *et al.*, 1998b) at a 1:5000 dilution. Antibodies against Pgk1 were used as a protein loading control (Molecular Probes). Anti-myc antibody was used at 1:1000 dilution for the Ski2 western blot analysis (gift from Eric J. Wagner's lab UTHealth).

Yeast two-hybrid assay

Yeast strain PJ69-4a was transformed with the first binding partner (bait), for example pAV744 (Trf5) or pAV705 (Empty vector). Transformants were selected on plates lacking leucine (SC-LEU). Single clones were streaked onto fresh SC-LEU plates. Likewise, PJ69-4α was transformed with the second binding partner (prey), for example pAV745 (Mtr4), pAV746 (Mtr4-archless), or pAV704 (empty vector) and streaked onto SC-TRP plates. Single clones of transformants were mated with each respective tester strain. Briefly, PJ69-4a [pAV744] was crossed to PJ69-4α [pAV746] to determined whether Trf5 interacts with archless Mtr4. After mating, diploids were selected for in plates lacking both leucine and tryptophan (SC-LEU-TRP). Diploids were grown overnight at 30°C in liquid SC-LEU-TRP media. Liquid cultures were then serially diluted and spotted onto media lacking adenine and media lacking histidine to screen for interaction as described in (Uetz *et al.*, 2000),

and control media (SC-LEU-TRP). Growth was assessed at 4 and 8 days. SC-HIS plates containing 10 mM 3-aminotriazole (3AT) were used. A positive control for the Y2H method was assessed from the interaction between Mec3 and Rad17, DNA damage checkpoint proteins that form a complex. Negative controls involved Y2H analysis with tester proteins against empty vector.

To test for β-galactosidase activity, the diploid Y2H strains were grown overnight at 30°C, diluted to A_{600} of 0.15 the next day and allowed to double twice. β-galactosidase activity was quantitated using the Beta-Glo reagent (Promega) per manufacturer's instructions on a Synergy MX automated microplate reader (provided by Kevin Morano's lab, UTHealth)

His3-nonstop growth assay

The His3-nonstop growth assay was performed as previously described (Schaeffer *et al.*, 2008). Briefly, a *ski2Δ* (yAV517) strain was transformed with the His3-nonstop reporter (pAV188). His3-ns reporter lacks in-frame stop codons and encodes the His3 protein (Schaeffer et al., 2008). The *ski2Δ* [pAV188] cells were then transformed with pAV878 (*SKI2*), pAV879 (*ski2-archless*), or pRS415 (Empty vector). The *ski2Δ* [pAV188] [pAV878/pAV879/pRS415] cells were serially diluted and replica plated onto plates with media lacking histidine, and control plates to select for double transformants (*i.e.* SC-LEU-URA).

Synthetic Lethality Growth Assay

yAV225 (*dcp1-2*/*ski2Δ*) was transformed with pAV876 (*SKI2*), pAV877 (*ski2-archless*), and pRS414 (empty vector with *TRP* marker). Transformations were plated at room temperature (20°). Single colonies of the transformants were selected, serially diluted and pronged onto SC-TRP plates. Replica plates were incubated at 20°C, 30°C, and 37°C.

Tandem Affinity Purification

Lysates were obtained as explained in the Western blot section. 300-500 µL of total cell lysates were incubated with 30µL of washed IgG sepharose beads for 2 hours in constant rotation at 4°C. Samples were centrifuged for 1 minute in a tabletop microfuge at 4°C, and the supernatant was moved to a new tube (flow-through). The beads were washed with 1mL of IP50, transferred to a clean tube, and incubated by rotation for 1 minute. Wash was centrifuged for 1 minute, supernatant saved in another tube, and washed again with 1mL of IP50. Washes and centrifugation steps were repeated twice with IP150 (same as IP50 except 150 mM KCl instead). Lastly, beads were resuspended in IP50 and 6X protein loading buffer. Beads were boiled and centrifuged prior to loading onto the gel.

Table 2.1

Name	Description	Marker	Insert	Reference
pAV125	*SKI2*-3X myc internally labeled, 2μ vector	LEU2	See ref.	(Brown et al., 2000)
pAV188	HIS3ns reporter	URA3	See ref.	(van Hoof et al. 2002)
pAV673	*MTR4* promoter, residues 1-1073, 3' UTR	*URA3*	See ref.	(Jackson et al. 2010)
pAV674	*Archless mtr4,* promoter, residues 1-614, linker region, 879-1073, 3'UTR	*LEU2*	See ref.	(Jackson et al. 2010)
pAV675	*MTR4* promoter, residues 1-1073, 3' UTR	*LEU2*	See ref.	(Jackson el al. 2010)
pAV706	*MTR4* promoter, residues 13-614, linker region, 879-1073, 3' UTR	*URA3*	PCR amplified from pAV675 using oAV743 and oAV759, digested and cloned into pAV67 (SpeI and SacI)	This study
pAV707	*MTR4* promoter, residues 90-1073, 3' UTR	*LEU2*	PCR amplified from pAV675 using oAV744 and oAV745, digested and cloned into pAV674 (SpeI and XbaI)	This study
pAV708	*MTR4* promoter, residues 13-1073, 3' UTR	*URA3*	PCR amplified pAV675 using oAV743 and oAV759, digested and cloned into pAV673 (EcoRI and XbaI)	This study
pAV712	*MTR4* promoter, residues 13-1073, 3' UTR	*LEU2*	Digested pAV708 and cloned into pAV675 (SacI and SpeI)	This study
pAV716	*MTR4* promoter, residues 1-1073, 3'UTR in 2μ plasmid	*URA3*	Digested from pAV675 cloned into pRS426 (SacI and SpeI)	This study
pAV717	*Archless mtr4* promoter, residues 1-614, linker, 879-1073, 3'UTR in 2μ plasmid	URA3	Digested from pAV674 cloned into pRS426 (SacI and SpeI)	This study
pOBD2/ pAV704	Yeast Two hybrid-DNA binding domain vector	*TRP1*	See ref.	(Uetz et al., 2000)
pOAD/ pAV705	Yeast Two hybrid-DNA Activation domain vector	*LEU2*	See ref.	(Uetz et al., 2000)
pAV745	*MTR4*, residues 1-1073 in Y2H vector	*TRP1*	Digested from pAV673 and cloned into pOBD2 (SmaI ad EcoRI)	This study
pAV746	*Archless mtr4*, residues 1-614, linker region, 879-1073 in Y2H vector	*TRP1*	Digested from pAV674 and cloned into pOBD2 (SmaI ad EcoRI)	This study
pAV744	*TRF5,* residues 53-199 in Y2H vector	*LEU2*	PCR amplified yeast genomic DNA with oAV767 and oAV768, digested and cloned into pOAD (PvuII and PstI)	This study

pAV747	*MTR4,* residues 13-1073 in Y2H vector	*TRP1*	PCR amplified using oAV803 and oAV809, cloned into pOBD2 (EcoRI and PstI)	This study
pAV748	*MTR4,* residues 609-1073 in Y2H vector	*TRP1*	PCR amplified using oAV810 and oAV809, cloned into pOBD2 (EcoRI and PstI)	This study
pAV749	*MTR4,* residues 912-1073 in Y2H vector	*TRP1*	PCR amplified using oAV808 and oAV809, cloned into pOBD2 (EcoRI and PstI)	This study
pAV750	*MTR4,* residues 1-325 in Y2H vector	*TRP1*	PCR amplified using oAV815 and oAV816, cloned into pOBD2 (EcoRI and PstI)	This study
pAV751	*MTR4,* residues 1-584 in Y2H vector	*TRP1*	PCR amplified using oAV814 and oAV816, cloned into pOBD2 (EcoRI and PstI)	This study
pAV752	*MTR4,* residues 1-910 in Y2H vector	*TRP1*	PCR amplified using oAV814 and oAV816, cloned into pOBD2 (EcoRI and PstI)	This study
pAV753	*MTR4,* residues 586-1073 in Y2H vector	*TRP1*	PCR amplified using oAV807 and oAV809, cloned into pOBD2 (EcoRI and PstI)	This study
pAV754	*MTR4,* residues 152-1073 in Y2H vector	*TRP1*	PCR amplified using oAV805 and oAV809, cloned into pOBD2 (EcoRI and PstI)	This study
pAV755	*MTR4,* residues 1-873 in Y2H vector	*TRP1*	PCR amplified using oAV812 and oAV816, cloned into pOBD2 (EcoRI and PstI)	This study
pAV756	*MTR4,* residues 1-607 in Y2H vector	*TRP1*	PCR amplified using oAV813 and oAV816, cloned into pOBD2 (EcoRI and PstI)	This study
pAV757	*MTR4,* residues 90-1073 in Y2H vector	*TRP1*	PCR amplified using oAV804 and oAV809, cloned into pOBD2 (EcoRI and PstI)	This study
pAV758	*MTR4,* residues 327-1073 in Y2H vector	*TRP1*	PCR amplified using oAV806 and oAV809, cloned into pOBD2 (EcoRI and PstI)	This study
pAV759	*TRF5,* residues 69-198 in Y2H vector	*LEU2*	PCR amplified pAV744 with oAV827 and oAV768, cloned into pOAD (PvuII and PstI)	This study
pAV760	*TRF5,* residues 84-198 in Y2H vector	*LEU2*	PCR amplified pAV744 with oAV828 and oAV768, cloned into pOAD (PvuII and PstI)	This study
pAV761	*TRF5,* residues 99-198 in Y2H vector	*LEU2*	PCR amplified pAV744 with oAV829 and oAV768, cloned into pOAD (PvuII and PstI)	This study
pAV762	*TRF5,* residues 53-184 in Y2H vector	*LEU2*	PCR amplified pAV744 with oAV830 and oAV767, cloned into pOAD (PvuII and PstI)	This study
pAV763	*TRF5,* residues 53-184 in Y2H vector	*LEU2*	PCR amplified pAV744 with oAV831 and oAV767, cloned into pOAD (PvuII and PstI)	This study
pAV764	*TRF5,* residues 53-154 in Y2H vector	*LEU2*	PCR amplified pAV744 with oAV832 and oAV767, cloned into pOAD (PvuII and PstI)	This study

pAV765	*TRF5,* residues 68-184 in Y2H vector	*LEU2*	PCR amplified pAV744 with oAV830 and oAV827, cloned into pOAD (PvuII and PstI)	This study
pAV766	*TRF5,* residues 118-199 in Y2H vector	*LEU2*	PCR amplified pAV744 with oAV836 and oAV768, cloned into pOAD (PvuII and PstI)	This study
pAV767	*TRF5,* residues 53-139 in Y2H vector	*LEU2*	PCR amplified pAV744 with oAV837 and oAV767, cloned into pOAD (PvuII and PstI)	This study
pAV768	*TRF5,* residues 53-124 in Y2H vector	*LEU2*	PCR amplified pAV744 with oAV838 and oAV767, cloned into pOAD (PvuII and PstI)	This study
pAV771	*RRP4,* residues 1-359 in Y2H vector	*LEU2*	PCR amplified pAV452 (*RRP4* ORF) with oAV819 and oAV820 (EcoRI and Pst1)	This study
pAV772	*RRP40,* residues 1-240 in Y2H vector	*LEU2*	PCR amplified pAV461 (*RRP40* (EcoRI and Pst1)ORF) with oAV821 and oAV822	This study
pAV794	*CLS4,* residues 1-292 in Y2H vector	*LEU2*	PCR amplified genomic DNA with oAV850 and oAV851, homologous recombination with pAV705 (NcoI and PstI on vector)-plasmid rescue	This study
pAV808	Promoter *MTR4,* residues 1-614, linker region, 879-1073 + residues 831-1085 of SKI2, *MTR4* 3'UTR	*LEU2*	Digested *MTR4-SKI2* arch from TOPO vector from Jackson and Johnson (pAV807) with HindIII and AatII cloned into pAV675	This study
pAV810	GFP-N, empty vector plasmid	*URA3*	See ref.	(Phillips & Butler, 2003)
pAV811	GFP-*RRP6,* residues 1-733	*URA3*	See ref.	(Phillips & Butler, 2003)
pAV812	GFP-*RRP6,* residues 1-440 and 486-733	*URA3*	See ref.	(Phillips & Butler, 2003)
pAV813	GFP-*RRP6,* residues 1-733, mutation D238A	*URA3*	See ref.	(Phillips & Butler, 2003)
pAV814	GFP-*RRP6,* residues 1-733, mutation D296A	*URA3*	See ref.	(Phillips & Butler, 2003)
pAV815	GFP-*RRP6,* residues 1-733, mutation Y361F	*URA3*	See ref.	(Phillips & Butler, 2003)
pAV816	GFP-*RRP6,* residues 1-733, mutation D457A	*URA3*	See ref.	(Phillips & Butler, 2003)
pAV854	*trf5,* residues 1-97 and 118-642, TAP tag	*LEU2*	PCR amplified genomic DNA with oAV906 and oAV870. PCR amplified genomic DNA with oAV871 and oAV869. Digested piece 1 with NotI and SpeI, digested piece 2 with SpeI and PstI. Cloned both pieces into pAV476 (TAP plasmid) (NotI and PstI)	This study
pAV876	SKI2 promoter, residues 1-1287,	*TRP1*	Same as 878 but in a TRP vector	This study

	3'UTR in CEN vector		backbone	
pAV877	*ski2-archless*. Same as pAV878, but residues 831 to 1085 deleted.	*TRP1*	Same as 879 but in a TRP vector backbone	This study
pAV878	SKI2 promoter, residues 1-1287, 3'UTR in CEN vector	*LEU2*	SKI2 coding region is from an *E. coli* expression plasmid from Jackson and Johnson. Added SKI2 promoter and 3'UTR PCR amplified with oAV823, oAV824, oAV825 and oAV826.	This study
pAV879	*ski2-archless*. Same as pAV878, but residues 831 to 1085 deleted.	*LEU2*	Same as pAV878	This study
pAV885	*TRF5,* residues 1-642, TAP tag	*LEU2*	Colony PCR yAV1304 (gifted strain with promoter,*TRF5,* 3'UTR on a plasmid) with oAV1039 and oAV1040 and cloned it into pAV854 (XbaI and BstAPI)	This study
pAV891	*TRF5,* residues 98-117, mutations on L101A, F107A, I108A	*LEU2*	Annealed oligos oAV876 and oAV877 and clones into pAV705 (PvuII and PstI)	This study
pAV892	*TRF5,* residues 98-117, mutations on E102A D103A, D106E	*LEU2*	Annealed oligos oAV 874 and oAV875 and cloned into pAV705 (PvuII and PstI)	This study
pAV899	*ski2-archless*-3Xmyc internally labeled, 2μ vector.	*LEU2*	Cloned *ski2-archless* (pAV879) with NdeI and XhoI into pAV125.	This study

Table 2.2

Strains	Genotype	References
yAV225	MATα, *trp1*, *leu2*, lys2, ura3, his4, *ski2*::LEU2, *dcp1*::*URA3*, [*dcp1-2*, *LYS2*]	(Jacobs Anderson & Parker, 1998)
yAV517	MATa, *his3Δ1, leu2Δ0, ura3Δ, met15Δ0, ski2Δ::*NEO	Open Biosystems
BY4742	*MAT**a**, his3Δ1, leu2Δ0, ura3Δ, met15Δ0*	(Giaever *et al.*, 2004)
yAV1151	MAT**α**, *his3Δ1, leu2Δ0, met15Δ0, mtr4*::NEO [*MTR4*, *URA3*]	(Jackson et al. 2010)
yAV1196	MAT**α**, *his3Δ1, leu2Δ0, met15Δ0, mtr4::*HYG [*MTR4*, *URA3*]	This study
yAV1144	*MAT**a**, his3Δ1, leu2Δ0, ura3Δ, met15Δ0, rrp6::*NEO	This study
yAV1233	MAT**α**, *his3Δ1, leu2Δ0, met15Δ0,rrp6::*NEO, *mtr4::*HYG [*MTR4*, *URA3*]	This study
PJ69-4a (yAV1170)	*MAT**a***, *trp1-901, leu2-3, ura3-52,his3-200, gal4Δ, gal80Δ, LYS2*::*GAL1-HIS3, GAL2::ADE2 met2::GAL7-LacZ*	(James *et al.*, 1996)
PJ69-4α (yAV1171)	MAT**α**, *trp1-901, leu2-3, ura3-52,his3-200, gal4Δ, gal80Δ, LYS2*::*GAL1-HIS3, GAL2::ADE2 met2::GAL7-LacZ*	(James *et al.*, 1996)
yAV1172	*MAT**a***, *trp1-901, leu2-3, ura3-52,his3-200, gal4Δ, gal80Δ, LYS2*::*GAL1-HIS3, GAL2::ADE2 met2::GAL7-LacZ* [pOAD, *LEU2*]	This study
yAV1173	Same as yAV1171 and transformed with [pOBD2, *TRP1*]	This study
yAV1174 (positive control Y2H)	MAT**α**, *trp1-901, leu2-3, ura3-52,his3-200, gal4Δ, gal80Δ, LYS2*::*GAL1-HIS3, GAL2::ADE2 met2::GAL7-LacZ* [*RAD17*, *TRP1*]	(James *et al.*, 1996)
yAV1175 (positive control Y2H)	*MAT**a***, *trp1-901, leu2-3, ura3-52,his3-200, gal4Δ, gal80Δ, LYS2*::*GAL1-HIS3, GAL2::ADE2 met2::GAL7-LacZ* [*MEC3*, *LEU2*]	(James *et al.*, 1996)
W303 (yAV1177)	*MAT**a**, ade2, his3, leu2, trp1, ura3, lys2*	(Abruzzi et al. 2007)
Y576 (yAV1178)	*MAT**a***, *ade2, his3, leu2, trp1, ura3*, *lys2, rrp6Δ::*NEO	(Abruzzi et al. 2007)
yAV1195	MAT**α**, *his3Δ1, leu2Δ0, met15Δ0, mtr4*::HYG [*MTR4*, *URA3*]	This study
yAV1233 (crossed yAV1195 and yAV1144)	MAT**α**, *his3Δ1, leu2Δ0, met15Δ0, mtr4*::HYG, *rrp6*::NEO [*MTR4*, *URA3*]	This study
yAV1303	MAT**α**, *his3Δ1, leu2Δ0, met15Δ0, trf4::*natMX*4, trf5::*KanMX6	(Holub et al. 2012)

	[pGAL-*TRF4*, blasticidin^R]	
yAV1304	MATα, *his3Δ1, leu2Δ0, met15Δ0, trf4::*natMX*4, trf5::*KanMX6 [pGAL-*TRF5*, blasticidin^R]	(Holub et al. 2012)
yAV1305	MATα, *his3Δ1, leu2Δ0, met15Δ0, trf4::*natMX*4, trf5::*KanMX6 [*TRF4*, *URA3*]	(Holub et al. 2012)
yAV1308	MATα, *his3Δ1, leu2Δ0, met15Δ0, mtr4*::NEO [*MTR4*, *LEU2*]	This study
yAV1309	MATα, *his3Δ1, leu2Δ0, met15Δ0, mtr4*::NEO [*mtr4-archless*, *LEU2*]	This study
yAV1335	MATα, *his3Δ1, leu2Δ0, met15Δ0, trf4::*natMX*4, trf5::*KanMX6 [*TRF5-TAP*, *LEU2*]	This study
yAV1336	MATα, *his3Δ1, leu2Δ0, met15Δ0, trf4::*natMX*4, trf5::*KanMX6 [*trf5Δ98-117-TAP*, *LEU2*]	This study

Table 2.3

Oligo name	Purpose	Sequence
oAV743	cloning	5’ CGC GAG AAT TCA TGA CAC CTG TTG AGC TTC CTA CAG 3’
oAV744	cloning	5’ CGC GAT CTA GAG GCC ATG AAT TCC CTT CGT ATA TAA TCT ATA TTT C 3’
oAV745	cloning	5’ GGA TCC ACT AGT CTC TCC CAG 3’
oAV759	cloning	5’ CAT TTA CCC TCT TAT GCT CAG CGA TAG GCG 3’
oAV767	cloning	5’-GAA TTC CAG CTG ACC ACC ATG GCT ATC GAC GTT GAA GAC GAT GAC-3’
oAV768	cloning	5’-AGA TCT CTG CAG TTA TCT ATT TCT GCA CTT GAT TTC GT-3’
oAV803	cloning	5’ CGC GAG AAT TCA CCA CCA TGA CAC CTG TTG AGC TTC CTA CAG 3’
oAV804	cloning	5’ CGC GAG AAT TCA CCA CCA TGT CTA GAG AAG TGG ATG CAT CGA AAG G 3’
oAV805	cloning	5’ CGC GAG AAT TCA CCA CCA TGC CTT TCC AGG ACA CTG CAA TCT CAT 3’
oAV 806	cloning	5’ CGC GAG AAT TCA CCA CCA TGT TAC AAC ATT ACC TGT TTC CAG 3’
oAV807	cloning	5’ CGC GAG AAT TCA CCA CCA TGG GAT ATA ATA TGA TTT TGA AC 3’
oAV808	cloning	5’ CGC GAG AAT TCA CCA CCA TGT CTA GTG GTG ATG AAC TGT TAC 3’
oAV809	cloning	5’ CGC GAC TGC AGC TAT AAA TAC AAA GAA CCA GCA 3’
oAV810	cloning	5’ CGC GAG AAT TCA CCA CCA TGT TCT TCC AAT TTC AAA ACG 3’

oAV811	cloning	5’ AGA TCT CTG CAG TTA TTC ACA TGC AAC TCT ACC TTT CAG 3’
oAV812	cloning	5’ AGA TCT CTG CAG TTA TGA TTC TGA AAT TTT GCG TTT CAA TTG 3’
oAV813	cloning	5’ AGA TCT CTG CAG TTA AGA ATG CTC CAA CAT AAA CTC CGG AGA 3’
oAV814	cloning	5’ AGA TCT CTG CAG TTA TAA GTG AAA AGC CGA GTC TAG CCT ATC 3’
oAV815	cloning	5’ AGA TCT CTG CAG TTA AGG AGT TGG ACG GAA ATT TGT GTA GAC 3’
oAV816	cloning	5’ CGC GAG AAT TCA CCA CCA TGG ATT CTA CTG ATC TGT TC 3’
oAV819	cloning	5’ CGC GAG AAT TCA CCA CCA TGT CCG AAG TTA TCA 3’
oAV820	cloning	5’ CGC GAC TGC AGT TAG TTG CCG TTA CC 3’
oAV821	cloning	5’ CGC GAG AAT TCA CCA CCA TGT CTA CGT TCA TAT TCC C 3’
oAV822	cloning	5’-CGC GAC TGC AGC TAC TCC TCC TTG ACC G-3’
oAV823	cloning	5’ ATA TAA GAT CTC GAC TGA GAA GAA TGA GCA CCA 3’
oAV824	cloning	5’ ATA TAG GCC ATT GTA GAA GAT CCA T 3’
oAV825	cloning	5’ ATA TAG ATG AGC AGG GCT CAA GAG 3’
oAV826	cloning	5’ ATA TAG AGC TCG AAG TAG ATT TAG GAG C 3’
oAV827	cloning	5’ GAA TTC CAG CTG ACC ACC ATG ATG GAA AAT GAT AAA AGT GAT GTC 3’
oAV828	cloning	5’ GAA TTC CAG CTG ACC ACC ATG GTC ACA TCA AGT GAA GAT GAA CAA AG 3’
oAV829	cloning	5’ GAA TTC CAG CTG ACC ACC ATG AAC AAT TCT CTC GAA GAT AAT CAA G 3’
oAV830	cloning	5’ AGA TCT CTG CAG TTA AAC GAA ATC TTT GAT TTC TGA AG 3’
oAV831	cloning	5’ AGA TCT CTG CAG TTA TTG TTT CGA ATG ACA ATG ATT TCT TAT C 3’
oAV832	cloning	5’ AGA TCT CTG CAG TTA GCA AGC GCT TTC AGT GTT CAG AG 3’
oAV836	cloning	5’ GAA TTC CAG CTG ACC ACC ATG ACA GAA CAA ATA AAG GAA GAT GAT GAT G 3’
oAV837	cloning	5’ AGA TCT CTG CAG TTA AAC TTC ATG TTC ATC GGT CAA C 3’
oAV838	cloning	5’ AGA TCT CTG CAG TTA ATC TTC CTT TAT TTG TTC TG 3’
oAV850	cloning/homologous recombination	5’ AAA CCC AAA AAA AGA GAT CGA ATT CCA GCT GAC CAC CAT GGC ATG CAA TTT TCA G 3’
oAV851	cloning/homologous recombination	5’ GCG GGG TTT TTC AGT ATC TAC GAT TCA TAG ATC TCT GCA GTC AAA AAG GTT TGG CAC A 3’
oAV854	cloning	5’ CTG CCA CCA ATG AAC AAT TCT CTC GAA GAT AAT CAA GAT TTT ATT GCT TTT TCG GAC AGC TCT GAA GAT GAA TAA CTG CA 3’
oAV855	cloning	5’ GTT ATT CAT CTT CAG AGC TGT CCG AAA AAG CAA TAA AAT

		CTT GAT TAT CTT CGA GAG AAT TGT TCA TTG GTG GCA G 3’
oAV869	cloning	5’ GCG GCG CTG CAG CAA GAG CCT GGC CTT TAG AGA GCC 3’
oAV870	cloning	5’ GCG GCG ACT AGT TCT TTT GCT AGA TTC TGC CCT TTG TTC 3’
oAV871	cloning	5’ GCG GCG ACT AGT GAA CAA ATA AAG GAA GAT GAT GAT G 3’
oAV874	cloning	5’ CTG CCA CCA ATG AAC AAT TCT CTC GCT GCT AAT CAA GCT TTT ATT GCT TTT TCG GAC AGC TCT GAA GAT TAA GAA TAA CTG CA 3’
oAV875	cloning	5’ GTT ATT CAT CTT CAG AGC TGT CCG AAA AAG CAA TAA AAG CTT GAT TAG CAG CGA GAG AAT TGT TCA TTG GTG GCA G 3’
oAV876	cloning	5’ CTG CCA CCA ATG AAC AAT TCT GCT GAA GAT AAT CAA GAT GCt GCT GCT TTT TCG GAC AGC TCT GAA GAT GAA TAA CTG CA 3’
oAV877	cloning	5’ GTT ATT CAT CTT CAG AGC TGT CCG AAA AAG CAG CAG CAT CTT GAT TAT CTT CAG CAG AAT TGT TCA TTG GTGGCA G 3’
oAV906	cloning	5’ TAT TAT GCG GCC GCC CAC AAA GTA CTA CAT CTA TGG TCT 3’
oAV1039	cloning	5’ CAT ACA AAA CGT ATG CGT 3’
oAV1040	cloning	5’ ACC CAG GTT ATC TAG AAC G 3’
oRP993	Northern (5’ ETS)	5’-CGA ACG ACA AGC CTA CTC G-3’
oAV777	Northern (5.8S)	5’- TTT CGC TGC GTT CTT CAT C-3’
oAV224	Northern (SRP)	5’-GTC TAG CCG CGA GGA AGG-3’
oAV849	Northern (snR33)	5’-AGG AAC CGA CTC AAA CCG G-3’
oAV908	Northern (pre-snR33)	5’-AAG TTT TGC AAATCG ATT GTC C-3’
oAV909	Northern (snR44)	5’ GTA AGA AGC ATT TCC ACA TGG G 3’
oAV910	Northern (U14)	5’ TCC TAC CGT GGA AAC TGC G 3’
oAV911	Northern (pre-U14)	5’ GAT ACT ACA GTA TAC GAT CAC TC 3’
oAV912	Northern (U4)	5’ CGG ACG AAT CCT CAC TGA TA 3’
oAV913	Northern (pre-U4)	5’ CAG TCC CTT TGA AAG AAT GAA T 3’
oAV1036	Northern (snR38)	5’-GAGAGGTTACCTATTATTATTACCCATTCAGACAGGGATAACTG-3’

Chapter 3: The arch domain of the exosome cofactors, Mtr4 and Ski2, is required for nuclear and cytoplasmic exosome functions.

INTRODUCTION

Several cofactors of the exosome have been identified, however, only two are required for all the known functions of the exosome in their respective cellular compartments. Mtr4 and Ski2 are the only RNA-dependent ATPases involved in promoting exosome functions. Mtr4 is an essential cofactor of the nuclear exosome, while Ski2 is a nonessential protein and cytoplasmic cofactor. Mtr4 and Ski2 are collectively required for all the degradation and processing functions of the exosome. It is unclear, however, why the exosome requires these specific cofactors; it is also unknown what makes Mtr4 and Ski2 unique among the many ATPases and helicases in the cell.

The crystal structures of Mtr4 and Ski2 provide a clue regarding the function of these exosome cofactors. The structures of Mtr4 and Ski2 contain all the conserved 'helicase core' domains (RecA1, RecA2, Winged Helix, and C-terminal helical bundle domain or DSHCT domain) and these fold into a globular helicase core (Weir *et al.*, 2010, Jackson *et al.*, 2010). These four domains are characteristic of the many other DNA and RNA helicases of varying functions, including the DNA helicase Hel308 and splicing factor Prp43 (Walbott *et al.*, 2010, Richards *et al.*, 2008). One unique structural feature of these two cofactors is the novel arch domain. The arch domain of Mtr4 and Ski2 rises above the helicase core (see figure 3.1) beginning and ending at the winged helix domain of Mtr4 and Ski2 (Jackson et al., 2010, Weir et al., 2010, Halbach *et al.*, 2012). The arch sequence is highly conserved amongst Mtr4 and Ski2 of different species. Critical residues promote the bend of the arch among other structural features, yet there is low sequences

similarity between the arch domain of Mtr4 and Ski2 proteins (Jackson et al., 2010). One important difference observed when comparing these crystal structures was the absence of the KOW motif in the Ski2 arch. The KOW motif was initially described as a 27-residue region with alternating hydrophilic and hydrophobic residues and an invariant glycine in the middle (Ponting, 1996). The KOW motif is conserved and important among ribosomal proteins and transcription factors throughout all three kingdoms of life. Furthermore, the KOW motif in Mtr4 has been shown to interact with *in vitro* transcribed tRNA (Weir et al., 2010). It is not surprising that Ski2 lacks this motif, since Ski2 does not interact with rRNAs or tRNAs in the cytoplasm. The lack of sequence similarity between the arch domains of Mtr4 and Ski2 provides further evidence that the structure of the arch may be necessary for general exosome function, while specific residues may be providing a level of substrate specificity.

To determine whether these arch domains are required for exosome functions, I created *mtr4-archless* and *ski2-archless* strains and analyzed whether removal of the arch affected exosome function in the nucleus and in the cytoplasm, respectively. Data presented here indicates that the arch domain of Mtr4 and Ski2 is indeed required for exosome functions in both cell compartments. Importantly, the arch domain of Mtr4 is involved in cell functions that promote optimal cell growth. Additionally, the arch domain of Mtr4 promotes a certain level of substrate specificity by targeting specific substrates of Rrp6, the nuclear exosome-associated exonuclease. To date, the arch domain of Ski2 seems to be required for all of the cytoplasmic functions of the exosome.

Figure 3.1

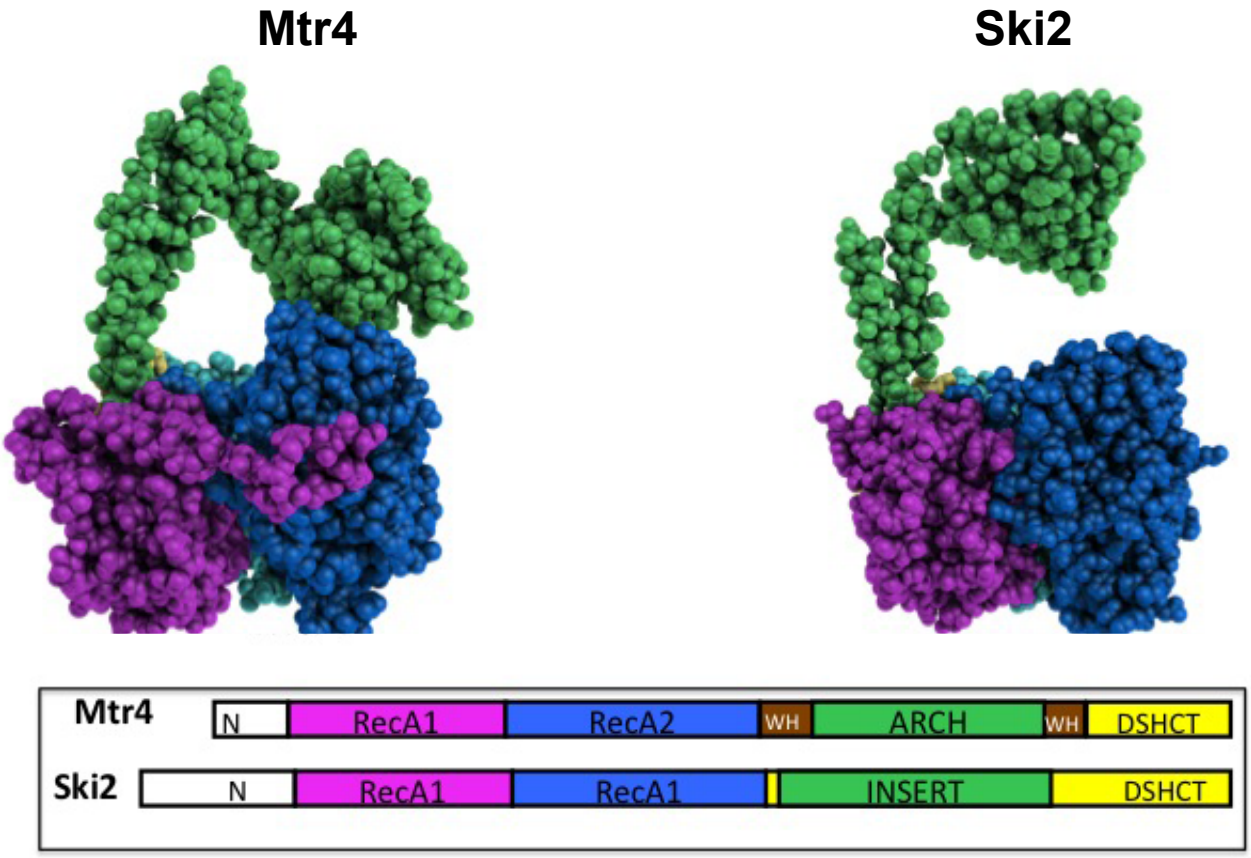

Figure 3.1 A side-by-side comparison of the crystal structures of Mtr4 and Ski2
In this side view of the structure of the exosome cofactors the RecA1 and RecA2 domains of the helicase core are shown. Mtr4 and Ski2 form the helicase core with two other domains, Winged Helix and Dob1(Mtr4)/Ski2/Hel308 C-terminal domain (DSHCT). There are conserved structural features with previously described DExD/H helicase. However the arch domain is unique to Mtr4 and Ski2.The arch domain emerges from the core at the Winged helix domain. The rest of the domains are shown below in a depiction of the primary structures of Mtr4 and Ski2. The structure of the N-terminal regions of either protein have not been solved.

RESULTS

The arch domain of Mtr4 is required for one processing and one degradation function of the nuclear exosome

To determine whether the arch domain is important for Mtr4 function, an *mtr4-archless* strain was constructed in the yeast *Saccharomyces cerevisiae* (see materials and methods). Unlike mutants that disrupt the catalytic functions of Mtr4, removing the arch domain did not cause cell death (Jackson *et al.*, 2010). The *mtr4-archless* strain, however, is slow-growing (Figure 3.2). Importantly, the growth defect seen in the *mtr4-archless* strain is not due to the lack of or lower expression of the Mtr4-archless protein (figure 3.5). Mtr4-archless is expressed at levels similar to the endogenous Mtr4 protein. The *mtr4-archless* strain does confer a temperature or cold-sensitive growth phenotype.

The ATPase and helicase functions of Mtr4 were tested in the Mtr4-archless protein *in vitro*; these data showed that the arch domain is not involved in the catalytic roles of Mtr4 (Jackson et al., 2010, Weir et al., 2010). To identify which functions of the exosome were causing the slow growth phenotype *in vivo*, RNA was isolated from *MTR4*, *mtr4-archless*, and strains with a point mutation in one of the three catalytic subunits of the nuclear exosome. I analyzed mutants disrupting the Rrp44 exonuclease activity (the *rrp44-exo*$^{-}$ mutant), the Rrp44 endonuclease activity (the *rrp44-endo*$^{-}$ mutant), and the exosome-associated exonuclease Rrp6 (*rrp6*Δ). Surprisingly, the *mtr4-archless* RNA defects resembled those seen in an *rrp6*Δ strain, rather than the general exosome defects seen in the *rrp44*-exo^{-} (Figure 3.3 compare lanes 2 with lanes 3 and 4). Removal of the arch domain of Mtr4 caused

accumulation of the 5.8 rRNA intermediate, 5.8S+30 (Jackson et al., 2010). Additionally, the cleavage product of the 35S pre-rRNA maturation pathway, 5' External Transcribed Spacer (5'ETS), also accumulated in the *mtr4-archless* strain. 5'ETS is a commonly tested exosome degradation target that accumulates in both *rrp44*-exo$^-$ and *rrp6*Δ mutant strains, however, the pattern of intermediates that accumulate between these exosome mutants are different (Figure 3.3 compare lanes 3 and 4). The *mtr4-archless* strain accumulates only the full-length 5'ETS degradation product, which occurs in the *rrp6*Δ strain and not in the *rrp44*-exo$^-$. These data suggest that the arch domain of Mtr4 is required for two exosome functions. Taken together with the *in vitro* results, I conclude that the arch's exosome-mediated function does not involve the helicase activity of Mtr4 (Weir et al., 2010, Jackson et al., 2010).

"The arch domain of Mtr4 is not required for all Rrp6 functions

The *mtr4-archless* strain has RNA processing and degradation defects that resemble those in an *rrp6Δ* strain. Our initial prediction involved that the arch domain of Mtr4 is required for all of Rrp6 functions. To test whether all of the Rrp6 functions require the arch domain of Mtr4, I analyzed the processing of snR33. Small nucleolar RNA processing requires the core exosome and Rrp6 in separate steps. In *rrp6Δ* strains many snoRNAs are extended by three or four nucleotides. Some of these extra nucleotides are encoded within the snoRNA gene, while others are added by the TRAMP complex (Allmang et al., 1999a, Grzechnik & Kufel, 2008, van Hoof *et al.*, 2000a). In addition to these *rrp6*-specific short 3' extensions, several different mutations in the core exosome, as well as *rrp6Δ,* lead to snoRNA species that carry longer encoded 3' extensions. Some of these 3' extended species are polyadenylated in a process that requires both TRAMP and Pap1 (Allmang et al., 1999a, Grzechnik & Kufel, 2008, Schneider et al., 2009, van Hoof et al., 2000a). To test whether the catalytic activity of Rrp44 was involved in snoRNA processing, I analyzed the same three exosome mutants (*rrp44*-exo-, *rrp44*-endo-, and *rrp6*Δ). Figure 3.4 shows that *rrp44-exo*$^-$ accumulates long 3' extended snR33 species, while the *rrp44-endo*$^-$ mutant

has no defect in the maturation of snR33. Unlike what was seen for 5.8S rRNA processing and 5'ETS degradation, the effect of *mtr4-archless* on processing of snR33 does not resemble the effect of *rrp6Δ*. The *mtr4-archless* strain does not accumulate snoRNAs with 2-3 nts extensions or long polyadenylated snoRNAs. I conclude that the arch domain of Mtr4 is only required for specific Rrp6 functions" and it is indeed promoting the substrate specificity of the exosome.

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"The arch domain of Ski2 is required to promote the cytoplasmic functions of the exosome

Although Ski2 and Mtr4 homology includes both the core helicase domains and the arch domain, the sequence similarity between the two arch domains is low (~34%)(Jackson et al., 2010). Moreover, it is not known whether the arch domain of Ski2 is functional. Like in Mtr4, *in vitro* activities of Ski2-archless are similar to the wild-type enzyme, including the capacity to form a Ski complex with Ski3 and Ski8 (Halbach *et al.*, 2012). To investigate the role of the arch domain in one of the cytoplasmic functions of the exosome, I performed two different assays: the synthetic lethality growth assay and the His3-nonstop growth assay. First, Ski2 is required for one of the two redundant pathways for mRNA decay. Mutations that disrupt *SKI2* function are synthetically lethal with mutations that inactivate the decapping enzyme. Therefore, I analyzed whether *ski2*-archless could rescue the synthetic lethality of the *dcp1-2, ski2*Δ strain which contains a temperature sensitive mutation that inactivates the decapping enzyme (Jacobs Anderson & Parker, 1998). As shown in Figure 3.6, the *dcp1-2*, *ski2Δ* synthetic lethality cannot be rescued by the *ski2-archless* plasmid. Importantly, western blotting indicated that Ski2-archless is expressed at levels similar to wild-type Ski2 (Figure 3.7).

The second assay for Ski2 function is based on the preferential degradation of mRNAs that lack a stop codon (nonstop mRNAs) by the cytoplasmic exosome. A strain with *SKI2* deleted in the chromosome was first transformed with the His3-nonstop (His3-ns) reporter plasmid and then with either the *SKI2*, *ski2-archless*, or empty vector plasmids. In cells with a defect in nonstop decay, such as *ski2*Δ, the His3-ns reporter mRNA is stable and such cells are able grow in media lacking histidine. Conversely, cells with functional Ski2 quickly degrade the aberrant His3-ns mRNA, and therefore are unable to grow in media lacking the amino acid. The His3-nonstop growth assay in Figure 3.8 shows growth in cells expressing the Ski2-archless mutant in plates lacking histidine (nonstop assay, left panel). These

two assays indicate that the arch domain of Ski2 is required for two exosome-mediated functions, nonstop degradation and normal mRNA decay." The ATPase and helicase activity has not been assessed for the Ski2-archless protein.

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Figure 3.2

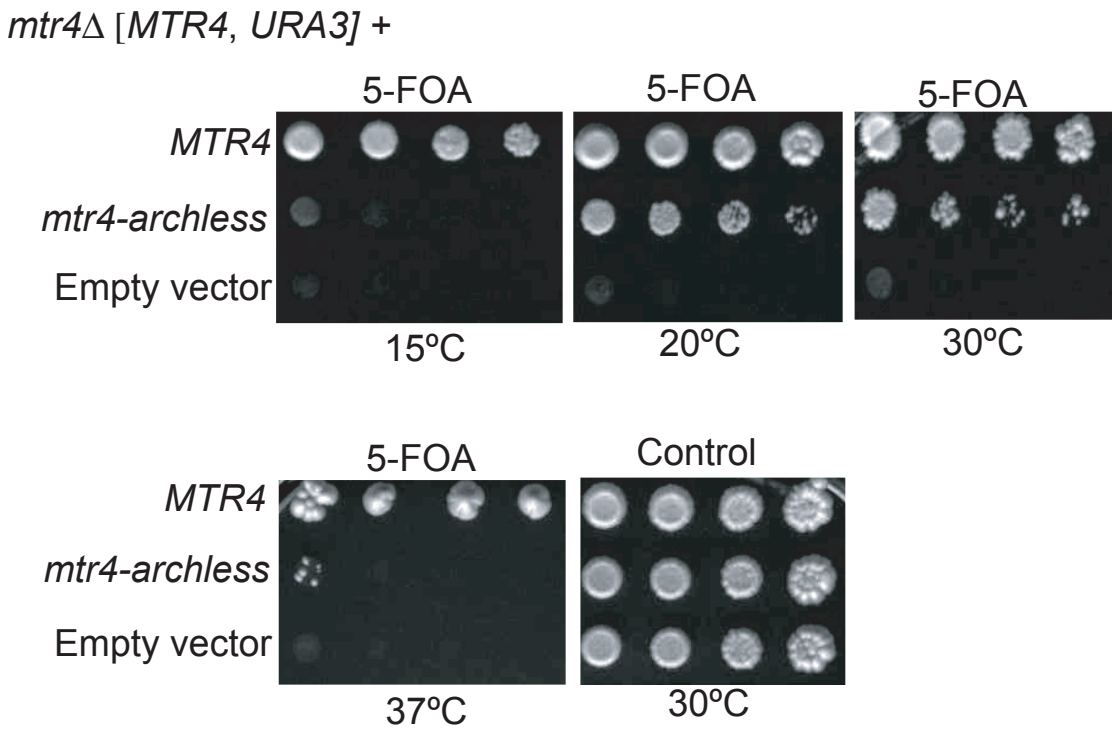

Figure 3.2 *mtr4-archless* growth assay:
*mtr4*Δ transformants containing *MTR4* with a *URA3* selectable marker and either *MTR4*/*mtr4-archless* or empty vector with a *LEU2* selectable marker were serially diluted onto 5-FOA plates and grown at different temperatures for 3 days.

Figure adapted from Jackson et al. 2010, and used with permission from *EMBO J*.

Figure 3.3

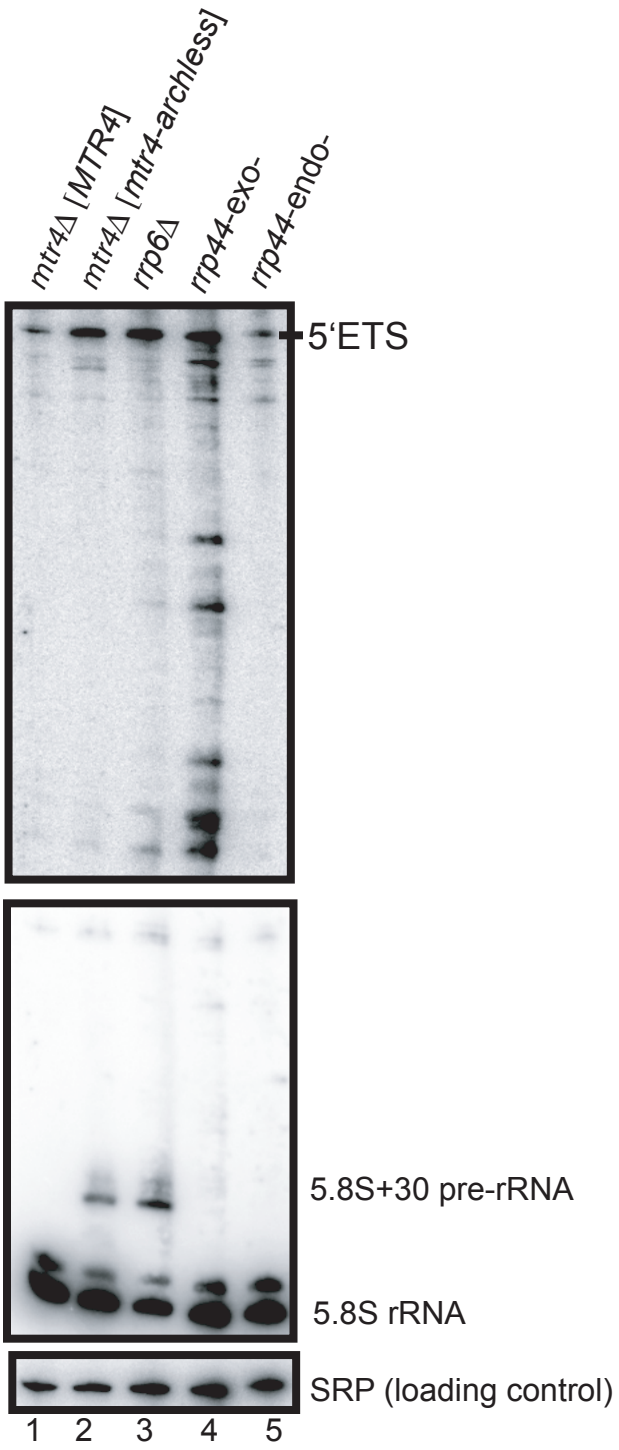

Figure 3.3: RNA analysis of mtr4-archless and exosome mutants:
Total RNA was isolated from *MTR4*, *mtr4-archless*, and exosome mutants, Northern blot was performed. Oligos complementary to 5’ETS, 5.8S, and SRP were used and are described in table 2.3.

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Figure 3.4

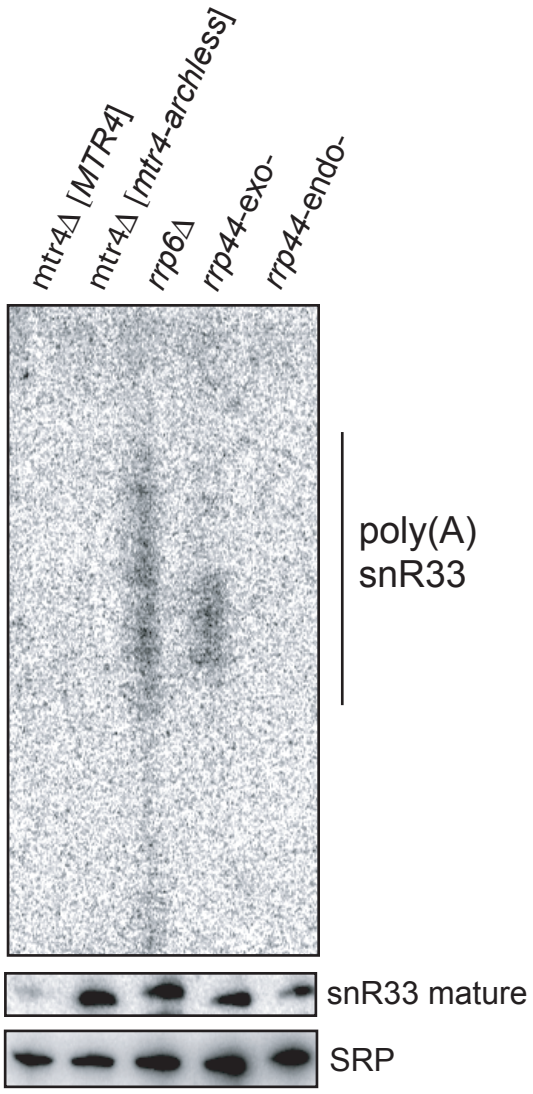

Figure 3.4 snoRNA analysis of mtr4-archless and exosome mutants:
Similarly to figure 3.4, RNA was isolated from the strains described and Northern blot analysis was performed.

Figure 3.5

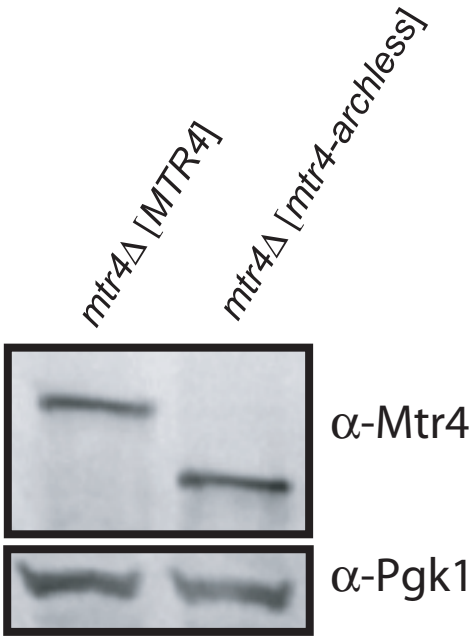

Figure 3.5 Western blot of Mtr4 and Mtr4-archless
Protein was isolated from *MTR4* and *mtr4-archless* cured strains. Protein levels are similar between the wild-type and mutant Mtr4 protein.
Pgk1- is 3-phosphoglycerate kinase protein, which is used as an internal control.

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Figure 3.6

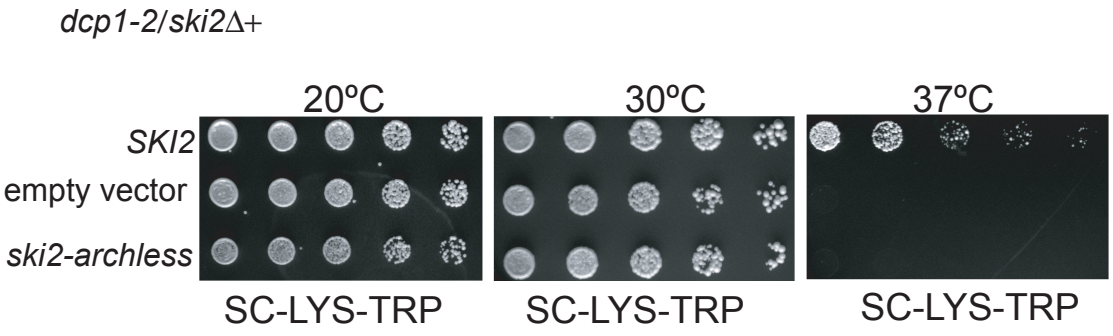

Figure 3.6 Synthetic lethality growth assay of ski2-archless: The *ski2Δ/dcp1-2* double mutant was transformed with *SKI2*/*ski2-archless* and empty vector plasmids.Transformants were serially diluted and pronged onto SC-LYS-TRP and incubated at 20ºC, 30ºC, and 37ºC (nonpermissive temperature). Growth in the nonpermissive temperature indicates that the Ski2 construct promotes normal mRNA decay.

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Figure 3.7

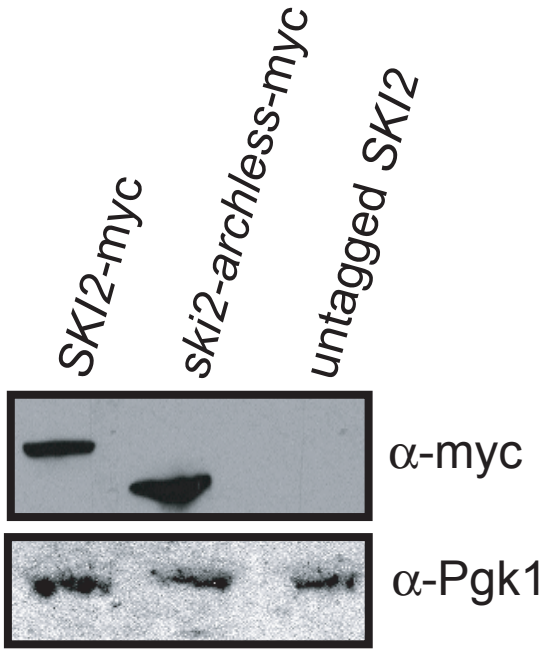

Figure 3.7 Western blot analysis of Ski2 and Ski2-archless: Ski2 and Ski2-archless were tagged internally upstream of the arch domain, and western blot analysis was performed.

Figure 3.8

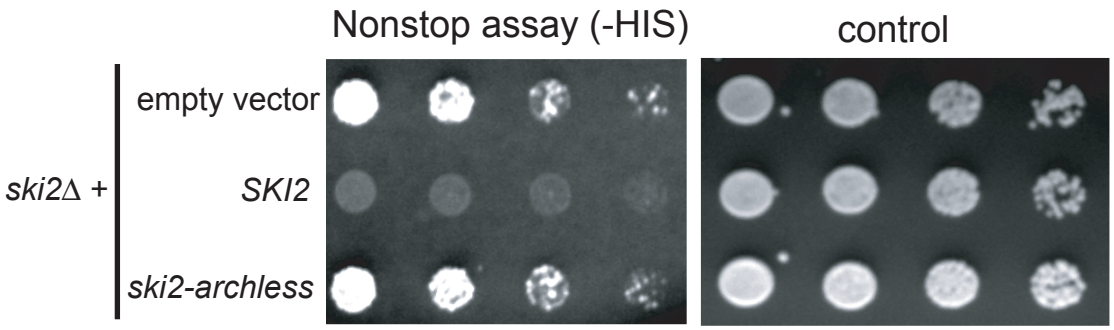

Figure 3.8 His3 Nonstop growth assay of *ski2-archless*: *ski2*Δ strains transformed with *SKI2/ski2-archless* and empty vector were further transformed with the His3nonstop reporter. Growth on plates lacking histadine indicates that nonstop decay is nonfunctional.

Mtr4-Ski2arch confers a small growth advantage when compared to the *mtr4-archless* strain

Although the arch domains of Mtr4 and Ski2 have low sequence similarity, I wanted to determine whether certain conserved structural features of the arch domain of Ski2 could be sufficient to restore normal growth rates in the *mtr4*Δ [*mtr4-ski2arc*h] strain. The *mtr4-ski2arch* strain was constructed by cloning the arch domain of Ski2 into the *mtr4-archless* plasmid and introducing this plasmid into *mtr4*Δ [*MTR4*, *URA3*] cells through a standard plasmid shuffle technique (Materials and Methods). Growth assays on 5-FOA indicate that although *mtr4-ski2arch* complements *mtr4*Δ, it does not restore growth to wild-type levels (figure 3.9). Surprisingly, there may still be a slight growth advantage over the *mtr4-archless* strain, which is more evident in the slower growing *mtr4*Δ/*rrp6*Δ double mutant (which will be discussed in Chapter 4). This slight growth advantage may indicate that certain conserved features (likely structural) of the arch may promote the general functions of the exosome. This complementation also indicates that Mtr4-Ski2arch is expressed.

The arch domain of Ski2 is not sufficient to promote nonstop mRNA decay

The *mtr4-ski2arch* strain was further tested for one of the cytoplasmic functions of the exosome. It is possible that the arch domain together with the

Figure 3.9

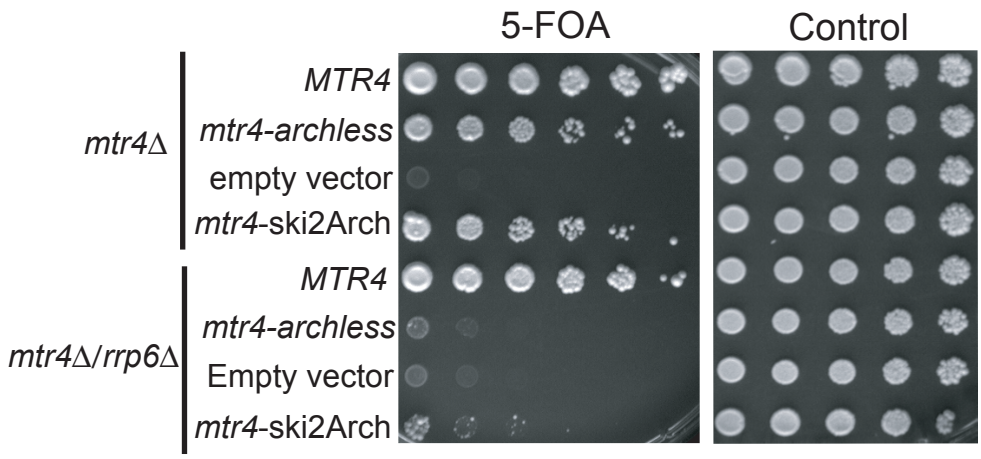

Figure 3.9 Growth assay to compare the *mtr4-archless* and *mtr4-ski2arch* strains:
Similarly to the *mtr4-archless* complementation analysis, cells were serially diluted and assayed for growth on 5-FOA. Strains with *mtr4-ski2arch* show a slight growth advantage. The *mtr4Δ/rrp6Δ* double mutant is included to show the more pronounced growth advantage with the *mtr4-ski2arch* strain.

helicase core of Mtr4, may be sufficient to promote nonstop mRNA decay. The *mtr4-ski2arch* does not promote nonstop mRNA decay (figure 3.10). However, this result was expected because Ski2 requires its N-terminus to interact with the rest of the Ski complex (Brown *et al.*, 2000, Wang *et al.*, 2005). Additionally, Ski2 localizes to the cytoplasm and Mtr4 to the nucleus, therefore it is possible that Mtr4-Ski2arch does not travel to the cytoplasm. Further experiments are needed to confirm whether the arch of each Mtr4 or Ski2 contain any conserved features that can promote the other's functions.

Figure 3.10

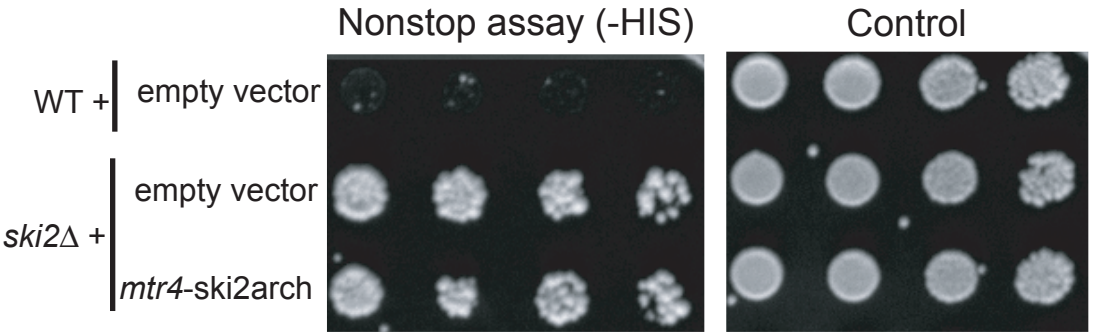

Figure 3.10 Nonstop growth assay in the *mtr4-ski2arch* strain:
The *mtr4-ski2arch* strain was tested for nonstop decay function. The arch domain of Ski2 is not sufficient to promote exosome-mediated nonstop mRNA decay.

CONCLUSIONS AND FUTURE DIRECTIONS

The arch domain of Mtr4 and Ski2 contain important features that allow them to promote exosome function in the nucleus and in the cytoplasm.

The results discussed in this chapter have shed light on the function of a novel domain in the exosome cofactors, Mtr4 and Ski2. The data indicate that the arch domain of the helicases Mtr4 and Ski2 is necessary for proper exosome function. It is unclear however, why the arch domain is needed for each of these cofactors. Structural data and sequence alignments indicate that the structural features of the arch are conserved throughout Mtr4 and Ski2 proteins, therefore it is the arch domain is acting as an important protein fold that contributes to the interaction with the exosome or its substrates. Some of the results discussed in Chapter 5 and 6 also indicate that the arch domain of Mtr4 may be contributing to the interaction of Mtr4 with other proteins, such as the TRAMP subunits. Additionally, certain regions of the Mtr4 arch have been shown to be involved in binding specific types of RNA (namely hypomodified tRNA) *in vitro* (Weir et al., 2010). Furthermore, point mutations within the KOW motif of the arch cause different RNA defects and growth phenotypes than the *mtr4-archless* strain (preliminary data not shown), which suggests that the arch domain of Mtr4 may have several sequence and structural features that contribute to different Mtr4 functions. Conversely, the arch domain of Ski2 promiscuously binds both double and single stranded mRNAs *in vitro* (Halbach et al., 2012). A more detailed analysis of

the arch domain of each of these proteins is necessary to dissect the specific functions certain regions attribute to these helicases.

The arch domains make Mtr4 and Ski2 unique ATPases and exosome cofactors

Mtr4 and Ski2 are categorized as part of the Ski2-family of helicases because of their DEVH motif in the helicase core domains (see figure 1.4a). After analyzing the crystal structures and the data presented here, I believe that these can be further subcategorized as 'arched helicases'. The arch domain sets each of these helicases apart from the rest of the Ski2-family of helicases in the cell. Furthermore, the fact that both of these 'arched helicases' require the arch to exclusively promote exosome-mediated functions, indicate that the arch domain is one of the features (if not THE feature) that makes these ATPases exosome-specific.

Several follow-up experiments should be considered to analyze which features of the arch allow the RNA helicase to promote exosome-mediated functions. First, RNA should be isolated from the *mtr4-ski2arch* strain and analyzed through northern blot for RNA processing and degradation defects. The *mtr4-ski2arch* strain provided a slight growth advantage over the *mtr4-archless* strain, therefore it would be interesting to identify which exosome functions can be restored when supplying Mtr4 with the arch domain of Ski2. Second, the level of expression and localization of Mtr4-Ski2arch should be examined through fluorescence microscopy by fluorescently tagging the *MTR4*, *mtr4*-archless, and *mtr4*-Ski2arch strains. Only by confirming the localization of the chimeric protein in the cytoplasm, can one conclude that Mtr4-Ski2arch is unable to promote nonstop decay because it lacks

the rest of the Ski2 protein. Further experiments introducing the arch domain of Mtr4 into other nuclear helicases, such as splicing factors Prp28 or Sub2, could unveil whether other helicases can promote the nuclear functions of the exosome. Likewise, the arch domain of Ski2 can be introduced into cytoplasmic helicases. However it is already known that Ski2 complex assembly requires the N-terminus of the protein, therefore it is unlikely that this will work for the cytoplasmic functions of the exosome.

Chapter 4: The core domain of Mtr4 can activate exosome function in an Rrp6-independent manner.

INTRODUCTION

The processing functions that Rrp6 promotes are exosome core independent, while the degradation functions require both Rrp6 and the exosome's catalytic subunit, Rrp44

Rrp6 is an exoribonuclease that localizes to the nucleus and nucleolus of eukaryotic cells. Together with Rrp44's exo- and endonuclease activity, Rrp6 provides the nuclear exosome with its third catalytic activity. Rrp6 readily co-purifies with all the other exosome subunits, including the catalytic subunit Rrp44 (Gavin *et al.*, 2002, Krogan *et al.*, 2004). However, crystal structures of the exosome do not include Rrp6, and how it is oriented relative to the core of the exosome and to the Rrp44 active sites is currently unknown.

Rrp6 is an important component of the nuclear exosome complex. Rrp6 cleaves the pre-rRNA 5.8+30; 5.8S+30 is the product of the 7S pre-rRNA previously cleaved by core exosome (Briggs *et al.*, 1998, Allmang *et al.*, 1999a, Phillips & Butler, 2003). Additionally, Rrp6 plays a role in sno/snRNA maturation by trimming the last few nucleotides to form the mature small RNA (Allmang et al., 1999a, Allmang et al., 1999b, van Hoof et al., 2000b, Allmang et al., 2000, Briggs et al., 1998, Phillips & Butler, 2003). Furthermore, Rrp6 degrades aberrant/pre-RNA substrates tagged through polyadenylation by the TRAMP complex (see below). It is unclear how Rrp6 contributes to exosome function and whether Rrp6 affects the catalytic activity of the core exosome, or whether Rrp6 is involved in substrate targeting. *In vitro* these two ribonucleases degrade RNA using two different patterns; Rrp6 degrades RNA in a distributive mode, while Rrp44-exo does so processively (Liu *et al.*, 2006, Dziembowski *et al.*, 2007, Burkard & Butler, 2000).

RNA defects observed in the *rrp6*Δ differ from the ones in the *rrp44* and other exosome core point mutants and depletions. The *rrp6Δ* mutants accumulate the discrete 5.8+30 rRNA species, while *rrp44* only accumulates intermediates between 7S and 5.8S+30 pre-rRNAs (Briggs et al., 1998, Allmang et al., 1999a, Allmang et al., 2000). The pre-snoRNA species that appear in these two exosome mutants are also distinct; *rrp6*Δ contains two different lengths of snoRNA species, while *rrp44* only accumulates the longer polyadenylated aberrant snoRNA (Allmang et al., 1999a, van Hoof et al., 2000b). Furthermore, Rrp6 is involved in the degradation of specific aberrant poly(A) pre-rRNA substrates; Rrp6, with the help of Mtr4, targets 23S, 21S, and the internal transcribed spacer 1 (ITS1)(Callahan & Butler, 2008).

There is, however, some redundancy in the functions Rrp6 and the core exosome promote; *rrp6*Δ/*rrp44-exo-* double mutants cause synthetic growth and RNA defects (Dziembowski et al., 2007, Schneider et al., 2009, Schaeffer *et al.*, 2009). Additionally, both *rrp6* and exosome mutants accumulate poly(A)+ RNAs in the nucleus (Das et al., 2003, Kadowaki *et al.*, 1994). Mutations that disrupt Rrp6-core exosome interaction have been shown to affect only the degradation functions of the aberrant rRNA, and not the Rrp6-specific processing functions (Callahan & Butler, 2008). It is unclear why the core exosome requires Rrp6's exonuclease activity for specific processing functions. Furthermore, together Rrp6 and Rrp44 degrade aberrant transcripts, and the mechanism behind this cooperative function is not understood.

Rrp6 degrades poly(A)+ RNAs tagged by the TRAMP complex

The TRAMP complex is involved in tagging aberrant/pre-RNAs for Rrp6- and exosome-mediated degradation. The TRAMP complex is composed of Mtr4, one of non-canonical poly(A) polymerases, and one RNA binding proteins (Vanacova *et al.*, 2005, LaCava *et al.*, 2005). *In vitro* reconstitutions using TRAMP and the exosome purified through the Rrp6 subunit show that TRAMP enhances the degradation functions of the exosome (LaCava et al., 2005, Vanacova et al., 2005). Although still unclear, there are reports that identified that only the catalytic activity of Rrp6 was enhanced in this *in vitro* reconstitution assay (Callahan & Butler, 2009).

Some reports suggest that Mtr4 and Rrp6 may work together to promote RNA degradation functions (Callahan & Butler, 2008). Genetic analysis by Abruzzi et al. showed that increased amounts of Mtr4 could suppress the slow growth phenotype of an *rrp6*Δ strain (Abruzzi *et al.*, 2007), indicating that Mtr4 and Rrp6 may have some overlapping functions. Additionally, previous results indicated that the arch domain may play an important role in promoting the function of Rrp6 (Chapter 3, (Jackson *et al.*, 2010)).

To determine whether these overlapping functions required the arch domain of Mtr4, molecular and genetic analysis were undertaken and are described in this chapter.

RESULTS

"Synthetic defects indicate that the arch domain of Mtr4 can function independently of Rrp6

To further resolve the relation between the functions of the arch domain of Mtr4 and Rrp6, I undertook a genetic analysis and compared the growth of the *rrp6Δ* and *mtr4-archless* mutants to the growth of an *rrp6Δ, mtr4-archless* double mutant" using standard plasmid shuffle technique. "Two observations suggest that the slow growth of *mtr4-archless* is not caused by a defect in Rrp6 activation. First, if the slow growth of *mtr4-archless* was caused by reduced activity of Rrp6, then *rrp6Δ* should lead to a growth defect at least as severe as *mtr4-archless*. I did not observe this, and instead the *mtr4-archless* growth defect is much more severe than that of *rrp6Δ* (compare the 2nd and 4th rows of Figure 4.1). Second, the hypothesis that the arch is needed to assist Rrp6 predicts that deleting the arch in a strain already lacking Rrp6 would have no additional phenotypic effect. Instead, the *rrp6Δ/mtr4-archless* double mutant grows much slower than either of the single mutants (Compare row 5 of Figure 4.1 to rows 2 and 4). This synthetic growth defect indicates that the arch domain of Mtr4 and Rrp6 have overlapping functions but that each can function independently of the other."

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The arch domain of Mtr4 and Rrp6 only function together in the processing of the 5.8S rRNA but not in the snoRNA processing pathway.

I "have previously shown that *mtr4-archless* and *rrp6Δ* result in similar 5.8S rRNA processing defects (Jackson et al., 2010). Specifically, both of these mutants accumulate a 5.8S processing intermediate that retains approximately 30 nucleotides of its 3' extension (Chapter 3). To test whether the synthetic growth defect of an *rrp6Δ, mtr4-archless* mutant was related to this shared rRNA processing defect, I compared 5.8S rRNA processing in the double mutant to that in both single mutants by Northern blot analysis. Figure 4.2 shows that the 5.8S rRNA defect in the double mutant is no more severe than in the single mutants (compare lanes 2 and 5). This is consistent with the suggestion that the arch domain and Rrp6 acts in the same pathway, and indicates that the slower growth of the double mutant is not correlated with worsening of this processing defect."

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The *mtr4-archless*/*rrp6*Δ double mutant was also tested for snoRNA processing and degradation functions. In the case of snoRNA processing and degradation, the defects are synergistically increased in the double mutants. The mature forms of U14 and snR33 snoRNAs are extended by more than 3 nucleotides in the double mutant compared to the single *rrp6*Δ mutant (compare lanes 5 and 6 to lane 7 in Figure 4.3). Additionally, figure 4.3 shows that the amount of the longer polyadenylated species of snR33 also increase in the *mtr4-archless*/*rrp6*Δ double mutant compared to the single *rrp6*Δ mutant. These results indicate that for the snoRNA processing and degradation functions, the arch domain of Mtr4 and Rrp6 are acting in two different pathways. Some surprising results included the differential amounts of snoRNAs obtained in all these different strains, the two *mtr4-archless* strains seem to be accumulating larger amounts of mature snoRNA compared to the wild-type strains. The significance of these results remains to be studied.

> "Overall, the results of the double mutant analysis suggest that the arch domain of Mtr4 and Rrp6 function in similar pathways, but there is not a complete overlap of these functions."
>
> Klauer and van Hoof 2012b

Figure 4.1

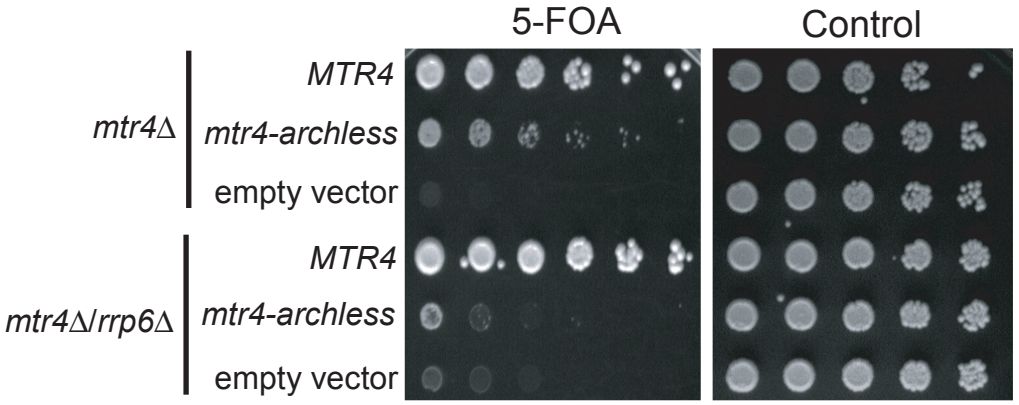

Figure 4.1 *rrp6Δ/mtr4-archless* double mutant has a synthetic growth defect compared to the *mtr4-archless* strain:
The original *mtr4*Δ[*MTR4*,*URA3*] strain was crossed to the *rrp6*Δ strain to produce the *mtr4*Δ [*MTR4*, *URA3*] /*rrp6*Δ double mutant.The plamid shuffle technique to construct these mutant strains was performed as previously described in figure 3.2.

Figure 4.2

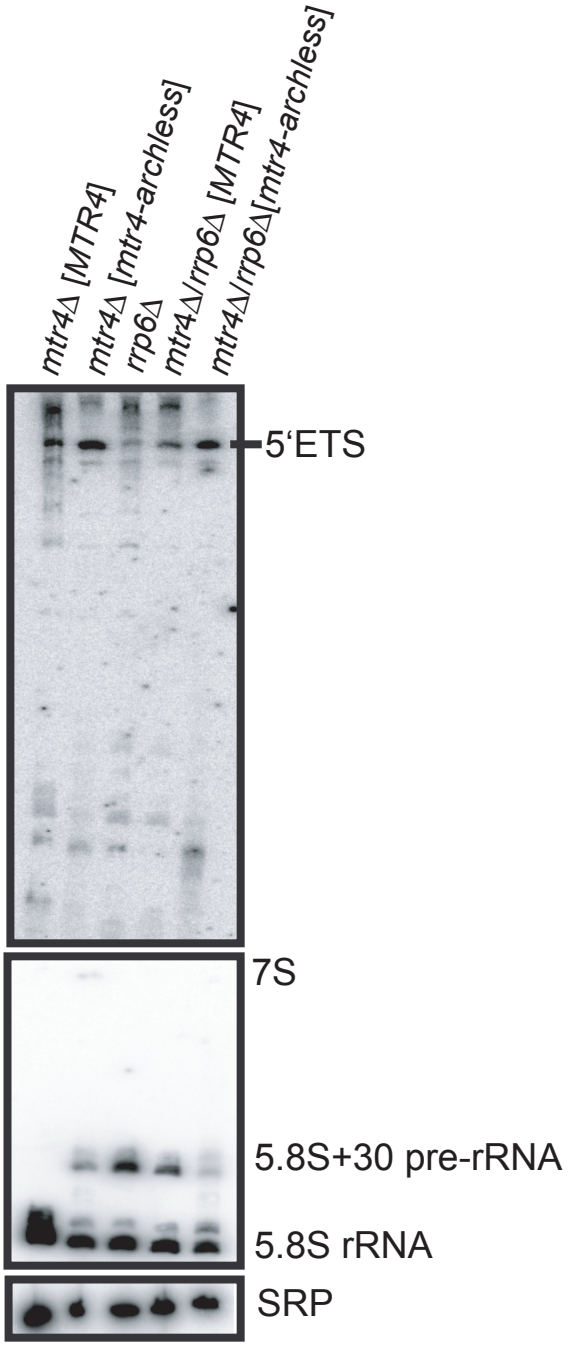

Figure 4.2 RNA analysis of the *mtr4-archless/rrp6*Δ double mutant strain:
Total RNA was isolated from the strains described, and Northern blot analysis was performed.
The *rrp6*Δ strain is the parental strain that was crossed with either the *MTR4* or *mtr4-archless* strains to create the complemented strain and the double mutant, respectively.

Figure 4.3

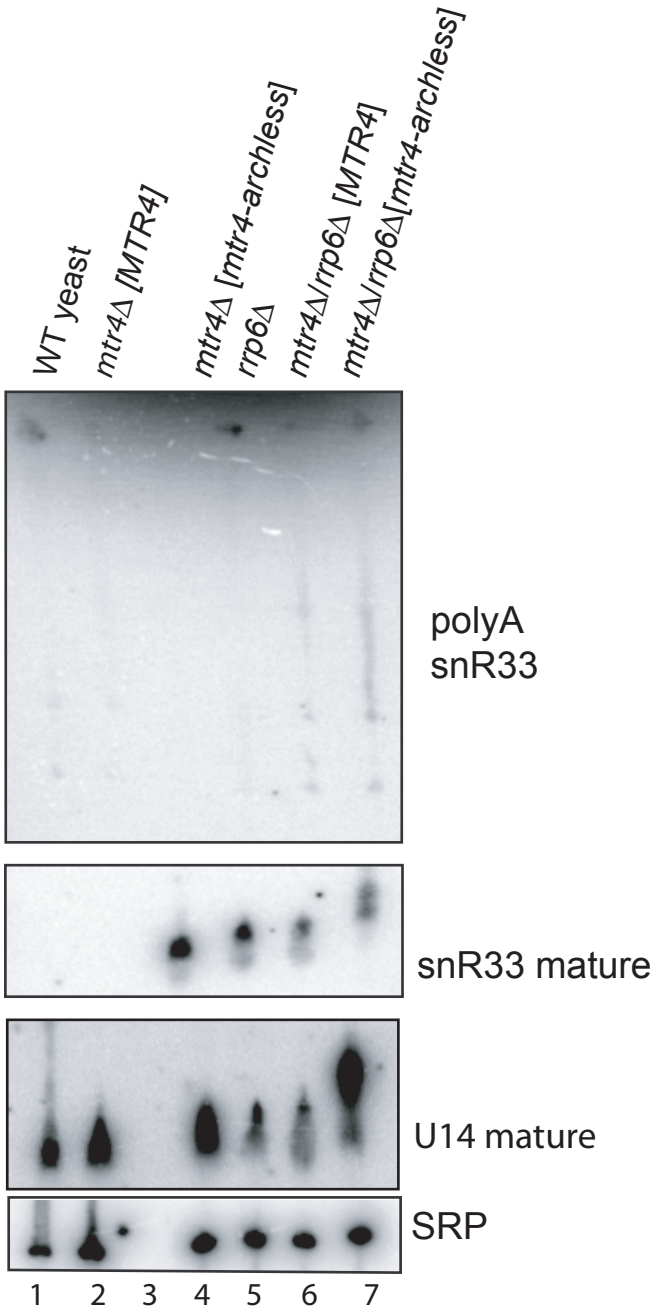

Figure 4.3 snoRNA analysis of the *mtr4-archless/rrp6*Δ double mutant
Total RNA was isolated and Northern blot analysis performed to detect extended species of two different snoRNAs.

The synthetic growth defect of the *mtr4-archless*/*rrp6Δ* is not caused by loss of catalytic activities of Rrp6.

To determine whether the slow growth defect of the *mtr4-archless* strain was exacerbated due to the loss of Rrp6's catalytic activity, the *mtr4-archless*/*rrp6*Δ double mutant was complemented with plasmids encoding catalytically inactive Rrp6 proteins. Figure 4.4 shows that the catalytically-inactive *rrp6* mutants suppress the synthetic growth defect to the levels of the *mtr4-archless* single mutant. Therefore, the synthetic growth defect observed in the *mtr4-archless*/*rrp6*Δ double mutant is not due to the loss of Rrp6's catalytic activity. These results provide further proof that Mtr4 (specifically its arch domain) and presumably Rrp6 have functions beyond their catalytic activities, and these functions are required for optimal growth.

"Overexpression of the core domains of Mtr4 is sufficient to suppress the growth defect of *rrp6Δ*

Previously, a high-copy suppressor screen showed that Mtr4 from a high-copy plasmid reduced the growth defect of the *rrp6Δ* strain (Holub et al., 2012). To further understand the genetic interactions between *RRP6* and *MTR4*, I analyzed whether this high-copy suppression required the arch domain of Mtr4. The high-copy suppressor screen was done in a different yeast background strain (W303) from the one used in our previous experiments (BY4741), because *rrp6*Δ in W303 results in a much more severe growth defect. Particularly, the *rrp6Δ* strain in the W303 background grows slowly at both 37°C and at room temperature. Thus to fully understand the genetic interactions between *rrp6Δ* and *mtr4-archless*, I expressed either wild-type *MTR4* or *mtr4-archless* from a high-copy plasmid in the wild-type and *rrp6Δ* W303 strain. Transformants were serially diluted, spotted onto plates, and incubated at room temperature 20°C, 30°C, and 37°C on media lacking uracil to select for transformants with the high-copy plasmid. Figure 4.5a shows that overexpression of wild-type and Mtr4-archless fully suppresses the growth defect of *rrp6Δ* at the non-permissive temperature, indicating that the arch domain is not necessary to restore the specific Rrp6-dependent functions that promote growth at the non-

permissive temperature. The western blot in Figure 4.5b confirms that both Mtr4 and Mtr4-archless are overexpressed at similar levels in this yeast strain."

"Overexpression of Mtr4 restores specific *rrp6Δ* RNA defects

It was previously reported that although overexpressing Mtr4 suppresses the growth defect in an *rrp6Δ,* it does not restore the defects in 5.8S rRNA and snR38 processing (Abruzzi et al., 2007). To determine whether other functions were restored in the *rrp6*Δ strain with each of the overexpressed Mtr4 versions, I performed Northern blot analysis. Total RNA was isolated from transformants for each of these strains: *rrp6Δ* overexpressing Mtr4, *rrp6Δ* overexpressing Mtr4-archless, and *rrp6Δ* with high-copy empty vector. Additionally, control strains that had the wild-type *RRP6* gene were transformed with each of the high-copy plasmids (*MTR4*, *mtr4-archless,* and empty vector) were analyzed. Overexpression of Mtr4 in the *rrp6Δ* strain did not affect the accumulation of 5.8S+30 or snR38 with short 3' extensions, as previously reported (Abruzzi *et al.*, 2007). However, in these same strains the accumulation of longer polyadenylated forms of snR33 was almost completely suppressed (Figure 4.6b, compare fourth and fifth lanes) and accumulation of 5' ETS degradation intermediates was partially suppressed (Figure 4.6a, compare fourth and fifth lanes). Overexpressing archless reduced the *rrp6Δ* defects to a lesser extent than overexpressing wild-type Mtr4 (Compare fifth and sixth lanes in Figure 4.6a and 4.6b). Overall, our results suggest that overexpressing Mtr4 in *rrp6Δ* does not affect the accumulation of snoRNA species with short 3' extensions but does suppress the accumulation of longer 3' extended species. Overexpressing *mtr4-archless* does so to a smaller extent but must suppress some other defects sufficiently to fully restore growth."

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Figure 4.4

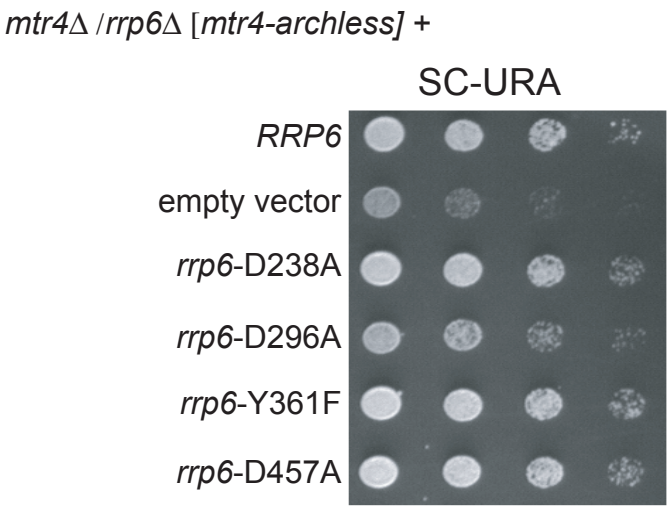


Figure 4.4 Growth rate comparison between *mtr4-archless/rrp6*Δ complemented with catalytically inactive *RRP6*:
Plasmids encoding Rrp6 mutant proteins with the catalytic residues mutated. Expression of catalytically inactive Rrp6 suppresses the synthetic growth defect of the *mtr4-archless/rrp6*Δ double mutant.

Figure 4.5

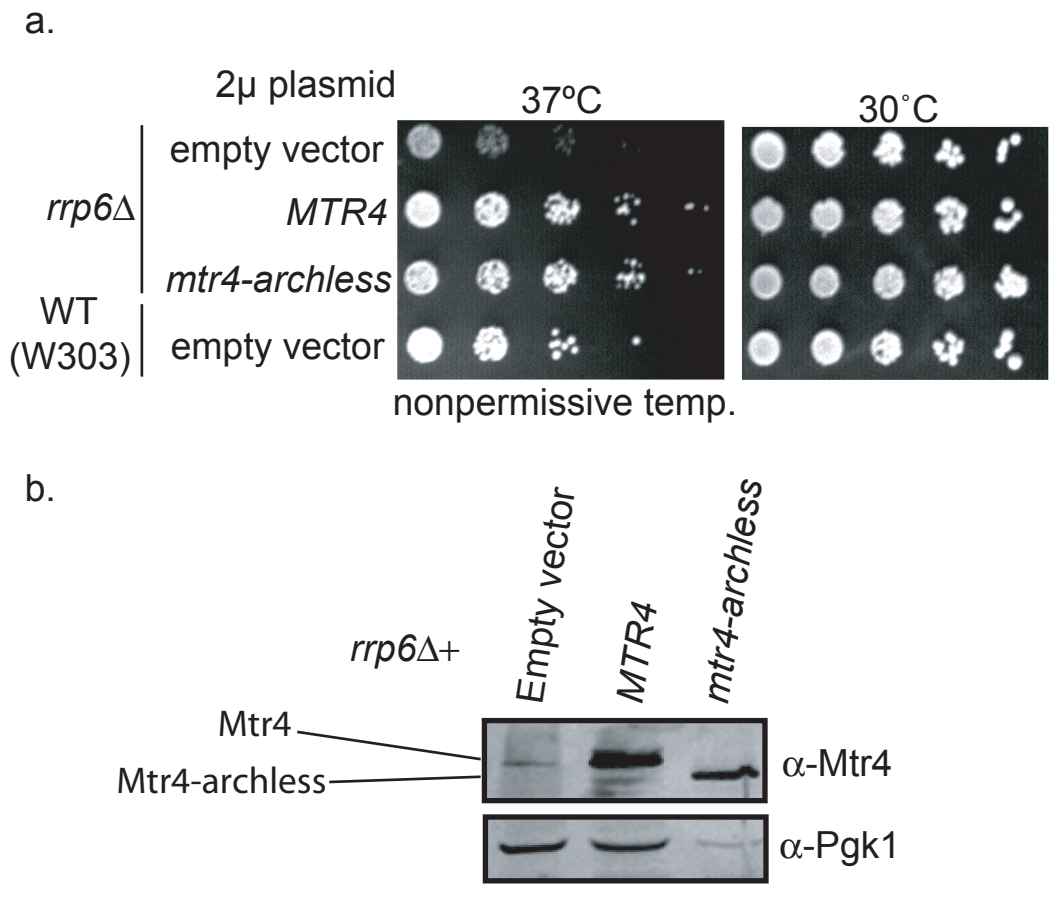


Figure 4.5 Overexpression of Mtr4 and Mtr4-archless suppresses the growth defect of an *rrp6*Δ strain: **a.** *MTR4* and *mtr4-archless* were cloned into a high copy vector to promote their overexpression. High copy plasmids were introduced into the *rrp6*Δ strain and parental wild-type strain. **b.** Western blot analysis indicates that Mtr4 and Mtr4-archless are overexpressed to similar levels in the *rrp6*Δ strain.

Figure 4.6

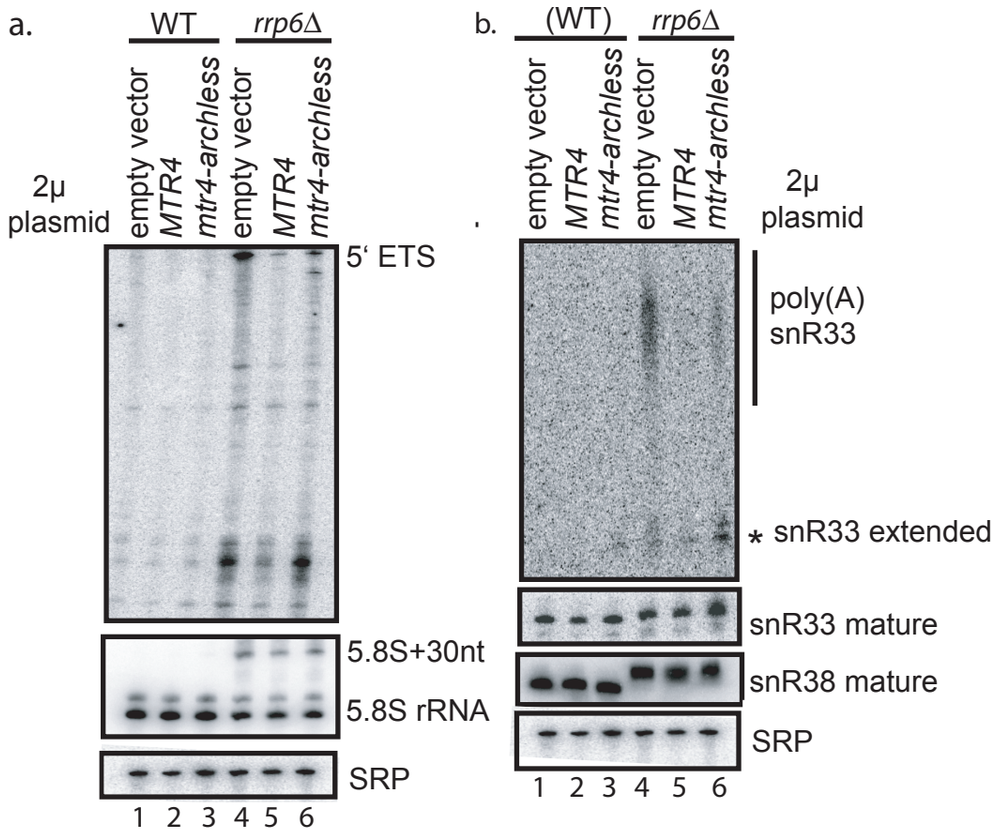


Figure 4.6 RNA analysis of the *rrp6*Δ strains overexpressing Mtr4 and Mtr4-archless:
Total RNA was isolated from the parental and *rrp6*Δ strain transformed with high copy plasmids encoding Mtr4 and Mtr4-archless. Northern blot analysis was performed as previously described.

Overexpressing Rrp6 does not restore the *mtr4-archless* growth defects, and it is toxic to the cells

Overexpressing Mtr4 and Mtr4-archless suppressed *rrp6*Δ temperature sensitivity. To test whether the converse was also true, the *mtr4-archless* strain was tested for complementation when Rrp6 was overexpressed. Figure 4.7 shows that overexpressing Rrp6 (GPD-Rrp6 (2μ plasmid)) did not restore the *mtr4-archless* slow growth to wild-type levels, however, it caused a toxic effect to wild-type and *mtr4-archless* cells. Different levels of overexpression were tested, including different combinations of high and low copy plasmids and promoters with different levels of expression (from highest expression to lowest: GPD-Rrp6 (CEN) TEF2-Rrp6 (2μ and CEN), CYC1-Rrp6 (2μ and CEN)). Only GPD-Rrp6 in the high copy plasmid, and to lesser extent, the TEF2-Rrp6 in the high copy plasmid, caused the increased growth defect in the *mtr4-archless* strain (data not shown). These results indicate that overexpressing Rrp6 does not restore the arch function in the *mtr4-archless* strain. This result suggests that the function the arch promotes occurs upstream of Rrp6 in the pathway. Furthermore, controlling Rrp6 protein levels seems to be crucial for optimal cell growth.

Figure 4.7

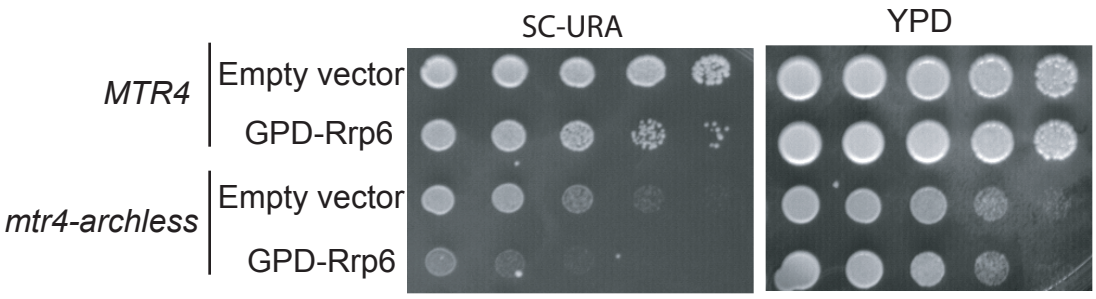


Figure 4.7 Overexpressing Rrp6 does not suppress the *mtr4-archless* growth defect:
RRP6 was cloned into a high-copy plasmid under the control of a high expression GPD promoter. Unlike the overexpression of Mtr4 in the *rrp6*Δ strain, overexpressing Rrp6 does not suppress the *mtr4-archless* strain slow growth phenotype.

CONCLUSIONS AND FUTURE DIRECTIONS

In addition to the results observed in Chapter 3, I have identified an interesting genetic interaction between Mtr4 and Rrp6. As described in Chapter 3, the arch domain of Mtr4 is required for two important Rrp6 functions, 5.8S processing and 5'ETS degradation. However, the arch domain plays no obvious role in the processing and degradation of snoRNAs. Here, I have shown that the arch domain of Mtr4 and the exonuclease Rrp6 independently promote a currently unidentified function required for optimal growth. It was also determined that defects in 5.8S processing are not the cause of the synthetic slow growth defects observed in the *mtr4-archless*/*rrp6*Δ strain. Furthermore, there is an increased accumulation of longer extended snoRNA species in the *mtr4-archless*/*rrp6*Δ double mutant. It is unclear whether these species are functional, and whether they are causing the observed synthetic growth defect in the double mutant. The increased accumulation of these mature snoRNAs in the *mtr4-archless* and *mtr4-archless*/*rrp6*Δ double mutant compared to the levels seen in the wild-type and *rrp6*Δ mutant should be further investigated. A more sensitive approach, such as qRT-PCR, should be taken when measuring the levels of mature and extended snoRNA species, since they are not extremely abundant. Previous experimental attempts using 5' end-labeling of nucleotides and Northern blot used to analyze other exosome and Rrp6 substrates, such as CUTs, did not detect these additional substrates. These substrates need to be tested through qPCR or utilizing internal labeling of the probe followed by Northern blot.

Other RNA functions disrupted in the *mtr4-archless*/*rrp6*Δ double mutant have to be further identified. There is a possibility that the synthetic growth phenotype is caused by a non-exosome function. Deep sequencing of the *mtr4-archless* and *mtr4-archless*/*rrp6*Δ double mutant, including non-poly(A) RNAs, will provide a global look at the RNA defects caused by removal of the arch domain, or in the *mtr4-archless*/*rrp6*Δ strains. Deep sequencing and tiling array analysis have been done previously for the *rrp6*Δ strain, as well as deletions and depletions of the Rrp6 cofactor, Rrp47, and other exosome subunits (Chekanova *et al.*, 2007, Kiss & Andrulis, 2010, Houalla *et al.*, 2006). Comparing the deep sequencing data obtained from our single and double mutant to the previously published data will provide a more complete overview of which RNAs require specific exosome subunits and cofactors. It will also be possible to identify which substrates affected by removal of the arch domain of Mtr4 correlate with other exosome mutants. Recently, a new technique called cross-linking and analysis of cDNAs (CRAC) was described in a publication by Schneider et al.. CRAC utilizes *in vivo* RNA-protein cross-linking followed by deep-sequencing to identify the regions in the RNA substrates that are critical for nuclease-RNA interaction. This study identified different binding patterns of interaction between exosome subunits and Rrp6. Furthermore, Mtr4 was shown to crosslink with the RNA that was threaded through the core of the exosome (Schneider et al., 2012). CRAC is a powerful tool that can be exploited to understand the function of the arch domain of Mtr4 when compared to the data obtained for Rrp6. Specifically, the *mtr4-archless* strain can be analyzed through this CRAC method in order to identify whether it loses binding to specific RNA

substrates (More detail in chapter 7 and figure 7.1). There are many genome-wide analyses for the *rrp6*Δ strain, but the RNA field lacks an unbiased global look at the *mtr4-archless* mutant. To understand the function of the arch domain, as well as the cooperative function with Rrp6, one needs to identify all of the substrates of the arch domain of Mtr4.

The slow growth defect observed in the *rrp6*Δ strain at the non-permissive temperature is not caused by the accumulation of 5' ETS or processing intermediates 5.8S rRNA and snoR33.

It is still unknown which Rrp6 functions cause temperature sensitivity in the *rrp6*Δ strain. Overexpressing the core of Mtr4 was sufficient to suppress the slow growth defect of the *rrp6*Δ. The 5.8S rRNA and the snoRNA processing are not restored in the overexpressing strains, which indicates that disruption of these two functions does not cause the slow growth defect. Moreover, overexpressing Mtr4-archless suppressed the snoRNA and 5'ETS degradation defects to a lesser extent than Mtr4. Since overexpressing both Mtr4 constructs suppresses the temperature sensitivity to wild-type levels, it is unlikely that these degradation functions are involved in the growth defects. As previously explained, a more unbiased and global approach comparing these three overexpressing strains (empty vector, Mtr4, Mtr4-archless) would provide a crucial insight into the function that causes the slow growth defect in the *rrp6*Δ strain.

RNA metabolism involve some pathways with overlapping functions while others require specific ribonucleases

The dynamic interaction between Rrp6 and Mtr4 shows that the exosome-associated proteins are involved in many redundant pathways of RNA decay and processing. Specific pathways, namely 5.8S processing, require both Rrp6 and the arch domain of Mtr4. Other RNA substrates, for example 5'ETS, can be degraded by either Rrp6, or cleaved by another nuclease through activation by the arch domain of Mtr4. In the second case, Mtr4 does not work cooperatively with Rrp6, indicated by the different pattern of 5'ETS degradation. Additionally, I can exclude that this unknown ribonuclease is Rrp44 because the pattern of 5'ETS degradation intermediates in the *rrp44-exo-* strain does not occur in the *rrp6*Δ or *mtr4-archless* strains. Furthermore, the double mutant (*mtr4-archless*/*rrp6*Δ) accumulates additional cleavage intermediates for the 5'ETS substrate. Analysis of one specific exosome degradation substrate, 5' ETS, confirms that exonucleases are redundant in the degradation of RNA substrates, and that there are specific targeted regions in the RNA substrate prone to cleavage by different ribonucleases.

Exosome subunits and cofactors must be tightly regulated for efficient cell functions

The differential growth and RNA intermediates observed in strains overexpressing Mtr4, Mtr4-archless, or Rrp6 indicate that the levels of these proteins must be tightly regulated. Overexpressing Mtr4 and Mtr4-archless restores normal growth in the *rrp6*Δ strain; this indicated that increased levels of Mtr4 can

activate a redundant function by ribonucleases or other proteins substituting for Rrp6. Although, *mtr4-archless* does not cause a dominant negative phenotype, overexpressing Mtr4-archless in the wild-type and *rrp6*Δ strains causes the accumulation of additional extended RNA species of snoRNAs and 5.8S processing (Figure 4.7, see bands with an asterisk and data not shown). Furthermore, overexpressing Rrp6 is toxic to the cell, possibly due to increased or misregulated cleavage by Rrp6. Importantly, the molecules for each exosome subunit per cell vary significantly from one exosome subunit to the next (Ghaemmaghami *et al.*, 2003). Overall, the protein levels of Mtr4 and Rrp6, and likely the rest of the exosome subunits, seem to be extremely important for cell function, as well as, understudied. It would be interesting to learn about the different expression levels of the exosome subunits, Mtr4, and Rrp6 under different growth conditions. Understanding the regulation of these proteins will shed light on their specific contributions in the complicated RNA degradation and processing pathway. It may even be possible to detect sub-complexes of the exosome if analyzed under different growth conditions.

Chapter 5: Mtr4 uses multiple contacts to interact with the TRAMP and exosome subunits

INTRODUCTION

A Yeast Two-hybrid screen identified protein-binding partners of Mtr4, Trf4 and Trf5

Mutants of *mtr4* had been identified in two independent reports to cause rRNA defects similar to exosome mutations (de la Cruz et al., 1998a, Kadowaki et al., 1994). To further characterize Mtr4 and find its binding partners, a Yeast Ywo-Hybrid (Y2H) screen was performed using Mtr4 as bait (LaCava *et al.*, 2005). The only two significant hits identified were Trf4 and Trf5. The Y2H analysis was able to narrow down the putative binding region of Trf5 (53-199) (more of this in Chapter 6). However, since it used the entire *MTR4* ORF, the screen did not provide any evidence regarding the Trf4/5-binding region of Mtr4. Air1 and Air2, the remaining TRAMP subunits, were later discovered through immunoprecipitation experiments of epitope-tagged Mtr4 and Trf4/5 followed by Mass spectrometry (Vanacova et al., 2005).

To identify the TRAMP and exosome binding regions within Mtr4 *in vivo*, N- and C-terminal truncations, as well as the Mtr4-archless construct, were analyzed using the same Y2H technique. Recently, a manuscript was published showing that ΔN truncations of Mtr4 complement an *mtr4*Δ while disruption of the helicase core does not. Equivalent truncations N- and C-terminal truncations also showed that Mr4 interacts with Trf4 through its RecA1 domain.(Holub et al., 2012) The data from Vanacova's lab agrees with the Y2H and complementation experiments described in this chapter.

Similarly, N- and C-terminal truncations of Mtr4 were tested against the cap subunits of the exosome. Rrp4, Rrp40, and Csl4 have putative RNA binding domains and are positioned at the site of RNA entry in the channel formed by the PH ring of the exosome (Liu *et al.*, 2006, Bonneau F., 2009, Lorentzen *et al.*, 2008). To date, there have not been any other studies analyzing the interaction of Mtr4 with the exosome subunits. In this chapter, I attempt to determine the protein regions Mtr4 required to physically associate with the cap subunits of the exosome. Furthermore, this will be the first experimental evidence of direct physical association between Mtr4 and the core exosome *in vivo*.

The arch domain of Mtr4 was shown to have an important role in the exosome-mediate functions of Mtr4. To determine whether the *mtr4-archless* growth defect is due to the loss of some protein-binding partner, Mtr4-archless was also included in the Y2H analysis with Trf5 and the exosome cap proteins. The arch domain of Mtr4 is not involved in promoting Trf5 (TRAMP) binding, however it does have a role in modulating Mtr4-TRAMP interaction. The dynamics between Mtr4-archless and Trf5 will be discussed.

RESULTS

The arch domain of Mtr4 is not required for Trf5-Mtr4 interaction, however it may modulate the affinity of this association

As previously described, removing the arch domain of Mtr4 causes a variety of RNA and growth defects. A possible cause for the slow growth and RNA aberrancies of the *mtr4-archless* strain could be a disruption in binding to another protein partner. To test whether Mtr4-archless continues to interact with the TRAMP subunits, Y2H analysis was utilized. Mtr4 and Mtr4-archless were translationally fused to the Gal4 DNA binding domain, while one of the subunits of TRAMP, Trf5, was fused to the Gal4 activation domain. A positive interaction between the two proteins tested can be qualitatively identified by assaying the growth of the Y2H diploid strains containing both fused proteins on media lacking adenine. Likewise, the strength of this interaction can be semi-quantitatively measured by adding increasing amounts of the competitive inhibitor of His3 protein, 3-amino triazole (3-AT). Furthermore, using the third reporter in these Y2H strains, LacZ, physical interaction between the two partners can be accurately quantified using β-galactosidase assay. All three of these reporters will assay the extent of the interaction between Mtr4, Mtr4-archless and Trf5 was determined. The controls used for this initial Y2H analysis include the DNA checkpoint complex proteins Mec3 and Rad17 as a positive control, and empty vectors as negative controls. The Y2H assay has a propensity for false positives, thereby the experiment also includes additional negative controls, Trf5 + EV, Mtr4+ EV, and Mtr4-archless + EV.

Figure 5.1 shows that removing the arch domain of Mtr4 does not hinder its ability to associate with Trf5, in fact it promotes tighter binding between the two proteins. The Mtr4-archless/Trf5 strain grows well in media lacking adenine, and continues to grow at very high concentration of the inhibitor 3-AT. The Mtr4/Trf5 diploid is grows up to concentrations in media with 50 mM 3-AT, while Mtr4-archless/Trf5 does so in media with 100 mM of 3-AT. The Mtr4-archless/Trf5 interaction can be quantified as three times stronger than the interaction between Mtr4 and Trf5 based on liquid β-gal assays. The increased affinity between Mtr4-archless and Trf5 does not seem to be due to structural changes in Mtr4 because previously published reports have shown similar affinities in the mutant and wild-type protein with Trf5 *in vitro* (Weir et al., 2010). Additionally, an *in vivo* pull-down experiment using tandem affinity purified Mtr4-archless showed that Mtr4-archless did not lose interaction to any of the TRAMP subunits tested (Trf4, Air1, and Air2)(Holub et al., 2012). The increased interaction between Mtr4-archless and Trf5 was not discovered in either of these two published results.

To confirm that the tighter association is not due to increased expression of the Mtr4-archless in the Y2H strain, the protein levels of Mtr4 and Mtr4-archless were assessed through western blotting. As shown in figure 5.2 both constructs are expressed at similar levels in the Y2H strain, as seen with the BY4741 strain described in Chapter 3. The fact that Mtr4-archless is expressed at similar levels and it has similar affinity to Trf4 *in vitro*, indicates that there may be a third protein mediating this dynamic interaction *in vivo*.

Figure 5.1

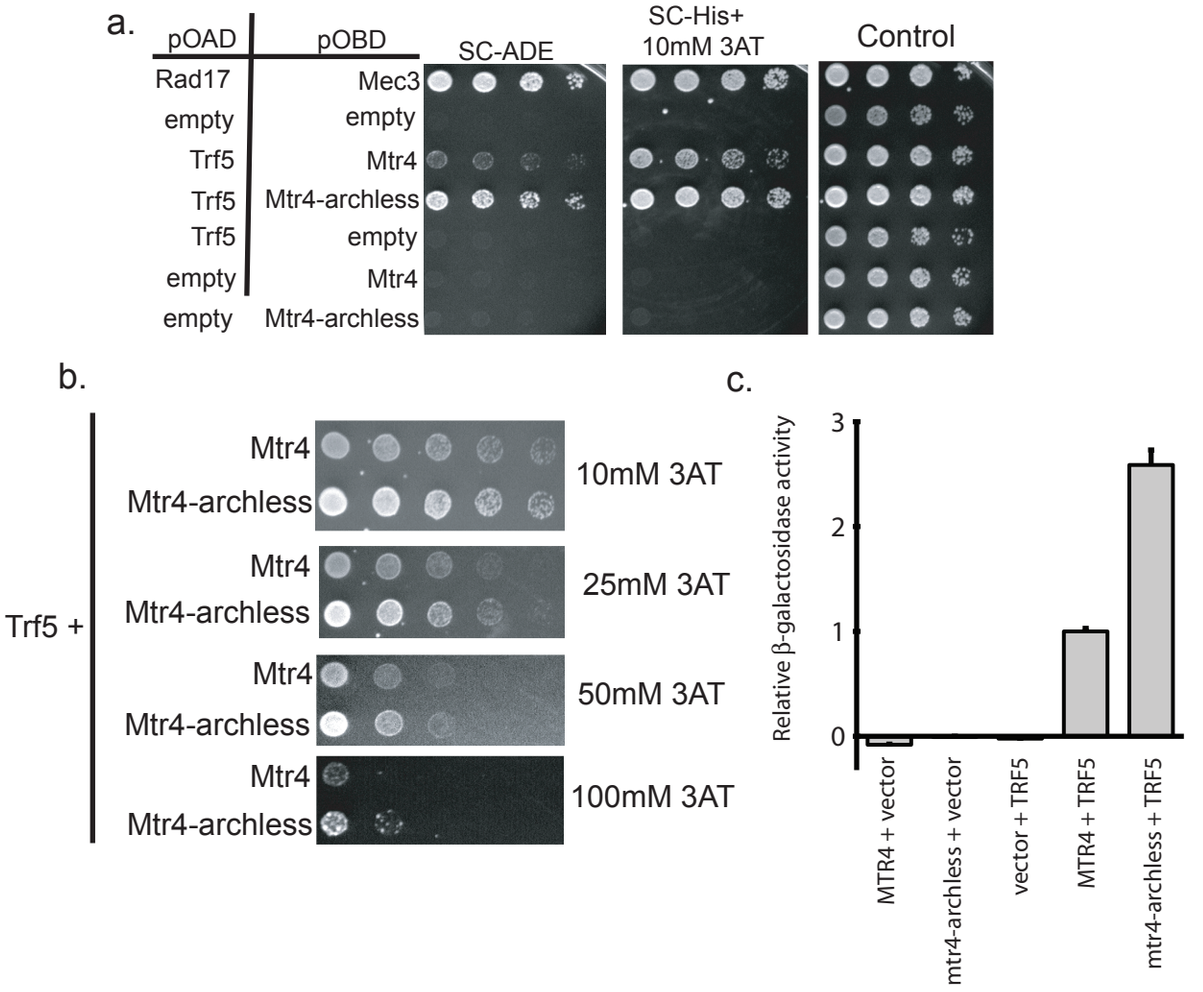


Figure 5.1 Mtr4-archless interacts with higher affinity than Mtr4 to Trf5:
Y2H analysis was performed using all three methods described in the text **a.** qualitative growth assay **b.** semi-quantitative competitor growth assay and **c.** quantitative B-galactosidase assay. Briefly, opposite mating type Y2H strains were transformed each with the tester plasmid encoding the interacting partner. Transformants were crossed and diploids were selected. Diploids were serially diluted and plated on the selective plates, or grown in liquid cultures. Pictures were taken at day 3.

Figure 5.2

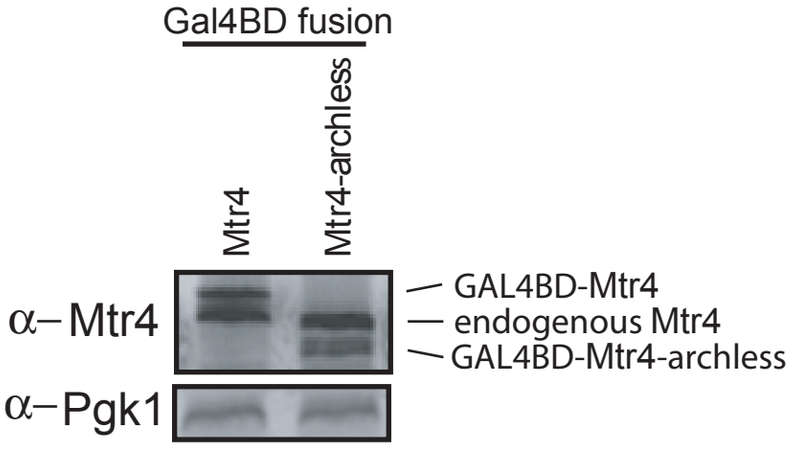


Figure 5.2 Gal4BD-Mtr4 and Gal4BD-Mtr4-archless are expressed at similar levels:
Western blot analysis was performed in the Y2H strains to confirm that the increased interaction of the Gal4BD-Mtr4-archless was not due to an artifact, such as higher protein expression compared to the full-length protein in the Y2H strains.

RecA1 and RecA2 domains of Mtr4 interact with the TRAMP subunit Trf5

The arch domain is not required for promoting the Mtr4-Trf5 interaction. To identify the region in Mtr4 that promotes Trf5 interaction, terminal truncations of Mtr4 were tested against Trf5 in the Y2H analysis. Figure 5.3 and 5.4 depicts the cartoon images of the truncations of Mtr4 next to the Y2H growth assays, including the positive and negative controls. The physical association between Mtr4 and Trf5 was maintained as long as the RecA1 domain was intact. Truncations at the C-terminus of the protein did not promote the interaction with Trf5, possibly because the tertiary structure of the helicase core is disrupted. There was a slight interaction between ΔC874-Mtr4 and ΔC326-Mtr4 with Trf5, indicating that there may be multiple contacts between Mtr4 in its tertiary structure and that exposing a larger surface on the RecA1 and A2 domains may promote a better interaction with Trf5. RecA1, and possibly together with the RecA2 domain, may be forming an interface where Trf5 associates. These data correlate with the immunoprecipitation experiment in the Vanacova paper, where it showed that deletions disrupting RecA1 and RecA2 in Mtr4 no longer interaced with Trf4 (Holub et al., 2012). Using antibodies against the Gal4 DNA binding domain, I show that most of the truncations created for this experiment are expressed in the cell (Figure 5.5).

Figure 5.3

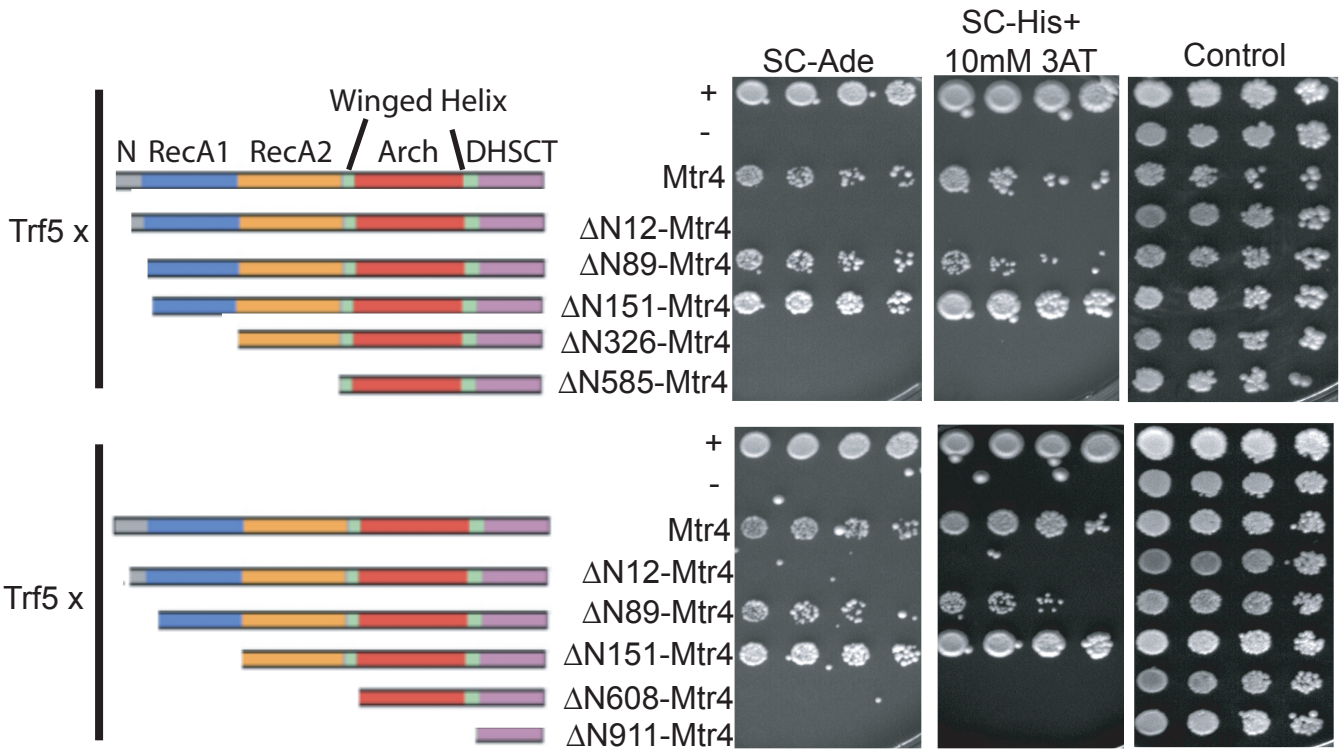


Figure 5.3 N- terminal truncations of Mtr4 show that there are mutiple contacts between Mtr4 and Trf5:
N-terminal truncations were constructed and tested for binding with Trf5 using the Y2H growth assays.

Figure 5.4

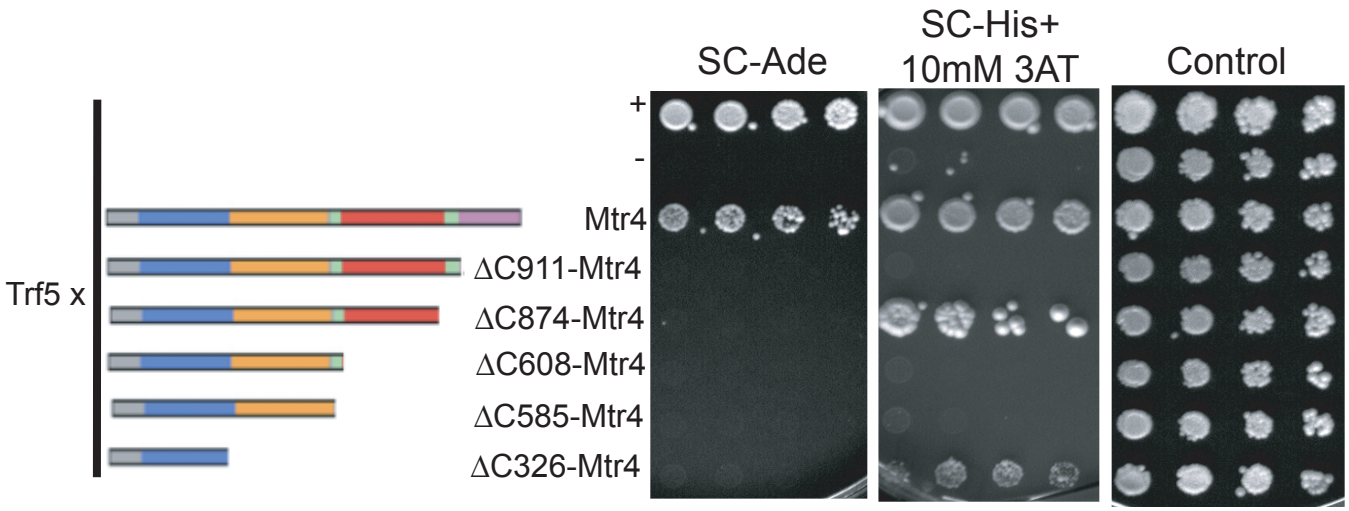


Figure 5.4 Y2H analysis of C-terminal truncations of Mtr4 with Trf5

Y2H analysis using C-terminal truncations of Mtr4 against Trf5. None of the C-terminal truncations promote interaction with Trf5. Plates are show at day 4 for YPD and day 7 for SC-HIS 3AT.

Figure 5.5

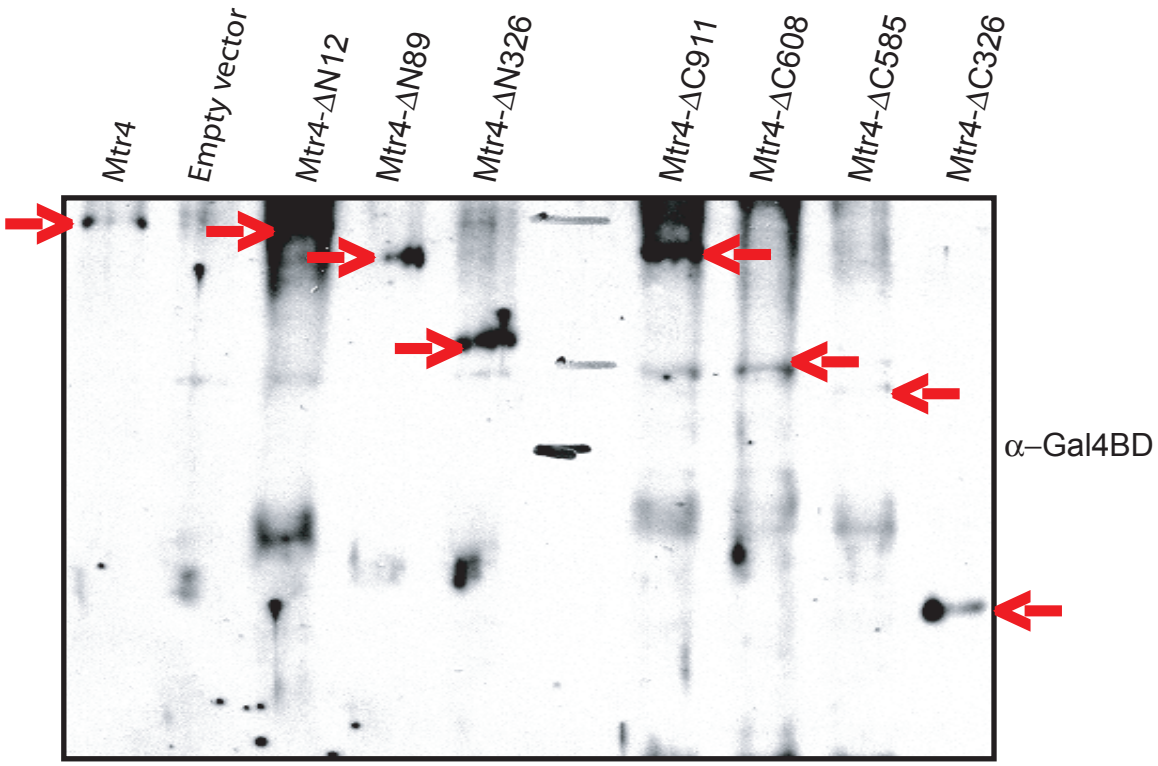


Figure 5.5 ΔN and ΔC constructs of Mtr4 mutant protein are expressed in the Y2H strain:
Arrows point at the expected size and band corresponding to the truncated protein. Cross-reactivity has been previously observed when using antibodies against Gal4BD.

The N-terminal region of Mtr4 is dispensable for the essential function of Mtr4

A surprising observation was the lack of interaction between ΔN12-Mtr4 and Trf5 (Figure 5.3). The N-terminal region of Mtr4, particularly the first 12 amino acids, is highly conserved (5.12). All three solved crystal structures of Mtr4 lack roughly the first 90 residues, making this an uncharacterized region. The fact that ΔN12-Mtr4 lost interaction with Trf5 indicates that this flexible and conserved region may have important implications in TRAMP and Mtr4 function. To test whether N-terminal truncations of Mtr4 had defects in Mtr4 function, ΔN12, ΔN89-*mtr4*, and ΔN12-*mtr4-archless* were constructed. ΔN12 and ΔN89-*mtr4* complement the *mtr4*Δ to wild-type levels, while ΔN12-*mtr4-archless* complemented the deletion strain to *mtr4-archless* levels (Figure 5.6). Therefore, the N-terminus of Mtr4 does not seem to be required for optimal growth, nor does it exacerbate the archless growth defect. RNA defects were also analyzed through northern blotting. Deletions at the N-terminus of Mtr4 do not cause any obvious processing or degradation defects (Figure 5.7). The cleavage product 5'ETS may be increased slightly, however there have been varying reports of how variable 5' ETS levels can be from experiment to experiment. Moreover, deletion of the N-terminus combined with removal of the arch do not cause any significant RNA defects beyond the ones seen in the *mtr4-archless* mutant strain (Figure 5.7). It is unclear why the ΔN12-Mtr4 loses interaction with Trf5 in the Y2H analysis, or whether this is an artifact of the experiment. Nevertheless, this region is dispensable for the essential function of the exosome, as well as 5.8S rRNA processing, snR33 processing and degradation, and degradation of the 5'ETS.

Figure 5.6

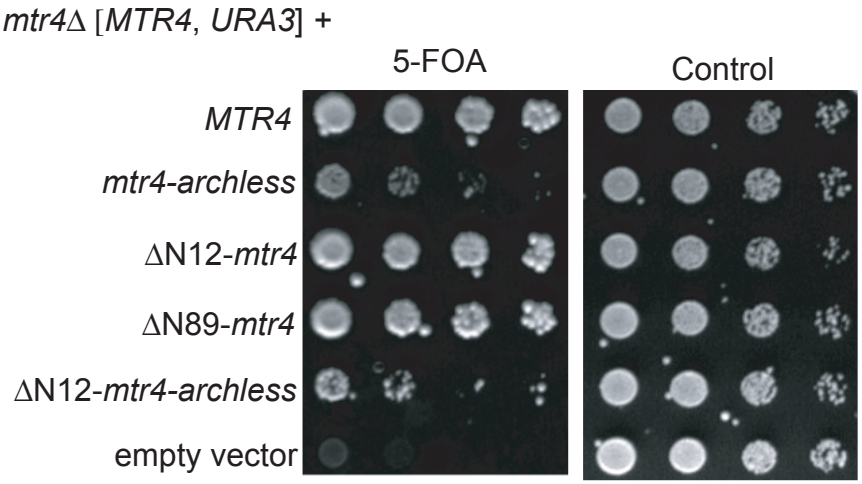


Figure 5.6 The N-terminus of Mtr4 is not required for the essential function of Mtr4:
Complementation analysis of ΔN-terminal truncations of Mtr4 indicate that the N-terminus is not required for optimal growth, since ΔN12-*mtr4* and ΔN89-*mtr4* complement the *mtr4*Δ strain to wild-type levels.

Figure 5.7

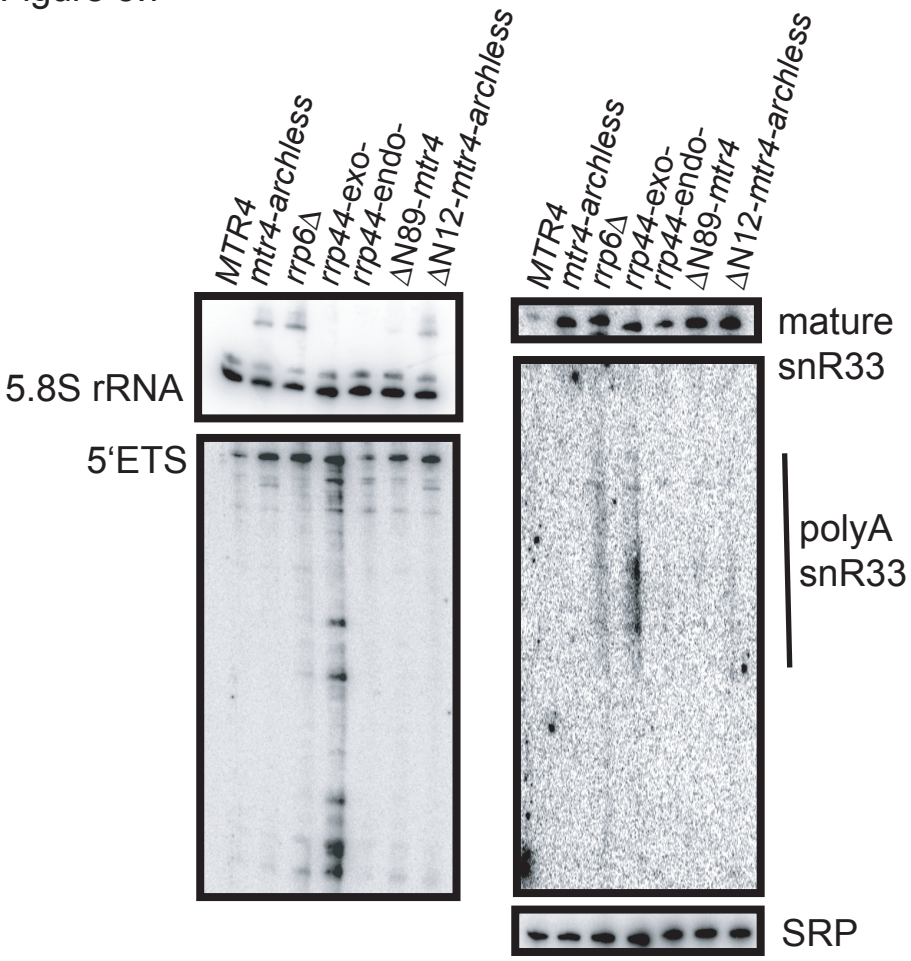


Figure 5.7 There are no obvious RNA defects in the Δ*N-mtr4* mutant strains:
RNA was isolated from the cured ΔN12 and ΔN89-*mtr4* strains and compared to *mtr4-archless* and exosome mutants. Further, ΔN12-*mtr4-archless* does not have any synthetic RNA defects in addition to the single *mtr4-archless* RNA defects.

Mtr4 utilizes multiple regions to interact with the exosome cap subunits, Csl4, Rrp4, and Rrp40

The N- and C-terminal truncations, as well as the Mtr4-archless were also tested for interaction with each of three exosome cap subunits through Y2H as previously described. Figure 5.8 shows that although most N- and C-terminal truncations of Mtr4 have some growth in the selective Y2H plates, only the full-length protein promotes a significant level of protein-protein interaction with Csl4. In the case of Rrp4, the only truncation that is still able to interact with the exosome protein is ΔN12-Mtr4 (Figure 5.9). Additionally, most of the truncations of Mtr4 do not maintain the helicase-Rrp40 interaction. ΔN- and ΔC-terminal truncations that somewhat expose the RecA1 and RecA2 domains (namely ΔN326 and ΔC326) seem to continue to make modest contact with the exosome protein, however not to the levels of the wild-type protein or archless. Furthermore, this is the first report of Mtr4/exosome interaction *in vivo*. Interestingly, when tested with the exosome cap subunits, Mtr4-archless promotes a more robust interaction in comparison to its wild-type counterpart (Figures 5.8, 5.9 5.10). A summary with the interaction profile of the different Mtr4 truncations is shown in figure 5.11.

Figure 5.8

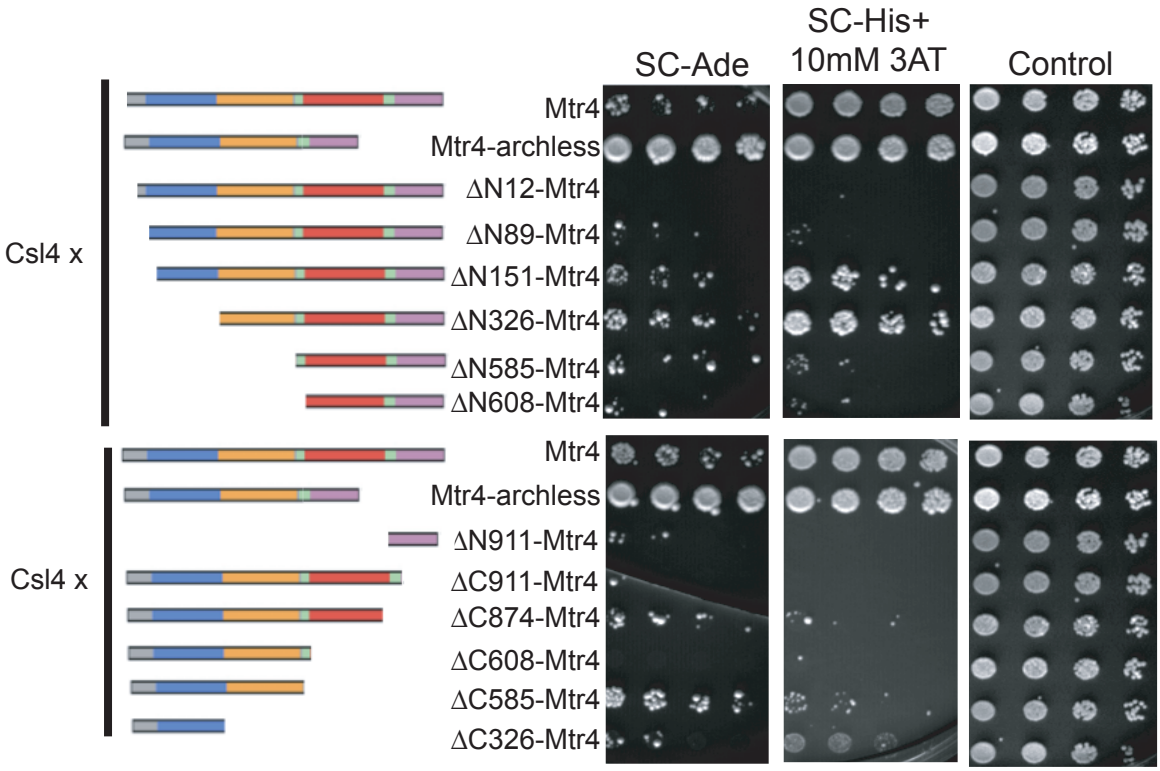


Figure 5.8 ΔN- and ΔC-terminal truncations of Mtr4 show multiple contacts between Mtr4 and the exosome cap binding protein, Csl4:
Similarly to the Y2H analysis between mutants of Mtr4 and Trf5, interaction with Csl4 was tested.

Figure 5.9

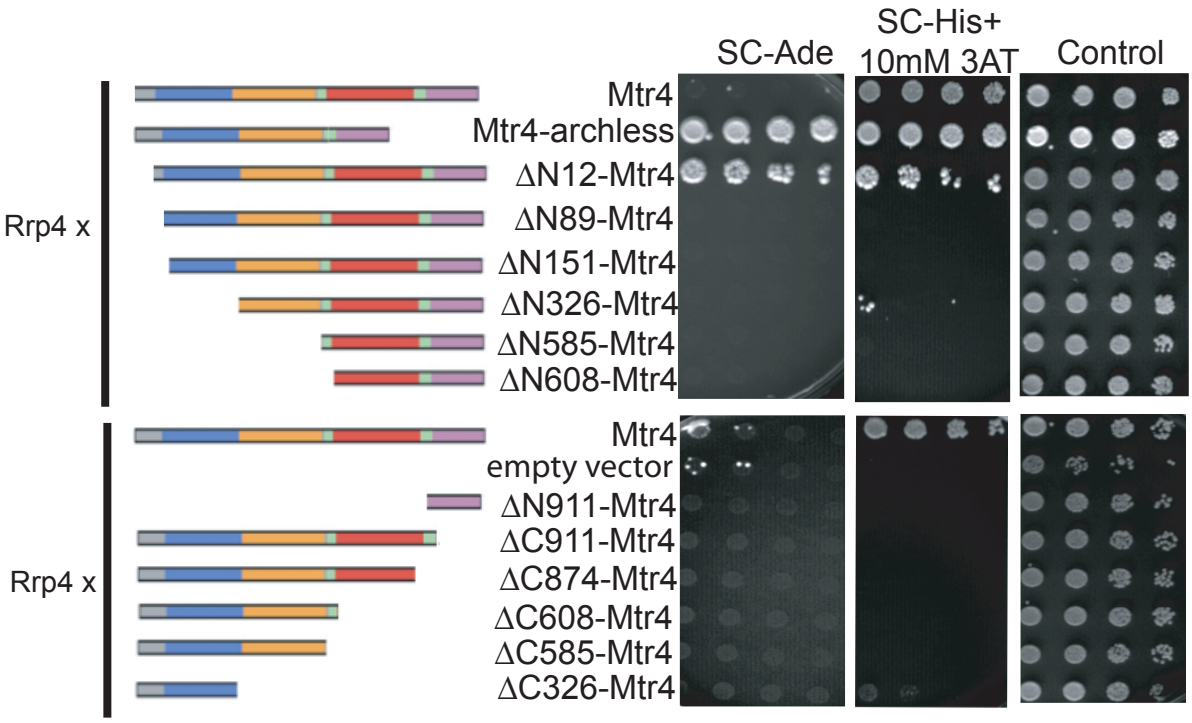


Figure 5.9 N- and C-terminal truncations of Mtr4 disrupt binding with exosome protein Rrp4:
Y2H analysis was also used to test interaction with Mtr4 and RNA bindind protein Rrp4.

Figure 5.10

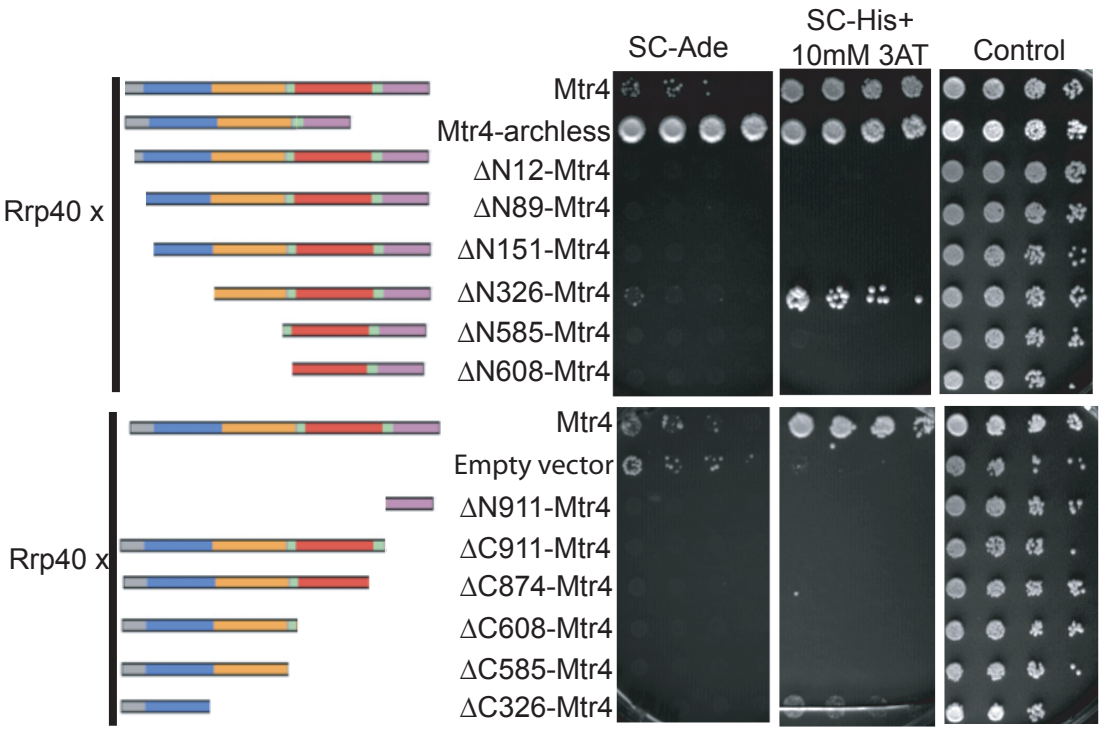
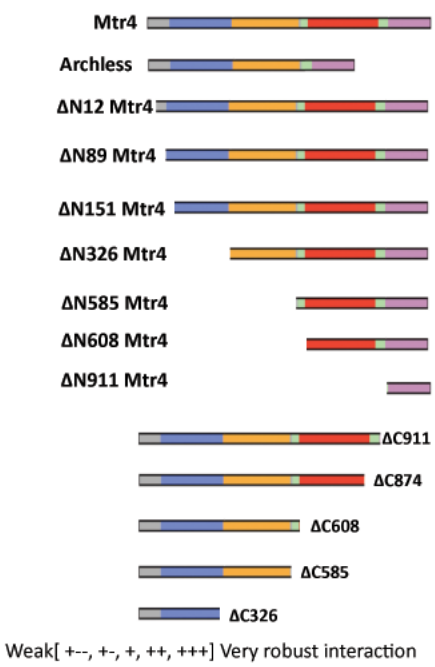


Figure 5.10 N- and C-terminal truncations of Mtr4 tested for interaction with Rrp40

Figure 5.11



	Trf5	Csl4	Rrp4	Rrp40	Expression
Mtr4	++	+	+	+	Yes
Archless	+++	++	++	++	Yes
ΔN12 Mtr4	-	-	+	-	Yes
ΔN89 Mtr4	++	-	-	-	Yes
ΔN151 Mtr4	+++	+-	-	-	Yes
ΔN326 Mtr4	-	+	-	+-	Yes
ΔN585 Mtr4	-	+--	-	-	Yes
ΔN608 Mtr4	-	+--	-	-	Yes
ΔN911 Mtr4	+--	-	-	-	Yes
ΔC911	-	-	-	-	Yes
ΔC874	-	-	-	-	No
ΔC608	-	-	-	-	No
ΔC585	-	+-	-	-	Yes
ΔC326	+-	+--	+--	+-	Yes

Figure 5.11 Summary of the Y2H interactions between ΔN and ΔC-Mtr4 and Trf5, Csl4, Rrp4, Rrp40

CONCLUSIONS AND FUTURE DIRECTIONS

Mtr4 uses different regions of the protein to make multiple contacts with Trf4/5 and exosome subunits

The Y2H analysis described here as well as published immunoprecipitation and *in vitro* reconstitutions (Holub et al., 2012, Weir et al., 2010) have shown that Mtr4 uses specific residues where it interacts with TRAMP subunits, Trf4/Trf5, and others where it interacts with each exosome subunit. For Trf5, RecA1 and RecA2 domains seem to be important for establishing Trf5 association. Csl4 appears to make multiple contacts with Mtr4, but requires the folded tertiary structure of the helicase core to interact fully, and it does not require the first 12 amino acids of the N-terminus. Rrp4 and Rrp40 make different contacts with Mtr4 than with Csl4, both requiring the N-terminus of Mtr4. However, for all three exosome cap proteins it is crucial that the entire tertiary structure of the Mtr4 is maintained for a stable interaction. It is unclear, however, whether this interaction between Mtr4 and Trf5 is direct. Biochemical assays, using purified proteins, should be used to determine this type of interaction.

The N-terminus of Mtr4 may have important implications for Mtr4 function, but it is not involved in growth promoting function of Mtr4

It is still unclear what contributions the N-terminus of Mtr4 provides. It is very likely that it contributes to specific protein-protein interaction, as seen with Trf5, Rrp4, and Rrp40, which lose Y2H interaction with Mtr4 upon the first N-terminal truncation. N-terminal truncations of Mtr4, as shown here, do not have

growth or RNA defects, nor do they exacerbate the archless growth phenotype. An important follow-up experiment would include testing the Trf5-TAP construct in the ΔN12-*mtr4* strain and confirming that ΔN12-Mtr4 truly loses interaction with Trf5.

An alternative approach to identifying the function of the N-terminus of Mtr4 could include a less drastic change than the deletion of the entire N-terminus. Site-directed mutagenesis of specific conserved residues in the N-terminus of Mtr4 may slightly affect the function of the N-terminus without activating other redundant mechanisms in Mtr4. For example, by disrupting just the acidic residues such as the aspartic acids and the glutamic acid, depicted in red in the weblogo in figure 5.12, in order to determine whether the N-terminal function of Mtr4 is disrupted. Conversely, the hydrophobic residues, depicted in black, can also be disrupted. These point mutants can then be analyzed by complementation of the Δ*mtr4* and Δ*mtr4*/Δ*rrp6* strains, northern blot analysis of RNA intermediates, and protein-protein interaction experiments. Finally, compensatory mutations should restore any affected Mtr4 functions and will further conclude that a specific fold in the N-terminus of Mtr4 contributes to its function.

There are several lines of evidence indicating that the N-terminus of Mtr4 is contributing to Mtr4's function. First, the N-terminus is highly conserved throughout Mtr4 homologs from different species. The N-termini of other fungi, such as *Schizosaccharomyces pombe*, *Ustilago maydis*, and *Neurospora crassa* also contain the hydrophobic residues flanked by the acidic amino acids. The Mtr4 of other non-fungal eukaryotes such as *Drosophila melanogaster*, *Trypanosome brucei*, *Caenorhabiditis elegans*, and humans, also displays a similar amino acid pattern of

hydrophobic and acidic residues (see alignment in figure 5.12). Second, the data in this chapter show that the N-terminus is indeed important for the physical associations of Mtr4 to its binding partners. Third, the N-terminus of Mtr4 was disordered in all three published crystal structures, indicating that it may be a flexible component of Mtr4's structure. The N-terminus of Mtr4 may be providing the rest of the protein with an extended arm that contributes to the protein-protein interaction of Mtr4. It will be interesting to identify how the N-terminus of Mtr4 is contributing to this protein-protein interaction, and the N-terminus should be further analyzed.

Figure 5.12

a.

```
ScMtr4     1 ---MDSTDLFDVFEETP--------------------------
NcMtr4     1 -----MDDLFEVFEEQPRAQKKRKASPDVEMADGAAAAAPVANA-
CnMtr4     1 ------------------------MSSSAPSDPPLSASSLTVKH
UmMtr4     1 ---MDPDGLFDFLAPAEAAPSPAASSGSSPSKKQRKRSKAAAAS-
SpMtr4     1 MSENSTDSKNFQFSEGSRESSND---------------------
AtMtr4     1 ---------------------------------------------
DdMtr4     1 ---MEDDDLFSVFDTKKTNKNKRTREATAAADEKENGKWIKPEES
HsMtr4     1 MADAFGDELFSVFEGDSTTAAGTKKDKEKD---------------
DrMtr4     1 MADAFGDDLFSVFDEEQAGSS------------------------
CiMtr4     1 -MAGFGDEIFNVFDEESDEKS------------------------
DmMTR4     1 ---MDIEELFDCFDEVTPSVPPPALNQEEKPSGSSASKKGNKRQ-
CeMtr4     1 -----MADLFDEFIEPGVKNEIMDVEEAPENPMEKLDSAKKRQAR
Tb         1 ---------------------------------------------
Tg         1 -MADAVDDLFAVFGGGDSSSSLGSSVESPNIAVGPGGESASACRE
```

b.

Figure 5.12 Sequence alignment and conservation patterns of the N-terminus of Mtr4:
a.The Mtr4 of different species were aligned and the highly conserved residues have been marked.
b. Weblogo depicts the level of conservation of each residue. Bigger letters indicates a high level of conservation.

Chapter 6: The Mtr4-TRAMP interaction is not essential for life

INTRODUCTION

After transcription, RNAs undergo several different modifications and cleavages before becoming fully mature RNAs. The pathways involved in these post-transcriptional modifications are tightly regulated. At the same time, a variety of surveillance mechanisms exists to ensure the fidelity of this RNA maturation process and the gene expression in the cell. The RNA exosome complex is a major player in several of these surveillance mechanisms in the nucleus and in the cytoplasm of eukaryotic cells. Specifically, the exosome requires a complex of proteins called the TRAMP complex to perform these surveillance functions (LaCava et al., 2005).

TRf4/5 **A**ir1/2 **M**tr4 **P**olyadenylation (TRAMP) Complex is composed of three proteins, one non-canonical poly(A) polymerase (Trf4/5), one RNA binding protein (Air1/2), and the RNA-dependent ATPase Mtr4 (LaCava et al., 2005). There are three different types of TRAMP; TRAMP4 refers to a TRAMP complex containing the polymerase Trf4, TRAMP5 describes the complex containing Trf5 (Houseley & Tollervey, 2006). TRAMP4 can further associate with one of two RNA-binding proteins, Air1 or Air2. TRAMP5 only associates with Air1 (Houseley & Tollervey, 2006, Wyers et al., 2005).

The TRAMP complex is involved in degrading aberrant or premature forms of rRNAs, snoRNAs, snRNAs, tRNAs, and mRNAs (Wang et al., 2008, Wyers et al., 2005, Kadaba et al., 2004, Vanacova et al., 2005, LaCava et al., 2005, Davis & Ares, 2006, Egecioglu *et al.*, 2006, Houalla et al., 2006). Together with TRAMP, the exosome also targets two types of noncoding RNAs with unknown functions: the

capped and noncoding Cryptic Unstable Transcripts (CUTs), and the stable, unannotated transcripts (SUTs)(David *et al.*, 2006, Wyers et al., 2005).

Individual subunits of the TRAMP seem to also function in pathways independently of the rest of the TRAMP complex. For example, Mtr4 promotes important processing reactions by the exosome, such as 5.8S rRNA and snoRNA maturation, discussed in Chapter 3 and 4 (de la Cruz et al., 1998a, van Hoof et al., 2000b). Additionally, a mutation of *TRF4* was originally identified to be synthetic lethal with a deletion of the gene encoding DNA topoisomerase I (*top1*)(Castano *et al.*, 1996a). DNA **t**opoisomerase I **r**elated **f**unction **4/5** (Trf4/5) have subsequently been shown to be involved in other DNA metabolic events, such as chromatid segregation, DNA condensation, and telomeric cleavage (Castano et al., 1996a, Castano *et al.*, 1996b, Wang *et al.*, 2000, Edwards *et al.*, 2003, San Paolo *et al.*, 2009). Furthermore, Trf4 is also involved in heterochromatin silencing, intron degradation, and rDNA copy number control (Berretta *et al.*, 2008, Buhler *et al.*, 2007, Camblong *et al.*, 2007, San Paolo et al., 2009).

TRF4 and *TRF5* are duplicated genes that encode proteins with 56% identity. Previous studies have focused on Trf4, however Trf5 seems to exhibit a level of substrate specificity for TRAMP and the exosome (San Paolo et al., 2009). While strains with single *trf4Δ* and *trf5Δ* knock-outs do not exhibit a slow growth phenotype, the double mutant *trf4*Δ/*trf5*Δ is synthetic lethal (Castano et al., 1996b). Therefore, there is an overlap in the essential function of these polymerases. Furthermore, it is yet-to-be determined what exactly the essential function of the TRAMP complex, or the Trf proteins, is.

Recent truncation analyses of Mtr4, Air2, and Trf4 proteins were used to analyze the regions of protein-protein interaction in these TRAMP subunits. Two groups identified that zinc knuckles 4 and 5 were important for not only the function of the Air protein but association with Trf4 as well (Fasken et al., 2011, Holub et al., 2012). In Trf4, truncations at the unstructured N- and C-terminus (ΔN181-Trf4, ΔC463-Trf4 and ΔN181/ΔC463-Trf4) affected the essential function of Trf4, as well as the RNA degradation functions of the TRAMP complex by the exosome (Holub et al., 2012). It is unclear how the N-and C-termini of Trf4, which do not include the conserved catalytic domains, contribute to the essential function of Trf4 or TRAMP. These recently published studies do not identify the region of interaction in Trf4/5 that mediates Mtr4 interaction. In Mtr4, truncations that disrupt the helicase core of the protein are not viable and lose interaction with TRAMP *in vitro* (Holub et al., 2012). The loss of Trf4/5 interaction in these truncations is observed *in vivo* through Y2H (Chapter 5).

Here, I utilize the data obtained in the original Y2H screen that identified Trf4 and Trf5 as Mtr4 protein partners and further narrow down the region of protein interaction (LaCava et al., 2005). In this case, I utilize Trf5, since few studies have been done specifically in this homolog of Trf4 (Houseley & Tollervey, 2006, San Paolo et al., 2009). I identified a 20-residue region in Trf5 that was required and sufficient for interaction with Mtr4. This region seems to be highly conserved in Trf5 and Trf4 proteins of different species. Furthermore, deletion of this important region does not affect complementation of the *trf4*Δ/*trf5*Δ double mutant. The fact

the Mtr4-TRAMP interaction can be disrupted and that the cells remain viable, indicates that assembly of the TRAMP complex may not be essential for life.

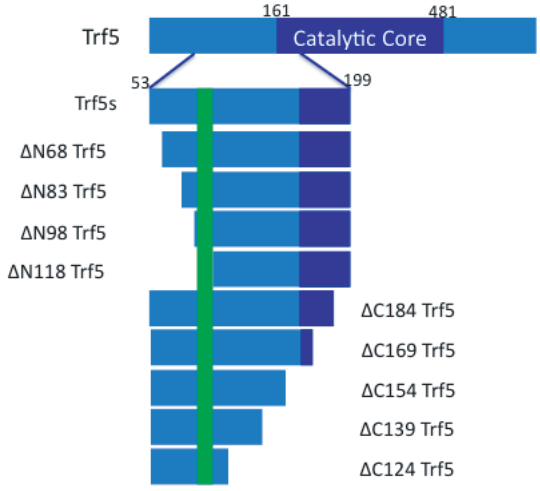
RESULTS

Residues 98-117 of Trf5 form the minimal region of interaction with Mtr4 *in vivo*

The original Y2H screen, using Mtr4 as bait, found a minimal region of interaction in the clones encoding different regions of Trf5 (LaCava et al., 2005). Clones containing the residues 53-199 promoted the association between Trf5 and Mtr4. A minimal region was not evident in Trf4. Using this minimal region in Trf5, which is termed Trf5s, I created N- and C-terminal truncations deleting 15 residues at a time. Figure 6.1 shows that 65 residues can be truncated at the N- of residues 53-199 of Trf5, and 124 residues can be truncated at the C-terminus of 53-199 of Trf5 without disrupting the interaction with Mtr4 (ΔN118 and ΔC124-Trf5s). Once the protein was truncated to residue 118 at the N-terminus, an almost complete disruption of the interaction with Mtr4 was observed. From the Y2H analysis I narrowed down the region of interaction with Mtr4 to be residues 98-117 (Figure 6.1 and data not shown).

Interestingly, residues 98-117 form part of the only conserved patch of amino acids outside of the catalytic and structured regions in Trf4 and Trf5 (Hamill *et al.*, 2010) (Figure 6.2).

Figure 6.1



Trf5 construct	Y2H interaction with Mtr4
Trf5s	+++
ΔN68	+++
ΔN83	+++
ΔN98	+++
ΔN118	+
ΔC184	+++
ΔC169	+++
ΔC154	+++
ΔC139	+++
ΔC124	+++

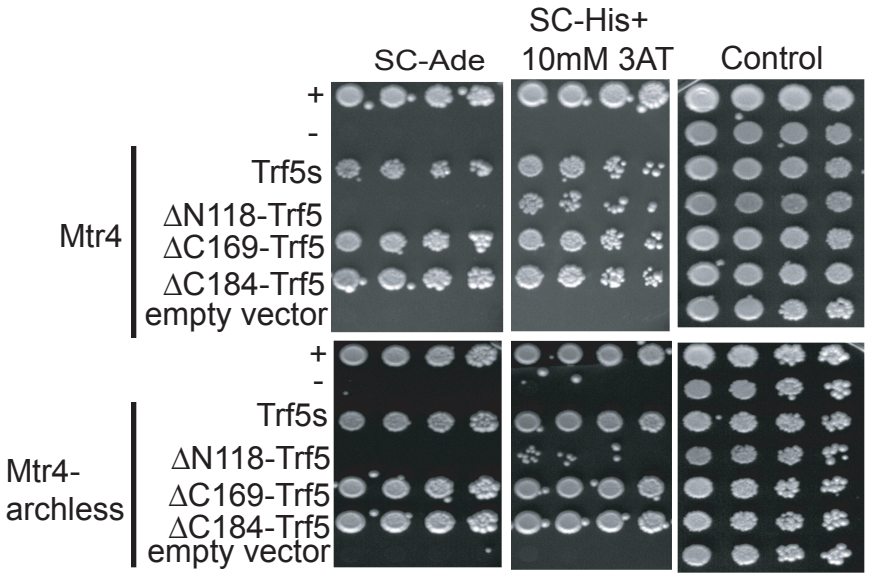


Figure 6.1 The 98-124 of Trf5 region is important for interaction with Mtr4 and Mtr4-archless:
ΔN and ΔC mutations of Trf5 were constructed to narrow down the minimal region of interaction with Mtr4. Previously it was shown that the 53-199 region of Trf5 was sufficient for interaction with Mtr4. Systematic truncations of Trf5 showed that all minimally Trf5 requires the 98-124 residues to interact with Mtr4 and Mtr4-archless.

Residues 98-117 are sufficient for interaction with Mtr4

The minimal region (98-117) is important for promoting the interaction with Mtr4, however, it was unclear whether this region could be sufficient for the interaction with Mtr4. To answer this question, a construct containing only this 20-mer patch of Trf5 was cloned into the Y2H vector and tested for interaction with Mtr4. Figure 6.3 shows that two independent clones of Trf5-98-117 maintain interaction with Mtr4 to the extent of the Trf5s (53-199). Previously, I showed that the mutant protein Mtr4-archless maintained a higher affinity to Trf5 (Klauer & van Hoof, 2012b, Klauer, 2012). This increased interaction with Mtr4-archless is also recapitulated with the Trf5-98-117 peptide.

Residues 98-117 in Trf5 are critical for the interaction between Mtr4 and Trf5 *in vivo*

N- and C-terminal truncations of the 98-117 region of Trf5 showed that disrupting this region affected binding to Mtr4. However, it was not determined whether this specific region was solely responsible for the physical association between Trf5 and Mtr4. For this purpose, an internal deletion in the full-length protein was created and cloned into the Y2H vector. Trf5Δ98-117 lost interaction completely with Mtr4 in the Y2H analysis shown in Figure 6.4. Conversely, mutations in the conserved amino acids (LFI and EDD) did not disrupt the interaction with Mtr4 in Figure 6.5.

Figure 6.2

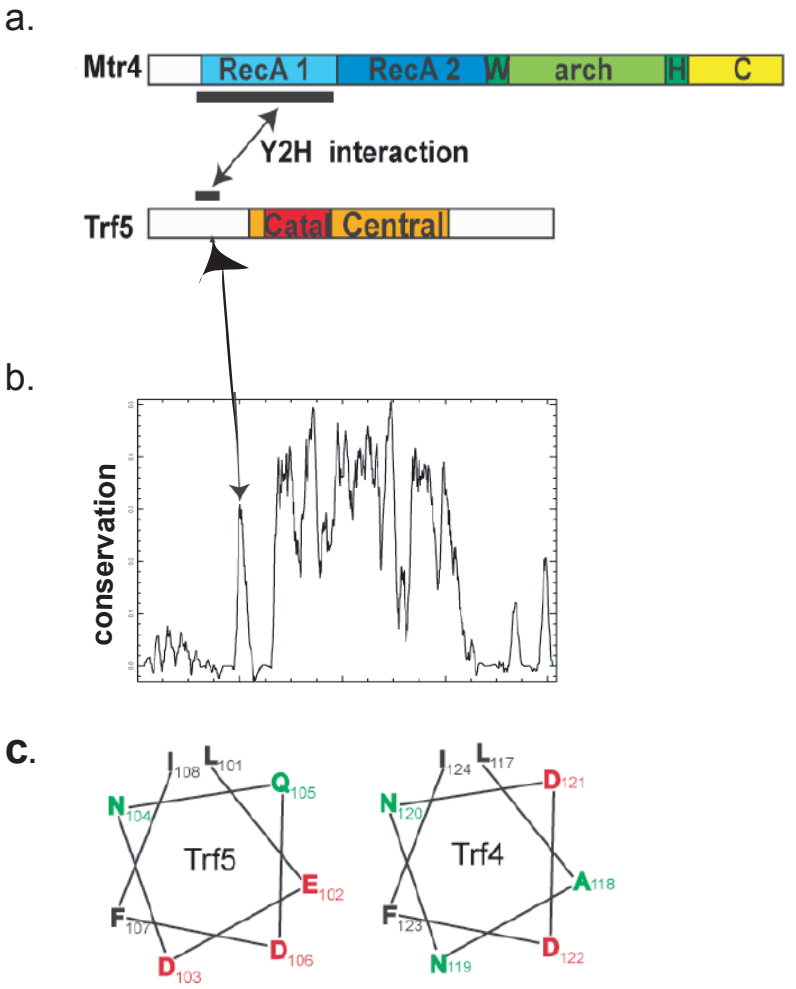


Figure 6.2 Schematic of the location, conservation and predicted structure of residues 98-124 of Trf5:
a. Cartoon depiction of the domains of Mtr4 and Trf5, particularly pointing at the minimal Mtr4 binding region of Trf5. **b.** Height of the peaks indicates the level of conservation. **c.** Helical Wheel predictions of the folding pattern of these conserved residues of Trf5 indicate that an amphipathic helix is formed.

Figure 6.3

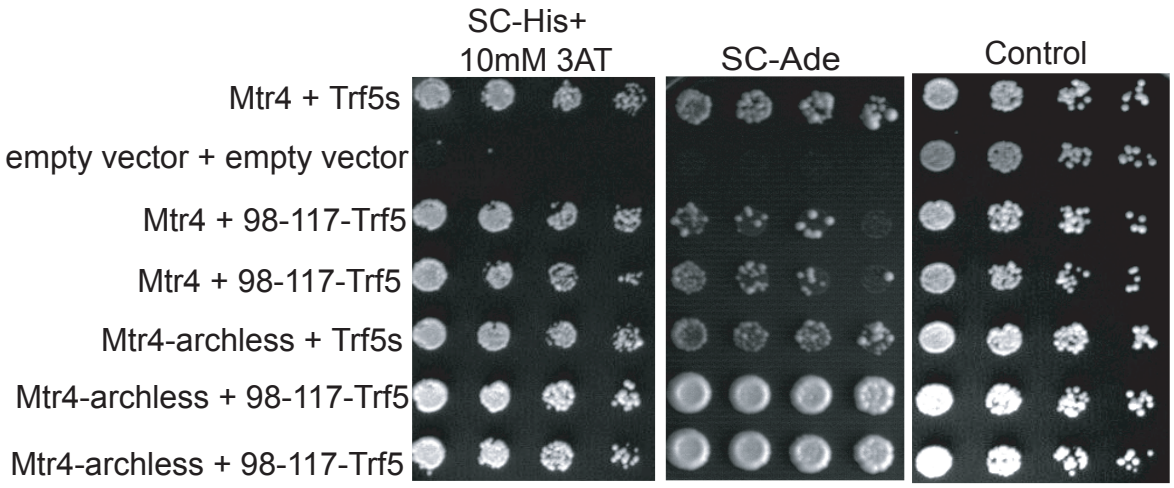


Figure 6.3 Y2H analysis of the minimal Mtr4 binding region of Trf5:
The region encoding residues 98-117 was cloned into the Y2H vector and testedfor binding against Mtr4 and Mtr4 archless. The minimal binding region is residues 98-117 and it is sufficient to promote Y2H interaction with Mtr4.

Figure 6.4

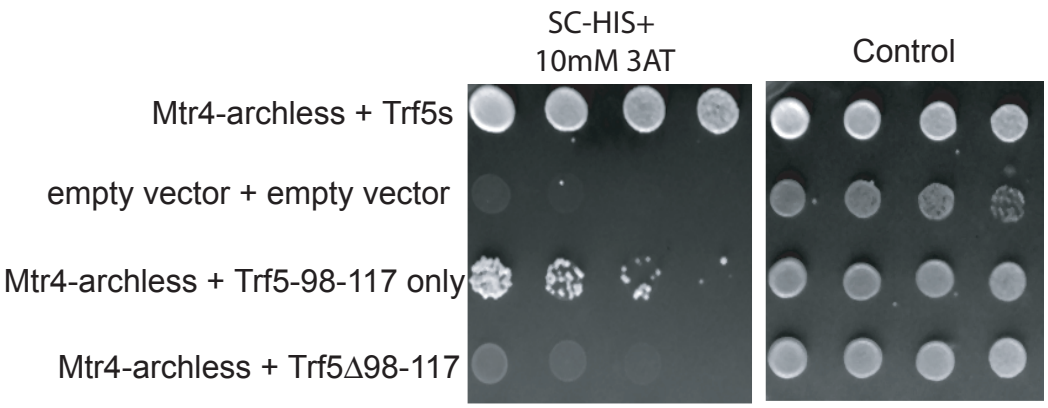


Figure 6.4 Residues 98-117 are required for the interaction with Mtr4:
Y2H analysis of construct containing only the 98-117 residues of Trf5 compared to the full-length protein missing those 20 residues.

Figure 6.5

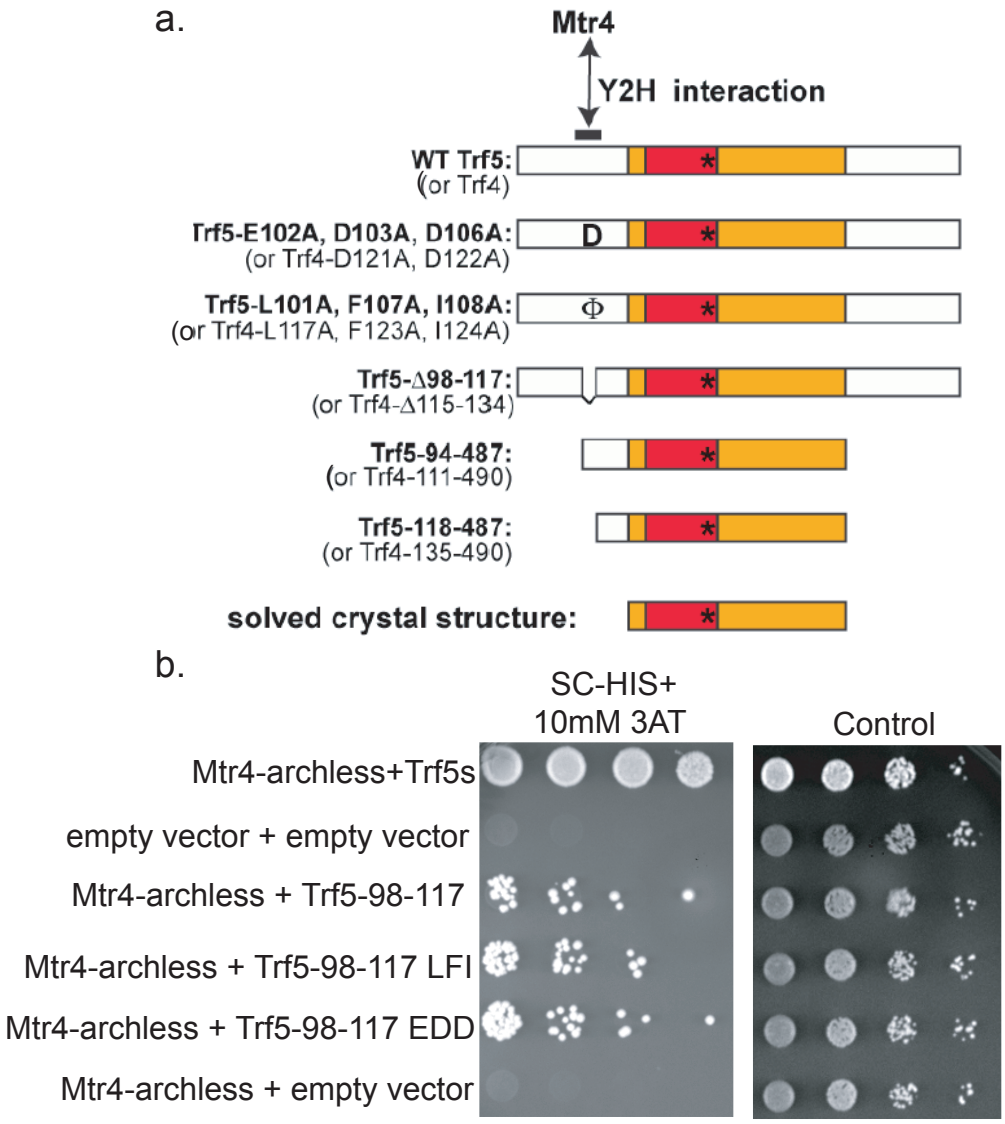


Figure 6.5 Conserved residues that were predicted to form the amphipathic helix are not required for interaction with Mtr4:
a. Cartoon depiction of the conserved residues that were mutated. Additionally, it depicts the additional constructs that will be and have been tested for interaction with Mtr4.
b. Y2H analysis of the mutated minimal region of interaction with Mtr4 in Trf5.

Residues 98-117 are not essential for cell viability

To confirm that deleting residues 98-177 in the conserved patch of residues does not affect protein expression, complementation and Western blot analysis were performed. The *trf5*Δ98-117 was introduced into a *trf4*Δ/*trf5*Δ double mutant through the standard plasmid shuffle technique. Figure 6.6a shows that deleting this region does not affect cell viability, since *trf5*Δ98-117 complements the double mutant to wild-type levels. Additionally, using α-protein A antibodies, the levels of Trf5Δ98-117-TAP (Tandem Affinity Purification tag) were assessed, and the mutant protein is expressed at levels similar to the full-length Trf5-TAP protein (Figure 6.6b).

Trf5Δ98-117-TAP was expressed at levels similar to wild type and complemented the double mutant, therefore it was possible to confirm protein-protein interaction through TAP followed by Western blot using the *trf4*Δ/*trf5*Δ [*TRF5*-TAP] and *trf4*Δ/*trf5*Δ[*trf5*Δ98-117-TAP] strains. Figure 6.7 indicates that immunoprecipitation of the Trf5-TAP (as described in materials and methods) precipitates Mtr4, as expected, however, the deletion mutant Trf5Δ98-117 does not. This immunoprecipitation confirms that this 20-mer region is crucial in mediating the interaction between Trf5 and Mtr4. Most interestingly, the essential function of Trf5 does not require this region of the protein.

Figure 6.6

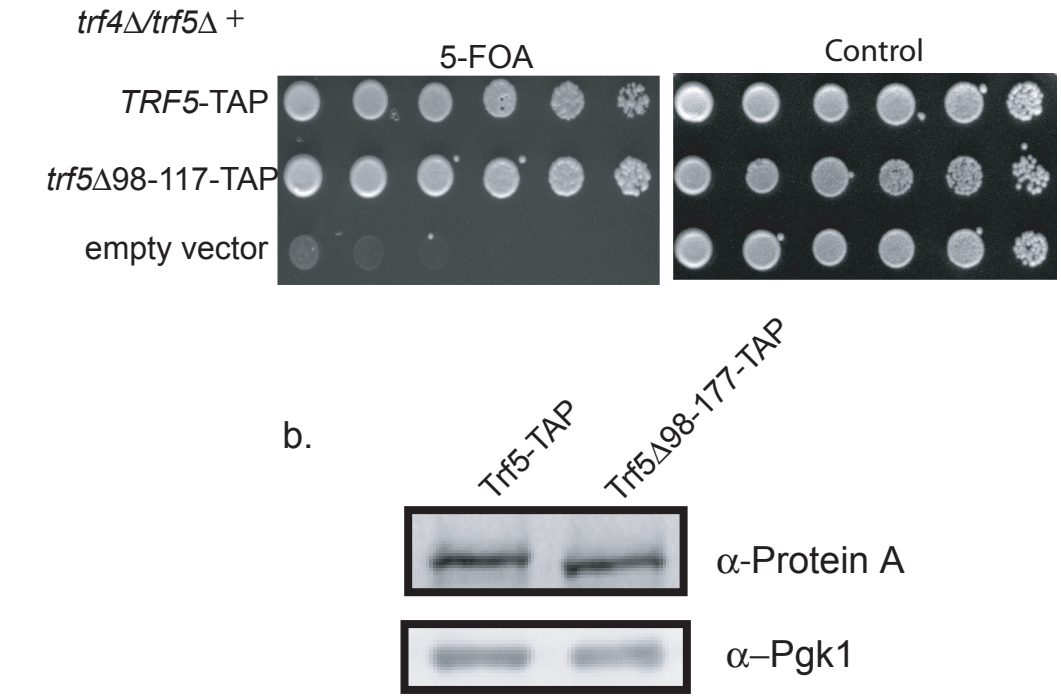


Figure 6.6 Deleting residues 98-117 of Trf5 does not cause growth defects in the *trf4Δ/trf5Δ* strain:
a. Mutant strain *trf4Δ/trf5Δ* [*TRF4, URA3*] was transformed with plasmids encoding either Trf5-TAP, Trf5Δ98-117-TAP and TAP empty vector with the LEU2 selectable marker. Growth was assayed on 5-FOA media to counterselect for the *TRF4-URA3* plasmid. b. Western blot indicates that the mutant protein is expressed at similar levels to the full-length Trf5 protein.

Figure 6.7

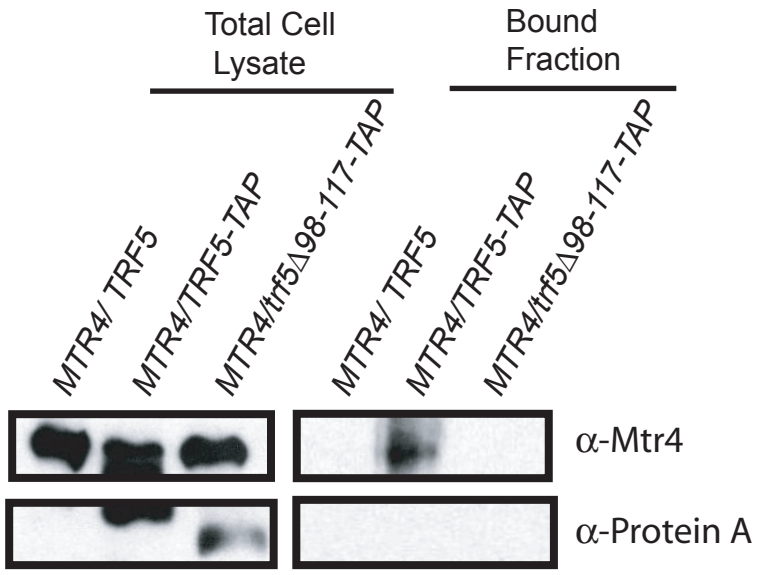


Figure 6.7 TAP confirms that residues 98-117 are crucial for the interaction between Trf5 and Mtr4: Total protein isolation was performed as described in the materials and methods. Cell lysates were incubated with IgG sepharose beads and washed as described in the methods section. Pull-down fractions were boiled for 5 minutes to release the proteins bound to the beads, centrifuged to separate from the TAP-tagged protein and loaded onto a polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane and western blot analysis was performed. Loading control was run off the gel, however, Pgk1 has shown to disassociate from the Trf5 pulldown by the second wash (data not shown).

CONCLUSIONS AND FUTURE DIRECTIONS

In this chapter, I describe the analysis of a specific region that mediates the interaction between Trf5 and Mtr4. Residues 98-117 of Trf5 are shown to be required and sufficient for the interaction with Mtr4. Previous reports have shown that disruption of the equivalent region in the homolog Trf4 (ΔN181-Trf4) disrupts TRAMP function. However, deleting the whole N-terminus of Trf4 did not complement a *trf4*Δ/*trf5*Δ double mutant (Holub et al., 2012). Conversely, in the case of Trf5, I tested only an internal deletion of the residues which mediate Mtr4 interaction. I found that the *trf5*-Δ98-117 mutant complements the *trf4*Δ/*trf5*Δ strain, indicating that Mtr4-Trf5 interaction is not essential for cell viability. Like in Trf4, the rest of the N-terminus of Trf5 may be necessary for the essential function of the protein, however this essential function may not involve TRAMP. To confirm that this is the case, equivalent internal deletions in Trf4 should be made. In the case of Trf4, deletions of residues 115-134 should be tested for Y2H, complementation, and co-precipitation experiments. Additionally, entire truncations of the N- and C-terminal domains of Trf5 should also disrupt the essential function of Trf5. N- and C-terminal truncations can be made similarly to the Y2H constructs described here, however, using the Trf5-TAP as template instead of the Trf5s. These two experiments will show that the N-terminal region of Trf5 serves two purposes, one, interaction with TRAMP and two, the essential function of Trf5. I expect that a deletion in the equivalent region of Trf4 will show disruption of Mtr4 binding and will complement the *trf4*Δ/*trf5*Δ double mutant.

Most interestingly, the results discussed in this chapter indicate that TRAMP function may not be essential as previously thought. The fact that the *trf4*Δ/*trf5*Δ strain is inviable suggests that TRAMP integrity is necessary for cell viability. Here, I show that this may not be the case. One alternative possibility is that TRAMP function may not require a strong interaction with Mtr4. Therefore the Trf5-Mtr4 interaction in the TRAMP complex may not be required, as long as Trf5 interacts with the Air protein. It is important to test whether disruption of this region affects Air protein interaction. Alternatively, the essential function of the Trf proteins may also arise from a cell function unrelated to TRAMP, possibly the pathways involved in DNA and chromatin integrity. This result is also consistent with the observation that poly(A) polymerase activity is not essential for life(San Paolo et al., 2009). In order to find out which hypothesis is correct, the RNA metabolism defects of the *trf5*Δ98-117 mutant should be analyzed. Northern blot analysis of the *trf5*Δ98-117 deletion mutant should shed light into whether TRAMP function is indeed disrupted when Mtr4 does not stably associate with the complex. I predict that this mutant strain will accumulate CUTs and extended snoRNA and snRNA species. If TRAMP function is not disrupted, then it indicates that Mtr4 interaction is dispensable for TRAMP function *in vivo.* Nevertheless, this is the first line of evidence suggesting that TRAMP function is not essential for life.

Chapter 7: Final Conclusions and Perspectives

The arch domains of Mtr4 and Ski2 are required for the nuclear and cytoplasmic functions of the exosome.

The work presented in this dissertation shows that the novel arch domain of the exosome cofactors, Mtr4 and Ski2 is important for promoting exosome functions in the cytoplasm and the nucleus. Mutant protein lacking the arch domain exhibit defects in RNA processing and degradation by the exosome complex. In the case of Mtr4-archless, only specific exosome functions are disrupted, while in the case of Ski2 all cytoplasmic functions tested required its arch domain. It is unclear, however, how the arch domain is contributing to the exosome-mediated functions of these helicase cofactors. Several follow-up experiments are needed to identify the role the arch domain of Mtr4 and Ski2 is playing in the cell.

Determining the function of the arch domain of Mtr4

To understand how the arch domain is contributing to Mtr4 function, several detailed examinations of Mtr4-archless should be taken. *In vitro* biochemical assays showed that the Mtr4-archless mutant protein retains its helicase activity (compare unwinding constant of 0.066±0.005 for Mtr4 and k'_{unw}= 0.058±0.008 for Mtr4-archless)(Jackson et al., 2010). Furthermore, the ATPase activity of Mtr4 is also unchanged regardless of the presence of the arch domain (Jackson et al., 2010). Data described in chapter 5 showed that the arch domain of Mtr4 may have a role in modulating the protein-protein interaction of the helicase. To confirm that one of the functions of the arch domain is involved in binding other proteins, HA-immunoprecipitation of Mtr4 and Mtr4-archless could be used. Recently, Mtr4 and

Mtr4-archless constructs have been N-terminally tagged with the HA-epitope. Initial pull-down experiments reproducibly detect coomassie stainable amounts of Mtr4 and other proteins. Thus far, there is no obvious difference in the protein pattern obtained between the precipitate from the *MTR4* and the *mtr4-archless* strains. This is however not surprising considering that the initial pull-down experiment that identified the other TRAMP subunits, did not detect them through Coomassie staining, but rather through mass spectrometry (LaCava et al., 2005). Likewise, proteins bound to HA-Mtr4 and HA-Mtr4-archless should be analyzed by mass spectrometry to identify any difference in binding partners between the two constructs. I hypothesize that Mtr4-archless may be interacting with an additional binding partner(s), which is (are) disrupting the ability to switch between TRAMP-independent and TRAMP-dependent functions. This prediction is supported by the data obtained to date. First, Mtr4-archless interacts with the TRAMP subunit, Trf5, with higher affinity *in vivo*. Second, Mtr4-archless has defects in functions that require Mtr4 and not the remainder of the TRAMP complex. Third, other protein interactions, namely with the exosome subunits, are also increased by removal of the arch.

There is also the possibility that the arch domain is involved in promoting the nucleic acid interactions of Mtr4. To compare the RNA interactions between the *MTR4* and *mtr4-archless* strains, a new technique called CRAC should be utilized. CRAC is an *in vivo* RNA-protein cross-linking technique that identifies specific binding regions of RNAs by using the His6,TEV cleavage, Protein A-tag(HTP) at the N-terminus of the protein, UV-crosslinking the cells, precipitating the protein, and

sequencing the transcripts in the precipitate (Granneman *et al.*, 2009)(Figure 7.1). Previous experiments have used CRAC to identify specific RNA targets of the exosome. Using the split-CRAC technique one can also identify targets of different parts of a specific protein. Split-CRAC has previously been used to identify substrates of the *rrp44*-exo and endo domains, respectively (Schneider *et al.*, 2012). Split-CRAC uses a precision protease (PP) cleavage site where one can split the protein in two and separate the cross-linked products in two pools (i.e N-terminus and C-terminus). By using both, the CRAC and split-CRAC technique, substrates of both Mtr4 and the arch domain of Mtr4 can be identified. Since the helicase core of Mtr4 is attached to the arch domain at one end, two PP cleavage site can be added to the winged-helix domain at the beginning and at the end of the arch domain (Figure 7.1b). Separating the arch and core domains will provide a specific and global look at the substrates of the two large regions of Mtr4 (see figure 7.1b for a schematic). Additionally, Mtr4-archless can also be analyzed through this technique. By comparing the RNAs that associate with Mtr4 versus Mtr4-archless, one can identify whether Mtr4-archless is defective at binding specific RNA substrates. This data set can be compared to the arch-only data set (split-CRAC). A global analysis of all the RNA substrates of Mtr4, and specifically of the arch domain of Mtr4, will give the RNA field a better perspective about the functions of the arch domain of Mtr4.

In addition to RNA-protein cross-linking assays, RNA deep-sequencing of the *MTR4* and *mtr4-archless* strains should also be compared. Recent transcriptome-wide analyses of *rrp44*-exo, *rrp44*-endo, and *rrp6*Δ mutants (including double and triple mutants) have been reported (Gudipati *et al.*, 2012). This alternative approach

will complement the CRAC analysis with additional indirect targets of Mtr4 and the arch domain of Mtr4. If removing the arch domain of Mtr4 affects the function of another protein, which in turn causes the accumulation of an RNA intermediate, this specific RNA would show up in the deep-sequencing but not in the CRAC analysis. By using this unbiased approach, it will be possible to identify additional RNA targets of Mtr4 and specifically of its arch domain. Both of these unbiased and global approaches will provide additional evidence of the role of the arch domain of Mtr4 in the RNA pathways of the cell. Additionally, if the essential role of the arch domain involves cellular pathways not related to the exosome, RNA targets that belong to such pathways will be enriched in the RNA-sequence dataset from the *mtr4-archless* strain.

Determining the function of the arch domain of Ski2

Here, I have shown that the arch domain of Ski2 is also important in the RNA degradation functions of the cytoplasmic exosome. Unlike Mtr4-archless, which only has defects in specific nuclear exosome functions, the arch domain of Ski2 seems to be involved in all of the cytoplasmic functions of the exosome tested so far. Previously, Ski2 has been shown to be a promiscuous RNA binding protein, interacting with both double and single RNA stranded substrates *in vitro*; therefore it is unlikely that Ski2 alone promotes substrate specificity of the cytoplasmic exosome (Halbach et al., 2012). To identify what RNA pathways require the arch domain of Ski2, CRAC analysis can also be used. Additionally, pull-down experiments followed by mass spectrometry will also unveil the protein-partners of

the Ski2-archless mutant protein. Ski2 and Ski2-archless have already been internally tagged with a myc-epitope, therefore it is possible to perform the same experiment described above for Mtr4 and identify differential binding partners, in this case, for Ski2-archless. Similarly, Ski2-archless can be analyzed through the yeast two-hybrid analysis to determine whether Ski2-archless interacts with higher affinity with the Ski complex *in vivo*. These three experiments will contribute to a more complete look at the *ski2-archless* phenotype, before making any more specific predictions.

Figure 7.1

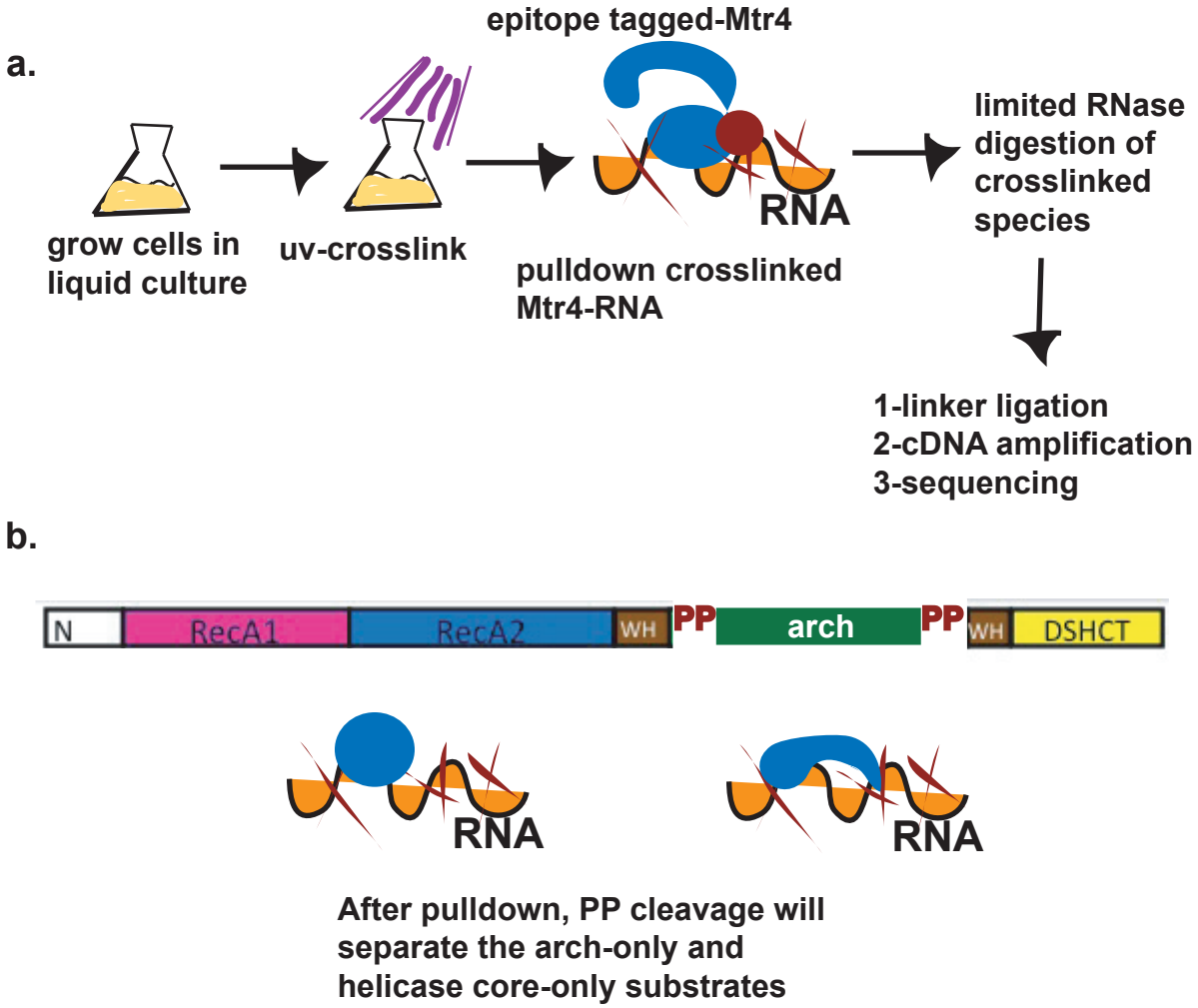


Figure 7.1 Schematic of the proposed CRAC and Split-CRAC technique to identify additional targets of Mtr4 which require the arch domain:
a. CRAC technique will identify RNAs that associate with Mtr4 and Mtr4-archless at the genome-wide level. b. Adding PP cleavage site upstream and downstream of the arch domain will allow for further purification of the core and arch domain of Mtr4. Split CRAC will distinguish between arch and core specific substrates, and identify the overlap.

Both normal and nonstop mRNA decay require the arch domain of Ski2, however it is unclear whether the arch domain is required for the antiviral activities of Ski2. Ski2 was initially identified as mutant strain that causes the super-killer (*ski*) phenotype when infected with the Killer-virus (Widner & Wickner, 1993). To determine whether the antiviral function of the Ski complex requires the arch domain of Ski2, the *ski2-archless* strain can be analyzed by the yeast killer assay (Schaeffer *et al.*, 2008).

Removing the equivalent domain affects specific functions of each of the exosome cofactors

Removal of the arch domains of Mtr4 and Ski2 has shown to be required for the functions of these cofactors. However, it is important to note that the Mtr4-archless defects disrupted only specific substrates of Mtr4, while Ski2-archless seems to affect all Ski2's substrates. Namely, the *mtr4-archless* phenotype is different from *mtr4* depletions, where general exosome defects are observed, while *ski2-archless* behaves just like *ski2*Δ. The experiments described above will also determine whether the arch domain of Ski2 is required for all the functions of Ski2 or, like in the case of Mtr4, whether it only promotes a specific Ski2 role.

The arch domain of Mtr4 has a genetic relationship with Rrp6.

Chapter 4 showed that the defects observed in the *mtr4-archless* strain specifically resembled those of *rrp6*Δ, rather than other exosome mutants, suggesting that the arch domain of Mtr4 and Rrp6 act cooperatively in the same

pathway. In contrast, complementation analysis of the double mutant *mtr4-archless*/*rrp6*Δ indicate that Mtr4 and Rrp6 act in separate and possibly redundant pathways. Not surprisingly, global exosome analyses have shown that Rrp6 and the exonuclease Rrp44 have both cooperative and independent roles in the cell. Rrp6 and Rrp44 have been shown to synergistically degrade CUTS and SUT, while having independent roles in the processing and degradation of snoRNAs and introns (Gudipati et al., 2012). Through our molecular and genetic analysis and through process of elimination I have begun to determine which substrates require the arch domain of Mtr4, Rrp6, or both. Data presented here indicate that 5.8S processing requires both the arch domain of Mtr4 and Rrp6, because the levels of 5.8S+30 are not increased in the *mtr4-archless/rrp6*Δ double mutant as compared with the single *mtr4-archless* mutant strain. However, figure 4.2 showed a lesser accumulation of 5.8S+30 in the single *mtr4-archless* and double *rrp6Δ/mtr4-archless* strains than in the single *rrp6*Δ strain. This decreased accumulation of 5.8S pre-rRNA appears to occur due to defects in the rRNA processing pathway upstream of the 5.8S+30 cleavage step, which are presumably caused by removal of the arch domain of Mtr4. Nevertheless, the similar levels of 5.8+30 in the *mtr4-archless* and *mtr4-archless/rrp6*Δ indicate that this specific processing step requires both the arch domain of Mtr4 and the exonuclease Rrp6. In the case of 5' ETS degradation it is not so clear, I can conclude that either the arch domain of Mtr4, Rrp6, or the exonuclease of Rrp44 are involved in the degradation of this cleavage product. In the case of the processing of snoRNAs, while the arch domain is not required, Rrp6 is crucial. Both Rrp44 and Rrp6 degrade long poly(A) snoRNAs, however the arch

domain of Mtr4 is not necessary for this RNA function. It is interesting to note that although certain functions use both Rrp44 and Rrp6, the patterns of the intermediates observed in the northern blot are strikingly different (i.e 5'ETS and snoRNA degradation). Different degradation patterns are often observed between depletions of several different exosome subunits, indicating that different exosome proteins, like Rrp44 and Rrp6, attack different regions of the RNA substrate. It is still unclear what the contribution of the arch domain of Mtr4 is for either Rrp6 or Rrp44, and additional experiments are necessary to understand the dynamic relationship between the cofactor and the ribonuclease.

Determining the cooperative and overlapping function of the arch domain of Mtr4 and Rrp6

Chapter 4 has shown that 5.8S processing requires the arch domain of Mtr4 and Rrp6 in a single linear pathway. To identify additional substrates that involve the cooperative interaction between the arch domain of Mtr4 and Rrp6, the following experiments should be performed. The deep-sequencing data is readily available for the *rrp6*Δ strain (Gudipati et al., 2012), and it was proposed here to analyze *mtr4-archless.* A third strain, the *mtr4-archless*/*rrp6*Δ double mutant, can be compared to either of the single mutants. RNA precursors that accumulate similarly in the single mutant (*rrp6*Δ and *mtr4-archless*) and in the double mutant shall be identified as substrates of the cooperative function of the arch domain of Mtr4 and Rrp6. Conversely, extended RNA species that show up in the single mutants and

increase in the double mutant indicate that these are substrates of two synergistic pathways, one mediated by the arch domain of Mtr4 the other by Rrp6. Further, RNAs that accumulate in the single mutant correspond to Rrp6-only, or arch-only mediated functions (for example snR33 is an Rrp6-only substrate). A model with all the functions of Mtr4 previously described is summarized in Figure 7.2.

Is the arch domain of Mtr4 mediating a physical interaction with Rrp6?

The previously described HA-Mtr4/Mtr4-archless pull-down could confirm the interaction between Mtr4 and Rrp6, and whether this interaction is mediated by the arch domain. Previous high-throughput proteomic analyses have shown that Mtr4 and Rrp6 physically interact (Krogan *et al.*, 2004, Peng *et al.*, 2003). Additionally, entire complex pull-downs have shown a physical interaction between Mtr4 and Rrp6. To identify whether this interaction is occurring, one can compare the capabilities of Mtr4 and Mtr4-archless to interact with Rrp6. First, Rrp6 should be fused to the Gal4 activation domain and tested against the already constructed Mtr4 and Mtr4-archless Y2H plasmids. This initial experiment will confirm the physical interaction between Mtr4 and Rrp6 and present a quick look at the binding capabilities of Mtr4-archless and Rrp6.

If a difference in binding between the mutant and full-length protein is found, a biochemical assay should be undertaken to determine whether this interaction is direct. Mtr4 and Mtr4 archless should be tagged with glutatione S-transferase tag and expressed in *E. coli* for purification through glutathione beads. Previously, Mtr4 and Mtr4-archless proteins have been successfully purified (Jackson *et al.*, 2010,

Figure 7.2

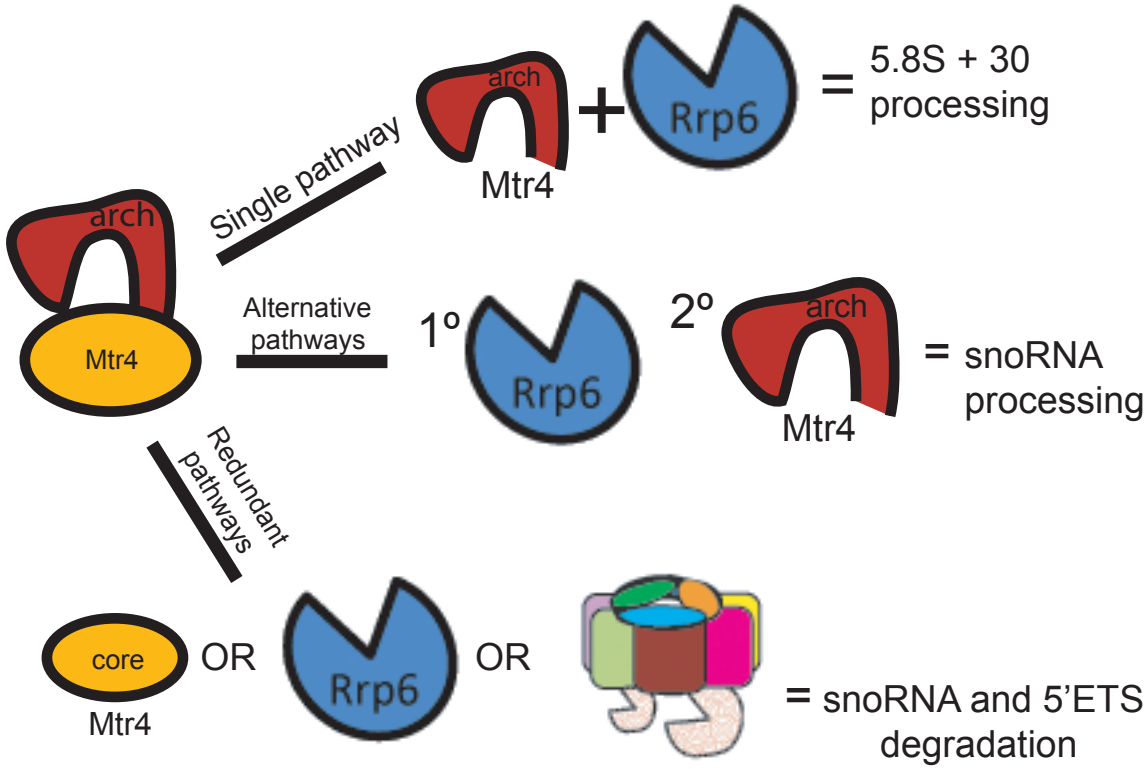


Figure 7.2 Model of the role of Mtr4 and the arch domain of Mtr4 in RNA metabolism:
Mtr4 is involved in a variety of RNA processes.Certain RNA functions require the arch domain of Mtr4, while the core of Mtr4 is sufficient for others. First, the arch domain and Rrp6 are both required for the 5.8S rRNA trimming step. Second, the arch domain is only required for snoRNA processing if Rrp6 is absent.Third, general degradation of RNAs by the exosome can be promoted through several redundant pathways.

Weir *et al.*, 2010). Pure protein (from both constructs) should be incubated with total cell lysate from a wild-type strain containing His-Rrp6, empty vector, and an *rrp6*Δ strain. Pure Mtr4 and Rrp6 will provide the RNA field with the first evidence of direct physical interaction (non-high-throughput) between Mtr4 and Rrp6. The precipitate obtained from the *rrp6*Δ lysate can present further evidence into the growth defects observed in the *mtr4-archless*/*rrp6*Δ strain. There is a possibility that in the absence of Rrp6, the protein-protein interaction capabilities of Mtr4 are disrupted, and that removing the arch exacerbates the defect. The complicated genetic interactions between the core and the arch domain of Mtr4 with Rrp6 could be mediated through physical association and this needs to be analyzed in more detail.

Determining the growth promoting function of the arch domain of Mtr4 (and Rrp6)

Chapters 3 and 4 have shown that the arch domain of Mtr4 is required for the optimal growth of yeast cells. The *rrp6*Δ has a temperature sensitive growth defect in certain yeast strain backgrounds. Furthermore, the double mutant *mtr4-archless*/*rrp6*Δ has synthetic growth defect at 30°C. It has yet to be determined what RNA or cellular defects are causing these growth phenotypes. From the data presented here, I can extrapolate which functions are NOT causing the slow growth defect. The *mtr4-archless* single mutant causes a level of 5.8S+30 accumulation similar to the *rrp6*Δ at 30°C. Since the *rrp6*Δ BY strain does not have growth defects at 30°C, I can conclude that the growth defect is not cause by the accumulation of this extended rRNA. Both *mtr4-archless* and *rrp6*Δ continue to accumulate mature

5.8S to the levels of WT strains. Furthermore, it is unlikely that 5.8S+30 is causing significant growth defects in the cell, because 5.8S+30 are actively assembled into the ribosome and cause no polysome defects (Briggs *et al.*, 1998). The accumulation of the second substrate tested, 5'ETS, is suppressed in *rrp6*Δ strains overexpressing Mtr4 and Mtr4-archless. While overexpression of full-length Mtr4 caused a complete suppression of 5'ETS accumulation, Mtr4-archless promoted the degradation of this substrate to a lesser extend. The *rrp6*Δ strains overexpressing Mtr4 and Mtr4-archless grew at the levels of wild-type *RRP6*, which does not correlate with the differential amount of 5'ETS obtained. I conclude that 5'ETS also does not cause the growth defect in either *mtr4-archless* or *rrp6*Δ strains. The same conclusion can be drawn for the poly(A) snoRNA products. Thus far, it is not clear what the "essential" function of these and other exosome proteins. I believe that the transcriptome-wide analysis proposed above will get us closer to identifying which substrates are required for optimal growth. It is becoming increasingly difficult to look at mutants of these proteins on a substrate-by-substrate basis, because there are too many redundant pathways involved in the RNA metabolic mechanisms. Alternatively, there may not be one essential Mtr4 function, but rather that the simultaneous disruption of several important RNA metabolic functions causes the lethality in the *mtr4*Δ. Transcriptome-wide analysis will provide further evidence of either possibility.

Mtr4 makes multiple different contacts with TRAMP and Exosome subunits

The arch domain of Mtr4 is not required for the interaction with the TRAMP complex, however it has an important role in modulating physical association with Mtr4

Yeast two-hybrid analysis of N and C-terminal truncations of Mtr4 has given us an initial look at the regions of protein-protein interaction of Mtr4. Moreover, the Y2H results presented here correlate with the recently published immunoprecipitation experiments of N- and C-terminal truncations of Mtr4 (Holub et al., 2012). Here I show that the arch domain of Mtr4 is not required to initiate the physical association with the TRAMP subunit, Trf5, but it seems to play a role in modulating this interaction. Mtr4-archless had a higher affinity with Trf5 than full-length Mtr4 *in vivo*. Interestingly, this increased affinity was not evident when Mtr4-archless was immunoprecipitated from yeast cells (Holub et al., 2012). *In vitro* TRAMP complex reconstitutions did not identify the increased protein interaction between Mtr4-archless and Trf5 (Weir et al., 2010). The discrepancy between the immunoprecipitation and Y2H results may indicate that additional proteins may be needed to maintain or disrupt the Mtr4-Trf5 physical association. The immunoprecipitation incubation conditions may have not been favorable for binding of these additional substrate(s) therefore the increased interaction between Mtr4-archless and Trf4 may have not been evident. Additionally, Trf4 and Trf5 may behave differently and the increased interaction between Mtr4-archless and the Trf protein may only happen in the case for Trf5. This raised the possibility that Trf4 and Trf5 may bind to Mtr4 with different affinities and the arch domain may be modulating this interaction. To determine whether Trf4 interacts with Mtr4-

archless with increased affinity *in vivo*, Trf4 should be included in the Y2H analysis. The comparison between Trf4 and Trf5 association with Mtr4-archless will grant additional evidence regarding the dynamics of protein binding between TRAMP4 and TRAMP5. Furthermore, it will provide a role for the arch domain in the protein binding of TRAMP4 and Mtr4.

Multiple domains/regions of Mtr4 are mediating the binding to TRAMP and the cap subunits of the exosome

Several conclusions can be drawn from the Y2H analysis of N- and C-terminal truncations of Mtr4. First, the N-terminal region of Mtr4 is important for TRAMP complex and exosome binding. ΔN12-Mtr4 does not interact with Trf5, Rrp4, and Rrp40. ΔN89-Mtr4 loses interaction with the other cap subunit of the exosome, Csl4. It is interesting to note that although the ΔN12-Mtr4 lacks Trf5 interaction, the ΔN89-Mtr4 restores the Trf5 interaction. While surprising, this result is highly reproducible. An explanation for this phenomenon could be that Mtr4 has "back-up" regions of interaction with the TRAMP subunit. Therefore, once the entire N-terminus is disrupted as in the ΔN89-Mtr4, then an additional region of interaction may be utilized for Trf5 binding, in this case the RecA1 domain. The ΔN12-Mtr4 may not disrupt the structure of the N-terminus to the extent of the deletion, thereby not requiring the redundant RecA1 binding site. The N-terminus could normally block the RecA1 binding site and if completely removed, it exposes the RecA1 region for Trf5 binding. I think that this is a very interesting observation indicating that the N-

terminus may have a very specific mechanism of binding to Trf5, which is not observed for the other exosome subunits tested.

Second, Csl4 makes multiple contacts with the helicase core of Mtr4. Csl4 shows low but reproducible levels of protein interaction with many of the ΔN and ΔC constructs of Mtr4. Csl4 continues to exhibit positive Y2H results with truncations up to ΔN608-Mtr4 and ΔC326-Mtr4, indicating that Csl4 makes contacts with residues throughout the surface of the helicase core. Interestingly, the other two exosome subunits tested do not exhibit this same pattern of interaction. This observation leads to the third conclusion, that there are multiple regions of protein-protein interaction in the structure of Mtr4.

The Y2H analysis is the first line of evidence of Mtr4-exosome binding in yeast *in vivo*. Follow-up experiments should include TAP experiments of the exosome cap subunits. Csl4-TAP, Rrp4-TAP, and Rrp40-TAP have already been constructed, and the plasmids encoding these constructs can be transformed into the ΔN12-*mtr4,* and the *Δ*N89-*mtr4* strains. In the case of the longer truncations, which do not support cell viability, a tagged version (i.e His6X or HA) of the truncation can be expressed in trans with the endogenous *MTR4.* After the exosome subunit is precipitated, western blot analysis with an anti-HA antibody can be used instead of the anti-Mtr4 antibody to detect the co-precipitate. TAP will be used to confirm the Y2H results. To identify the minimal binding region of Mtr4 to Trf5, Rrp4, or Rrp40, further truncations can be done to the N-terminus and the RecA1 domain and analyzed by Y2H analysis. Additionally, once the minimal region is identified, oligos encoding the residues can be cloned into the Y2H vector to show

whether the region is also sufficient for Mtr4-Trf5/Rrp4/Rrp40. In the case of Csl4, there are multiple contacts between Cls4 and Mtr4, therefore it will be difficult to identify the specific residues. Mutagenesis of the residues exposed to the "top" surface of the helicase core of Mtr4 (i.e the RNA entry side) may provide some insight into the physical interaction between Mtr4 and Csl4.

Determining the function of the N-terminus of Mtr4

Protein alignments of Mtr4 of different species (Figure 5.12) show that the first 12 amino acids of Mtr4 are highly conserved. It is unclear what the function of the unstructured N-terminus is. Y2H analyses provide us a clue regarding the role of the N-terminus in the interaction with Trf5, and the exosome subunits, Rrp4 and Rrp40. The full complementation of the ΔN12-*mtr4* mutant in the *mtr4*Δ strain indicates that disrupting the Trf5-Mtr4 interaction does not affect the normal growth of the cells, nor does it disrupt 5.8S rRNA processing, or 5'ETS degradation. Additionally, it does not exacerbate the *mtr4-archless* phenotype. It is possible that deleting all the 12 conserved residues may activate other redundant mechanisms. In order to see a phenotype for an N-terminal mutant, point mutants should be analyzed. The conserved residues of the first 12 amino acid of the N-terminus of Mtr4 contain aspartic and glutamic acids flanking leucine, phenylalanine and valine. First, I propose to mutate each of the acidic residues to alanines and arginines and analyze the mutant for *mtr4Δ* complementation, RNA defects, and binding to Trf5 by the Y2H assay. I hypothesize that the charge of the amino acid contributes to the

binding to Trf5. Mutating these residues to either alanine or the positively charged Arginine will disrupt the association with Trf5.

Second, the hydrophobic residues can also be mutated because there is also the possibility that the N-terminus of Mtr4 forms an amphipathic helix. By mutating pheylalanine and valine to alanine or Serine, the clustering of hydrophobicity should be disrupted and affect the structure of the N-terminus. These mutants can also be tested for growth, RNA, and protein-protein interaction. The N-terminus of Ski2 has been shown to be important for protein-protein interaction with the Ski complex proteins. Specifically, Ski2 requires its N-terminus to interact with the scaffolding protein Ski3. Thus, Ski3 bridges the interaction with the Ski8 homodimer (Wang *et al.*, 2005). I believe that the N-terminus of Mtr4 has an important role in the function of Mtr4, similarly to the N-terminus of Ski2, and it must be studied.

The interaction between the TRAMP complex and Mtr4 may not be required for cell viability

Here, I have shown that although Mtr4 has multiple domains of interaction with Trf5 and the exosome cap proteins, Trf5 only requires a specific 20 amino acid patch to associate with Mtr4. I narrowed down the Mtr4 binding pocket to residues 98-117 of Trf5 which are outside of the catalytic domains and have a high level of conservation in both Trf5 and Trf4 proteins. The 20-mer peptide is sufficient to interact with Mtr4 both *in vivo* and *in vitro* (Y2H and *pers. comm* Sean Johnson's Lab). The fact that the 20-mer peptide interacts with purified Mtr4 *in vitro* provides evidence that the physical interaction between Mtr4 and Trf5 *in vivo* is direct.

Furthermore, deleting these 20-amino acids completely interferes with the interaction with Mtr4 through Y2H and immunoprecipitation assay.

The most interesting finding is that the loss of interaction between Trf5 and Mtr4 seen in the *trf5*Δ98-117 complements the *trf4*Δ/*trf5*Δ to wild-type levels. It has yet to be determined whether TRAMP function is affected when Mtr4 does not associate with the other TRAMP subunits. To analyze whether Mtr4 needs to physically associate with the Trf protein to function properly, RNA defects need to be analyzed. TRAMP is involved in the degradation of aberrant RNAs by the exosome. RNA can be isolated from the *trf5*Δ98-117 strain, and northern blot performed. The accumulation of 23S pre-rRNA, CUTs, and poly(A) snoRNAs can then be analyzed. If these degradation products accumulate in the *trf5*Δ98-117 strain, then it is required that Mtr4 binds to Trf5 for TRAMP to function normally. If this is the case then this will be the first line of evidence showing that TRAMP function is not essential for life. If TRAMP function is not disrupted then Mtr4 continues to coordinate TRAMP functions even when not physically associated with the Trf protein. In this case, TRAMP function may still be essential for life.

Determining whether Trf4 also requires this 20-mer region to interact with Mtr4

These experiments need to also be recapitulated with Trf4. Trf4 contains an equivalent region that contains this highly conserved patch of residues. Residues 118-134 should be internally deleted from the protein and tested for complementation in the *trf4*Δ/*trf5*Δ strain and protein-protein interaction with

Mtr4. I hypothesize that the same result will be true for Trf4, however, it is important to confirm that both of these TRAMP subunits associate similarly towards Mtr4. A contrasting result may indicate that Trf4 and Trf5 associate differently with Mtr4. Recently, it was shown that an N-terminal truncation of Trf4 (ΔN181-Trf4), which lacks the conserved patch of residues, does not complement the *trf4*Δ/*trf5*Δ strain (Holub et al., 2012). Several possible conclusions can be drawn. First, the loss of Mtr4 interaction, in the case of Trf4, disrupts cell viability. Second, residues at the far N-terminus (outside of the 20 residues) of Trf4 may be involved in promoting an important function essential for growth. Third, both the 118-134 and the N-terminal region of Trf4 are essential for growth. By repeating the genetic, Y2H, and TAP experiments in the equivalent Trf4 internal deletion, the correct conclusion can be drawn.

Analyzing the phenotype of an *mtr4-archless*/*trf5*Δ98-117 strain

The data obtained regarding *mtr4-archless* suggested that the arch domain may be modulating the protein-protein interaction capabilities of Mtr4. Specifically, Mtr4-archless interacts with other proteins with an increased affinity relative to Mtr4. Furthermore, the analysis of the *trf5*Δ98-117 mutant indicated that it loses binding to Mtr4. Thus, a combined *mtr4-archless/trf5*Δ98-117 strain should suppress the *mtr4-archless* growth phenotype. I propose that the increased interaction between Mtr4-archless to Trf5 should be affected when using a Trf5 that lacks the Mtr4-interacting residues. If the double mutant complements an *mtr4*Δ strain, then it will indicate that the increased protein binding directly causes the

slow growth phenotype. Moreover, if the growth and RNA defects are not restored, it may indicate that the Mtr4-archless interacts with Trf5 somewhere outside of the conserved 20-mer patch, or that the binding dynamics of Mtr4 don't directly affect the cell viability. Co-immunoprecipitation analysis will be able to distinguish between these two possibilities. The Trf5Δ98-117 is already TAP tagged, therefore, it can be affinity purified from *mtr4-archless/trf5*Δ98-117 cells and western blot using Mtr4 antibodies performed. The precipitate can be compared side-by-side with the *MTR4* strains (just like Figure 6.7) to quantify the levels of Mtr4 precipitate. This analysis will also identify which one of the proteins is the major "binder" of the two. Most importantly, these experiments will confirm whether the cell growth defect is caused by Mtr4-archless being sequestered by Tr4/5.

Concluding Remarks

The findings presented in this dissertation have advanced our understanding of Mtr4, Ski2, the TRAMP complex, and the exosome. Specifically, I have identified and characterized the novel arch domain of Mtr4, and to a lesser extent, Ski2. The arch domains contribute to the function of the helicase, specifically promoting exosome-mediated functions. However, it is still unclear why the exosome requires cofactors with this special domain. Follow-up experiments need to be performed to understand the role and mechanism of this accessory domain, which is only present in these two exosome helicase cofactors. Further, I have described a *trf5* mutant that does not interact with Mtr4. This *trf5 strain* will provide the RNA and exosome field a tool for studying Mtr4 functions both TRAMP-dependent and independent.

Additionally, we will be able to dismiss the possibility that the essential function of the exosome involves its degradation functions, placing us one step closer to finding the holy grail of exosome function.

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VITA

Ana Alejandra (Ale) Klauer-King was born in Lima, Peru, the daughter of Armando S. Klauer and Ana Maria Klauer. Ale immigrated to the United States in November of 2000. She went to Westside High school and graduate with honors and the Biology award in 2003. Ale decided attended Texas A&M University, where she studied Genetics and Microbiology and performed bench research on quorum sensing in *Salmonella typhimirium* at Texas A&M. Ale graduated in 2007 with a B.S. in Microbiology from Texas A&M University in College Station. Ale joined UT-Graduate School in Biomedical Science in Houston in August of 2007 and chose the Microbiology and Molecular Genetics program. She joined the laboratory of Ambro van Hoof in July 2008, where she characterized cofactors of the RNA exosome. She has enjoyed her graduate school experience very much.

In January of 2013, Ale will begin the Postdoc Professionals Master Program (PPM) at the Keck Graduate Institute in Claremont, California. Ale hopes to pursue a career in the business of biotechnology and drug development.