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Nrd1 Interacts with the Nuclear Exosome for 3′ Processing of RNA Polymerase II Transcripts

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Summary

The exosome complex is involved in multiple RNA processing and degradation pathways. How exosome is recruited to particular RNA substrates and then chooses between RNA processing and degradation modes remains unclear. We find that the RNA binding protein Nrd1, complexed with its partners Nab3, Sen1, and cap binding complex, physically interacts with the nuclear form of exosome. Nrd1 stimulates the RNA degradation activity of the exosome in vitro. However, Nrd1 can also block 3′ to 5′ degradation by the exosome at some Nrd1 binding sites. Nrd1 mutations share some phenotypes with exosome mutants, including increased readthrough transcription from several mRNA and sn/snoRNA genes. Therefore, Nrd1 may recruit exosome to RNA and influence the choice between processing and degradation. Since Nrd1 is known to bind RNA polymerase II and be important for sn/snoRNA 3′ end processing, Nrd1 may link transcription and RNA 3′ end formation with surveillance by the exosome.

Introduction

Gene expression involves extensive coordination between multiple processes. One point of coordination is the processing of pre-mRNA 3′ ends. In addition to being essential for transcript maturation, polyadenylation links transcription and mRNA export to the cytoplasm (reviewed in Jensen et al. [2003]). In *Saccharomyces cerevisiae*, defects in mRNA 3′ end formation cause accumulation of aberrant transcripts in foci near the sites of transcription (Hilleren et al., 2001; Libri et al., 2002). Therefore, release of newly synthesized mRNA from the transcription site may not be a passive process but instead may be a checkpoint for mRNA integrity (Jensen et al., 2001, 2003, 2004).

An important component of nuclear mRNA surveillance is the exosome complex, which consists of multiple 3′ to 5′ exoribonuclease subunits. This complex functions in both degradation of aberrant mRNAs (Bousquet-Antonelli et al., 2000; Mitchell and Tollervey, 2000; Hilleren et al., 2001; Butler, 2002; Symmons et al., 2002; Jensen et al., 2003) and maturation of some noncoding RNAs (see Perumal and Reddy [2002], Raijmakers et al. [2004]). Nuclear exosome differs from its cytoplasmic counterpart by the presence of the extra subunits Rrp6, Rrp47/Lrp1, and Mtr4 (Mitchell et al., 2003; Saguez et al., 2005). The other ten "core" exonuclease subunits are in both nuclear and cytoplasmic exosomes. In the absence of Rrp6, aberrant pre-mRNAs are no longer retained at transcription foci (Hilleren et al., 2001; Jensen et al., 2001; Libri et al., 2002). Further linking transcription and mRNA surveillance, the nuclear exosome in *Drosophila* directly associates with the transcription elongation factors Spt5 and Spt6 (Andrulis et al., 2002).

The noncoding snRNAs and snoRNAs are transcribed as longer precursors by RNA polymerase II (RNApII). They undergo endonucleolytic cleavage by RNase III followed by exosome-mediated 3′ end trimming (Perumal and Reddy, 2002). Pre-mRNAs are also internally cleaved, by polyadenylation factors rather than RNase III, and the new 3′ ends are then polyadenylated (reviewed in Tollervey [2004], Luo and Bentley [2004]). Polyadenylation factors recognize specific RNA sequences in the transcript (Zhao et al., 1999). Cotranscriptional 3′ end processing is facilitated by association of the C-terminal domain (CTD) of RNApII largest subunit Rpb1 with the polyadenylation factors (Proudfoot et al., 2002). Proper 3′ end formation of snRNAs and snoRNAs also requires the CTD and the CTD kinase Ctk1 (Steinmetz et al., 2001) as well as several of the factors involved in mRNA 3′ end formation (Fatica et al., 2000; Dheur et al., 2003; Morlando et al., 2002).

How does exosome distinguish RNAs destined for 3′ end trimming from substrates that are to be completely degraded? We find that Nrd1 can recruit nuclear exosome to RNA substrates and may influence the choice between degradation and 3′ end trimming. These findings reinforce the concept of crosstalk between mRNA surveillance, processing, and transcription termination.

Results

In Vitro Activities of Purified Nuclear Exosome

To study how exosome interacts with aberrant and normal 3′ ends of mRNAs, nuclear exosome complex was purified from *S. cerevisiae* using TAP (tandem affinity purification)-tagged Rrp6 subunit (Figure 1A). Purified exosome was incubated with two different *GAL7* RNA substrates (Figure 1D) (Chen and Moore, 1992; Gross and Moore, 2001). Two distinct outcomes were observed. RNA with a 3′ terminus corresponding to that of properly cleaved *GAL7* transcript (*GAL7-9*) was degraded to short products that ran off the gel (Figure 1D and Figure S1 (in the Supplemental Data available with this article online), see also Figure 3C). In contrast, an RNA substrate with sequences extending beyond the normal polyadenylation site (*GAL7-1*) gave rise to a discrete stable fragment ~30 nt shorter than the input RNA, which was designated the "strong stop" product (Figure 1B).

The resistance of the strong stop product could be explained by sequence specificity of the degradation reaction or by a stable secondary structure element that blocks exosome passage. The latter seems unlikely, based on the low GC content and secondary structure predictions of the *GAL7-1* RNA (Zuker, 2003). The exosome subunit Mtr4 is a predicted ATP-dependent RNA

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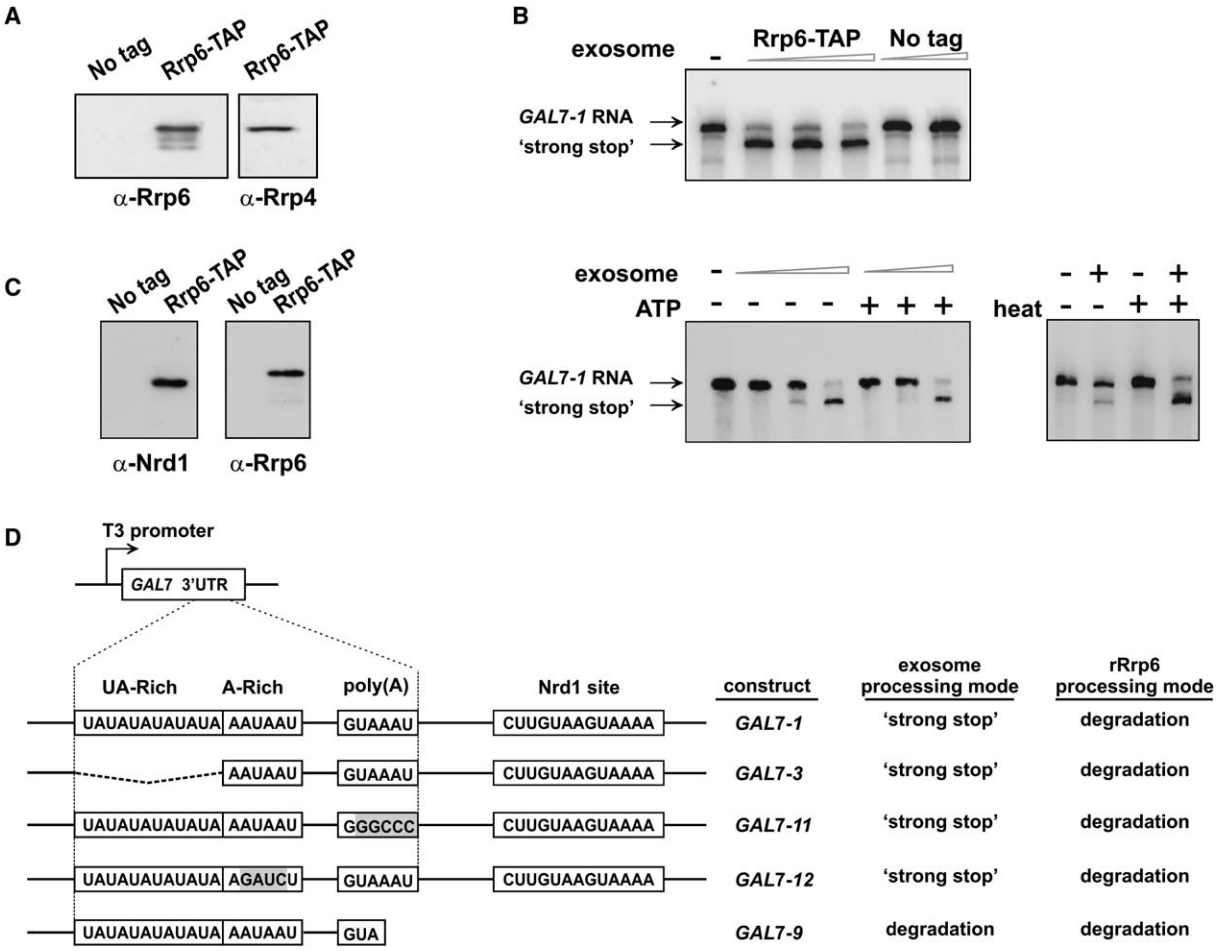


Figure 1. Purification and Activity of Rrp6-TAP Exosome

(A) Rrp6-TAP-associated proteins were assayed with antibodies against Rrp6 and Rrp4 (Mitchell et al., 1997, 2003). A mock purification was carried out with an untagged strain (No tag) as a negative control.

(B) A *GAL7-1* RNA was incubated with increasing amounts of Rrp6-TAP exosome or a mock purification from an untagged strain (upper panel). The effects of ATP or prior heat denaturation (80°C) on the reaction were tested (lower panels).

(C) Rrp6-TAP exosome was assayed for Nrd1 by immunoblotting.

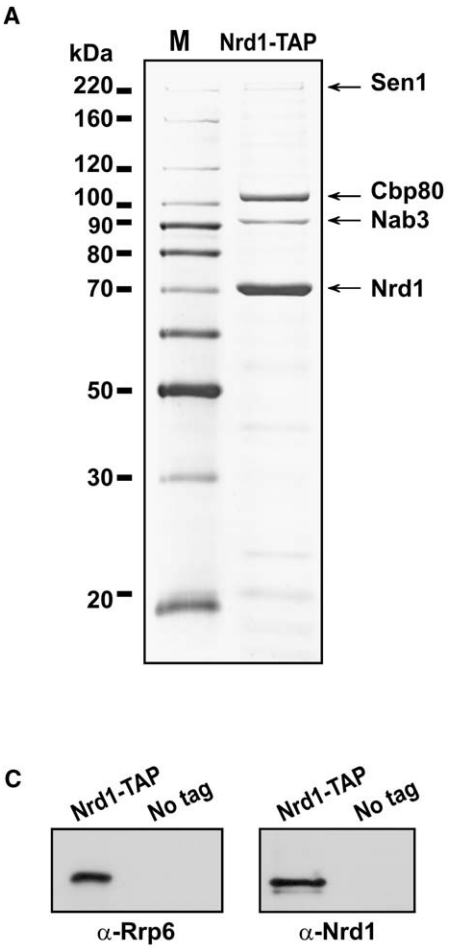
(D) Schematic representation of the *GAL7* 3′UTR region and RNA substrates used in degradation assay using Rrp6-TAP or recombinant Rrp6. Characterized 3′-end processing sequence elements (UA-rich, A-rich, poly(A) site) and Nrd1 sequence containing the strong stop are boxed. The mutated nucleotides are shaded gray.

helicase, but the addition of ATP did not affect the rate or specificity of the reaction (Figure 1B). To further rule out secondary structure, the *GAL7-1* RNA was heat denatured, followed by quick chilling on ice. This treatment did not reduce the strong stop but did increase the overall reaction efficiency (Figure 1B).

To test the role of RNA sequence, exosome was incubated with additional variants of the *GAL7* RNA substrate. RNAs lacking the UA-rich sequence (*GAL7-3*) or with nucleotide substitutions in the A-rich sequence (*GAL7-12*) or the polyadenylation signal (*GAL7-11*) gave products similar to *GAL7-1* (Figure 1D and Figure S1). Therefore, any exosome sequence specificity is distinct from the sequences that contribute to polyadenylation. Interestingly, recombinant Rrp6 efficiently degraded all the substrates with no strong stop product, indicating that there is no general block to exonuclease activity on the RNA (Figure 1D and data not shown). Mitchell et al. (1997) reported that purified exosome has low in vitro activity, while individual exosome subunits are very active exonucleases. Taken together, the above data indicate that the strong-stop signal located downstream of the polyadenylation site is recognized by the intact nuclear exosome or an associated protein.

Nrd1 Is Associated with Exosome

The 3′ end of the strong stop product was mapped by ligation-mediated RT-PCR. Sequencing of 24 cDNAs showed that exosome digestion stops within a window ~100–106 nt downstream of the poly(A) signal at the sequence …CUUGUAAGUAAAA-3′ (mapped 3′ ends are underlined). This sequence is similar to that recognized by the Nrd1 protein (CUUUGUAAAACGGU as defined by Steinmetz and Brow [1998]; GUAA/G as defined by Carroll et al. [2004]). Nrd1 has RNA and pol II CTD binding domains and it is important for transcription termination of sn/snoRNA genes (Steinmetz and Brow, 1996, 1998;



B

	Protein	Number of peptides	Protein function
Nrd1 complex	Sen1	57	RNA helicase, sn/snoRNA processing
	Nab3	42	sn/snoRNA termination
	Cbp80	32	Cap-binding
	Cbp20	5	Cap-binding
	Nrd1	27	sn/snoRNA termination
RNA pol II complex	Spt5	24	transcription factor
	Rpb1	24	RNApol II subunit
	Rpb2	19	RNApol II subunit
	Rpb3	9	RNApol II subunit
	Rpb5	3	RNApol II subunit
	Rpb4	2	RNApol II subunit
RNA processing complex	Rrp6	15	nuclear exosome
	Rrp44	5	exosome core
	Rrp4, Csl4, Rrp45, Rrp43	8	exosome core
	Air2	9	Trf4 cofactor
	Trf4	7	poly(A) polymerase
	Pab1	5	poly(A) binding
	Rnt1	3	sno/snRNA processing, endonuclease

Figure 2. Purification of Nrd1-Associated Proteins

(A) SDS-PAGE analysis of proteins purified from a Nrd1-TAP strain. Molecular weight markers were run in parallel (lane M).

(B) List of the proteins associated with Nrd1 as identified by mass spectrometry. The full list of identified peptides is included in Supplemental Data.

(C) Immunoblot analyses of precipitates from the nontagged strain (BY4741) and Nrd1-TAP strains with antisera specific to Rrp6 and Nrd1.

Conrad et al., 2000; Steinmetz et al., 2001). We tested whether Nrd1 was present in the TAP-tagged exosome preparation. Indeed, anti-Nrd1-specific antibodies (Steinmetz and Brow, 1998) readily detected Nrd1 copurifying with exosome (Figure 1C). The strong stop product could be a consequence of Nrd1 binding to the RNA and impeding further degradation by exosome. Recombinant Nrd1 can bind to *GAL7-1* RNA, while binding to *GAL7-9* RNA had approximately 60-fold lower affinity (data not shown).

TAP-tagged Nrd1 was isolated from cells (Mitchell et al., 2003; Puig et al., 2001) and copurified with the putative RNA-helicase Sen1, the RNA binding protein Nab3, and cap binding complex (CBC) subunits Cbp80 and Cbp20 (Figures 2A and 2B and Supplemental Data). Sen1 and Nab3 are known to function with Nrd1, since mutant alleles of *nrd1*, *sen1*, or *nab3* exhibit termination defects in snoRNA transcription (Steinmetz et al., 2001). The RNA sequences 5′-CUU-3′ recognized by Nab3 and 5′-GUAAA-3′ recognized by Nrd1 (Carroll et al., 2004) are important for this pathway, since insertion of a Nrd1 recognition element into a normal mRNA gene leads to premature transcription termination (Steinmetz and Brow, 1996, 1998). A physical interaction between Nrd1 and Nab3 proteins has been observed previously by coimmunoprecipitation (Conrad et al., 2000).

Mass spectrometry identified other putative Nrd1-interacting proteins (Figure 2B and Supplemental Data), which included components of the transcription elongation complex (RNApII subunits and elongation factor Spt5). Also observed were multiple peptides from the nuclear exosome, including Rrp6, Rrp44, Rrp4, Rrp45, and Csl4. Also detected was the poly(A) polymerase Trf4 and its interacting partner Air2, which target RNAs for degradation by exosome (Kadaba et al., 2004; Vanacova et al., 2005; LaCava et al., 2005; Wyers et al., 2005). Also associated with Nrd1 was the endonuclease Rnt1 (RNase III). Interestingly, an interaction between Rnt1 and Sen1 was previously observed using both the yeast two-hybrid system and coimmunoprecipitation (Ursic et al., 2004). Rnt1 and Sen1 are involved in late stages of sn/snoRNA and U5S 3′ end processing (Rasmussen and Culbertson, 1998; Ursic et al., 2004). Also detected was the polyA binding protein PAB1.

Association of Rrp6 and Nrd1 in vivo was confirmed by coprecipitation and immunoblotting (Figures 1C and 2C). This association was unaffected by RNase A, suggesting that it is not RNA mediated (data not shown). The Nrd1-TAP fraction also contained several ribosomal proteins (see Supplemental Data), but these are common false positives in TAP purifications (Gavin et al., 2002; Ho et al., 2002). Although mass spectroscopy is not quantitative, the relative number of peptides and silver staining suggest that exosome and RNApII are substoichiometric to Nrd1.

Nrd1 Can Recruit Exosome to RNA

To test if Nrd1 association affects its activity, nuclear exosome was immunoprecipitated from wild-type and *nrd1* mutants via Rrp6 (Figure 3). Deletion of the Nrd1 RNA-recognition motif (RRM, aa 340–410) is lethal (Conrad et al., 2000), but the *nrd1-2* allele, which has a stop codon at residue 286, supports slow growth when expressed from a high-copy plasmid due to a small amount of readthrough (Steinmetz and Brow, 1998). The Nrd1-2 protein includes the CTD-interacting domain (CID, aa 1–140) (Yuryev et al., 1996) and an ArgSerGlu-rich segment that is phosphorylated in vivo (Conrad et al., 2000; Ficarro et al., 2002). The *nrd1*$\Delta^{CID(39–169)}$ allele lacks the Nrd1 CID, which is dispensable for cell viability (Steinmetz and Brow, 1998). Also used was a *nrd1-102* temperature-sensitive strain, which has a single-point mutation in the RRM (Conrad et al., 2000). Amounts of exosome immunoprecipitated from wild-type, *nrd1-2*, or *nrd1*$\Delta^{CID(39–169)}$ were roughly equivalent (Figure 3B). Also, Nrd1-102 protein copurified with Rrp6-TAP at levels similar to wild-type (Figure 3B).

Incubating *GAL7-1* RNA with exosome from wild-type or *nrd1*$\Delta^{CID(39–169)}$ strains produced the strong stop fragment, while *GAL7-9* RNA was degraded without this intermediate (Figure 3C). Exosomes from *nrd1-102* or *nrd1-2* were very defective in degradation. Addition of recombinant Nrd1 strongly stimulated activity of exosome from not only *nrd1*$\Delta^{CID(39–169)}$ and *nrd1-2* but also wild-type cells. To determine if Nrd1 also affected exosome processing of sn/snoRNA substrates, two U14 RNA precursors were treated with exosome from *nrd1-2* cells, either with or without recombinant Nrd1 (Figure 3D). In vivo, an snR190-U14 dimeric RNA must be cleaved by Rnt1 before exosome processing. This substrate was not efficiently digested by exosome in vitro. The second RNA substrate was a 5′- and 3′-extended U14 that resembles the product of Rnt1 cleavage. The addition of Nrd1 to this reaction produced a product with the size expected for properly processed U14.

GAL7 may not be a physiological substrate of Nrd1/exosome complex, so we assayed an RNA known to be regulated by Nrd1 in vivo. Nrd1 downregulates its own mRNA levels (Steinmetz et al., 2001). Interestingly, this feedback loop requires exosome and Nrd1 and Nab3 binding sites within the mRNA (J.T. Arigo and J. Corden, personal communication). In vitro degradation assays were performed with wild-type *NRD1* RNA or the *nrd1-218* variant in which all Nrd1 and Nab3 binding sites had been mutated. Wild-type RNA was degraded, while the mutant was largely resistant (Figure 3E). In agreement with these findings, the *nrd1-218* mRNA levels are significantly higher than wild-type in vivo (J.T. Arigo and J. Corden, personal communication). These results suggest that Nrd1 protein stimulates exosome activity, perhaps by recruiting exosome to substrate RNAs with Nrd1 binding sites.

Accumulation of Extended RNA Species in *NRD1* and Exosome Mutants

Nuclear exosome degrades improperly terminated, readthrough mRNA transcripts (Mitchell and Tollervey, 2000). To test whether Nrd1 contributes to this process, we analyzed RNAs in cells mutated for exosome or Nrd1. Depletion of Rrp6 or exosome core subunit Rrp4 resulted in accumulation of 3′-extended readthrough mRNAs for *RNA15* and *CYC1* (Figure 4A). A similar effect was seen with the *nrd1* mutant strains. The 3′-extended *CYC1* mRNA accumulation was particularly dramatic in the *nrd1-2* strain. A recent analysis of *NAB2* expression showed that exosome can downregulate properly processed mRNAs. Depletion of exosome components increased normal and 3′-extended *NAB2* mRNA levels (Roth et al., 2005). We confirmed this result and found that the most severe Nrd1 mutant, *nrd1-2*, also had increased levels of *NAB2* mRNA (Figure 4A). In contrast, extended *NAB3* and *SEN1* mRNAs did not accumulate in strains depleted of exosome components or in the Nrd1 mutants (Figure 4B). Therefore, the exosome may not degrade readthrough mRNAs at all genes, or there may be some genes that do not produce readthrough transcripts.

The effects of Nrd1 and exosome mutants were compared to a strain with a temperature-sensitive allele of the termination/polyadenylation factor Rna15 (Figures 4A and 4B). The 3′-extended species of *RNA15*, *CYC1*, and *NAB2* mRNAs were seen at relatively high levels at the permissive and even more so at the nonpermissive temperature. Interestingly, the *NAB2* readthrough product in the *rna15-2* strain was a discrete band rather than a smear as seen in the exosome mutants. Readthrough transcripts of *SEN1* and *NAB3* were not detectable in the *rna15-2* strain, while levels of properly processed mRNAs were greatly reduced, particularly after temperature shift. Although *rna15-2* may lead to readthrough transcription at these genes, these species may be rapidly degraded by exosome. Clearly, *nrd1* mutants behave differently from the *rna15-2* mutant.

The effect of inactivating exosome or Nrd1 on snoRNA processing was also examined (Figure 4C). Extended U14 RNAs were detected in *rrp6*Δ strains. Similar but not identical patterns were observed for *rrp4-1*, *nrd1-2*, and *nrd1*$\Delta^{CID(39–169)}$ strains, with only longer readthrough RNA species seen. This is consistent with Nrd1 and exosome both acting in U14 3′ end processing or termination. The intermediate-sized transcripts in the *rrp6* deletion strains suggest that Rrp6 and Rrp4 may have nonredundant roles in U14 trimming. Several groups (Allmang et al., 1999; van Hoof et al., 2000; Mitchell et al., 2003) reported extended, polyadenylated U14 species in strains lacking Rrp6. Surprisingly, the *rna15-2* mutant did not have any obvious defects, suggesting that Rna15 is not necessary for 3′ end processing of U14.

Torchet et al. (2002) found that deletion of *RRP6* can partially "rescue" defective mRNAs produced in an *rna14* mutant strain. We confirmed this, observing stabilization of *NAB3* transcripts in an *rna14-1/rrp6*Δ double

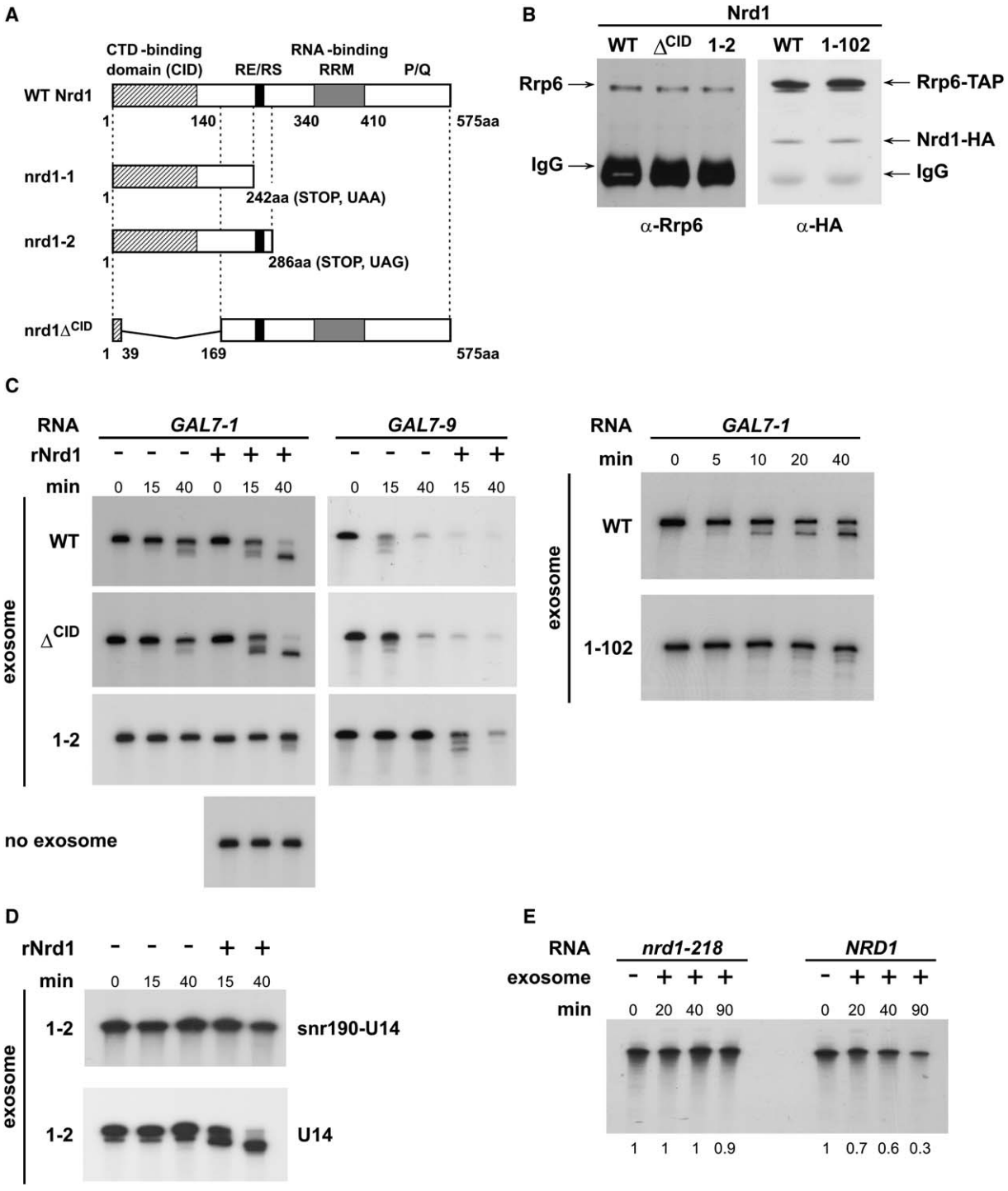


Figure 3. Nrd1 Stimulates Nuclear Exosome Exoribonuclease Activity In Vitro

(A) Schematic diagram of Nrd1 structural organization (from Steinmetz and Brow [1996]). The RNApII CTD binding (CID, aa 1–140), RNA binding (RRM, aa 340–410) domains, and RE/RS region (arginine-, serine-, and glutamate-rich, aa 245–265) are marked; P/Q is a proline-, glutamine-rich region (aa 500–575). Structure of Nrd1 mutants is shown, except for *nrd1-102*, which is a V379G point mutation in the RRM (Conrad et al., 2000).

(B) Equivalent amounts of Rrp6 immunopurified from wild-type, *nrd1-2*, and *nrd1*$\Delta^{CID(39-169)}$ mutant strains (left panel). The right panel shows that copurified Nrd1 and Rrp6 levels are similar in Rrp6-TAP purifications from wild-type and *nrd1-102* strains.

(C) Nrd1 stimulates exosome activity in vitro. *GAL7-1* and *GAL7-9* RNA substrates were incubated with α-Rrp6 immunopurified exosome from wild-type, *nrd1-2*, or *nrd1*$\Delta^{CID(39-169)}$ strains in the absence or presence of recombinant Nrd1 (3 pmol). The right panel shows a similar experiment with exosome from the *nrd1-102* strain.

(D) Nrd1 stimulates exosome snoRNA processing activity in vitro. Snr190-U14 and U14 RNA substrates were incubated with α-Rrp6 immunopurified exosome from *nrd1-2* cells for the indicated time in the absence or presence of rNrd1.

(E) Efficient exosome-mediated degradation of the *NRD1* mRNA requires Nrd1 and Nab3 binding sites. Exosome immunopurified from Nrd1 wild-type cells was incubated with a wild-type *NRD1* RNA or a mutated version lacking Nrd1 and Nab3 binding sites (*nrd1-218*, provided by J.T. Arigo and J. Corden).

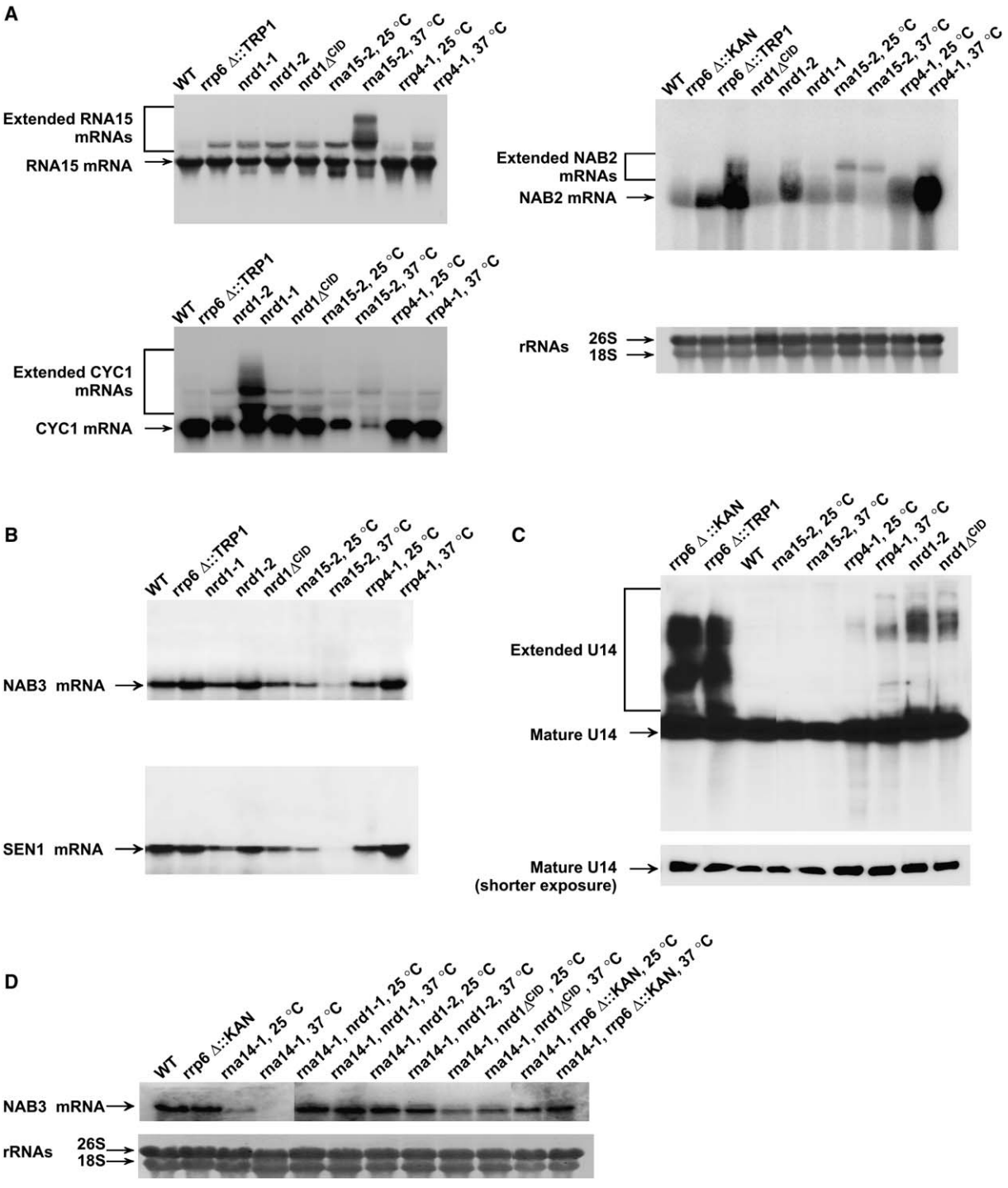


Figure 4. Effect of Exosome and Nrd1 Mutants on Various RNAs

Northern blot analysis was performed with total RNA from the indicated strains grown at 30° unless otherwise indicated. Positions of 3′-extended and mature mRNA species are indicated.

(A) Increased readthrough transcripts at *RNA15*, *CYC1*, *NAB2* genes. Methylene-blue stained 18S and 26S ribosomal RNAs are shown as a loading control.

(B) *NAB3* and *SEN1* transcripts are unaffected.

(C) Increased extended transcripts of the U14 snoRNA. The lower panel is a shorter exposure of the hybridized blot showing the mature U14 RNA band.

(D) Mutations in *RRP6* or *NRD1* rescue defective transcripts produced in an *rna14-1* strain at the nonpermissive temperature. Northern blot analysis of the *NAB3* mRNA from the indicated strains is shown.

mutant strain at the nonpermissive temperature (Figure 4D). Interestingly, a similar stabilization is seen when *rna14-1* is combined with the *nrd1* mutants.

Discussion

The exosome functions in multiple RNA processing and degradation pathways, but how exosome is recruited to particular RNA substrates and how the choice between processing and complete degradation is made remains unclear. Presumably, these outcomes are determined by recognition of particular RNA sequences or secondary structures. Here we report a physical connection between exosome and the sequence-specific RNA binding protein Nrd1. Although this interaction appears to be substoichiometric, several experiments suggest that it is functionally relevant. Recombinant Nrd1 stimulates the ability of exosome to degrade RNA in vitro. Furthermore, in vitro exosome degradation of a *GAL7* substrate paused at a tandem Nrd1 binding site. It will be interesting to determine whether the Nrd1 complex protects any RNA 3′ ends from exosome in vivo. Finally, mutations in either *NRD1* or exosome increase readthrough transcript levels at several mRNA and sn/snoRNA genes. These physical and functional associations suggest that the Nrd1 complex may recruit exosome to RNA substrates and influence whether exosome is in RNA "processing" or "degradation" mode (Figures 5A and 5B).

Purification of Nrd1-TAP isolated several interesting interacting proteins in addition to exosome subunits. Nab3 and Sen1 have previously been linked to Nrd1. The RNA binding protein Nab3 interacts genetically and physically with Nrd1 (Conrad et al., 2000). Nab3 recognition sites are often found in proximity to Nrd1 sites, and the two proteins may bind RNA cooperatively (Carroll et al., 2004). Mutations in *SEN1*, encoding a putative RNA helicase, cause transcription readthrough in the same system used to isolate *NRD1* mutants (Steinmetz et al., 2001). We find all three of these proteins and exosome in a complex with RNApII and the elongation factor Spt5. This echoes a report that exosome interacts with RNApII and the transcription factors Spt5 and Spt6 in *Drosophila* (Andrulis et al., 2002). This might be an elongation or termination complex for nonpolyadenylated RNAs.

Two other proteins connected to exosome were found at substoichiometric levels in the Nrd1 purification. The Trf4 poly(A) polymerase and its interacting partner Air2 polyadenylate rRNA, tRNAs, and sn/snoRNAs (Kadaba et al., 2004; Vanacova et al., 2005; LaCava et al., 2005; Wyers et al., 2005), and this modification targets the RNAs for destruction or processing by the exosome (van Hoof et al., 2000; Fang et al., 2004; Kadaba et al., 2004; Vanacova et al., 2005; LaCava et al., 2005; Wyers et al., 2005). It is not yet clear whether exosome is recruited by the poly(A) tail itself or via other proteins involved in this process. Also found in the Nrd1 fraction was the Rnt1 endoribonuclease (RNase III), which cleaves double-stranded RNA structures, in some cases generating a new 3′ end that is subsequently processed by the exosome (Chanfreau et al., 1997, 1998). Interestingly, Rnt1 interacts physically and functionally with Sen1 (Ursic et al., 2004). The CBC subunits Cbp80 and Cbp20 also copurified with Nrd1-TAP. Das et al. (2000, 2003) found that Cbp80 functions in a nuclear mRNA degradation pathway that also requires exosome. Deletion of *CBP80* suppresses mutant phenotypes caused by a crippled *CYC1* polyA site (the *cyc1-512* allele, see Zaret and Sherman [1982]), in part by stabilizing extended *CYC1* transcripts. Properly processed mRNAs can also be degraded in a Cbc80-dependent manner in the nucleus. The physical association of Cbp80 with Nrd1 and exosome may help explain these observations. This interaction may also explain why Nrd1 crosslinks to 5′ as well as 3′ ends of mRNA encoding genes (Nedea et al., 2003).

Nrd1, Nab3, and Sen1 are important for transcription termination by RNApII at sn/snoRNA genes (Steinmetz and Brow, 1996, 1998; Conrad et al., 2000; Steinmetz et al., 2001). Our data suggest that Nrd1 may also recruit and activate exosome at the 3′ end of sn/snoRNAs for subsequent trimming. The association between Nrd1 and exosome may help link termination and 3′ end processing at these nonpolyadenylated transcripts. These two functions may depend upon different domains of Nrd1. The RNA binding domain of Nrd1 is essential for viability and is probably required for recruitment/activation of exosome. Exosome purified from the *nrd1-2* strain was inactive for in vitro RNA degradation (Figure 3). The Nrd1 CID is not needed for this function; in vitro activity of exosome from *nrd1*$\Delta^{CID(39–169)}$ cells was comparable to that of exosome from a wild-type Nrd1 strain. In contrast, alteration of either the Nrd1 RRM or CID leads to defective sn/snoRNA transcription termination (Steinmetz and Brow, 1996, 1998; Conrad et al., 2000; Steinmetz et al., 2001).

Due to defects in termination and/or trimming, both Nrd1 and exosome mutations result in accumulation of 3′-extended U14 snoRNA transcripts (Figure 4C). Presumably, the *nrd1*$\Delta^{CID(39–169)}$ mutant has extended U14 RNAs because transcription termination requires the interaction of Nrd1 with the RNApII CTD. The mechanism of termination on sn/snoRNA genes is not clear, but it has been suggested that the nascent transcript is cleaved at sites that are often found just downstream of predicted Nrd1 and Nab3 binding sites (Morlando et al., 2002; Fatica et al., 2000). Sn/snoRNA termination apparently requires a subset of the proteins involved in cleavage of polyadenylated mRNAs (Morlando et al., 2002; Dheur et al., 2003), although we did not observe an effect of the *rna15-2* mutation on U14.

The role of Nrd1 in termination and 3′ end formation of mRNAs is less clear. *NRD1* mutants have mRNA effects distinct from mutants in the polyadenylation factor Rna15 (Figures 4A and 4B). Instead, *NRD1* mutants share some RNA phenotypes with exosome mutants, leading us to suggest that Nrd1 may aid exosome in recognizing certain mRNAs as targets for degradation. The *NAB2* mRNA is degraded by the nuclear exosome, with susceptibility mapping to a stretch of 26 adenosine residues near the 3′ end (Roth et al., 2005). In the *rna15-2* mutant, a discrete readthrough product is observed, presumably reflecting termination downstream. In contrast, exosome mutants show dramatically increased levels of *NAB2* mRNA, some of which appear to be extended. These transcripts presumably represent those that would normally be degraded by exosome. Interestingly, the *nrd1-2* mutant also stabilizes the *NAB2* mRNA.

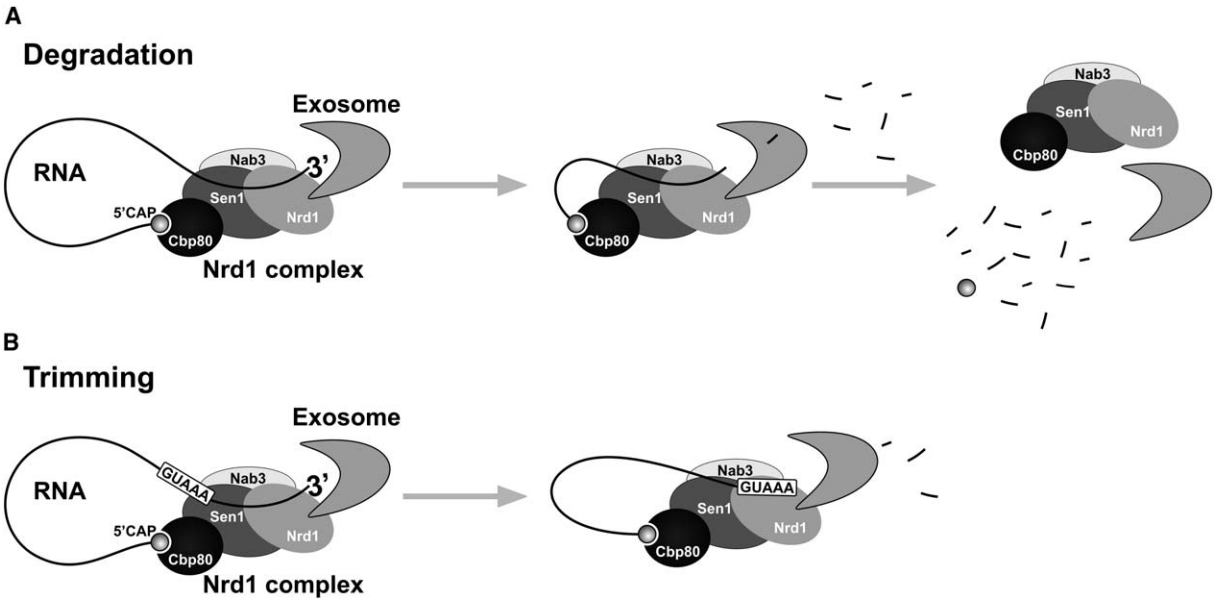


Figure 5. Model for Nrd1-Mediated Degradation and Processing/Trimming Modes of Exosome Activity

(A) In degradation mode, the Nrd1 complex may recruit exosome to an RNA substrate and promote its degradation. This pathway may be used for degradation of *NRD1* and *NAB3* mRNAs and for mRNAs that are not properly polyadenylated.

(B) In processing mode, Nrd1 complex recruits exosome, but processive degradation would be blocked at a particular Nrd1/Nab3 binding site. This pathway could be used for trimming sn/snoRNAs.

The *NRD1* mRNA is another likely physiological substrate for the Nrd1/exosome complex. Nrd1 protein binds to its own mRNA, leading to exosome-dependent downregulation (J.T. Arigo and J. Corden, personal communication). In vitro, immunopurified exosome/Nrd1 complex degrades the wild-type *NRD1* mRNA significantly faster than a derivative mRNA with all the Nrd1 and Nab3 binding sites mutated (Figure 3E). These results are consistent with Nrd1 recruiting exosome. It is unclear whether full-length, polyadenylated *NRD1* mRNA or a prematurely terminated species is the direct target of exosome degradation.

Exosome degrades mRNAs that are not polyadenylated, and it will be interesting to determine whether Nrd1 also plays a role in this process. Like exosome mutants, Nrd1 mutants show increased levels of *RNA15* and *CYC1* readthrough transcripts (Figure 4A). Torchet et al. (2002) showed that nonfunctional readthrough transcripts caused by a polyadenylation factor mutant are normally degraded by exosome, but can be partially rescued in exosome mutants. *NRD1* mutants rescue *NAB3* transcripts in an *rna14-1* mutant background, similar to *rrp6Δ* (Figure 4D). Therefore, Nrd1 may normally contribute to exosome-mediated degradation of improperly 3′-processed mRNAs. If so, the processing mode of exosome would simply differ from degradation mode by the presence of a block to complete exosome digestion (Figure 5).

Experimental Procedures

Yeast Strains and Plasmids

Strains used in this study are listed in Table S1. The coding sequences of Rrp6 and Nrd1 were PCR amplified using *S. cerevisiae* genomic DNA as template and primers Rrp6_ORF_up 5′-CCCAA TAAGTGCGCCATGGCTTCTGAAAATC-3′, Rrp6_ORF_down 5′-CG CTTGTTTTACACTCGAGCCTTTTAAATGACAGA-3′, Nrd1_ORF_up 5′-ACTAAACATCCCCATATGCAGCAGGACGA-3′, and Nrd1_ORF_down 5′-GGTGGAGTAAAGCTCGAGGCTTTGTTGTTGT-3′. The Rrp6_ORF_up primer also changed the tyrosine at position 2 of the Rrp6 protein to alanine. The PCR products were purified, digested with NcoI and XhoI (Rrp6) or NdeI and XhoI (Nrd1) and ligated into the corresponding sites of pET21d or pET41a, respectively. The recombinant plasmids (pET21d-Rrp6 and pET-Nrd1) were verified by sequencing. Full-length proteins were expressed and purified as previously described (Vasiljeva et al., 2001).

Tandem Affinity Purification

Cell extracts were prepared from *S. cerevisiae* grown in YPD medium to an optical density at 600 nm of 1.0. Cell pellets were frozen in liquid nitrogen in lysis buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, 0.5 mM dithiothreitol [DTT], 0.1% NP-40) containing the protease inhibitors 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM benzamidine, 1 mM leupeptin, 2 mM pepstatin A, and 2.6 mM aprotinin. Purification procedures were performed as described previously (Mitchell et al., 2003; Puig et al., 2001) except that immunoglobulin G (IgG)-Sepharose precipitates were treated with RNase A (2 μg/ml) for 1 hr at +4ºC and washed with lysis buffer before cleavage with with tobacco etch virus protease. After the calmodulin column step, purified proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by silver staining. Protein bands were identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry at Harvard's Taplin Mass Spectroscopy Facility. Polyclonal antibodies recognizing Rrp6 or Rrp4 (Mitchell et al., 2003) were provided by P. Mitchell. Polyclonal rabbit antiserum against Nrd1 protein (aa 169–560) was a gift from D. Brow and E. Steinmetz (Steinmetz and Brow, 1998).

RNA Synthesis and In Vitro RNA Processing

RNA synthesis and labeling was done essentially as described (Vasiljeva et al., 2000), except that reactions were performed in the presence of 1 mM of cap analog m7G(5′)ppp(5′)G and 50 μCi of [α-^{32}P] UTP. The RNA substrates corresponding to the *GAL7* 3′UTR region were produced using pJCGAL7-1, pJCGAL7-3, pJCGAL7-11, pJCGAL7-12 digested with AvaI (Chen and Moore, 1992; Gross and Moore, 2001) as DNA templates for T3 RNA polymerase.

To produce sn/snoRNAs, pT7U5P (Chanfreau et al., 1997), pT7UPSsnR190-U14 (Chanfreau et al., 1998), and pT7U14 3′ext (a gift from G. Chanfreau) digested by BamHI were used as templates for T7 polymerase.

RNAs corresponding to the 5′ 1100 nt of the *NRD1* mRNA were expressed using pJC_NRD1 and pJC_nrd1-218 plasmids linearized with SmaI as templates for T7 polymerase. pJC_NRD1 produces a wild-type Nrd1 transcript, while pJC_nrd1-218 is identical, except that all Nrd1 (GTAA/G) and Nab3 (TCTT) binding elements were mutated. These plasmids were generously provided by Jeff Corden.

In vitro reaction mixtures containing 20 mM HEPES-KOH (pH 7.5), 75 mM K-acetate, 2.5 mM Mg-acetate, 5 mM K_2HPO_4 (pH 7.6), 2% PEG 8000, 0.5 mM DTT, 50 pmol [α-^{32}P]-RNA, 0.8 U/μl of RNasin, and either affinity-purified exosome complex or anti-Rrp6 immunocomplex beads were incubated at 30ºC for 20–60 min. The reactions were quenched by the addition of a stop buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, 300 mM NaCl, 1% SDS. The reaction products were extracted with phenol/chloroform/isoamylalcohol (25:24:1), and chloroform and precipitated with 100% ethanol. RNA pellets were resuspended in formamide loading buffer (10 mM Tris-HCl [pH 7.5], 10 mM EDTA [pH 8.0], 95% deionized formamide) and analyzed by 7 M urea/6%–8% PAGE. The radioactive RNA bands were visualized by autoradiography or phosphoimager.

RNA Extraction and Northern Blotting

Total RNA was extracted by the hot phenol method (Schmitt et al., 1990). RNA transfer and Northern blot hybridization was performed as described previously (Sambrook and Russell, 2001). Formaldehyde-containing 1.2% agarose gels and urea-containing 6% PAGE were used to analyze the high (*NAB3*, *NAB2*, *SEN1*, *RNA15*, *CYC1*) and low molecular weight (U14) RNAs, respectively. Probes were generated from PCR products using Megaprime Kit (Amersham) and then purified on Sephadex G-50 microspin colums (BioMax). Membranes were hybridized with the probes for 24 hr at 42ºC and washed five to six times with 2xSSC (300 mM NaCl, sodium citrate 30 mM [pH 7.0]), 0.1% SDS for 20 min at 42ºC and exposed for phosphoimaging.

Chromatin Immunoprecipitations

Cells were incubated at 30ºC until OD_{595} 0.8. Formaldehyde cross-linking, chromatin preparation and immunoprecipitation, and quantitation of the PCR results were performed as described (Keogh and Buratowski, 2004).

Mapping of RNA 3′ Ends

RNA bands from the *GAL7* RNA processing assays were excised from the gel and eluted into buffer containing 20 mM Tris-HCl (pH 8.0), 500 mM ammonium acetate, 10 mM $MgCl_2$, 1 mM EDTA, 0.1% SDS at +4ºC overnight. 3′ ends were mapped by creation of full-length cDNAs as described by Lambden et al. (1992). The cDNAs were cloned into the pCR-TOPO vector (Invitrogen) and sequenced.

Supplemental Data

Supplemental Data include one figure and one table and can be found with this article online at http://www.molecule.org/cgi/content/full/21/2/239/DC1/.

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