

# Different nuclease requirements for exosome-mediated degradation of normal and nonstop mRNAs

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Two general pathways of mRNA decay have been characterized in yeast. In one pathway, the mRNA is degraded by the cytoplasmic form of the exosome. The exosome has both 3' to 5' exonuclease and endoribonuclease activity, and the available evidence suggests that the exonuclease activity is required for the degradation of mRNAs. We confirm here that this is true for normal mRNAs, but that aberrant mRNAs that lack a stop codon can be efficiently degraded in the absence of the exonuclease activity of the exosome. Specifically, we show that the endo- and exonuclease activities of the exosome are both capable of rapidly degrading nonstop mRNAs and ribozyme-cleaved mRNAs. Additionally, the endonuclease activity of the exosome is not required for endonucleolytic cleavage in no-go decay. In vitro, the endonuclease domain of the exosome is active only under nonphysiological conditions, but our findings show that the in vivo activity is sufficient for the rapid degradation of nonstop mRNAs. Thus, whereas normal mRNAs are degraded by two exonucleases (Xrn1p and Rrp44p), several endonucleases contribute to the decay of many aberrant mRNAs, including transcripts subject to nonstop and no-go decay. Our findings suggest that the nuclease requirements for general and nonstop mRNA decay are different, and describe a molecular function of the core exosome that is not disrupted by inactivating its exonuclease activity.

Dis3 | *Saccharomyces cerevisiae*

The core eukaryotic exosome contains nine subunits that are essential for viability, but catalytically inactive (1–4). The catalytic activity is provided by a 10th essential subunit, Rrp44p (2, 5, 6). This protein is similar to RNase II in that it contains three putative RNA binding domains that flank an RNB domain (3, 7–10). The RNB domain is responsible for the 3' exonuclease activity of the exosome (3). In addition, the N terminus of Rrp44p contains an endonucleolytic PIN domain. Either active site is sufficient for viability; however, simultaneous inactivation of both nuclease activities results in a lack of cell growth (11–13). Although the biological substrates of the Rrp44p endonuclease have not been fully elucidated, the synthetic lethality observed upon inactivation of the Rrp44p nucleases implies that they have overlapping functions. The exosome is involved in RNA processing and RNA degradation, and several of these reactions have been shown to be defective if the exonuclease activity of the exosome is disrupted by a point mutation [including 5.8S rRNA and snoRNA processing and 5' external transcribed spacer (ETS) and cryptic unstable transcripts (CUT) degradation]. In contrast, mutating the endonuclease active site of Rrp44p has minor or no effect on the exosome functions that have been tested (11–13).

The exosome is present in both the nucleus and the cytoplasm, and the cytoplasmic exosome plays a dual role in gene expression. First, the exosome is involved in one of two redundant decay pathways for normal mRNAs. The initiating step for decay of normal mRNAs is shortening of the poly(A) tail (14, 15). Removal of the poly(A) tail predominantly triggers cytoplasmic 5'-to-3' decay in yeast (16–19). Deadenylation can also trigger the degradation of an mRNA from its 3' end, in a process catalyzed by the cytoplasmic exosome (20).

The second role of the cytoplasmic exosome is to maintain the fidelity of gene expression by degrading aberrant mRNAs. Aberrant transcripts arise through mistakes in gene expression, including genetic mutations, defects in transcription or splicing, or premature polyadenylation at incorrect or cryptic sites. In one of the exosome-mediated mRNA surveillance pathways, mRNAs that lack in-frame termination codons are targeted to the nonstop decay pathway (21, 22). In the current model of nonstop decay, a translating ribosome stalls at the 3' end, which triggers exosome-mediated decay (22).

Whereas normal mRNAs are degraded mainly by exonucleases, a number of specialized mRNA decay pathways can be initiated by endonuclease cleavage [e.g., no-go decay, RNAi, and nonsense-mediated mRNA decay (23–31)]. For example, in no-go decay, mRNAs that have stalled ribosomes within their coding region are cleaved by an unknown endonuclease (25, 29). Similar cleavage products can be generated by inserting a ribozyme into an mRNA (32). Though these pathways are initiated by a variety of endonucleases, the fragments resulting from this cleavage are degraded by common exonucleases. Specifically, the 5' fragments are degraded by the cytoplasmic exosome, and the 3' fragments are degraded by the 5'-to-3' exonuclease Xrn1p (25–28, 32–34).

Understanding the molecular mechanisms that are responsible for the degradation of aberrant transcripts is needed to understand how these mRNAs are preferentially targeted for rapid degradation and how the fidelity of gene expression is maintained. Additionally, the recent identification of a second nuclease active site in the exosome means that the role of the Rrp44p endonuclease must be examined in the known functions of the exosome. To address these issues, we tested the role of the Rrp44p nuclease activities in general mRNA degradation and in mRNA surveillance. Here we report that nonstop mRNAs and ribozyme-cleaved mRNAs can be degraded by either of the Rrp44p nuclease activities. Additionally, we show that the Rrp44p endonuclease is not responsible for endonucleolytic cleavage in no-go decay, which suggests that endonuclease-mediated nonstop decay is distinct from no-go decay. Our results indicate that the exonuclease activity of Rrp44p is needed for the cytoplasmic exosome-mediated turnover of normal cellular transcripts, but not for the degradation of nonstop mRNAs.

## Results

**Individual Mutations That Disrupt the Endo- or Exonuclease Activity of Rrp44p Do Not Affect Expression of Nonstop Reporters.** Mutations that inactivate the cytoplasmic exosome stabilize transcripts that lack stop codons, suggesting that the exosome degrades such mRNAs (21, 22). However, the cytoplasmic exosome contains two RNase domains, and it is unknown which domain degrades

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nonstop transcripts. To investigate this question we initially analyzed expression of a *his3-nonstop* reporter mRNA. Cells that have a functional nonstop decay pathway rapidly degrade the *his3-nonstop* transcript, resulting in a lack of growth on media lacking histidine. In contrast, in a strain with a defect in nonstop decay, the *his3-nonstop* reporter is stable, which allows cells to grow on media lacking histidine (22). To determine whether the nuclease activities of Rrp44p are required for nonstop decay, a plasmid encoding a *his3-nonstop* reporter was transformed into an *rrp44Δ* strain that was complemented with a catalytically inactive endonuclease point mutant (*rrp44-D171A*, hereafter called *rrp44-endo<sup>-</sup>*) or a catalytically inactive exonuclease point mutant (*rrp44-D551N*, hereafter called *rrp44-exo<sup>-</sup>*). Similar to the wild-type strain, both the *rrp44-endo<sup>-</sup>* and *rrp44-exo<sup>-</sup>* point mutants failed to grow on media lacking histidine, indicating that the *his3-nonstop* mRNA was unstable. In contrast, as previously reported, deletion of the gene for the cytoplasmic exosome cofactor Ski7 allowed for growth on media lacking histidine, indicating that the *his3-nonstop* mRNA was stable in this strain (Fig. 1A) (22). These findings suggest that inactivation of the nuclease activities of Rrp44p, individually, does not affect the expression of a *his3-nonstop* allele.

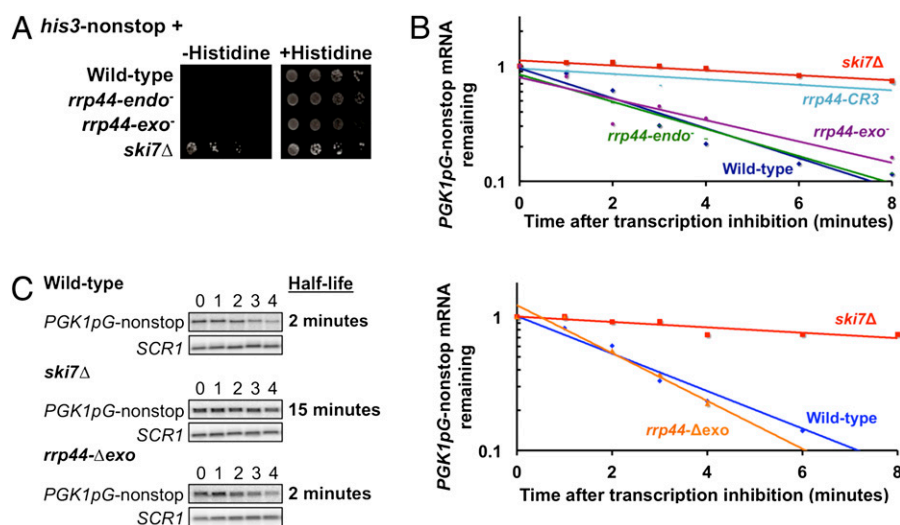
To examine a second nonstop mRNA and more directly determine whether the nuclease activities of Rrp44p are required to degrade nonstop mRNAs, we measured *PGK1pG-nonstop* mRNA decay rates in the *rrp44-endo<sup>-</sup>* and *rrp44-exo<sup>-</sup>* mutants. In this experiment, transcription of the *PGK1pG-nonstop* reporter was repressed by the addition of glucose, and RNA was isolated at various time points. These and subsequent mRNA stability measurements were done using at least two independent time-course experiments, and the average value at each time point is plotted in Fig. 1B. Similar to what was observed in the *his3-nonstop* growth assay, the *PGK1pG-nonstop* transcript was as unstable in the *rrp44-endo<sup>-</sup>* and *rrp44-exo<sup>-</sup>* mutants, as it was in the wild-type strain. In both the *his3-nonstop* and *PGK1-nonstop* assays, the *rrp44-endo<sup>-</sup>* and *rrp44-exo<sup>-</sup>* strains resemble a wild-type strain, and this phenotype is distinct from that of a *ski7Δ* strain. These results indicate that mutations that individually

disrupt either of the nuclease activities of Rrp44p do not affect the stability of nonstop transcripts.

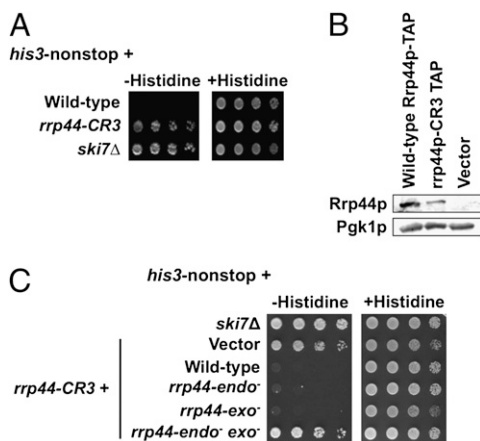
Our finding that the *rrp44-exo<sup>-</sup>* mutant did not affect stability on nonstop mRNAs is surprising, because published results indicate this mutation stabilizes normal transcripts (see below). One possibility is that the *rrp44-exo<sup>-</sup>* point mutation strongly reduces exonuclease activity but does not completely eliminate it. If this is true, then this strongly reduced activity may be sufficient for nonstop decay, but not for regular mRNA decay. To test this possibility we used a strain that completely lacks the RNB domain of Rrp44p. In this strain (*rrp44-Δexo*), the *PGK1pG-nonstop* transcript was also unstable, ruling out the possibility that any theoretical residual activity of the RNB domain in the *rrp44-exo<sup>-</sup>* mutant is sufficient for nonstop decay (Fig. 1C).

**Either the Endo- or Exonuclease Activity of Rrp44p Can Efficiently Degrade Nonstop mRNAs.** Because disrupting the catalytic activities of Rrp44p individually does not abrogate nonstop decay, our results suggest that neither of the catalytic activities are required for nonstop mRNA degradation. This could be because the two activities are redundant for nonstop decay, or because Rrp44p plays no role in nonstop decay. To test whether Rrp44p had any role in nonstop mRNA degradation, we analyzed the expression of nonstop reporters in one additional *RRP44* allele, *rrp44-CR3*. In this mutant, the three conserved cysteine residues of the N-terminal CR3 motif of Rrp44p have been mutated to serine. Although the biological function of this CR3 motif is not yet known, the *rrp44-CR3* mutant has a slow growth phenotype (12). As shown in Figs. 2A and 1B, the *rrp44-CR3* mutant suppressed the *his3-nonstop* allele and stabilized the *PGK1pG-nonstop* transcript. The Rrp44p-CR3 protein expression level is somewhat reduced compared with wild-type Rrp44p (Fig. 2B), and the defect in nonstop decay may be due to the sequence changes or the reduced expression of the Rrp44p-CR3 protein. In either case, this finding indicates that Rrp44p is required for nonstop decay.

The *rrp44-endo<sup>-</sup>exo<sup>-</sup>* double mutant is inviable, making it impossible to analyze nonstop decay in this strain. However, knowing that the *rrp44-CR3* mutation stabilizes nonstop mRNAs allowed us to test whether the endo- and exonuclease activities



**Fig. 1.** Mutations that disrupt the endo- or exoribonuclease activity of Rrp44p do not affect expression of nonstop reporters. (A) Strains containing the *rrp44-endo<sup>-</sup>* or *rrp44-exo<sup>-</sup>* mutations were transformed with a *his3-nonstop* reporter. Each of the indicated strains were serially diluted and spotted onto media lacking histidine to assay suppression of the *his3-nonstop* allele. (B and C) Strains containing the *rrp44-endo<sup>-</sup>*, *rrp44-exo<sup>-</sup>*, or *rrp44-CR3* mutations (B) or completely lacking the exonucleolytic RNB domain of Rrp44p (C) were transformed with a *PGK1pG-nonstop* reporter under the control of a galactose-inducible promoter. Expression of the reporter was repressed by the addition of glucose. Total RNA was isolated and *PGK1pG-nonstop* mRNA levels were analyzed by Northern blot analysis. Plotted is the mRNA remaining at each time point after correcting for loading differences using a probe specific for the RNA subunit of the signal recognition particle (*SCR1*).

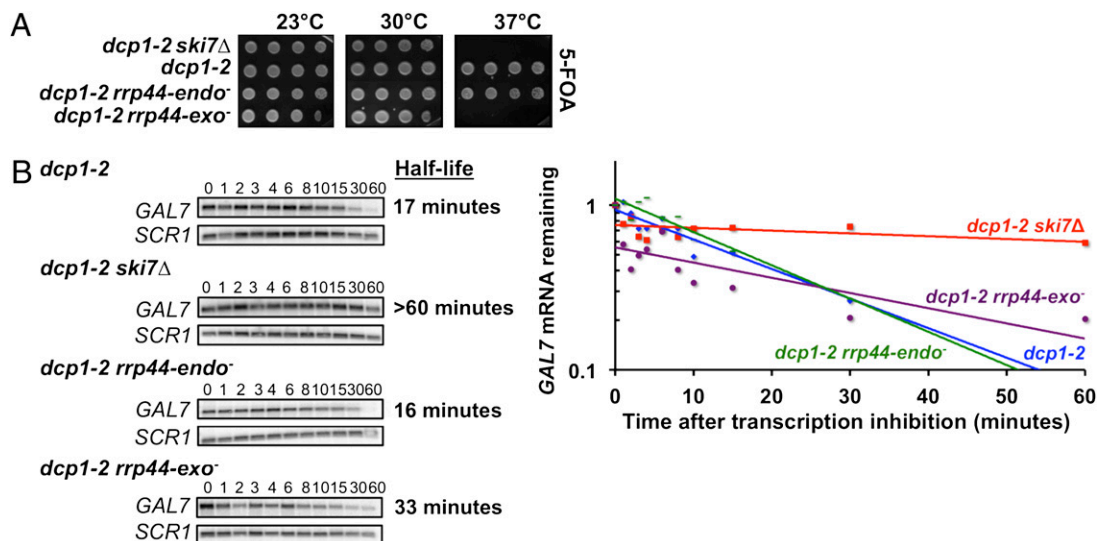


**Fig. 2.** Either the endo- or exoribonuclease activity of Rrp44p can efficiently degrade nonstop mRNAs. (A) Mutation of the CR3 region of Rrp44p affects expression of a *his3-nonstop* reporter. A strain containing the *rrp44-CR3* mutation was transformed with a *his3-nonstop* reporter. Each of the indicated strains were serially diluted and spotted onto media lacking histidine. (B) Protein expression of the Rrp44p-CR3 mutant was analyzed using Western blot analysis with antibodies specific for Protein A, and Pgk1p as a loading control. (C) The nonstop mRNA decay defect seen in the *rrp44-CR3* strain can be complemented by wild-type *RRP44* or by the *rrp44-endo*<sup>-</sup> or *rrp44-exo*<sup>-</sup> allele, but not by the double-mutant *rrp44-endo*<sup>-</sup>*exo*<sup>-</sup>.

are redundant for nonstop decay. Specifically, we attempted to complement the *rrp44-CR3* strain with wild-type *RRP44*, the *rrp44-endo*<sup>-</sup> mutant, the *rrp44-exo*<sup>-</sup> mutant, or the *rrp44-endo*<sup>-</sup>*exo*<sup>-</sup> double mutant. As shown in Fig. 2C, expressing *rrp44-endo*<sup>-</sup> or *rrp44-exo*<sup>-</sup> restored *his3-nonstop* mRNA decay to the *rrp44-CR3* strain, confirming that both of these alleles were functional in nonstop mRNA decay. Importantly, the *rrp44-endo*<sup>-</sup>*exo*<sup>-</sup> double mutant failed to restore nonstop decay (Fig. 2C), despite being expressed at levels similar to wild-type Rrp44p (11). We conclude that either the endo- or exonuclease activity of Rrp44p can efficiently degrade nonstop mRNAs.

**Exonuclease Activity of Rrp44p Is Required for the Exosome-Mediated Decay of Normal mRNAs.** The lack of a requirement for the Rrp44p exonuclease in nonstop decay is surprising given that two experiments implicate that this activity is required for general mRNA degradation. First, it was shown that the *rrp44-exo*<sup>-</sup> allele is synthetically lethal with an *xm1Δ* (13). Mutations that block exosome-mediated mRNA decay are synthetically lethal with defects in the 5'-to-3' mRNA decay pathway, such as an *xm1Δ* (20, 35, 36). Thus, a likely explanation for the *rrp44-exo*<sup>-</sup>*xm1Δ* synthetic lethality is that combining these mutations blocks both general mRNA decay pathways. However, Xrn1p and Rrp44 exonuclease activities are also both required for rRNA processing (3, 11–13), which suggests that other possible explanations for the reported synthetic lethality may exist. Unlike Xrn1p, the Dcp1p/Dcp2p decapping enzyme is thought to only be required for mRNA degradation (37, 38). Thus, to confirm and expand on the previous results, we tested synthetic lethality with a *dcp1* mutation. Because of its role in 5'-to-3' decay, a temperature-sensitive allele of *dcp1*, *dcp1-2*, is synthetically lethal with mutations in exosome subunits and cytoplasmic exosome cofactors at the nonpermissive temperature of 37 °C (20). Similar to the results obtained with an *xm1Δ* (13), the *rrp44-exo*<sup>-</sup> mutation was synthetically lethal with *dcp1-2* at 37 °C (Fig. 3A). In contrast, the *dcp1-2 rrp44-endo*<sup>-</sup> double mutant is viable at 37 °C. This indicates that the exonuclease activity of Rrp44p is needed for general 3'-to-5' mRNA decay.

The second published experiment implicating the Rrp44p exoribonuclease activity in general mRNA decay is that *MFA2pG* mRNA is stabilized, and *MFA2pG* decay products accumulate in a strain that is defective in decapping, depleted of wild-type Rrp44p, and also expresses the *rrp44-exo*<sup>-</sup> mutant (3). This suggests that the exonuclease activity of Rrp44p is required for mRNA decay, but whether the endonuclease activity is also involved was not addressed in this assay. Furthermore, Dziembowski et al. (3) did not address whether the *MFA2* decay intermediates were generated by the Rrp44p endo- or exonuclease activity. Therefore, we directly measured decay rates of the endogenous *GAL7* mRNA. The *GAL7* transcript is normally primarily degraded through the decapping pathway, but in a



**Fig. 3.** The exoribonuclease activity of Rrp44p is required for exosome-mediated decay of normal mRNAs. (A) Strains containing either the *rrp44-endo*<sup>-</sup> or *rrp44-exo*<sup>-</sup> mutation in combination with *dcp1-2* were serially diluted and grown at the indicated temperatures. (B) The stability of *GAL7* mRNA in the *dcp1-2 rrp44-endo*<sup>-</sup> or *dcp1-2 rrp44-exo*<sup>-</sup> mutants was examined by growing cells at 23 °C in media containing galactose. The 5'-to-3' decay pathway was inactivated by incubating cells for 1 h at 37 °C. Following the addition of glucose, RNA was isolated at the times indicated, and *GAL7* mRNA levels were analyzed by Northern blot analysis. Plotted is the mRNA remaining at each time point after correcting for loading differences using a probe specific for the RNA subunit of the signal recognition particle (*SCR1*).

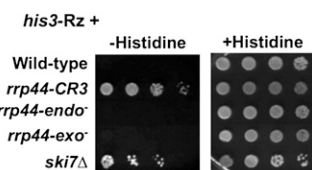
*dcp1-2* strain at 37 °C, this transcript is degraded by the cytoplasmic exosome (20). We assayed the stability of *GAL7* mRNA in *dcp1-2 rrp44-endo<sup>-</sup>* and *dcp1-2 rrp44-exo<sup>-</sup>* mutants. As expected, *GAL7* was more stable in the *dcp1-2 ski7Δ* and *dcp1-2 rrp44-exo<sup>-</sup>* strains, compared with the *dcp1-2* single mutant. In contrast, *GAL7* was not stabilized in the *dcp1-2 rrp44-endo<sup>-</sup>* mutant (Fig. 3B). Overall, the *dcp1* synthetic lethality and *GAL7* mRNA stability results confirm that the exonuclease activity of Rrp44p is needed for the exosome-mediated decay of normal mRNAs, and thus that the nuclease requirements for nonstop and general mRNA decay are different.

#### Either the Endo- or Exonuclease Activity of Rrp44p Can Efficiently Degrade an Endonucleolytically Cleaved mRNA.

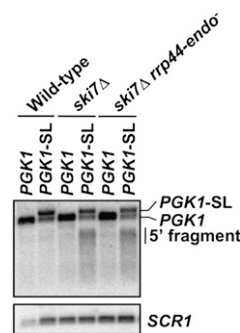
To determine whether the nuclease requirements for the degradation of other aberrant transcripts were similar to that of nonstop mRNAs, we analyzed the degradation of a ribozyme-cleaved transcript. Previous studies indicate that the 5' cleavage product resulting from ribozyme cleavage is rapidly degraded by the cytoplasmic exosome (32). To determine whether the nuclease activities of Rrp44p were needed for this mRNA decay pathway, a plasmid containing a hammerhead ribozyme inserted into the *HIS3* gene (*his3-Rz*) was transformed into the *rrp44-endo<sup>-</sup>* or *rrp44-exo<sup>-</sup>* mutants. Similar to the results obtained for nonstop decay, both the *rrp44-endo<sup>-</sup>* and *rrp44-exo<sup>-</sup>* mutants that contained *his3-Rz* failed to grow on media lacking histidine (Fig. 4), suggesting that inactivation of either of the nuclease activities of Rrp44p does not affect expression of *his3-Rz*. However, the *rrp44-CR3* mutant supported growth on media lacking histidine, indicating that Rrp44p is required for the rapid degradation of this reporter mRNA. Therefore, the nuclease requirements for the degradation of this hammerhead-cleaved mRNA mirror those for nonstop decay, suggesting that the nuclease requirements for exosome-mediated decay of several kinds of aberrant transcripts differ from that of normal mRNAs.

#### Endonuclease-Mediated Nonstop Decay Is Distinct from No-Go Decay.

Nonstop transcripts are thought to be recognized as aberrant because a ribosome is stalled at the 3' end of an mRNA (22). This is conceptually similar to no-go decay, which is the rapid endonucleolytic cleavage of mRNAs with an internally stalled ribosome (25). Furthermore, nonstop decay and no-go decay require the paralogs *SKI7* and *HBS1*, respectively (22, 25, 39). Given this similarity, we considered the possibility that Rrp44p may be the endonuclease responsible for no-go decay and that our nonstop mRNAs with stalled ribosomes at their 3' ends were also susceptible to no-go decay. It has previously been shown that the 5' cleavage product resulting from no-go decay of the *PGK1-SL* reporter mRNA accumulates in a *ski7Δ* strain and that 3' no-go decay cleavage products accumulate in an *xm1Δ*. Fig. 5 and Fig. S1A show that these cleavage products still accumulate when the endonuclease activity of Rrp44p is inactivated. These results suggest that the endonuclease activity of Rrp44p is not needed



**Fig. 4.** Mutations that disrupt the endo- or exoribonuclease activity of Rrp44p do not affect expression of a hammerhead ribozyme cleavage product. Strains containing the *rrp44-endo<sup>-</sup>*, *rrp44-exo<sup>-</sup>*, or *rrp44-CR3* mutations were transformed with a reporter containing a hammerhead ribozyme inserted into the *HIS3* gene. Each of the indicated strains were serially diluted and spotted onto media lacking histidine to assay suppression of the *his3*-ribozyme allele.

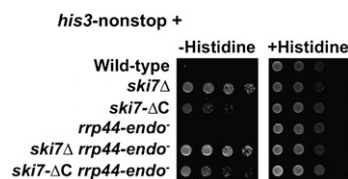


**Fig. 5.** Endoribonuclease-mediated nonstop mRNA decay is distinct from no-go decay. A wild-type strain, a *ski7Δ* strain, and a *ski7Δ rrp44-endo<sup>-</sup>* double-mutant strain were transformed with a *PGK1* or *PGK1-SL* reporters under the control of a galactose-inducible promoter. Total RNA was isolated from cultures grown in galactose, and *PGK1* mRNA levels were analyzed by Northern blot analysis. The RNA subunit of the signal recognition particle (*SCR1*) served as a loading control.

for the endonucleolytic cleavage in no-go decay. As detailed in *SI Results* and Fig. S1B and C, we also show that unlike nonstop decay, the *rrp44-CR3* mutation does not disrupt no-go decay. Combined, these findings suggest that endonuclease-mediated nonstop decay is distinct from the endonucleolytic cleavage in no-go decay.

#### Endonuclease Activity of Rrp44p Is Not Redundant with the C-Terminal Domain of Ski7p in Nonstop Decay.

In the current model of nonstop mRNA degradation, the C terminus of Ski7p recognizes a stalled ribosome with an empty A-site and recruits the exosome to rapidly degrade the nonstop transcript from the 3' end (22). One unresolved issue with this model is that the 3' end of the mRNA should be inaccessible to the exosome because it is buried in the decoding center of the ribosome. One solution to this problem may be that the C terminus of Ski7p triggers ribosome disassembly (Fig. S2) (22). A second solution may be that the Rrp44p endonuclease cleaves the transcript near the stalled ribosome, thereby releasing the very 3' end and its bound ribosome. As described in detail in *SI Results*, neither of these models is fully supported by published data (22) or the data described above. We therefore tested whether both mechanisms operate redundantly by introducing the *his3-nonstop* reporter into a strain containing a C-terminal truncation of Ski7p and the *rrp44-endo<sup>-</sup>* mutant (*ski7-ΔC rrp44-endo<sup>-</sup>*). As previously reported (22), the *ski7-ΔC* strain had a moderate effect on growth on media lacking histidine (Fig. 6, third row). Similarly, the *ski7-ΔC rrp44-endo<sup>-</sup>* mutant was able to only partially suppress of the *his3-nonstop* allele (Fig. 6, sixth row). In contrast, a complete deletion of *SKI7* (*ski7Δ*) was able to more effectively suppress the *his3-nonstop* allele. This suggests that the endonuclease activity of Rrp44p is not specifically required to initiate nonstop decay, but that the endo- and exonuclease activities of Rrp44p act in the same step of nonstop decay.



**Fig. 6.** The endoribonuclease activity of Rrp44p is not redundant with the C-terminal domain of Ski7p in nonstop mRNA decay. The indicated strains were transformed with a *his3-nonstop* reporter and analyzed as in Fig. 1A.

## Discussion

**Nuclease Requirements for Exosome-Mediated Degradation of Normal and Nonstop mRNAs Differ.** In this study we show that a mutation that inactivates the exonuclease activity of the cytoplasmic exosome inhibits its ability to degrade normal mRNAs, but has no effect on its ability to degrade nonstop mRNAs. We directly measured the ability of the cytoplasmic exosome to degrade the *GAL7* mRNA and found that this normal mRNA is stabilized when the exonuclease domain of Rrp44p is inactivated. The conclusion that the *rrp44-exo<sup>-</sup>* allele blocks exosome-mediated decay of normal mRNAs is further supported by the observations that this allele (*i*) is synthetically lethal with a decapping mutation (Fig. 3A) and an *xrn1Δ* (13), (*ii*) stabilizes *MFA2pG* mRNA in a decapping-defective strain, and (*iii*) causes accumulation of degradation intermediates of the *MFA2pG* mRNA (3). Although the *rrp44-exo<sup>-</sup>* allele blocks exosome-mediated decay of normal mRNAs, it has no effect on the expression of *his3-nonstop* and *his3-Rz* reporter genes or the degradation of a *PGK1pG-nonstop* reporter mRNA. Therefore, we conclude that the nuclease requirements for exosome-mediated mRNA decay depend on the mRNA substrate. Furthermore, the observation that *rrp44-exo<sup>-</sup>* and *rrp44-endo<sup>-</sup>* do not affect the expression of two different kinds of aberrant mRNA indicate that the degradation of several kinds of aberrant mRNAs are not affected by the *rrp44-exo<sup>-</sup>* mutation. More broadly, our findings describe a molecular function of Rrp44p that is exonuclease independent.

**Contributions of the Endo- and Exonuclease Activities of Rrp44p to Nonstop Decay.** We show that mutations that disrupt the endo- or exonuclease activity of Rrp44p do not affect the stability of nonstop mRNAs, but that the *rrp44-CR3* allele stabilizes nonstop transcripts. These data indicate that Rrp44p is required for nonstop decay, but that neither of its nuclease activities is required. Furthermore, the observation that *rrp44-endo<sup>-</sup>* or *rrp44-exo<sup>-</sup>*, but not the double *rrp44-endo<sup>-</sup>exo<sup>-</sup>* mutants, can complement *rrp44-CR3* indicates that either ribonuclease activity can rapidly degrade nonstop mRNAs.

If the endo- and exonuclease activities of Rrp44p can both degrade nonstop mRNAs, they could have different roles or identical roles in the overall process. For example, we initially considered the possibility that nonstop decay is initiated by the endonuclease activity, and that the exonuclease activity degrades the cleavage products. This model would predict that the *rrp44-exo<sup>-</sup>* mutation should have no effect of the stability of the full-length nonstop mRNA, but should stabilize decay intermediates. We have not been able to detect such decay intermediates, and therefore this model is unlikely. Another possibility we considered is that the endonuclease activity of Rrp44p contributes to the removal of the ribosome stalled at the 3' end, which makes the mRNA accessible to the exonuclease activity. However, this would predict that the *rrp44-endo<sup>-</sup>* allele stabilizes nonstop mRNAs either by itself or in combination with the *ski7-ΔC* allele, which we have shown does not occur (*SI Results* and Fig. S2). A third possibility is that the degradation of nonstop mRNAs by the Rrp44p endonuclease may mean that ribosomes stalled on nonstop mRNAs can also trigger no-go decay. However, this model

predicts that the *rrp44-endo<sup>-</sup>* mutation blocks no-go decay, which we show is not true. Furthermore, the *rrp44-CR3* allele blocks nonstop decay, but does not block the cleavage of no-go mRNAs (*SI Results* and Fig. S1). Having ruled out all of these alternatives, we propose that the two nuclease activities of the exosome act in the same step of nonstop decay.

**In Vivo Endonuclease Activity of the Exosome.** Previous studies suggested that the exonuclease activity of the exosome predominates over the endoribonuclease activity in vivo because the *rrp44-endo<sup>-</sup>* mutation has, at best, minor effects on various nuclear RNA processing and degrading activities of the exosome, whereas the *rrp44-exo<sup>-</sup>* mutation has clear effects (11–13). This suggests that the endonuclease activity is normally not very active in these processes. Our direct measurement of endonuclease-mediated decay of the *PGK1pG-nonstop* mRNA in an *rrp44-exo<sup>-</sup>* mutant shows that this endonuclease is sufficient to rapidly degrade an mRNA in vivo and that the contributions of the endo- and exonuclease activities of Rrp44p to exosome function are substrate dependent. We conclude that even though the Rrp44p endonuclease activity in vitro requires nonphysiological conditions (i.e., 3 mM Mn<sup>2+</sup>), in vivo, the activity is sufficient to rapidly degraded mRNAs.

**Multiple Endonucleases Are Involved in Specialized mRNA Decay Pathways.** Although most normal eukaryotic mRNAs are thought to be degraded by exonucleases (i.e., Xrn1p and the exonuclease activity of the exosome), several more-specialized mRNA decay pathways in diverse eukaryotes appear to use endonucleases. These pathways include no-go decay, which is initiated by an unknown endonuclease (25, 29); NMD, which can be initiated by SMG6 in *Drosophila* and human cells (26–28) or by PMR1 in human erythroid cells (23, 30); RNAi, which can be initiated by Argonaute (31); and the degradation of endoplasmic reticulum-localized transcripts, which is initiated by Ire1p during the human unfolded protein response (40). To this list of specialized endonuclease-mediated mRNA decay pathways, we can now add nonstop mRNA decay, although nonstop decay appears unique, in that in most of the above pathways an endonuclease appears to initiate mRNA degradation, but exonucleases degrade the bulk of the transcript.

## Materials and Methods

The yeast strains and plasmids used in these studies were created using standard techniques and are described in Tables S1 and S2. Synthetic lethality and *his3-nonstop* growth assays were performed essentially as described (22, 36). The half-life of the *PGK1pG-nonstop* reporter was determined essentially as described (22). Northern blots were hybridized with <sup>32</sup>P 5' end-labeled oligonucleotides listed in Table S3. Signals were detected and quantitated using a STORM PhosphorImager, and corrected for loading by quantitating the RNA subunit of the signal recognition particle, *SCR1*, or *ACT1*. Each mRNA stability measurement was done at least twice, using independent cultures. Average values of replicate experiments were graphed.

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# Supporting Information

Schaeffer and van Hoof 10.1073/pnas.1013180108

## SI Results

**Endoribonuclease-Mediated Nonstop Decay Is Distinct from No-Go Decay.** Endoribonuclease-mediated no-go decay generates unstable 5' and 3' cleavage products that are more easily detected in a *ski7Δ* and *xm1Δ* strain, respectively (1). To further address whether the endonuclease activity of Rrp44p is the endo-ribonuclease in no-go decay, the steady-state level of a *PGK1-SL* transcript was also measured in the *xm1Δ rrp44-endo<sup>-</sup>* strain. Similar to the results shown in Fig. 5, the 3' fragment is present in both the *xm1Δ* and *xm1Δ rrp44-endo<sup>-</sup>* strains (Fig. S14).

In a second assay to test whether endoribonucleolytic nonstop mRNA decay is the same as no-go decay, we examined the effect of the *rrp44-CR3* mutation. As shown in Figs. 1B and 2A, this mutation blocks decay of nonstop mRNAs by the Rrp44p endoribonuclease domain but, as shown in Fig. S1 B and C, does not block no-go mRNA cleavage.

Combined, these results show that the endoribonuclease activity of Rrp44p is not needed for the initial endoribonucleolytic cleavage event in no-go decay, and that endoribonuclease-mediated nonstop decay is distinct from the endoribonucleolytic cleavage in no-go decay.

**Mechanism of Ribosome Disassembly in Nonstop mRNA Decay Is Not Yet Known.** An unresolved issue of the current model of nonstop mRNA decay is that the 3' end of the nonstop mRNA should be inaccessible to the exosome—presumably it is buried in the decoding center of the stalled ribosome. One possibility is that Ski7p triggers ribosome disassembly (Fig. S2). The C terminus of Ski7p is similar to eRF3 and Hbs1. eRF3 is required for ribosome disassembly at termination codons, whereas Hbs1 is required for disassembly of stalled ribosomes in no-go decay (2). Therefore, the C terminus of Ski7p may be needed to remove the stalled ribosome to allow the nonstop transcript to be degraded. Consistent with this idea, a C-terminal truncation of Ski7p that lacks the region homologous to eRF3 inhibits nonstop decay without inhibiting other exosome functions. However, the stabilization of nonstop transcripts seen in a *ski7-ΔC* mutant is not as severe as that seen in a complete *ski7Δ* (3). This partial inhibition suggests that ribosome disassembly by Ski7p may be one way, although not the only way, to make the nonstop mRNA accessible to the degradation machinery. Another possibility is that the Rrp44p endoribonuclease cleaves the transcript near the stalled ribosome, which would release the 3' end of the nonstop mRNA and its bound ribosome (Fig. S2). This cleavage would form a new 3' end, lacking a stalled ribosome, which is accessible to the degradation machinery. This second possibility predicts that the *rrp44-endo<sup>-</sup>* mutant stabilizes nonstop mRNAs, which we have shown in Fig. 1 A and B not to be the case. A third possibility is a combination of the two, where the 3' end is mainly made accessible because the C terminus of Ski7p disassembles the ribosome, but that the endoribonuclease activity of Rrp44p can also remove the part of the mRNA with the stalled ribosome. This third model predicts that combining a C-terminal truncation of Ski7p with the *rrp44-endo<sup>-</sup>* mutant would completely block nonstop decay, and thus resemble *ski7Δ*.

## SI Materials and Methods

**Yeast Strains.** The yeast strains used in these studies are described in Table S1. In all crosses, haploid progeny spores were obtained by the hydrophobic spore isolation method as described (4). To

create a *dcp1-2Δ rrp44Δ* strain (yAV1143), the *rrp44Δ::NEO* disruption was first introduced into the diploid strain yRP1375 (5). The resulting strain was transformed with pAV361 and sporulated, which resulted in yAV1139. The *dcp1-2Δ* parent strain yRP1340 was crossed with yAV1139. The resulting diploid strain was sporulated, resulting in yAV1143.

A *ski7Δ rrp44Δ* strain with a *RRP44,URA3* plasmid was similarly created by starting with yAV987 and yAV1115. The resulting haploid double mutant was then transformed with *LEU2* plasmids that encoded either wild-type *Rrp44p* or the *Rrp44p-endo<sup>-</sup>* mutant. These transformants were then streaked onto media containing 5-fluoroorotic acid (5-FOA) to select for loss of the *RRP44, URA3* plasmid, resulting in yAV1179 and yAV1183.

Strain yAV1192 was created by transforming yAV1115 with a *LEU2* plasmid that encoded the Rrp44p-CR3 mutant. Transformants were then streaked onto media containing 5-FOA to select for loss of the *RRP44,URA3* plasmid, resulting in yAV1192. Strain yAV1116 (*rrp44Δ::NEO*) was created exactly like yAV1115 (6). yAV1116 was transformed with pAG32 (7) that was digested with SalI and ClaI, resulting in the *rrp44Δ::HYG* strain, yAV1121. yAV1121 was then crossed to the *xm1Δ::NEO* strain from the yeast knockout collection to generate yAV1189. yAV1189 was then transformed with *LEU2* plasmids that encoded either wild-type *Rrp44p* or the *Rrp44p-endo<sup>-</sup>* mutant. These transformants were then streaked onto media containing 5-FOA to select for loss of the *RRP44,URA3* plasmid, resulting in yAV1193 and yAV1194.

**Plasmids.** The majority *RRP44* plasmids used have been described previously (6) and are listed in Table S2. pAV179 was created by site-directed mutagenesis of pAV152 using the Kunkel method (8) and oRP1055 (5' TGTCAAATTCACACTAGTGGGTGGCAATGAATG).

**Yeast Growth Assays. Synthetic lethality growth assays.** Synthetic lethality growth assays were performed essentially as described (9). Briefly, plasmids encoding the *rrp44* D171A point mutation (*rrp44 endo<sup>-</sup>*) or the *rrp44* D551N point mutation (*rrp44 exo<sup>-</sup>*) were transformed into a *dcp1-2 rrp44Δ* strain containing a *URA3* plasmid encoding wild-type *RRP44*. Transformants were grown in SC-LEU-URA + 2% dextrose overnight at 23 °C. Cultures were diluted in the same selection media to an optical density at 600 nm of 0.8. Next, cells were serially diluted in 96-well plates by a factor of 5 and spotted onto media containing 5-FOA. Plates were incubated at 23 °C (permissive temperature), 30 °C, and 37 °C (nonpermissive temperature) for 2–5 d.

**His3-nonstop growth assays.** The *his3-nonstop* growth assay was performed essentially as described (3). Briefly, *rrp44* deletion strains containing *LEU2* plasmids encoding the *rrp44 endo<sup>-</sup>* or *rrp44 exo<sup>-</sup>* point mutations were transformed with a *URA3* or *LYS2* plasmid encoding a *his3-nonstop* reporter. This reporter contains a nonstop mutation at the end of the *HIS3* ORF. Transformants were grown in SC-LEU-URA + 2% dextrose or SC-LEU-LYS + 2% dextrose overnight at 30 °C. Cultures were diluted in the same selection media to an optical density at 600 nm of 0.8. Next, cells were serially diluted in 96-well plates and spotted onto media lacking histidine, SC-HIS-LEU-URA or SC-HIS-LEU-LYS. The plates were incubated at 30 °C for 2–5 d.



**Table S1. Yeast strains used**

Strain	Genotype	Ref.
BY4741	<i>MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ</i>	(1)
yAV1115	<i>MATa; his3Δ1; leu2Δ0; ura3Δ0; rrp44Δ::NEO [RRP44, URA3]</i>	(2)
yAV1192	<i>MATa; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; rrp44Δ::NEO [RRP44 (C47S, C52S, C55S), LEU2]</i>	This study
yRP1536	<i>MATα; leu2-3,112; lys2-201; trp1; ura3-52; cup1::LEU2/PGK1pGIMFA2pG; dcp1-2::TRP1; ski7Δ::NEO</i>	(3)
yAV1143	<i>MATα; leu2Δ0; trp1; ura3Δ0; dcp1-2::TRP1; rrp44Δ::NEO [RRP44, URA3]</i>	This study
yAV856	<i>MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; ski7Δ::NEO</i>	This study
yAV394	<i>MATa; leu2-3,112; lys2-201; trp1; ura3-52; cup1::LEU2/PGK1pGIMFA2pG; ski7::3HA:NEO</i>	(unpublished data)
yAV1179	<i>MATα; leu2Δ0; lys2Δ0; ura3Δ0; ski7Δ::HYG; rrp44Δ::NEO [RRP44, LEU2]</i>	This study
yAV1183	<i>MATα; leu2Δ0; lys2Δ0; ura3Δ0; ski7Δ::HYG; rrp44Δ::NEO [RRP44 (D171A), LEU2]</i>	This study
yAV1201	<i>MATα; leu2Δ0; lys2Δ0; ura3Δ0; ski7Δ::HYG; rrp44Δ::NEO [RRP44 (C47S, C52S, C55S), LEU2]</i>	This study
yAV1193	<i>MATα; leu2Δ0; ura3Δ0; xrn1Δ::NEO; rrp44Δ::HYG [RRP44, LEU2]</i>	This study
yAV1194	<i>MATα; leu2Δ0; ura3Δ0; xrn1Δ::NEO; rrp44Δ::HYG [RRP44 (D171A), LEU2]</i>	This study
yAV1199	<i>MATα; leu2Δ0; ura3Δ0; xrn1Δ::NEO; rrp44Δ::HYG [RRP44 (C47S, C52S, C55S), LEU2]</i>	This study

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**Table S2. Plasmids used**

Name	Description	Marker	Insert/origin of plasmid (ref.)	Parent plasmid
<b>Plasmid</b>				
pAV317	<i>RRP44</i> promoter	<i>URA3</i>	(1)	—
pAV318	<i>RRP44</i> 3' flanking region	<i>URA3</i>	(1)	—
pAV344	<i>RRP44</i> promoter, residues 1–1,001, <i>RRP44</i> 3' flanking region	<i>LEU2</i>	(1)	—
pAV361	<i>RRP44</i> promoter, residues 1–1,001, <i>RRP44</i> 3' flanking region	<i>URA3</i>	(1)	—
pAV364	<i>RRP44</i> promoter, residues 1–474, <i>RRP44</i> 3' flanking region	<i>LEU2</i>	(1)	—
pAV515	<i>RRP44</i> promoter, residues 1–1,001 (C47S, C52S, C55S), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	(1)	—
pAV503	<i>RRP44</i> promoter, residues 1–1,001 (D171A), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	(1)	—
pAV501	<i>RRP44</i> promoter, residues 1–1,001 (D551N), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	(1)	—
pAV539	<i>RRP44</i> promoter, residues 1–1,001 (D171A, D551N), <i>RRP44</i> 3' flanking region	<i>LEU2</i>	(1)	—
pAV656	<i>RRP44</i> promoter, residues 1–1,001 (D171A), <i>RRP44</i> 3' flanking region	<i>URA3</i>	Digest pAV503	pRS416
pAV657	<i>RRP44</i> promoter, residues 1–1,001 (D551N), <i>RRP44</i> 3' flanking region	<i>URA3</i>	Digest pAV501	pAV361
pAV740	<i>RRP44</i> promoter, residues 1–1,001 (D171A, D551N), <i>RRP44</i> 3' flanking region	<i>URA3</i>	Digest pAV539	pRS416
pAV139	<i>SKI7</i> promoter, residues 1–748, <i>SKI7</i> 3' flanking region	<i>URA3</i>	(2)	—
pAV152	<i>SKI7</i> promoter, residues 1–748 HA, <i>SKI7</i> 3' flanking region	<i>URA3</i>	yAV394	pAV139
pAV179	<i>SKI7</i> promoter, residues 1–748 (P265*) HA, <i>SKI7</i> 3' flanking region	<i>URA3</i>	oRP1055 mutagenesis	pAV152
pAG32	Hygromycin resistance cassette	-	(3)	—
<b>Reporter plasmids</b>				
pAV700	<i>his3-nonstop</i>	<i>LYS2</i>	Digest pAV188	pRS317
pAV188	<i>his3-nonstop</i>	<i>URA3</i>	(4)	—
pAV241	<i>his3-Rz</i>	<i>URA3</i>	(5)	—
pAV175	<i>GAL::pgk1-nonstop</i>	<i>URA3</i>	(6)	—
pRP469	<i>GAL::pgk1</i>	<i>URA3</i>	(7)	—
pRP1251	<i>GAL::pgk1-SL</i>	<i>URA3</i>	(7)	—

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