No-Go Decay mRNA cleavage in the ribosome exit tunnel produces 5′-OH ends phosphorylated by Trl1

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The No-Go Decay (NGD) mRNA surveillance pathway degrades mRNAs containing stacks of stalled ribosomes. Although an endoribonuclease has been proposed to initiate cleavages upstream of the stall sequence, the production of two RNA fragments resulting from a unique cleavage has never been demonstrated. Here we use mRNAs expressing a 3′-ribozyme to produce truncated transcripts in vivo to mimic naturally occurring truncated mRNAs known to trigger NGD. This technique allows us to analyse endonucleolytic cleavage events at single-nucleotide resolution starting at the third collided ribosome, which we show to be Hel2-dependent. These cleavages map precisely in the mRNA exit tunnel of the ribosome, 8 nucleotides upstream of the first P-site residue and release 5′-hydroxylated RNA fragments requiring 5′-phosphorylation prior to digestion by the exoribonuclease Xrn1, or alternatively by Dxo1. Finally, we identify the RNA kinase Trl1, alias Rlg1, as an essential player in the degradation of NGD RNAs.
The No-Go Decay (NGD) mRNA surveillance pathway degrades mRNAs containing stalled ribosomes. NGD occurs when translation elongation is blocked by the presence of stable intramolecular or intermolecular RNA structures, enzymatic cleavage, chemically damaged sequences or rare codons. This mRNA degradation process is dependent on translation and involves an endoribonuclease that cleaves just upstream of the stall sequence. Other mRNA surveillance pathways can also ultimately lead to NGD. For instance, transcripts synthesized without a stop codon due to premature polyadenylation have stalled ribosomes that are initially detected by the non-stop decay (NSD) pathway. NSD-targeted mRNAs are cleaved by an uncharacterized mechanism and become targets of NGD when ribosomes reach the new 3′-end and stall. NGD thus plays a key role in resolving translational issues potentially detrimental to cellular homeostasis. When mRNAs are truncated, the stalled ribosomes are rescued in a process mediated by the Dom34/Hbs1 complex that dissociates the ribosomal subunits. Their association with the 60S subunit is recognized by the ribosome quality control (RQC) pathway, leading to the rapid degradation of the nascent peptide. However, despite extensive study, the precise location of NGD cleavage and the mechanism of degradation of the resulting RNA fragment remain elusive.

In this paper, we focus on the fate of NGD-cleaved mRNAs, with an initial goal of mapping the sites of mRNA cleavage with accuracy. Two major obstacles to achieving this objective are that NGD fragments are rapidly attacked by 5′-3′ and 3′-5′ exoribonucleases after ribosome dissociation and that simultaneously blocking the 5′-3′ and 3′-5′ exoribonuclease decay pathways is synthetically lethal. It has been shown, however, that the stability of such mRNAs is largely dependent on the Dom34/Hbs1 complex. In dom34 mutant cells, ribosomes stalled at the 3′-end of truncated mRNAs inhibit the degradation by the exosome and facilitate the detection of sequential endonucleolytic cleavages upstream of the ribosomal stall site. Interestingly, dom34 and xrn1 mutations (inactivating the main 5′-3′ exonucleolytic degradation pathway) are not synthetic lethal. Moreover, NGD endonucleolytic cleavages still occur in the absence of Dom34 and xrn1 mutations by primer extension experiments with probe prA, Fig. 1a and Supplementary Fig. 1a). The upstream and downstream cleavage products are referred to as 5′-NGD and 3′-NGD RNAs, respectively (Fig. 1a). We indeed detected a ladder of 3′-NGD RNA fragments in dom34 mutant cells (Fig. 1b), in the presence or absence of active 5′-3′ or 3′-5′ exonucleolytic decay pathways, i.e. xrn1 or ski2 mutations, respectively. In agreement with the current NGD model in which endonucleolytically cleaved 3′-NGD cleavages are primarily degraded by the 5′-3′ exoribonuclease Xrn1, inactivation of the 5′-3′ RNA decay pathway (xrn1 mutant cells) produced a different ladder of 3′-NGD RNAs compared to WT or the ski2 mutant. This was confirmed by a higher resolution PAGE analysis followed by northern blotting (Fig. 1c). The PAGE analysis was completed by mapping the 5′-ends of the 3′-NGD RNA fragments in the dom34 and dom34/xrn1 mutants by primer extension experiments with probe prA (Fig. 1d). We showed that the truncated mRNAs produce several discrete 3′-NGD RNA bands (B1–B5) that can be mapped to single-nucleotide resolution. B5 (77 nts) and the major RNA species B1 (47 nts) visible in the dom34 mutant were not detected in the absence of the 5′-3′ exoribonuclease Xrn1 (Fig. 1b–d). B3 (68 nts) and B2 (65 nts) RNAs were exclusively observed in the xrn1 mutant cells, and B4 (71 nts) was detected in all four strains (Fig. 1c). In addition, we analysed a dcp2Δ decapitating mutant that totally blocks 5′-3′ exoribonucleolytic attacks on the 5′-end of mRNAs (Fig. 1b, c). The particularly strong growth defect of the dcp2Δ mutant may explain the decrease in the amount of NGD RNA detected but, interestingly, the fact that NGD RNAs remained detectable (Fig. 1c) suggests that an alternative pathway bypasses mRNA decapping for their production.

The sizes of the major B1 and B5 RNAs differ by 30 nts (Fig. 1d), consistent with the length of mRNA covered by an individual ribosome. We therefore surmised that the difference in size is most likely due to the presence of an extra ribosome protecting the B5 RNA species from 5′-3′ degradation by Xrn1, compared to B1. This prompted us to analyse the association of these 3′-NGD RNAs with ribosomes in sucrose gradients.

Ribosome association with 3′-NGD RNA fragments. We performed polysome analyses to assess the distribution of 3′-NGD RNAs in different ribosomal fractions in dom34 mutant cells (Fig. 1e, f). The B1 RNA (47 nts) was found to associate with monosomes (fractions 11–13) and disomes (fraction 15), with disomes being the theoretical maximal coverage of such a short RNA, as proposed by Gyuosh et al. and Simms et al. Indeed, the association of a 47-nt RNA species with disomes has been deduced from ribosome-profiling experiments and is explained by the approximate size of the trailing ribosome protecting a full ribosome footprint (28–30 nt) and the leading ribosome protecting a half footprint to the site of mRNA truncation (16–17 nts, with no RNA or an incomplete codon in the A-site). An additional ribosome would thus be expected to protect a
Fig. 1 Size characterization of 3′-NGD RNA fragments and ribosomal association. a Schematic view of the URA3Rz mRNA showing the ribozyme (Rz) site (see also Supplementary Fig. 1a). Translational start (AUG) and stop codons are indicated. RNA1 (in magenta) is the stop-codon-less mRNA following ribozyme cleavage. Probes prA, prB and prC used in northern blots analysis are indicated. 5′ and 3′-NGD RNAs are the products of NGD cleavage of mRNA1. The lightning flash represents the NGD endonucleolytic cleavage and probe prA is designed for the detection of all potential 3′-NGD RNAs (see also Supplementary Fig. 1a). 

b Agarose gel electrophoresis followed by northern blot showing levels of mRNA1 and 3′-NGD RNA fragments in the indicated strains. The ScR1 RNA served as a loading control.

c Analysis similar to b using 8% PAGE. The 5S rRNA served as a loading control.

d Primer extension experiments using probe prA to determine the 5′-end of 3′-NGD RNAs. B1, B2, B3, B4 and B5 RNAs shown in c are indicated with the corresponding size in nucleotides (nts) calculated by primer extension.

e Analysis of 3′-NGD RNA association with ribosomes in dom34 mutant cells. Upper, on ribosome profile, positions of 40S and 60S subunits, 80S and polysome are indicated. Panel below, 20 fractions were collected and extracted RNAs were analysed similarly to c. Bottom panel: 18S and 25S rRNAs are shown as references for ribosome and polysome sedimentation, using 1% agarose gel electrophoresis and ethidium bromide staining. f Distribution of the 3′-NGD RNAs analysed in e. For each fraction, levels of B1, B4 and B5 RNAs were plotted as a % of total amount of B1, B4 and B5 RNAs, respectively. The profile in e is reported in f. g Schematic view of the ribosome positioning on 3′-NGD RNAs combining information on size resolution, ribosomal association (Fig. 1), and RNase ribosomal protection of 3′-NGD RNAs (Supplementary Fig. 1). Codons are shown in black or magenta. A and P are ribosome A- and P-sites, respectively. Error bars indicate standard deviation (s.d.) calculated from three independent experiments. Source data are provided as a Source Data file.
~77-nt RNA. Accordingly, the B5 RNA (77 nts) was found to associate with two and three ribosomes, in fractions 15 and 16, respectively. The 71-nt B4 RNA was also associated with the disome and trisome peaks (Fig. 1e, f). We suspect that ribosomes do not stay stably bound to the different RNA species during sucrose gradient analysis, but this experiment allows us to propose that two ribosomes maximally associate with B1, while up to three can associate with B4 and B5 in vivo. This also prompted us to determine how these 3′-NGD RNAs were protected from Xrn1 and RNase I activities in cell extracts prior to centrifugation on sucrose cushions (Supplementary Fig. 1). These complementary experiments show that ribosomes persist and protect these RNA species from RNases in vitro and support results presented above (Supplementary Notes 1 and 2 and Supplementary Fig. 1a–i).

The results in Fig. 1 and Supplementary Fig. 1 allow us to infer the precise positions of ribosomes on B5 and B4 RNA species in the dom34 mutant backgrounds. Our experiments all converge to the conclusion that the B5 species corresponds to RNAs covered by trisomes (Fig. 1g). We also conclude that three ribosomes cover the 71-nt B4 RNA in dom34 mutant cell extracts as this species is resistant to Xrn1 (Supplementary Note 1), its 5′-region is protected from RNase I digestion in vitro (Supplementary Note 2) and a significant proportion remains associated with fractions corresponding to three ribosomes in sucrose gradients (Fig. 1e, f).

**Dxo1 trimming of 3′-NGD fragments in Xrn1-deficient cells.**

We strongly suspected that the B4 species was the original NGD product, and because B3 and B2 RNAs were exclusively detected in Xrn1-deficient cells, we speculated that these RNAs might be derived from B4 by an alternative 5′–3′ exoribonuclease. We therefore asked whether the 5′–3′ exoribonucleolytic activity of Dxo1, which plays an important role in 5′-end capping quality control22, might explain the presence of the B3 and B2 RNAs. Remarkably, deletion of both XRN1 and DXO1 genes in a dom34 background completely abolished the production of the B3 and B2 RNAs, and only the B4 3′-NGD species remained detectable by northern blot analysis (Fig. 2a) or in primer extension assays (Supplementary Fig. 2a). Complementation of the dom34/xrn1/ dxo1 mutant with wild-type Dxo1 restored B3 and B2 RNA production to a significant extent, but a catalytic mutant failed to do so (Fig. 2b). We took advantage of the almost exclusive presence of the B4 3′-NGD RNAs in dom34/xrn1/dxo1 mutant cells to ask how this RNA is protected by ribosomes by adding Xrn1 to cell extracts as described above (Supplementary Fig. 1b). Some of the B4 RNA was Xrn1-resistant (Supplementary Fig. 2b) in accordance with our hypothesis that a portion of this species is protected by three ribosomes in xrn1/dom34 cell extracts (Fig. 1g). Interestingly, the decrease in the amount of B4 RNA was correlated with an almost equivalent increase of a 47-nt species (Supplementary Fig. 2b, c), suggesting that disomes persist on the majority of the 3′-ends of B4 RNAs in dom34/xrn1/dxo1 cells in vivo. We thus propose that two populations of B4 RNAs co-exist in Xrn1-deficient cells in vivo, with one population covered by three ribosomes, especially in dom34 mutants, and the other only covered by two ribosomes, but having a 5′-protuding RNA extremity due to the absence of 5′–3′ exoribonucleases.

**Mapping of the primary NGD endonucleolytic cleavage site.**

The results described above suggest that the principal band detected in the absence of Xrn1 and Dxo1 (B4 RNA) is a specific 3′-product of NGD cleavage in our constructs (Fig. 2a). While B4 RNA resistance to Xrn1 in vitro (Supplementary Fig. 1b) could be explained by a third ribosome dwelling after cleavage, we also considered the possibility that its 5′-phosphorylation state could contribute to its stability, since both Xrn1 and Dxo1 require 5′-phosphorylated extremities to degrade RNA22,28. We therefore asked whether the B4 RNA naturally has a monophosphate (5′-P) or a hydroxyl group (5′-OH) at its 5′-end by treating RNA purified from dom34 cell extracts with T4 polynucleotide kinase in vitro (Fig. 3a), demonstrating that the B4 RNA has a 5′-OH extremity in dom34 cells. A portion of the abundant B1 RNA persisted during Xrn1 treatment in kinase buffer (Fig. 3a, and see the “Methods” section), but parallel Xrn1 digestion in optimal buffer confirms that B1 and B5 RNAs were totally digested (i.e., are fully mono-phosphorylated), while the B4 RNA remained resistant (Supplementary Fig. 3a). It has recently been demonstrated that the Hel2 ubiquitin-protein ligase is crucial for the activation of NGD cleavages29. Consistent with our hypothesis that the B4 species corresponds to the 3′-NGD cleavage product, this RNA is no longer produced in the hel2 mutant (Supplementary Fig. 3b).

By definition endonucleolytic cleavage of RNA results in the production of 5′- and 3′-RNA fragments. However, corresponding 5′- and 3′-fragments have never been definitively demonstrated in the case of NGD-targeted mRNAs. We thus searched for the corresponding 5′-NGD fragment for the B4 3′-NGD RNA. To map the 3′-end of 5′-NGD RNAs, total RNA preparations from ski2 and ski2/dom34 mutants were ligated to a pre-adenylated oligonucleotide linker using truncated RNA ligase to perform 3′-RACE (Fig. 3b)30,31. The ski2 mutant context was used to limit 3′-trimming of these RNAs in vivo, and RNAs were pre-treated with T4 polynucleotide kinase to modify 3′-phosphates or 2′-3′ cyclic phosphates to 3′-OH to permit RNA ligation31. The major RT-PCR product was of the expected size (66 bp; Fig. 3c and Supplementary Fig. 3c) and verified by sequencing the resulting clones (Fig. 3d). The identification of a matching 5′-NGD fragment for the B4 3′-NGD RNA, confirms that endonucleolytic event occurred at this precise position. The same procedure performed on RNAs isolated from ski2 mutants where Dom34 was still active yielded the same major PCR product, also verified by sequencing (Supplementary Fig. 3d). Thus, while the...
The fate of 5′-NGD RNAs. We anticipated that following NGD cleavage of mRNA1, ribosomes that had initiated translation on the 5′-NGD fragments would advance to the new 3′-end and the RNA be subjected to Xrn1 trimming, similar to the process that generates B1 and B5 (Fig. 4a). Since the B4 3′-NGD RNAs were cut in the +1 reading frame (Fig. 1g), upstream ribosomes on these 5′-NGD RNAs would be expected to stall with one nucleotide in ribosome A-site (Fig. 4a and Supplementary Fig. 4) and as result produce new RNA fragments 47′ + 1, 77 + 1 nts, protected by two or three ribosomes, respectively (see Supplementary Fig. 4). Indeed, in northern blots using probe prG, which is complementary to the new 3′-ends generated by NGD cleavage, we detected RNA fragments consistent with a 1-nt increase in size compared to those detected by prA on the same membrane (Fig. 4b). We mapped the 5′-ends of these new ribosome protected fragments by primer extension assays using prG (Fig. 4c and Supplementary Fig. 4). The detection of 48-nt (and 78-nt) cDNAs only in cells containing active Xrn1 (Fig. 4c) strongly suggests that the new NGD endonucleolytic products are covered by two and three ribosomes, respectively. The production of cDNAs of exactly the predicted sizes (48 and 78 nts) is an independent confirmation that the 3′-extremity of the 5′-NGD product corresponds precisely to the proposed NGD endonucleolytic cleavage site (Supplementary Fig. 4). Remarkably, the 3′-extremity of the 5′-NGD RNA was detected in the context of active 3′−5′ exonucleases, meaning that ribosomes run on and cover the 3′-extremity before any 3′−5′ attacks can occur. In summary, we propose that the B4 RNA is produced by endonucleolytic cleavage within the footprint of the third stalled ribosome and that at least two upstream ribosomes promptly protect the resulting 5′-NGD fragment from degradation by 3′−5′ exoribonucleases (Fig. 4d).

The 5′-OH endocleaved product is phosphorylated by Trl1. Despite the fact that a portion of the B4 species was found to be 5′-hydroxylated in dom34 cell extracts (Fig. 3a), a major fraction of the B4 RNA from dom34/xrn1 and dom34/xrn1/dxo1 cell extracts can be degraded by Xrn1 in vitro without prior 5′-phosphorylation (Supplementary Figs. 1b and 2b), suggesting that B4 accumulates as a 5′-phosphorylated species in this mutant background. A well-characterized factor with RNA kinase activity in yeast is the essential RNA ligase Trl131,32. Splicing of tRNAs is known to generate 5′-OH-intron RNAs which require Trl1 kinase activity to permit their degradation by Xrn1. We therefore asked whether the 3′-NGD B4 RNA fragments were substrates of Trl1. Since Trl1 is an essential gene, we used trl1Δ strains expressing pre-spliced intron-less versions of the 10 intron-containing tRNAs that restore viability33,34. If Trl1 were required for B4 degradation, loss of Trl1 function should increase the amount of B4 RNA. Remarkably, in trl1 versus Trl1 cells, we observed a 24-fold accumulation of the B4 RNA species and only a two-fold-decrease of B1 RNA levels, while the B5 RNA was barely detectable (Fig. 5a, b). We asked whether the accumulated B4 RNA had a hydroxyl group (5′-OH) at its 5′-end. RNAs were analysed by 12% PAGE allowing separation of 5′-hydroxylated from 5′-phosphorylated RNA35. B4 RNA migrated as two bands in Trl1 cells, whereas only the lower band was detected in trl1Δ cells. This species was fully shifted to the upper band after 5′-phosphorylation of RNA in vitro (Fig. 5c). We also verified that the B4 RNA was resistant to Xrn1 treatment in vitro. In contrast to B1 and B5 RNAs (Supplementary Fig. 5), B4 was completely degraded by Xrn1 only after 5′-phosphorylation by T4 kinase in vitro (Fig. 5d). We conclude that the B4 RNA accumulates as a fully 5′-OH species in the trl1Δ mutant. Thus, Trl1 is the major kinase involved in phosphorylating the B4 RNA following NGD cleavage.

NGD cleavage products derived from mRNAs containing rare codons. We asked whether we could identify endonucleolytic cleavages on another NGD-targeted mRNAs, using what we learned about this process on truncated mRNAs. We chose an mRNA containing four contiguous rare CGA codons, which we call (CGA)4-mRNA, as an NGD target (Fig. 6a and Supplementary Fig. 6a)3. Similar to the truncated mRNAs, ribosomes were shown to stall when decoding rare codons, producing 5′- and 3′-NGD RNAs (Fig. 6a). As previously demonstrated5,35,

![Flow chart illustrating the method used for 3′-RNA ligase RACE](https://example.com/image1.png)

**Fig. 3 Characterization of the endonucleolytic RNA fragments.** a Xrn1 digestion of total RNA extracts from dom34 mutant cells in the presence or absence of polynucleotide kinase (PNK) in vitro. 8% PAGE followed by northern blot analysis using probe prA. The 5S rRNA served as a loading control and 5.8S rRNA digestion control is made of total RNA from cell extracts (Fig. 3a), a major fraction of 5′-NGD RNA was detected in the context of 3′-OH. b Flow chart illustrating the method used for 3′-RNA ligase RACE. c PCR products obtained from 3′-RACE and separated on a 2% agarose gel. Purified DNAs for sequencing are indicated by an arrowhead. Prior to PCR, cDNAs were produced from total RNA from sk2, sk2/dom34 mutant cells expressing mRNA1. d Sequences obtained after 3′-RACE performed on sk2 and sk2/dom34 total RNA. 100% of sequenced clones (omitting a residual 5S rRNA-linker amplification detected) have this DNA sequence. 5′-NGD DNA sequence (in green) and linker sequence (in magenta). Below, the site of mRNA1 is shown before and after the cleavage producing the 3′-NGD RNA B4 and the 3′-extremity of the 5′-NGD RNA confirmed by 3′-RACE. Source data are provided as a Source Data file.
we detected 3′-NGD RNAs fragments in dom34 or DOM34 genetic contexts by northern blotting experiments using probe prB (Supplementary Fig. 6b). The precise identification of endonucleolytic cleavages by primer extension experiments is known to be challenging probably because, in contrast to truncated mRNAs, the positioning of ribosomes on contiguous rare codons is variable. We first asked whether we could detect 5′-NGD RNAs (Fig. 6a) using the same procedure as for NGD-targeted truncated RNAs (Fig. 4a, b). By probing the (CGA)₄-mRNA in a large region upstream of the four CGA codons (Supplementary Fig. 6a), we detected RNA bands using a probe prG to determine the 5′ end of RNAs (see also Supplementary Fig. 5). The detection of short RNA species by prH probe suggested that endonucleolytic cleavages occurred just downstream, in a region located ~70 nts upstream of the cluster of rare codons (Supplementary Fig. 6a). We thus set out to map the NGD cleavage sites on the (CGA)₄-mRNA, using 3′-RACE for the detection of the 3′-ends of 5′-NGD RNAs in ski2 and ski2/dom34 mutant cells (Supplementary Fig. 6c). We obtained major RT-PCR products of about 45 bp that were purified, cloned and sequenced (Fig. 6d and Supplementary Fig. 6d). The 3′-end sequences (Fig. 6e) formed three clusters, C1, C2 and C3 (Fig. 6g), that map to ~71 nts upstream of the second, third and fourth rare codon, respectively, consistent with cleavage within the footprint of the third ribosome as seen for the truncated mRNA1. No 3′-ends were detected within the region covered by two ribosomes, comforting the notion that disomes are not competent for NGD endonuclease activation. Xrn1 arrests mapping to 17–18 nts upstream of the A-site of the two first ribosomes positioned with either the second or third CGA codon in the A-site were also detected by primer extension assay (Fig. 6f–h). The strongest Xrn1 arrests corresponded to those where the lead ribosome contains the third CGA codon in the A-site (Fig. 6h), suggesting that the major stall occurs on this codon. Typically, Xrn1 is preferentially blocked 17 nts upstream of the first ribosomal A-site residue. We speculate that this 1-nt difference reveals distinct conformations of stalled ribosomes on rare codons versus
truncated mRNAs. All these results taken together suggest that the (CGA)$_n$-mRNA and truncated mRNA1 and mRNA2 are NGD-targeted in a highly similar process that results in cleavage within the footprint of the third ribosome, 71 nts upstream of the stall site for the leading ribosome.

Cleavages have been proposed by others to occur in the region covered by disomes using primer extension experiments$^{5,29}$. We thus analysed an mRNA containing rare codons with an identical ribosome stalling sequence to that previously examined$^5$ (Supplementary Fig. 6e). We demonstrate that primer extension arrests detected in the region covered by disomes are abolished in dox1/xrn1 mutant cells, suggesting that they are the products of subsequent trimming by these enzymes. In conclusion, our data suggests that stalled disomes on truncated mRNAs or on mRNAs containing short CGA repeats are poorly competent for NGD endonucleolytic cleavages. Endonucleolytic cleavages instead occur upstream of collided disomes, in agreement with other 3’-RACE analyses$^{27}$. Our data suggests that these cleavages first occur within the mRNA exit tunnel of the third stacked ribosome and those queuing further upstream.

**Discussion**

In this study, we first characterized the 3’-NGD RNA fragments produced near the 3’-end of truncated mRNAs that mimic natural cleaved mRNAs known to be NGD targets. One advantage of studying the 3’-NGD products of truncated mRNAs is that the precise positioning of stalled ribosomes results in 3’-NGD RNA fragments of specific sizes. Indeed, the precise identification of endonucleolytic cleavages is known to be challenging for mRNAs containing rare codons$^{5,29}$ because the positioning of ribosomes on multiple contiguous rare codons is variable. Using a ribozyme to efficiently generate precise 3’-ends within an open-reading frame, we were able to obtain detailed information about ribosome positioning on 3’-NGD RNAs, and provide the first precise mapping of the original site of endonucleolytic cleavage on an NGD substrate. Our model suggests that this cleavage occurs 71 nts from the 3’ end of the truncated mRNA, 8 nts upstream of the P-site codon in the third stacked ribosome (Figs. 1g and 7). This localizes the 5’-extremity of cleaved RNA fragment within the mRNA exit tunnel, 4 nts downstream of the expected nucleotide position of a canonical mRNA that emerges from the ribosome and becomes available for cleavage by RNase I in vitro, classically used in ribosome footprinting studies. A buried cleavage site is consistent with the idea that the NGD endonuclease might be the ribosome itself. However, we cannot fully exclude the possibility that the stalled ribosome allows access to an external nuclease with a specific conformation to penetrate this far into the mRNA exit tunnel.

Xrn1 treatment of various mutant cell extracts suggested that the predominant ribosome configuration on truncated mRNAs is disomes. Interestingly, the existence of disomes on truncated mRNAs has been previously reported in ribosome-profiling analysis$^{20}$ and stacking of two or more ribosomes has been proposed as a prerequisite for the activation of the endonuclease$^{27}$. The latter observation led to the proposition that ribosome collision triggers NGD cleavage upstream of disomes. Our data suggests that NGD endonucleolytic cleavage detected within the third stacked ribosome, suggests that the first two stalled ribosomes (disome) are not competent for the activation of the endonuclease activity (Fig. 7). Our 3’-RACE experiments did not amplify DNA products corresponding to RNAs matching the predicted sizes of NGD-cleaved RNAs within the second (41 nts) or first stalled ribosome (15 nts) (predicted sizes 95 and 125 nts).
nts, respectively), indicating that they do not occur to any significant level. The major ~65-bp RT-PCR product corresponded perfectly to an RNA cleaved 71nt upstream of the 3′-extremity of mRNA1, suggesting this is the primary site of NGD cleavage. This also suggests that the particular conformation of disomes on these mRNAs is incompatible with an NGD endonuclease activity cutting upstream of the ribosome P-site. The ability to induce this precise NGD cleavage appears thus to be a normal property of stalled ribosomes, with disomes (and monosomes) being exceptions.
We considered the possibility that the \textit{dom34} mutation may exaggerate the ribosome stalling and allow cleavage beyond what would naturally be observed. As discussed in the section Introduction, the analysis of NGD RNA fragments is facilitated by the \textit{dom34} mutation and is crucial for RNA stabilization when analysing truncated mRNAs by northern blotting experiments. In the presence of Dom34, and more efficient ribosome dissociation, the exosome would certainly be more actively involved once the first endonucleolytic cleavage event has occurred. Importantly, however, our 3'-RACE experiments confirmed the existence of 5'-NGD products with 3'-ends matching the 5'-extirpity of the 3'-NGD B4 RNA in cells containing Dom34 (Fig. 3c, d). Thus, NGD endonucleolytic cleavage does not occur naturally upstream of the ribosomal stall site and is not an artefact of \textit{dom34} genetic context.

These observations were used to map endonucleolytic cleavages that occur on a second NGD-target mRNA containing rare codons, also in a \textit{DOM34} genetic context. Endonucleolytic cleavages occurred 71nts upstream of the first residue in the leading ribosome A-site, in the region potentially covered by the third ribosome. Therefore, mRNAs containing rare codons are processed similar to truncated RNAs, although cleavage accuracy is slightly affected, correlating with specific Xrn1 arrests (Fig. 6f) that might be explained by a particular conformation of the first stalled ribosome.

It has been recently reported that Cue2 is the endonuclease that cleaves NGD/NSD targeted RNAs. Its action has been proposed to occur in the ribosome A site and therefore deviates significantly from our observations. We do not have a good explanation for this difference. However, previous experiments with the naturally occurring truncated HAC1 mRNA are consistent with our mapping of the endonucleolytic cleavage site. The HAC1 intron is known to be excised by Ire1, but RNA ligation can be incomplete and lead to a truncated but translated.
mRNA. Green and colleagues showed by ribosome-profiling analysis that ribosomes stall at the 3′-end of the first exon of the HAC1 mRNA, leading to an endonucleolytic cleavage ~70 nt upstream from the 3′-end. Consistent with this observation, our data suggests that the NGD endonuclease cleaves 71 nt upstream of the 3′-end of the truncated mRNAs (B4 RNAs, Fig. 1g). We also showed that the NGD endonuclease cleaves mRNAs in the +1 reading frame. As a consequence, upstream ribosomes run forward on the 5′-NGD mRNA and stall with 1 nt in the A-site (Figs. 1g and 7). This is in agreement with ribosome-profiling analysis reporting the predominance of short RNAs having one 3′-nucleotide in the ribosome A site.

We showed that the Trl1 kinase plays a role in NGD, and we propose that the resistance of the B4 3′-NGD RNA fragments to Xrn1 attacks in vitro and in vivo (Fig. 3a and Supplementary Figs. 1b and 3a) is a direct consequence of the 3rd ribosome preventing access to Trl1, with dissociation of this ribosome triggering the 5′-phosphorylation of the B4 RNA (Fig. 7). It is also possible that the leading ribosome dissociates, and that upstream ribosomes run fast to form a new disome with a 5′-protruding RNA (Fig. 7). Once the RNA is 5′-phosphorylated, the progressive 5′→3′ exonucleolytic activity of Xrn1 can degrade to produce the disome protected B1 RNA (Fig. 7). The fact that RNAs longer than B5, which accumulate in the trl1 mutant (Fig. 5a), are Xrn1-resistant (Fig. 5c and Supplementary Fig. 5) coupled with the absence of the B5 RNA upon Xrn1 inactivation (Fig. 1b–d) lead us to propose that the B5 RNA originates from 5′-OH RNAs cleaved within ribosomes upstream of the 3rd ribosome, are phosphorylated by Trl1 and are digested by Xrn1 until it bumps into downstream ribosomes (Fig. 7). A portion of the B1 species (Fig. 5a and Supplementary Fig. 5a) is detected upon Trl1 inactivation, however. Therefore, 5′-phosphorylation of 5′-hydroxylated B4 RNAs only accounts for a portion of B1 RNA production. In agreement, the hel2 mutation abolishes B4 RNA detection without impacting B1 and B5 RNA levels (Supplementary Fig. 3b). Mutations can thus reveal the existence of alternative pathways. In this regard, we learned that the inactivation of Xrn1 can lead to a Ddx1 trimming and production of B2 and B3 RNAs (Fig. 7). We cannot exclude the possibility that a portion of the B1 and B5 RNAs derive from alternative pathways, such as the canonical 5′→3′ decay pathway (i.e., decapping and Xrn1 5′→3′ digestion) or an uncharacterized endo- or exonucleolytic decay pathway (Fig. 7). Lower levels of NGD RNAs in a dcp2 mutant (Fig. 1b, c) do not contradict a decapping requirement in some of their production, but the poor growth of dcp2 mutant cells prevents us from drawing further conclusions. The potential existence of alternative pathways does not overshadow the strong impact of the trl1 mutant on NGD RNA production (Fig. 5).

We show that the NGD pathway produces RNAs bearing a 5′-hydroxyl group (Figs. 3a, 5 and 7). 5′-hydroxyl and 2′−3′ cyclic phosphate 3′-extremes are typically generated by metal-independent endoribonucleolytic reactions. We also show that the Trl1 kinase phosphorylates these 3′-NGD fragments to allow degradation by 5′−3′ exonucleotases. Thus, in addition to its role in RNA splicing and in phosphorylation of 5′-hydroxylated exon of the HAC1 mRNA, Trl1 is an important player in NGD pathway. This study also provides mechanistic insights that will help to go further in the comprehension of mRNAs surveillance pathways in connection to NGD.

**Methods**

**Yeast media, plasmids, strains, and oligonucleotides.** The media, strains of *S. cerevisiae*, oligonucleotides, synthesized DNAs and plasmids used in this study are described in the Supplementary Methods and Supplementary Tables 1, 2, 3 and 4, respectively.

**Northern blot analysis.** 8 OD600nm of exponentially growing cells were collected by centrifugation. RNA was extracted by adding on cell pellet 500 µl of buffer AE (50 mM Na acetate [pH 5.3], 10 mM EDTA, vortexing, and adding 500 µl of phenol previously equilibrated in buffer AE. After vortexing, the tubes were incubated at 65 °C for 4 min and then frozen in a dry ice–ethanol mix, followed by a 15-min centrifugation at room temperature. A second extraction with 300 µl of phenol–Tris–EDTA–chloroform was performed. RNA from 360 µl of supernatant was precipitated with 460 µl of 3 M Na acetate (pH 5.3) and 2.5 volumes of ethanol and centrifuged at 4°C for 30 min. The pellet was washed in 80% ethanol, dried at room temperature and resuspended in water. After electrophoresis, RNA was blotted onto a Hybond-N + membrane (Amersham) and hybridization to end-labelled oligoexonucleotides probes was carried out at 42°C overnight in BioQuick solution (Carl-roth). The Hybond-N + membrane was washed successively at 42°C in 5 × SSC-0.1% SDS and twice in 1 × SSC-0.1% SDS. Blots were exposed to PhosphorImager screens, scanned using a Typhoon FLA 9500 (Fuji), and quantified with ImageJ software.

**Gel electrophoresis for separation of RNA molecules.** Total RNA was resolved by 8% TBE-urea polyacrylamide or 14% TBE-Agarose gels, and to dissociate 5′-hydroxylated from 5′-phosphorylated RNAs total RNA was resolved by 12% TBE-urea polyacrylamide gels. All gels were followed by northern blot analysis.

**In vitro RNA digestion.** RNA digestion of 20 OD260nm of cell extracts were performed by using 1 unit of Xrn1 (Biolabs) in NEB buffer 3 at 25°C during 30 min unless otherwise indicated. NEB Buffer 3 was replaced by T4 PNK buffer (NEB) in kinase assays in the presence or absence of Xrn1 (Figs. 3a and 5d). For RNase I treatment, cell extracts, (20 µg of total RNA, 0.5 OD260nm units) were incubated with 0.5, 1, or 2 µl of RNase I (Invitrogen, 100 units/µl) 30 min at 25°C. For total RNA treatment, 5 µg of RNA were digested 30 min at 25°C. All RNAse treatments were followed by RNA extraction and northern blot analysis as described above.

**Polyosome analysis.** Yeast cells were grown exponentially to 0.8 OD600 at 28°C and the 200 ml cell culture was quickly cooled in ice water and then centrifuged and washed in 20 ml of buffer A containing 10 mM Tris pH 7.4, 100 mM NaCl and 30 mM MgCl2, and 0.5 mg/ml heparin. Heparin was only omitted for RNase I treatment. Cylcokhinemide was not used to prevent any drug-induced ribosome positioning. The cell pellet was resuspended in 0.6 ml of buffer A. A 1:4.5 amount of glass beads was added, and cell lysis was performed by bead beating twice for 30 s at each time (with intermittent cooling). After lysis, the cell extract was clarified for 5 min at full speed in a microcentrifuge. The equivalent of 20 OD260nm units of cell extract (80 µg of total RNA) was then layered onto linear 10–50% sucrose density gradients. Sucrose gradients (in 10 mM Tris–HCl [pH 7.4], 70 mM ammonium acetate, 30 mM MgCl2) were prepared in 12 × 89 mm polyallomer tubes (Beckman Coulter). Polyosome profiles were generated by continuous absorbance measurement at 254 nm using the Isco fraction collector. Gradients were collected in 20 fractions and processed for northern blotting as described above.

**Primer extension.** Radiolabelled primers (primers prA and prE for mRNA1, and primer pJ for (CGA)n-mRNA) were used and Maxima H Minus reverse transcriptase (ThermoFisher) was used to synthesize a single-stranded DNA toward the 5′-end of the RNA. The size of the labelled single-stranded DNA was determined relative to a sequencing ladder (ThermoFisher Sequenase sequencing kit) on 5% TBE-urea polyacrylamide gel. Oligonucleotides were radio-labelled with [γ-32P] ATP with the T4 polynucleotide kinase (NEB).

**3′-end RNA mapping.** Mapping was performed according to the 3′-RNA ligase-mediated RACE method with minor modifications: Total RNA preparations were first 3′-dephosphorylated using T4 PNK for 1 h at 37°C without ATP and pre-denatured linker (Universal mRNA cloning linker, NEB) ligation was performed during 4 h at 22°C in the presence of truncated RNA ligase 2 (NEB). Reverse transcriptase reactions were performed using primer preC complementary to the linker sequence. PCR primer preF specific to mRNA1, or primer prK specific to (CGA)n-mRNA, were used with primer preC in PCR reactions (Supplementary Figs. 3a and 6c). PCR products were purified, cloned into Zero Blunt TOPO PCR Cloning vector (Invitrogen), transformed and plasmids sequenced.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this Article.

**Data availability.** A Reporting Summary for this Article is available as a Supplementary Information file. The authors declare that all data supporting the findings of this study are available within the Article and its Supplementary Information files or from the corresponding author upon reasonable request. The source data underlying Figs. 1b–f, 2a, 2c, 3a, 4b, 4c, 5a–d and 6b–d, f and Supplementary Figures 1b, d–i, 2a–c, 3a, b, 5 and 6c are provided as a Source Data file in the Supplementary Information and at FigShare with DOI information (10.6084/m9.figshare.11317613).
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Author contributions

A.N., S.C., R.S.-F., J.H., C.T. and L.B. designed, performed and analysed data. L.B. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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