The H/ACA complex disrupts triplex in hTR precursor to permit processing by RRP6 and PARN

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Human telomerase RNA (hTR) is transcribed as a precursor that is then posttranscriptionally modified and processed. A fraction of the transcripts is oligoadenylated by TRAMP and either processed into the mature hTR or degraded by the exosome. Here, we characterize the processing of 3' extended forms of varying length by PARN and RRP6. We show that tertiary RNA interactions unique to the longer transcripts favor RNA degradation, whereas H/ACA RNP assembly stimulates productive processing. Interestingly, the H/ACA complex actively promotes processing in addition to protecting the mature 3' end. Processing occurs in two steps with longer forms first being trimmed by RRP6 and shorter forms then being processed by PARN. These results reveal how RNA structure and RNP assembly affect the kinetics of processing and degradation and ultimately determine the amount of functional telomerase produced in cells.
The catalytic protein subunit telomerase reverse transcriptase (TERT) and the RNA component telomerase RNA (TR) form the core of the telomerase enzyme. TERT reiteratively copies the template region of TR onto the chromosome ends to replenish terminal DNA repeats lost during DNA replication. In addition to providing the template for telomerase repeats, TR acts as a scaffold for the assembly of protein components that are part of the holoenzyme.

At the gross level, human telomerase RNA (hTR) can be divided into two domains. The 5′ domain folds into an evolutionarily conserved pseudoknot that harbors the template region and a triple helix structure that contributes to telomerase activity. The 3′ half of hTR closely resembles a box H/ACA domain. This structure is composed of two RNA hairpins connected by a single-stranded hinge, referred to as H box (ANANNA). The second hairpin is followed by a 3′ single-stranded tail containing the sequence motif ACA. Like other H/ACA RNAs, the H/ACA domain of hTR is associated with the four H/ACA box proteins dyskerin, NHP2, NOP10, and GAR1. Mutations in the H/ACA domain of hTR are associated with the four H/ACA box proteins linking poly(A)-specific short primary transcripts are also generated has remained increased in these cells. Oligo-adenylation is predominantly syndromes.

This structure is composed of two RNA hairpins connected by the sequence motif ACA. Like other H/ACA RNAs, the H/ACA domain of hTR is associated with the four H/ACA box proteins dyskerin, NHP2, NOP10, and GAR1. Mutations in the H/ACA domain of hTR have been identified in patients with dyskeratosis congenita, aplastic anemia, and idiopathic pulmonary fibrosis. These have been shown to cause defects in processing and localization of RNA, resulting in accelerated telomere shortening. Similarly, mutations in the protein components that interact with this domain also lead to a reduction in the steady-state level of hTR and are associated with the same spectrum of degenerative syndromes.

In vertebrates, H/ACA snoRNAs are typically encoded within introns, and mature via splicing, intron debranching, and exonucleolytic processing. In contrast, hTR is transcribed as an independent gene by RNA polymerase II and is subsequently processed into the 451-nt mature form. While the promoter of hTR is well-characterized, the mechanism of transcription termination and processing remain poorly understood. Several independent studies have demonstrated the existence of hTR transcripts extending beyond the mature end at position 451. Some 3′-extended forms are only a few nucleotides (nts) longer than the mature hTR, whereas others exceed 1500 nts in length. Their accumulation in cells depleted of the cap-binding component RRP40, respectively, from cultured cells (Supplementary Figure 1a) and analyzed the distribution of hTR ends by RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) coupled with Illumina sequencing. The analysis revealed distinct categories of 3′-extended RNAs. Knockdown of PARN resulted in an increase of RNA terminating at positions 454–457, coincident with a reduction in the fraction of shorter transcripts ending at positions 452–453 (Fig. 1a, top panel and Supplementary Figure 1b, top panel). Knockdown of RRP40 and RRP6 resulted in an increase in the fraction of longer hTR transcripts with 3′ termini mapping beyond position 460, consistent with a reduction in the fraction of short hTR transcripts that terminate at positions 452–459 (Fig. 1a, middle and bottom panels and Supplementary Figure 1b, middle and bottom panel). The accumulation of 460± isofoms was more pronounced in the RRP6 knockdown than the RRP40 knockdown. Consistent with prior reports, knockdown of PARN caused a reduction in mature hTR, whereas knockdown of RRP6 or RRP40 resulted in an increase in the mature form.

To gain further insights into the mechanisms of PARN action, we generated a series of synthetic RNA oligonucleotide substrates. PARN has a strong preference for degrading homopolymers of adenosine over other nucleotides. However, it was not known whether following removal of an oligo-A tail, the enzyme would also remove genome-encoded sequences from hTR to generate the mature 3′ end. Oligo (A) sequences were efficiently trimmed from all RNA substrates (Fig. 1b). Further degradation was observed for the mature form (ending at position 451) and exS forms (ending at position 453 or 455). In contrast, an exL form (ending at 460) was resistant to PARN even at higher concentration (lanes 25–32). PARN also degraded mature and exS substrates not carrying an oligo (A) tail but did not act on the exL RNA. A time course reveals that adenylated versions of mature and exS were more rapidly deadenylated by PARN than exL (Supplementary Figure 2a). The inefficient processing of exL RNA is not simply a consequence of exL being a longer RNA, as exL was still resistant to PARN after removing 17 nts from the 5′ end of the substrate (Fig. 1c). These results show that PARN preferentially acts on exS in vitro, consistent with the in vivo observation that exS forms accumulate following PARN depletion.

The observed substrate specificity pointed toward sequence-dependent attenuation of PARN. The enzyme has been shown to exhibit a tenfold preference for oligo (A) over oligo (U), with oligo (C) and oligo (G) being even poorer substrates. Surprisingly though, the exS (455) and the exL (460) forms both terminate in two uridines. The sequences diverge only further upstream: (456-CGC-458) in exL and (451-CAG-453) in exS. To determine whether GC-rich sequence inhibits PARN, we...
introduced A452C, A452G, and G450A mutations into exS substrates (Fig. 1d and Supplementary Figure 2b and 2c). A452C generates the sequence CCG and A452G generates CGG. In particular, A452C strongly decreased the efficiency of deadenylation and degradation by PARN, with A452G causing modest attenuation of PARN (Fig. 1e, and Supplementary Figure 2c). By contrast, deadenylation and degradation of G450A was slightly more efficient than wild type (Fig. 1e, compare lanes 3 and 9). These results indicate that PARN activity is affected by sequence context at a distance of several nucleotides.
The H/ACA RNP attenuates processing by PARN at position 451. Although in vitro processing assays revealed pronounced effects of sequence on PARN processivity, slight attenuation was observed at the position of the mature 3’ end (Fig. 1b). Clearly, sequence context alone is not sufficient for PARN to generate the 451-nt mature RNA. To examine whether folding of the RNA into the two-hairpin structure of an H/ACA box RNA is needed, we in vitro synthesized the H/ACA domain of hTR (starting at nucleotide 206) as mature, exS and exL forms (Fig. 2a). Incubation of recombinant PARN with H/ACA domain exS plus oligo (A) tail resulted in products shorter than the mature form, demonstrating that the hairpin structure alone is insufficient to attenuate PARN at position 451 (Fig. 2b, lanes 1–6). In contrast, the mature form was produced during incubation with whole cell extract (lanes 7–12), even when the extract was supplemented with excess recombinant PARN (lanes 13–18). To confirm that the 3’ processing of the hTR precursor is mediated by PARN, extracts were prepared from cells subjected to PARN knockdown and control siRNA (Supplementary Figure 3a, lane 2). The 3’ processing of the precursor into mature hTR was substantially reduced in extracts from PARN knockdown cells (Fig. 2c, lanes 5–8 and Supplementary Figure 3b and 3c). Although deadenylation still occurred completion in less than 30 min, further processing was inefficient, and after 120 min, less than 25% had been converted into the mature form. These results further support a role for PARN in converting exS into the mature form.

As binding of the H/ACA complex is critical for hTR stability in vivo, we next asked whether this complex was responsible for attenuating PARN at position 451 in vitro. Following incubation of mature, exS and exL in extracts on ice, all forms of wild-type but not C408G mutant hTR were recovered by immunoprecipitation with anti-DKC1 (Fig. 2d). Consistent with previous results, siRNA-mediated knockdown of dyskerin reduced the steady-state level of hTR in cells by tenfold (Supplementary Figure 4a and 4b). Incubation of oligo-adenylated hTR (nucleotides 206–461+5A) in control extracts at 37 °C confirmed that this form is stable over 2 h following rapid deadenylation (Fig. 2e, lanes 1–4). In contrast, this RNA was rapidly degraded in the dyskerin knockdown extracts (Fig. 2e, lanes 5–8 and Supplementary Figure 6), indicating that the in vitro assay recapitulates the protective role of the H/ACA RNP bound to hTR in cells.

RRP6 and dyskerin are required for the processing of exL. Recombinant PARN alone failed to degrade exL oligonucleotides (Fig. 1b). Similarly, incubation of the exL form of the H/ACA domain (460+5A) resulted in deadenylation but lack of further processing, although a smear indicating some degradation at the later time points (Fig. 3a, lanes 1–6). In contrast, incubation with whole cell extract produced the mature form (Fig. 3a, lanes 7–18), indicating that 3’ processing of exL requires activities in addition to PARN. This idea was also supported by the observations that PARN knockdown resulted in an increase in the fraction of hTR exS with minimal effect on exL (Fig. 1a, top panel). In contrast, knockdown of RRP6 resulted in a pronounced increase in exL, indicating a role for this nuclease in the processing of longer forms (Fig. 1a, middle panel).

To examine the requirement of RRP6 in processing or degradation of exL, we prepared extracts from cells that had been subjected to RRP6 siRNA (Supplementary Figure 3a, lane 3). Oligo-adenylated exS was still processed into mature hTR in the knockdown extract (Fig. 2c, lanes 9–12 and Supplementary Figure 3b and 3c). In contrast, oligo-adenylated exL was deadenylated but not processed into mature hTR following knockdown of RRP6 (Fig. 3b and Supplementary Figure 5). Together with the in vivo 3’ end sequence analysis, these results argue for exL first being processed by RRP6 into exS, which then becomes a substrate for PARN to generate the mature form. This raises the question of what determines whether exL is degraded or processed into exS? In vivo, cotranscriptional assembly of the H/ACA core complex on hTR is thought to be critical for protecting hTR from degradation. To examine the effect of H/ACA RNP assembly on exL processing, oligo-adenylated exL (nucleotides 206–461+5A) was incubated in the siRNA-control and dyskerin knockdown extracts, respectively (Fig. 4a). Whereas deadenylation was unaffected by the presence or absence of dyskerin (Fig. 4a, compare lanes 2 and 6), the processing into the mature form was abolished in the dyskerin knockdown extracts (Fig. 4a, lanes 5–8). Although the exL form was partially degraded in the absence of dyskerin, it is important to note that the exL form is threefold more stable than mature hTR in the absence of dyskerin (Fig. 4b and Supplementary Figure 6). The deficiency in 3’ processing following dyskerin knockdown was not caused by a reduction in PARN or RRP6 (Supplementary Figure 4a). These observations demonstrate that the H/ACA core complex is not only essential for the protection of hTR from degradation but is also required for processing of exL into exS. This indicates that an RNA structure or an RNA binding protein selectively stabilizes the exL forms when not bound by the dyskerin complex.

Tertiary RNA interactions stabilize exL. To dissect the basis for the increased stability of exL in DKC1 knockdown extracts, we examined the stability of oligo-adenylated RNAs ending at positions 459, 460, and 461, respectively (Fig. 4c). All three substrates were deadenylated, but the additional two nucleotides at the 3’ terminus of 461 resulted in notable stabilization relative to the RNA ending at position 459 (compare lanes 4 and 12). Increased resistance to degradation by 3’–5’ exonucleases could be mediated by RNA secondary structure involving the 3’ terminal sequence. To test this idea, we utilized an RNA crosslinking method in which the modified ribonucleotide 4-thiouridine (4sU) was introduced into the exL (461) form at positions 459, 460, and 461, respectively (Fig. 4d). Under exposure to UV light, 4sU reacts with nearby RNA bases, and crosslinked products can be separated from uncrosslinked RNA by denaturing gel electrophoresis. Two main conjugate bands (XL-1 and XL-2) appeared specifically after UV-irradiation (Fig. 4d). The crosslinked...
**Fig. 2** PARN processing is attenuated by the H/ACA RNP. 

**a** Schematic of the hTR fragment used in the in vitro assay. 

**b** 32P-labeled hTR fragments (nucleotides 206 to 455 with oligo A tails) were incubated with 5.0 nM of recombinant PARN alone or in HeLa cell extracts without or with 5.0 nM of recombinant PARN at 37 °C for the indicated times. RNA was purified and resolved by a 6% polyacrylamide/8 M urea gel. 

**c** The 32P-labeled in vitro transcribed hTR fragments (nucleotides 206 to 455 with oligo A tails) was incubated in cell extracts (20 μg) prepared from siRNA-treated cells as indicated. The reaction was performed at 37 °C for the indicated times. RNA was purified and resolved by a 6% polyacrylamide/8 M urea gel. 

**d** Wild type or C408G mutant hTR fragments (nucleotides 206 to 451, 453, 455, and 460 with or without oligo A tails) were in vitro transcribed in the presence of α-32P-UTP and incubated in HeLa cell extracts on ice for 10 min. The reaction mixture was immunoprecipitated with antibodies against dyskerin. RNA was purified and resolved by a 6% polyacrylamide/8 M urea gel. 

**e** The 32P-labeled in vitro transcribed hTR fragments (nucleotides 206 to 451 with oligo A tails) were incubated in cell extracts (20 μg) prepared from siRNA-treated cells as indicated. The reaction was performed at 37 °C for the indicated times. RNA was purified and resolved by a 6% polyacrylamide/8 M urea gel.
products were most abundant when 4sU was introduced at position 461 (lane 6). To identify the interaction site, crosslinked products were gel-purified and mapped by primer extension. We were unable to map the positions crosslinked in XL-2, which may represent interactions between two RNA molecules. Remarkably, though, the XL-1 band revealed intramolecular crosslinks at two specific locations, the H box and the UCU sequence between the P4.2 and P5 stems (Fig. 4e, lane 6). As the UCU sequence cannot base pair with the UUU sequence at the 3′ end of hTR (461), the crosslinks are indicative of triple base interactions such as U–A●U found in triple helix structures (where ● denotes the Hoogsteen face and – denotes the Watson–Crick face).

**Role of the tertiary interactions in hTR biogenesis.** Engaging the 3′ end and the H box in tertiary RNA interactions would not only impair 3′ exonucleolytic degradation, but also compete with H/ACA RNP assembly on the H-box. To assess the effects of stabilizing or destabilizing this new structure, two mutants were created. The U460C mutant strengthens a triple base interaction site while disrupting the potential for triple helix formation. Where U460C decreased dyskerin binding, the GG375/6AU mutations resulted in increased dyskerin binding (Supplementary Figure 8). UV-crosslinking with 4sU at position 461 produced an enhanced XL-1 signal for U460C as expected for a stabilized structure (Fig. 5b). Mapping of the crosslink sites corroborated enhanced interaction between the exL 3′ end and the UCU sequence starting at position 327 (Fig. 5c). Although the GG375/6AU mutant showed a strong crosslink near XL-1 (Fig. 5b), mapping revealed simple base pairing with the H box and no detectable tertiary interaction with the 327-UCU sequence (Fig. 5c).

Subjecting the U460C mutant to the in vitro processing assay resulted in a 30% increase in degradation compared to wild type (Fig. 5d, e). Similarly, introduction of hTR containing the U460C mutation into VA13 cells resulted in a 20% decrease in the steady-state level of the mature hTR in vivo (Fig. 5f, g). This is the first mutation downstream of position 451 shown to affect the steady-state level of the RNA, suggesting that mutations downstream of position 451 could also be associated with premature telomere shortening in humans. The GG375/6AU mutation had the opposite effect on hTR processing, causing an approximately twofold increase in the mature form in vitro (Fig. 5d, lanes 9–12 and Fig. 5e) and an ~1.6-fold increase in vivo (Fig. 5f, g). In
Fig. 4 A triplet helix structure forms in exL. 

**a** The $^{32}$P-labeled in vitro transcribed hTR fragments were incubated in extracts prepared from 293T cells subjected to dyskerin knockdown and control siRNA for the indicated times. RNA was purified and resolved by a 6% polyacrylamide/8 M urea gel. 

**b** The $^{32}$P-labeled in vitro transcribed hTR fragments were incubated in dyskerin knockdown extracts for the indicated times. RNA was purified and resolved on a 6% polyacrylamide/8 M urea gel. The hTR signal was quantified using a phosphoimager. The ratio of each RNA species was normalized to both time 0 and the loading control, Actin. Mean values were calculated from three biological replicate experiments as shown in Supplementary Figure 6. Bars represent the standard error. For the mature form, 50% is degraded after 22.4 min, for exL after 69.9 min. 

**c** The $^{32}$P-labeled in vitro transcribed hTR fragments were incubated in dyskerin knockdown extracts for the indicated times. RNA was purified and resolved on a 6% polyacrylamide/8 M urea gel.

**d** Schematic showing an hTR fragment with a single nucleotide replaced with 4-thiouridine (4sU) for positions 459, 460, and 461 used for the in vitro UV crosslinking assay. Nucleotides modified with 4sU are shown in red. The primers used for primer extension are shown in blue. RNA crosslinks were induced by irradiation with UV light. The crosslinked products were resolved on a 6% polyacrylamide/8 M urea gel.

**e** Specific signals corresponding to XL-1 were gel-purified and mapped by primer extension. Asterisks mark non-specific RT pause sites.
aggregate, these results reveal that the exL form of hTR can adopt a triple helix structure. Conversion of the exL form into exS requires binding of the H/ACA complex to shift the balance from degradation to maturation. **Discussion**

The correct processing of TR and the assembly of the RNP are critically important for enzymatic activity and telomere length homeostasis. Here, we have shown that hTR 3′ end processing...
The importance of the triple helix structure in biogenesis of hTR. a Schematic of the proposed triple helix structure formed in exL. Introduced nucleotide replacements in the H box motif are depicted. b Wild type, U460C, and GG375/6AU hTR transcripts with a single radioactive phosphate followed by a single photoactivatable 4SU at position 461 were irradiated with UV light, followed by separation on a 6% polyacrylamide/8 M urea gel. c Specific signals corresponding to XL-1 were gel-purified and mapped by primer extension. Asterisks mark non-specific RT pause sites. d and e In vitro hTR processing assays were performed with the 32P-labeled hTR fragments as indicated in a. RNA was purified and resolved on a 6% polyacrylamide/8 M urea gel. The bar graph depicts mean fold change for mature form relative to wild-type samples and normalized to Actin. Mean values were calculated from in vitro hTR processing assays of three biological replicates. Bars represent the standard error. Significance of change in mature form between samples was calculated with a two-sided Student’s t-test; p values: 0.002462 (U460C); 0.001484 (GG375/6AU). Dots represent data points from individual experiments. f and g VA13 cells were transfected with vectors containing wild type, U460C, or GG375/6AU mutant. Total RNA was prepared and subjected to northern blot. A probe against neomycin served as control for transfection efficiency and loading. The bar graph illustrates the mean fold change for hTR levels relative to wild-type samples and normalized to neomycin. Mean values were calculated from northern blot experiments of three biological replicates. Bars represent the standard error. Significance of change in mature form relative to wild-type samples was calculated with a two-sided Student’s t-test; p values: 0.000989 (U460C); 0.000239 (GG375/6AU). Dots represent data points from individual experiments.

Fig. 6 Model. Schematic illustrating the multiple roles of the H/ACA complex and the different functions for RRP6, the exosome, and PARN in human telomerase RNP biogenesis.
reverse transcription quantitative polymerase chain reaction (RT-qPCR) of hTR implicated the core exosome (RRP40) and its two associated exonucleases, RRP6 and RRP44/DIS3, in the turnover of hTR extending by at least 50 nts beyond the mature 3′ end. In one study, knockdown of RRP40 stabilized 3′ extended hTR sixfold more than knockdown of RRP6. The implication of the NEXT complex in the turnover of longer forms of hTR supports the idea that degradation occurs via NEXT targeting the exosome to hTR, whereas processing may involve RRP6 alone or in complex with other factors.

It has become clear that RNA triple helix structures affect many aspects of RNA biology and may be far more common than previously thought. They have been found in diverse functional contexts. For instance, the catalytic activity of telomerase depends on a triple helix in the pseudoknot domain of hTR. Furthermore, a triple helix in the SAM-II riboswitch creates a binding pocket for S-adenosylmethionine, and triple helix structures have also been implicated in protecting 3′ ends of RNAs from exonucleolytic degradation. In the case of the Kapossi’s sarcoma-associated herpes virus polyadenylated nuclear RNA, a protein–RNA triple helix involves the poly(A) tail. In MALAT1 RNAs, a triple helix structure involving the non-polyadenylated 3′ end increases RNA stability and stimulates translation. The tertiary RNA interactions involving the 3′ end of hTR exL, the H box, and an internal loop sequence does not fit into any of these functional paradigms. Although the structure inhibits rapid nuclear RNA decay as shown for the analogous structure in MALAT1, its presence in hTR is ultimately in direct competition with canonical processing. Functionally, it appears that the structure transiently protects exL from rapid degradation and creates a window of opportunity for the H/ACA complex to bind and remove the triple helix in the process. Consistent with dyskerin binding resolving the triple helix structure, exL has a notably longer half-life in extracts from dyskerin-depleted cells than exS or the mature form.

Independent of the formation of the triple helix structure, PARN is inhibited at the boundary of exL and exS by sequence context. Attenuation of both nucleases by sequence and structure allows time for conformational rearrangements and RNP assembly, thereby coupling successful H/ACA RNP formation to the final processing steps. This creates an effective quality-control pathway for hTR biogenesis but makes the process of generating sufficient telomerase to ensure long-term telomere length homeostasis sensitive to defects in a large number of RNA processing factors. Ongoing association studies will undoubtedly uncover additional loci associated with telomopathies.

**Methods**

**In vitro deadenylation.** Trace amounts of 5′-32P-labeled (3000 cpn µl−1) and 250 nM unlabeled hTR RNA oligo were incubated with the indicated concentrations of recombinant PARN (Origene, cat. # TP037220) in a buffer containing 20 mM Tris-HCl (pH 7.5), 50 mM KCl, 2.5 mM MgCl2, 50 µg ml−1 BSA (NEB), and 40 U RNasin plus RNase inhibitor (Promega). A 10-µl reaction mixture was incubated at 37 °C for 30 min, and the reaction was stopped by the addition of 2× RNA loading dye containing 99% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, and 0.1 M EDTA. The samples (5 µl) were separated on 20% denaturing acrylamide sequencing gels containing 7 M urea at 80 W for 3 h for long hTR RNA oligos and 1.5 h for short hTR oligos. RNA oligos used for in vitro deadenylation assays are listed in Supplementary Tables 1 and 2.

**Preparation of hTR RNA substrates and in vitro hTR processing assay.** In vitro transcription reactions were carried out in 1× transcription buffer (Promega), 0.5 mM each for ATP, CTP, and GTP, 0.1 mM UTP, 0.66 µM α-32P-UTP (3000 Ci mmol−1, 10 mM Ci ml−1, PerkinElmer), 1 µg DNA template, 40 U Rnase plus RNase inhibitor (Promega), and 20 units T7 RNA polymerase (Promega). Reaction mixtures (10 µl) were incubated at 37 °C for 2 h followed by the addition of an equal volume of formamide-loading dye. Full-length RNA products were purified on 8% polyacrylamide/8 M urea gels. Primers used to generate DNA templates are listed in Supplementary Table 3. For preparation of the loading control actin mRNA, a similar reaction was carried out except that pSP6Ach6-88 was used as DNA template and 18.9 units of Sp6 RNA polymerase (Promega) instead of T7 RNA polymerase.

In vitro hTR processing reactions (10 µl) were carried out at 37 °C in a buffer containing 20 mM Tris-HCl (pH 7.5), 50 mM KCl, 2.5 mM MgCl2, 50 µg ml−1 BSA (NEB), 40 U Rnaseplus plus RNase inhibitor (Promega), 30 nM 32P-labeled hTR RNA, and either indicated amounts of recombinant PARN or 20 µg of whole cell extract, or both. Reactions were stopped by the addition of 10 µl stop buffer (10 mg ml−1 protease K in 0.5% SDS, 40 mM EDTA, 20 mM Tris-HCl, pH 7.5, and 1000 µM ρ-32P-labeled actin mRNA) and incubation at 37 °C for 20 min followed by extraction with phenol/chloroform pre-equilibrated with 50 mM NaOAc (pH 5.0), and ethanol precipitation. RNA was dissolved in 70% formamide dye and analyzed on 6% polyacrylamide (19:1) gels containing 8 M urea.

**Constructs and site-directed mutagenesis.** A fragment of DNA containing 1.5 kb upstream and 2 kb downstream of the major transcription start site of hTR was cloned into pACGFP1-1 using Xhol and BamH1 sites; this plasmid was named pMG80. Mutations were introduced using QuickChange II XL Site-Directed Mutagenesis Kit (Agilent). Oligonucleotides used for site-directed mutagenesis are listed in Supplementary Table 4.

**Cell culture, transfection, and sample preparation.** WI-38 VA-13 subline 2RA cells (ATCC CCL-751) were cultured in E-MEM medium (ATCC, 30-2003) supplemented with 10% fetal bovine serum (Sigma-Aldrich) at 37 °C, 5% CO2. Cells were transfected with 12 µg of plasmids using Fugene HD (Promega) for 72 h. Plasmids used for transfection are listed in Supplementary Table 5. HeLa cells were cultured in DMEM medium (Life Technologies, 11995-065) containing 10% fetal bovine serum (Sigma-Aldrich) at 37 °C, 5% CO2. HeLa or 293T cells were treated with 20 nM or 40 nM siRNA for 72 h using Dharmafect 1 (Dharmacon). siRNAs used in this study are listed in Supplementary Table 6.

**Western blot.** For western blotting analysis, cells were lysed in a solution containing 2× LDS sample buffer (Life Technologies), 0.78% β-mercaptoethanol (v/v) (J.T. Baker), and 4% SDS. Protein lysate was loaded onto a 4–12% Bis-Tris gel (Invitrogen) and transferred to a nitrocellulose blotting membrane (GE Healthcare Life science, 10000012), Low-fat milk (5%) in TBS buffer was used as a blocking reagent. Antibodies used in this study are listed in Supplementary Table 7.

**Northern blot.** Cells were collected in Trizol reagent (Ambion, Life Technologies) according to the manufacturer’s instructions, followed by treatment with DNase I (New England Biolabs) at 37 °C for 30 min. Total RNA was extracted once with phenol/chloroform equilibrated with 50 mM NaOAc (pH 5.0), then ethanol precipitation and either indicated amounts of recombinant PARN or 20 µg of whole cell extract, or both. Reactions were stopped by the addition of 10 µl stop buffer (10 mg ml−1 protease K in 0.5% SDS, 40 mM EDTA, 20 mM Tris-HCl, pH 7.5, and 1000 µM ρ-32P-labeled actin mRNA) and incubation at 37 °C for 20 min followed by crosslinking in a Stratalinker (Stratagene, 254 nm, 120 ml). Hybridizations were carried out in Church-Gilbert buffer at 65 °C (for hTR, probes were generated by nick translation of a polymerase chain reaction (PCR) fragment with 32P-dCTP and 42 °C (for oligonucleotide probe BLoi2948 against neocymin, labeled with 32P-ATP by T4 PNK kinase). Oligonucleotide sequences are listed in Supplementary Table 8.

**RNA ligase-mediated 3′ RACE and deep sequencing.** Library preparation was based on a published protocol. Oligonucleotides used for the library preparation are listed in Supplementary Table 9. Ligations reactions contained 1.5 µg total RNA, 13 nM RNA ligase buffer, 3.75 µg ECG000D, 7.4% DMSO, 10 U T4 RNA ligase I, 0.7 µM ATP, and 3.7 µM linker (BLoi5511). The ligation reaction mixture was incubated at 16 °C for 18 h and inactivated at 65 °C for 15 min. About 30 µl of reverse transcription reaction contained ligation reactions, 1× First Strand buffer, 1.6 µM RT primer (BLoi5573), 0.5 µM dNTPs, 5 mM DTT, 40 U RNasin plus RNase inhibitor (Promega), and 300 U SuperScript III (Invitrogen) and was incubated at 55 °C for 60 min. A total of 10 U Rnase H was directly added into the reverse transcription reaction, followed by incubation at 37 °C for 20 min and then heat-inactivation at 70 °C for 15 min. The first round PCR was performed with 0.5 µM BLoi5573 and BLoi5574 in 50 µl volumes containing 1× Phusion HF buffer, 0.2 mM dNTPs, and 1× Phusion Hot Start II polymerase (Thermo Scientific). First-round PCR products were purified by PCR purification kit (QIAGEN). For the second-round PCR reactions, 5 µl of 10×-diluted first-round PCR product was used as a template; 0.5 µM BLoi4666 was used as the forward primer for all samples and reverse primers BLoi4668, BLoi4669, BLoi4779, BLoi4785, BLoi4782, and BLoi4783 were used for si-Ctrl (PARN), si-Ctrl (RPP6), si-RPP, si-Ctrl (RPP40), and si-RPP40, respectively. Amplicons were purified by PCR purification kit (QIAGEN) and the primer dimers were removed using the
Pippin Prep System (Sage Science). The samples were quantified by Qubit and Bioanalyzer, multiplexed and split between two lanes of a RapidSeq flow cell for sequencing. The library samples were sequenced on the Illumina HiSeq using RapidSeq-250 bp single-end reads. A 10-nl molecular barcode was used to remove PCR duplicates. After quality filtering, 0.7 to 1.0 million reads were analyzed per sample. To pass the filter, a read required a minimum match of 20 nt to hTR reference sequence and 10 nts to the linker sequence. For each filtered read, the most 3′ hTR reference coordinate between hTR-1366-641 was identified by matching 20 nts closest to the 3′ end allowing for two mismatches not including the two most 3′ bases. Nucleotides found between the reference hTR and the linker sequence were considered non-templated nucleotide additions (NTNA). All reads were included in the 3′ end analysis irrespective of the presence or absence of NTNA.

Preparation of 45S hTR substrate. DNA templates for in vitro transcription were generated by PCR. BL016336 and BL016790 as listed in Supplementary Table 3 were used in a PCR reaction to generate the template for the 5′ hTR fragment. The 3′ RNA fragments that contain a single photoactive 4sU were purchased from GE Dharmacon and are listed in Supplementary Table 10. The 3′ fragment was labeled with 32P at the 5′ end using T4 polynucleotide kinase. hTR substrates containing a single 32P-labeled 4sU were produced by ligation of a 3′ fragment to the 3′ end of the in vitro-transcribed 5′ fragment using T4 RNA ligase 2 (New England Biolabs, NEB). A split DNA oligonucleotide (BL016792 as listed in Supplementary Table 10) was mixed with the 5′ and 3′ fragments at a ratio of 2:1:3 (Split:5′ fragment:3′ fragment) for ligation in a buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 1 mM DTT, 1 mM ATP, 2 μM 32P-RNA plus RNase inhibitor (Promega), and 1 unit μl−1 T4 RNA ligase II. The ligated products were gel-purified by electrophoresis on a 6% acrylamide sequencing gel containing 8 M urea.

Crosslinking analysis. The 4sU–hTR substrates (6 μM) were incubated at 37 °C for 10 min in buffer containing 20 mM Tris-HCl (pH 8.5), 50 mM KCl, 2.5 mM MgCl2, 50 μg ml−1 BSA (NEB), and 40 U RNasin plus RNase inhibitor (Promega). The reaction mixture was spread onto a piece of parafilm covering an ice-cold aluminum block. Droplets were UV-irradiated for 20 min at a distance of ~2 cm with a 365-nm UV Lamp (Model UVGL-25, UVP Inc.). The crosslinking products were gel-purified by primer extension. Primer extension products were mapped by primer extension, pMG80 as template, and components of the primer extension kit. Primer extension products identified by electrophoresis on a 6% acrylamide sequencing gel containing 8 M urea.
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**Author contributions**
C.-K.T. and P.B. designed the experiments; C.-K.T. performed most of the experiments; H.-F.W. generated all the western blots; C.-K.T. and H.-F.W. performed the RLM-RACE experiments; and M.R.S. analyzed the Illumina data. All authors analyzed the data, and C.-K.T. and P.B. wrote the manuscript.

**Additional information**
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