JCI The Journal of Clinical Investigation

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J Clin Invest. 2016;126(9):3377-3382. https://doi.org/10.1172/JCI87547.

Brief Report Genetics

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Posttranscriptional manipulation of TERC reverses molecular hallmarks of telomere disease

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Introduction

The telomerase RNA component (TERC) is the essential noncoding RNA template and scaffold of the telomerase holoenzyme (1–3). TERC levels are limiting for telomerase activity and telomere maintenance and thus are a critical determinant of self-renewal (4, 5). The *TERC* locus is recurrently amplified in cancer (6, 7), and genetic mutations that reduce TERC result in telomere diseases such as dyskeratosis congenita (DC) and idiopathic pulmonary fibrosis (IPF) (8–10). Manipulating TERC RNA levels could therefore be useful for altering telomerase and self-renewal in degenerative and malignant disorders, but until recently, factors regulating its accumulation in cells have been unknown.

Genetic studies in patients with telomere diseases (11–13) have led to the discovery that the poly(A)-specific ribonuclease (PARN) is required for the removal of oligo(A) tails and/or genomically encoded 3′ extensions from nascent TERC RNA transcripts (14). Independent studies have also identified components of RNA quality control pathways that regulate TERC 3′ end maturation and support a role for PARN in removing oligo(A) tails from TERC transcripts (15, 16). Nuclear RNAs with oligo(A) tails are recognized and targeted for destruction by the exosome (17). In keeping with this, we and others have found that TERC transcripts in *PARN*-mutant cells show aberrant 3′ oligo-adenylation, decreased half-life, and reductions in steady-state levels (13, 14). Collectively, these results indicate that PARN is required for the removal of nontemplated, posttranscriptionally acquired oligo(A) tails that destabilize nascent TERC RNA transcripts.

PARN has been shown to deadenylate noncoding RNAs such as small nucleolar RNAs and microRNAs (miRNAs) that are adenylated by the noncanonical poly(A) polymerase PAP-associated domain–containing 5 (PAPD5; also known as TRF4-2) (18, 19). PAPD5 acts within the TRAMP complex (TRF4-2, AIR2, MTR4) to add short oligo(A) extensions to nuclear RNAs, leading to their destruction by the exosome (17, 20). Recent experiments using RNA interference in immortalized cell lines indicate that *PAPD5* inhibition reduces oligo-adenylation of TERC and increases steady-state RNA levels (15, 16, 21). We hypothesized that counteracting TERC oligo-adenylation would balance the effects of PARN deficiency in patient cells and restore telomere homeostasis. In the current study, we show that TERC is limiting for telomere maintenance in *PARN*-mutant cells and that *PAPD5* inhibition is sufficient to counteract the effects of PARN deficiency by restoring TERC levels, telomerase activity, and telomere elongation. Our results demonstrate that manipulating posttranscriptional processing of TERC RNA can affect telomere biology in DC patient cells.

Results and Discussion

To directly assess the effects of PARN deficiency on telomere maintenance, we disrupted *PARN* in HEK293 cells using lentivirus-encoded shRNAs and continuously cultured the transduced cells under antibiotic selection. In *PARN* knockdown cells, using 2 independent shRNAs, we observed telomere attrition over time as compared with that in control cell lines (Figure 1A). When we overexpressed a *PARN* cDNA that is not susceptible to knock-

Conflict of interest: B. Boyraz, D.H. Moon, M. Segal, and S. Agarwal are listed as coinventors on a provisional patent application that includes data presented in this work. Provisional patent numbers 62/242,970 and 62/308,427.
Submitted: March 14, 2016; **Accepted:** June 2, 2016.
Reference information: *J Clin Invest*. 2016;126(9):3377–3382. doi:10.1172/JCI87547.

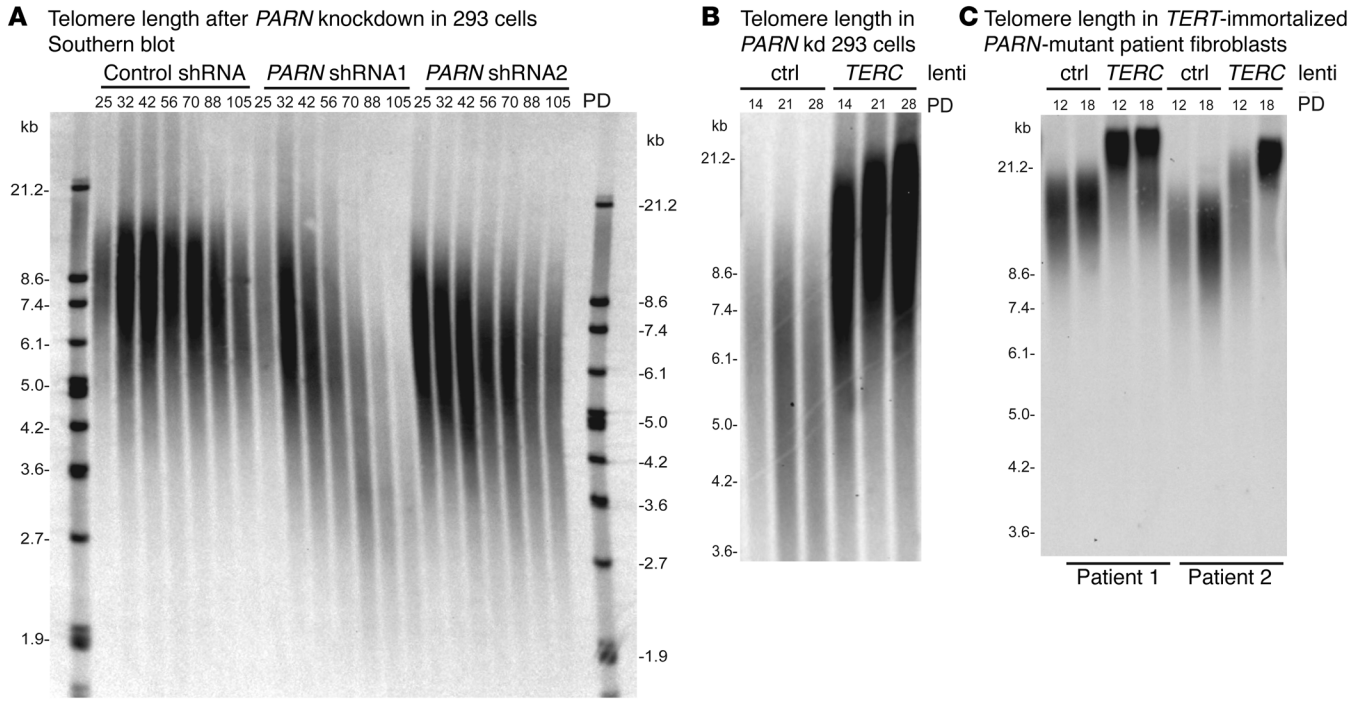

Figure 1. *TERC* rescues telomere length in PARN-deficient cells. (**A**) Telomere restriction fragment (TRF) length in HEK293 cells infected with shRNAs targeting luciferase (control) versus *PARN*. PD, population doubling. (**B**) TRF in *PARN*-knockdown HEK293 cells with lentivirus encoding *TERC* versus vector (ctrl). (**C**) TRF of immortalized, *PARN*-mutant patient fibroblasts with lentivirus encoding *TERC* versus vector.

down by the shRNA, we observed increased telomere length as compared with that in control cells (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI87547DS1). Next, we evaluated the effects of restoring *PARN* expression on telomere maintenance in patient cells. In TERT-immortalized fibroblasts from patients with DC caused by *PARN* loss-of-function mutations, we found that *PARN* expression restored telomere elongation (Supplemental Figure 2). These results confirm that PARN deficiency compromises telomere maintenance and indicate that PARN is limiting for telomere maintenance in human cells, despite the presence of TERT.

We have previously suggested that PARN deficiency causes telomere disease primarily via direct effects on TERC (14). It has also been proposed that reduction of mRNA transcripts encoding other factors, including dyskerin, TRF2, and RTEL1, may impair telomere maintenance in *PARN*-mutant patient cells (12). To determine whether PARN deficiency affects telomere elongation primarily via effects on TERC, we stably overexpressed *TERC* via lentiviral vectors in PARN-deficient cells and analyzed telomere length over time. We found that expression of *TERC* in *PARN*-knockdown HEK293 cells led to rapid elongation of telomeres (Figure 1B and Supplemental Figure 3A). Similarly, we found that overexpression of *TERC* in TERT-immortalized fibroblasts from patients with *PARN* mutations led to elongation of telomeres (Figure 1C). TERC has been shown to be limiting for telomere elongation in cells carrying DC-associated variants of dyskerin (22). To determine whether dyskerin deficiency plays a role in *PARN*-mutant cells, we ectopically expressed *DKC1* cDNA in *PARN*-knockdown HEK293 cells, but did not observe a restoration of telomere length (Supplemental Figure 3B). In *PARN*-knockdown HEK293 cells with or without *TERC* overexpression, we did not observe changes in dyskerin, TRF2, or TERT levels (Supplemental Figure 4). These data demonstrate that PARN deficiency impairs telomere maintenance via a direct impact on TERC and suggest that enhancing TERC will restore telomere elongation in *PARN*-mutant cells.

Prior studies indicate that PARN deadenylates noncoding RNAs that have undergone posttranscriptional 3′ adenylation by PAPD5 and thus promotes maturation rather than degradation of noncoding RNAs (18, 23). We hypothesized that if the primary role of PARN in telomere biology is to counteract oligo-adenylation and degradation of TERC, then *PAPD5* inhibition should be sufficient to restore TERC processing and increase telomerase activity in human cells. To investigate this, we disrupted *PAPD5* in HEK293 cells using lentivirus-encoded shRNAs and performed rapid amplification of complementary DNA ends (RACE) to characterize TERC 3′ ends (Figure 2A and Supplemental Figure 5). With *PAPD5* knockdown, we observed an alteration in the size distribution of TERC 3′ ends in that the intensity of the amplicon corresponding to the mature form of TERC was increased relative to the immature, extended form of TERC that predominates in *PARN*-knockdown cells (Figure 2B). By deep sequencing of 3′ RACE products, we found that *PAPD5* inhibition significantly reduced the proportion of genomically extended TERC species that were oligo-adenylated as well as the average oligo(A) tail length of nascent TERC transcripts (Figure 2, C–D). Conversely, both the percentage of mature TERC (Figure 2 C) and steady-state levels of TERC transcripts were increased in *PAPD5*-knockdown cells (Figure 2E; lanes 1 and 3). When we overexpressed *PAPD5* cDNA in cells (Supplemental Figure 6), we found that total TERC

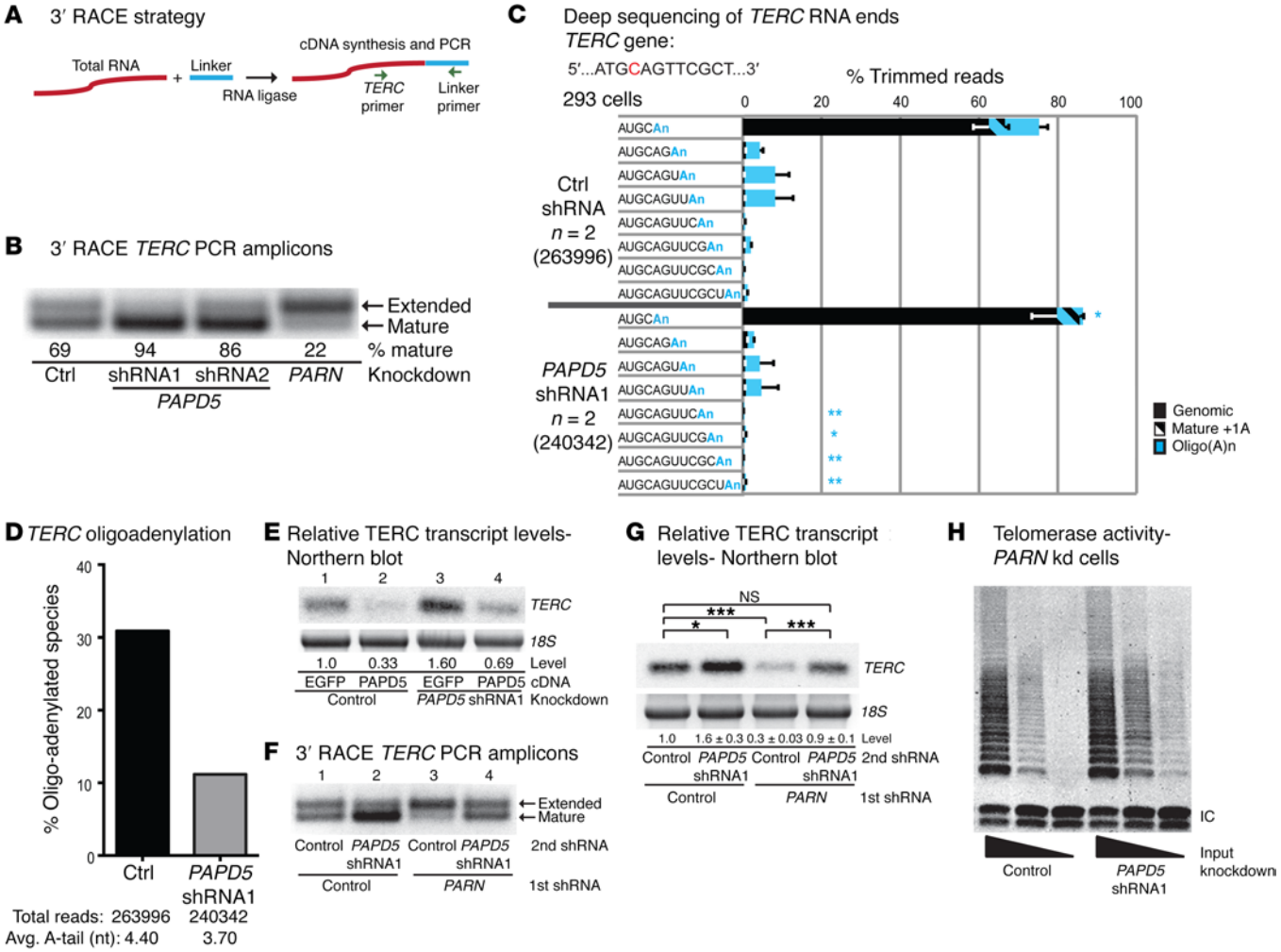

Figure 2. *PAPD5* inhibition restores TERC and telomerase activity in PARN-deficient cells. (**A**) Strategy for 3′ RACE. (**B**) Agarose gel electrophoresis of 3′ RACE TERC products from HEK293 cells transduced with lentivirus encoding shRNA against *PAPD5*, *PARN*, or luciferase (ctrl). $n = 4$. (**C**) RACE products were subjected to deep sequencing and aligned to the *TERC* gene. Canonical TERC terminus is shown in red. Genomically encoded termini are in black, mature TERC with a single A (which may or may not be genomically encoded) is hatched, and oligo(A) additions of any length (n) are in solid blue. Total trimmed reads in parentheses. $n = 2$. $^*P \le 0.05$; $^{**}P \le 0.01$. (**D**) Oligo(An) species as a proportion of total reads in control versus *PAPD5* knockdown cells. The average A-tail length in nucleotides is indicated. (**E**) Northern blot of TERC from HEK293 cells with shRNA against *PAPD5* versus luciferase, followed by transfection with plasmids expressing *PAPD5* versus *EGFP* cDNAs. Loading control, ethidium bromide staining of 18S rRNA. $n = 3$. (**F**) RACE amplicons from HEK293 cells with shRNA directed against *PARN* versus luciferase, followed by shRNA against *PAPD5* or luciferase. $n = 3$. (**G**) Northern blot of TERC from HEK293 cells with shRNA against *PARN* versus luciferase, followed by shRNA against *PAPD5* versus luciferase. $n = 3$. $^*P \le 0.05$; $^{***}P \le 0.001$; NS: not significant. (**H**) Telomerase activity (TRAP) in PARN-deficient HEK293 cells after control versus *PAPD5* knockdown, using 3-fold dilutions of input cell extract. IC, internal control amplification standard. $n = 3$.

transcript levels were decreased to approximately one-third of those found in control cells within 48 hours (Figure 2E; lanes 1 and 2). In *PAPD5*-knockdown cells, expression of *PAPD5* cDNA (which is codon optimized and insensitive to *PAPD5* shRNAs) also reduced TERC levels to less than those found in control cells (Figure 2E; lanes 3 and 4). These data confirm that PAPD5 negatively regulates TERC via oligo-adenylation of nascent RNA transcripts.

To establish the relationship between PAPD5 and PARN in posttranscriptional regulation of TERC, we disrupted PAPD5 in the setting of PARN deficiency. By 3′ RACE, *PARN* knockdown results in an accumulation of extended TERC forms (Figure 2F; lane 3 versus lane 1). We found that *PAPD5* inhibition in PARN-deficient cells restored the distribution of TERC 3′ ends to that observed in control cells (Figure 2F; compare lanes 4 and 1). By deep sequencing of 3′ RACE products, we found that the proportion of mature TERC was significantly increased after *PAPD5* knockdown in PARN-deficient cells (65% versus 32%). Moreover, the increased proportion (~60%) of oligo(A) TERC species found in PARN-deficient cells was decreased by 3-fold after inhibiting *PAPD5*, and the average length of oligo(A) tails was also reduced (Supplemental Figure 7). By Northern blot, we found that steady-state levels of TERC transcripts were increased by *PAPD5* inhibition in both normal and PARN-deficient cells (Figure 2G). Notably, TERC was restored in PARN-deficient cells by *PAPD5* knockdown to levels found in control cells (Figure 2G). To determine the effects of changes in TERC on telomerase function, we performed the telomerase repeat amplification protocol (TRAP) assay and found that *PAPD5* knockdown increased telomerase activity in PARN-deficient cells (Figure 2H). These data

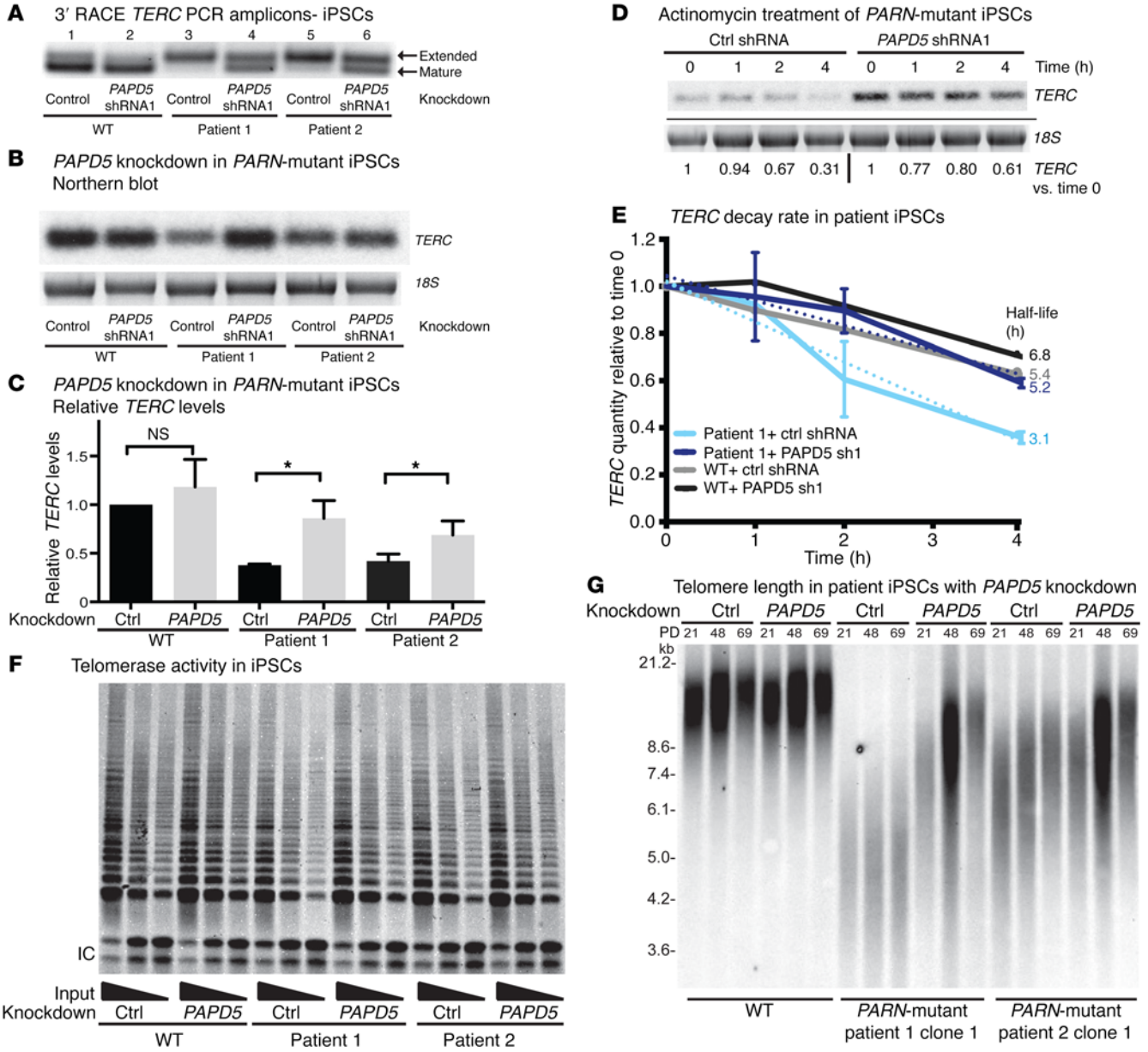

Figure 3. *PAPD5* inhibition rescues TERC maturation, telomerase activity, and telomere length in *PARN*-mutant patient cells. (**A**) 3′ RACE TERC amplicons from normal (WT) versus *PARN*-mutant iPSCs after lentiviral shRNA directed against *PAPD5* versus luciferase (ctrl). (**B**) Representative Northern blot of TERC RNA from WT versus *PARN*-mutant iPSCs after lentiviral shRNA directed against *PAPD5* versus luciferase. (**C**) Relative TERC levels from Northern blots (**B**) are quantified relative to WT cells with control knockdown $^*P \le 0.05$; NS: not significant. $n = 3$. (**D**) Representative Northern blot of TERC levels in WT and *PARN*-mutant iPSCs after lentiviral shRNA directed against *PAPD5* versus luciferase at 0–4 hours following actinomycin treatment. TERC levels are normalized relative to time 0. (**E**) Graph of TERC decay rate. Dashed line reflects slope determined by simple linear regression. (**F**) TRAP assay in iPSCs after lentiviral shRNA against *PAPD5* versus luciferase. $n = 3$. (**G**) TRF of WT and *PARN*-mutant patient iPSCs after lentiviral shRNA against *PAPD5* versus luciferase over time in culture.

indicate that PAPD5 and PARN mediate opposing and nonredundant effects in the posttranscriptional maturation of nascent TERC transcripts. Despite the presence of other poly(A) polymerases and deadenylases in cells, the manipulation of these 2 factors substantially affects TERC levels and cellular telomerase activity.

Our results indicate that TERC is limiting in PARN-deficient cells due to inadequate deadenylation of nascent TERC transcripts and that this effect can be reversed by inhibiting the major adenylating activity, PAPD5. To determine whether inhibiting posttranscriptional adenylation by PAPD5 could reverse molecular features of telomere disease in *PARN*-mutant DC patient cells, we transduced induced pluripotent stem cells (iPSCs) from 2 DC patients harboring pathogenic *PARN* mutations (14) with lentiviral vectors encoding *PAPD5* shRNA. By 3′ RACE, *PARN*-mutant patient cells show a predominance of extended TERC forms (Figure 3A; lanes 1, 3, and 5). We found that this altered distribution of TERC species in *PARN*-mutant patient cells was normalized by *PAPD5* knockdown, as evidenced by an increase in the proportion of amplicons corresponding to the mature form of TERC (Figure 3A and Supplemental Figure 8). Accordingly, by Northern blot, we

found that *PAPD5* inhibition rescued total TERC levels in patient iPSCs (Figure 3, B and C and Supplemental Figure 8). The increased TERC steady-state levels were due to higher stability of RNA transcripts, which we demonstrated by inhibiting Pol II transcription using actinomycin D in patient iPSCs with or without *PAPD5* inhibition. When we compared decay rates by Northern blot, we found that *PAPD5* knockdown increased the half-life of TERC transcripts in *PARN*-mutant patient iPSCs compared with cells expressing control shRNA (Figure 3, D and E). Collectively, these results indicate that *PAPD5* inhibition is sufficient to restore TERC processing, stability, and steady-state levels in the setting of *PARN* mutations.

We next asked whether *PAPD5* inhibition would be sufficient to rescue telomerase activity and telomere elongation. Using TRAP assays, we found that *PAPD5* inhibition in *PARN*-mutant patient iPSCs increased cellular telomerase activity (Figure 3F). Remarkably, when we assayed telomere length by Southern blot, we found that *PAPD5* knockdown in *PARN*-mutant patient iPSCs led to a rapid increase in telomere lengths, approximating those found in control iPSCs (Figure 3G and Supplemental Figure 8). Patient iPSCs with stable *PAPD5* knockdown showed continuous self-renewal in culture. We next performed a global analysis of transcriptional changes after *PAPD5* knockdown by RNA sequencing. In patient iPSCs, we found only a small number of transcripts (11 genes) whose levels were altered to a degree that exceeded the increase in TERC; of those, only noncoding RNAs were commonly affected more than would be predicted by chance (Supplemental Tables 1–3 and Supplemental Dataset). Taken together, our data indicate that *PAPD5* inhibition restores TERC levels, telomerase activity, and telomere length in cells from patients with *PARN* mutations.

We previously showed that PARN functions in the terminal stages of 3′ deadenylation and end trimming for productive maturation of nascent TERC transcripts (14), suggesting that low TERC levels are sufficient to explain telomere disease in patients with *PARN* mutations. Consistent with this hypothesis, we show here that TERC expression can restore telomere length in PARN-deficient cell lines and *PARN*-mutant patient cells. Similar findings are seen in cells from patients with mutations in *TERC* itself or in *DKC1* (22, 24). These results support the hypothesis that TERC deficiency underlies defective telomere maintenance in patients with *PARN* mutations. Given our findings of increased oligo-adenylation and destabilization of TERC RNA species in the setting of *PARN* mutations, we focused on reversing this posttranscriptional modification as a means of normalizing TERC levels.

Our results indicate a critical role for PAPD5 in regulating human TERC biogenesis, a discovery that stems from the findings of *PARN* mutations in patients with telomere diseases (11, 12). We found that PAPD5 oligo-adenylates and destabilizes nascent TERC transcripts in patient cells, corroborating and extending recent findings in cell lines (15, 16). From modulating PARN and PAPD5 individually or in combination, we found that their functions in TERC maturation are largely nonredundant despite the presence of other poly(A) polymerases and deadenylases in the cell. Our data further show that *PAPD5* inhibition increases TERC to levels sufficient to augment telomerase activity and telomere length in *PARN*-mutant patient cells, but without altering the majority of other RNAs across the transcriptome to a similar degree and without compromising cellular viability. We propose a model where the balance of PARN and *PAPD5* activities serves to establish TERC steady-state levels, which in turn plays a major role in determining telomerase activity and telomere maintenance in TERT-expressing cells, such as stem cells and cancer cells (Supplemental Figure 9).

Our results provide an example of how enzymes that regulate noncoding RNA processing might be targeted to reverse disease phenotypes. Although noncoding RNAs play a variety of roles in diseases, prospective therapies generally depend on delivery of the RNA or antisense strategies (25). We and others have shown that ectopically expressing TERC is sufficient to extend telomere length in cells from patients with various forms of DC (22, 24, 26), but that approach poses major challenges for clinical translation. The results presented here identify PAPD5 as a component of the posttranscriptional machinery potentially amenable to pharmacological inhibition, to restore telomere maintenance in patients with DC and IPF caused by *PARN* mutations. More broadly, our results suggest an unanticipated therapeutic window for PARN and PAPD5 manipulation that might be exploited to alter telomerase activity and self-renewal in a range of degenerative and malignant diseases (Supplemental Figure 9).

Methods

Methods are detailed in Supplemental Methods and Supplemental Tables 4 and 5. RNA sequencing (RNA-seq) data were deposited in the NCBI's Gene Expression Omnibus (GEO GSE81507).

Statistics. The data as presented as mean with SD. Two-tailed Student's *t* test was performed to generate *P* values, and values less than or equal to 0.05 were considered significant.

Study approval. The study was approved by the Boston Children's Hospital Institutional Review Board.

Author contributions

BB and SA conceived the study, analyzed data, prepared figures, and wrote the manuscript. BB, MS, DHM, MZM, and AA executed experiments, analyzed data, and prepared figures. AKT analyzed 3′ RACE deep sequencing data. PC analyzed RNA-seq data.

Acknowledgments

We thank the patients and families for participation in research. The work was funded by NIH R01 DK107716-01 and Pedals for Pediatrics, Dana-Farber Cancer Institute (to SA); T32 HL007574 (to DHM); and the Scientific and Technological Council of Turkey (to BB).

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