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Brief Report



HEMATOPOIESIS AND STEM CELLS

# Posttranscriptional modulation of TERC by PAPD5 inhibition rescues hematopoietic development in dyskeratosis congenita

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KEY POINTS

- ***PAPD5* inhibition stabilizes *TERC*, rescues telomerase, and lengthens telomeres in X-linked DC hESCs.**
- **Modulation of *PAPD5* improves definitive hematopoietic development from hESCs with a pathological mutation in dyskerin.**

**Reduced levels of *TERC*, the telomerase RNA component, cause dyskeratosis congenita (DC) in patients harboring mutations in TERC, PARN, NOP10, NHP2, NAF1, or DKC1. Inhibition of the noncanonical poly(A) polymerase *PAPD5*, or the exosome RNA degradation complex, partially restores *TERC* levels in immortalized DKC1 mutant cells, but it remains unknown if modulation of posttranscriptional processing of *TERC* could improve hematopoietic output in DC. We used human embryonic stem cells (hESCs) with a common dyskerin mutation (DKC1_A353V), which have defective telomere maintenance and reduced definitive hematopoietic potential, to understand the effects of reducing EXOSC3 activity, or silencing PAPD5-mediated oligoadenylation, on hematopoietic progenitor specification and function in DC. Reduction of *EXOSC3* or *PAPD5* levels in DKC1 mutant hESCs led to functional improvements in *TERC* levels and telomerase activity, with concomitant telomere elongation and reduced levels of DNA damage signaling. Interestingly, the silencing of *PAPD5*, but not *EXOSC3*, significantly restored definitive hematopoietic potential in DKC1 mutant cells. Mechanistically, we show that *PAPD5* inhibition is sustained in differentiated CD34$^+$ cells, with a concomitant increase in mature, functional, forms of *TERC*, indicating that regulation of *PAPD5* is a potential strategy to reverse hematologic dysfunction in DC patients. (*Blood*. 2019;133(12):1308-1312)**

## Introduction

Patients with dyskeratosis congenita (DC) harbor mutations in telomere maintenance genes[1,2] and suffer morbidity from bone marrow failure.[3] Several of the mutations found in DC cause reduced *TERC* levels, resulting in telomerase impairment.[4-9] Although overexpression of *TERC* increases hematopoietic output from DC cells,[10] it is not a viable approach for patients. The discovery that *TERC* degradation by the exosome complex can be controlled by its oligoadenylation status, through modulation of *PAPD5* (noncanonical poly(A) polymerase 5), opened a new avenue of opportunity for clinical intervention in DC.[11-14] However, it remains unknown if the reduction of *TERC* decay by modulation of *PAPD5* or the exosome[11-15] could restore hematopoietic potential in DC, a crucial end point in this disease.

We used human embryonic stem cells (hESCs) to assess the effect of silencing *PAPD5* or the Exosome Component gene 3 (*EXOSC3*) on primitive and definitive hematopoietic potential of DC. We used hESCs harboring a common DKC1_A353V mutation, which recapitulates key aspects of the hematopoietic defects of DC.[10] We show that silencing of *PAPD5* or *EXOSC3* increases telomerase activity, elongates telomeres, and reduces γH2AX in DKC1_A353V hESCs. However, only the silencing of *PAPD5* and not *EXOSC3* restored definitive hematopoietic potential in DKC1 mutants. Our data give strong support for the development of therapeutics targeting the posttranscriptional regulation of *TERC* by *PAPD5* in patients with mutations that impair *TERC* stability.

## Study design

H1 (WA01) hESCs were maintained as described.[10] DKC1_A353V, WT_shEXOSC3, WT_shPAPD5, DKC1_A353V_shEXOSC3, and DKC1_A353V_shPAPD5 hESCs were engineered using CRISPR/cas9 or zinc-fingers genome editing. In vitro hematopoiesis was performed as described.[16,17]

## Results and discussion

We and others have established that the hematopoietic differentiation of DKC1_A353V hESCs recapitulates major phenotypes of DC.[10,18] To determine if *TERC* levels could be posttranscriptionally

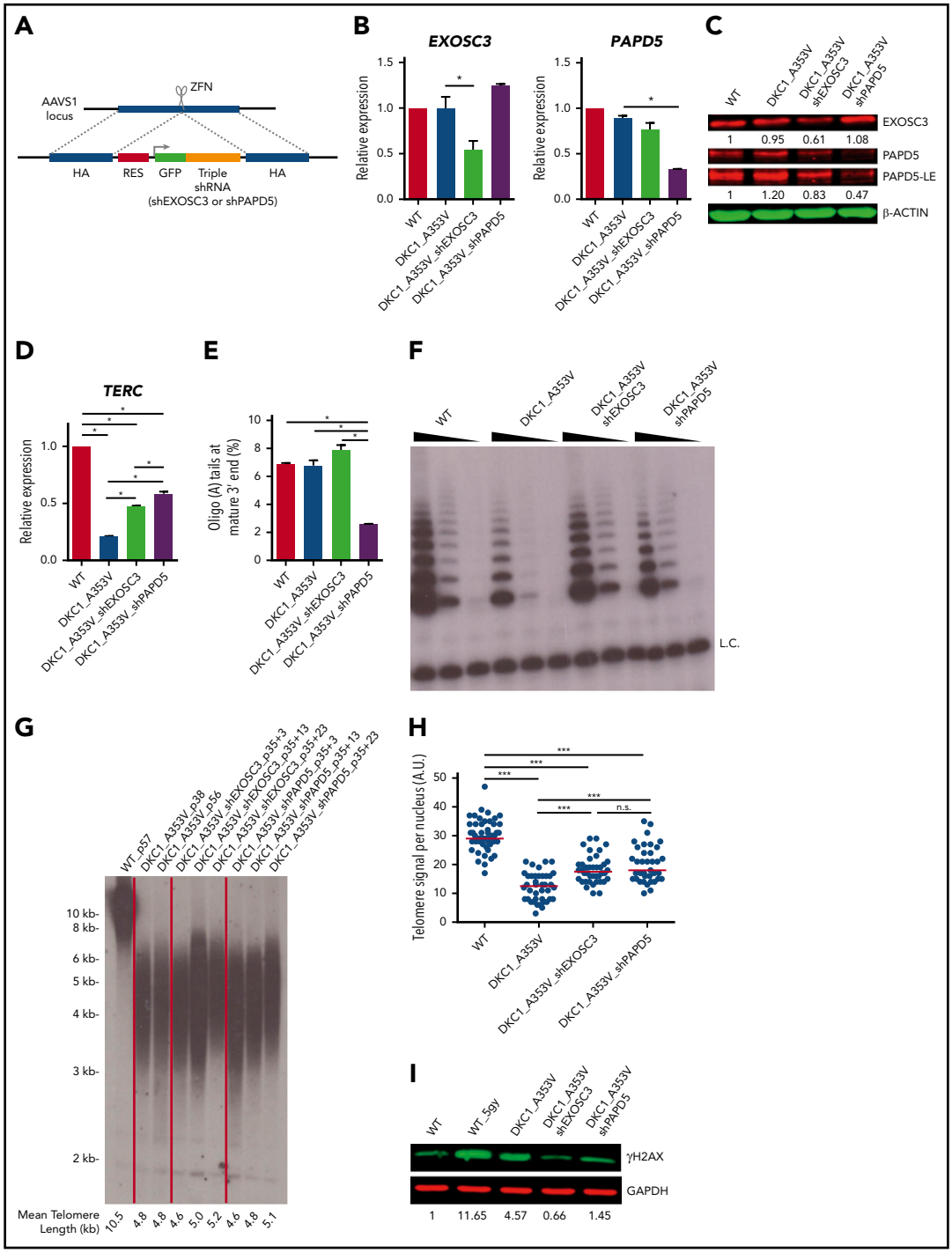


**Figure 1. Modulation of *EXOSC3* and *PAPD5* rescue telomere integrity in DKC1_A353V hESCs.** (A) Schematic depicting shRNA cassette insertion into the AAVS1 locus of hESCs. shRNA sequences used for each cassette are described in supplemental Methods and supplemental Table 1. HA, homology arm; RES, resistance cassette. (B) Quantification of *EXOSC3* (left) and *PAPD5* (right) levels in WT, DKC1_A353V, DKC1_A353V_shEXOSC3, and DKC1_A353V_shPAPD5 by quantitative reverse transcription polymerase chain reaction. (C) Western blot for EXOSC3 and PAPD5 in WT, DKC1_A353V, DKC1_A353V_shEXOSC3, and DKC1_A353V_shPAPD5 hESCs. LE, long exposure. β-Actin is shown as loading control. Quantification of band intensities is shown (relative to β-actin). (D) Quantification of *TERC* in WT, DKC1_A353V, DKC1_A353V_shEXOSC3, and DKC1_A353V_shPAPD5 by quantitative reverse transcription polymerase chain reaction. (E) Relative abundance of oligoadenylated reads at mature 3′ end of TERC in WT, DKC1_A353V, DKC1_A353V_shEXOSC3, and DKC1_A353V_shPAPD5 hESC. (F) Telomerase activity by telomere repeat amplification protocol in WT, DKC1_A353V, DKC1_A353V_shEXOSC3, and DKC1_A353V_shPAPD5 hESCs. Range of concentrations represents fourfold serial dilutions. L.C., loading control. (G) Telomere length analysis by telomere restriction fragment (TRF) of WT, DKC1_A353V, DKC1_A353V_shEXOSC3, and DKC1_A353V_shPAPD5 hESCs at different passages. Passage numbers are described for each lane. For shEXOSC3 and shPAPD5 transfected cells, passage numbers reflect passage at transfection (35), plus number of passages since transduction. Quantification of mean telomere length is shown. (H) Quantification of interphase quantitative fluorescence in situ hybridization analysis, cells at same passage number as panel G. At least 40 nuclei were analyzed in each cell line. (I) Representative immunoblot analysis of γH2AX in WT, DKC1_A353V (passage 57), DKC1_A353V_shEXOSC3 (passage 35+28), and DKC1_A353V_shPAPD5 (passage 35+28) hESCs. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as a loading control. Numbers indicate band intensity relative to GAPDH. All experiments were conducted using n = 3, mean ± standard error of the mean, $*P \leq .05$, unless otherwise indicated. Statistical analysis was performed using 1-way analysis of variance followed by Tukey's post hoc test or by Bonferroni posttest in panel H. In panel H, $***P \leq .0001$. n.s., not significant; ZFN, zinc finger nucleases.

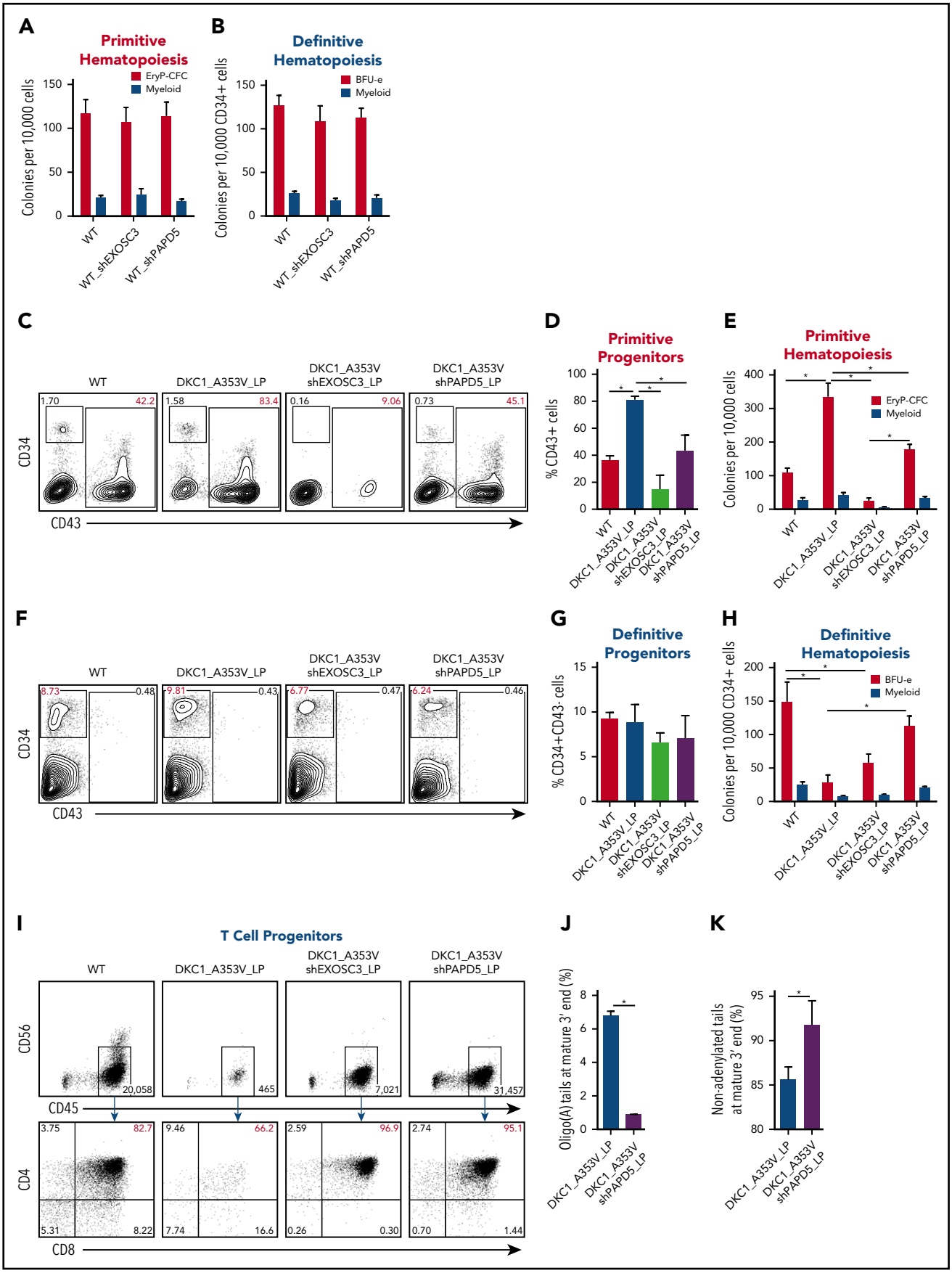


**Figure 2.**

regulated in hESCs with clinically relevant mutations in DKC1, we treated genetically engineered DKC1_A353 hESCs[10] with small interfering RNAs against *EXOSC3* or *PAPD5*. Transient silencing of *EXOSC3* or *PAPD5* increased *TERC* levels (supplemental Figure 1A-B, available on the *Blood* Web site), prompting us to constitutively silence these genes. We targeted the AAVS1 safe-harbor locus[19] of both wild-type (WT) and DKC1_A353V hESCs with short hairpin RNAs (shRNAs) against PAPD5 and EXOSC3 (Figure 1A), creating WT_shPAPD5, WT_shEXOSC3, DKC1_A353V_shPAPD5, and DKC1_A353V_shEXOSC3 hESCs. These cells showed significantly reduced levels of *EXOSC3* and *PAPD5* messenger RNAs (Figure 1B; supplemental Figure 1C) and protein (Figure 1C).

*TERC* levels were significantly increased by constitutive silencing of *PAPD5* or *EXOSC3* in DKC1_A353V but not in WT hESCs (Figure 1D; supplemental Figure 1D). Targeted RNA sequencing at the 3′ end of *TERC* showed that DKC1_A353V_shPAPD5 cells have a significant reduction in the percentage of oligo(A) species at the mature (Figure 1E) and extended (supplemental Figure 2A) forms of *TERC*, when compared with WT, DKC1_A353V, and DKC1_A353V_shEXOSC3 hESCs. This demonstrates silencing of *PAPD5* and *EXOSC3* in hESCs rescues *TERC* levels by reducing its 3′ adenylation- and exosome-mediated degradation. The DKC1_A353V mutation by itself did not cause a change in the mature *TERC* composition in terms of oligo(A) reads (supplemental Figure 2B), supporting a model where any unassembled *TERC* is rapidly degraded.[14]

Modulation of 3′ oligoadenylation by *PAPD5*, as well as the inhibition of *EXOSC3*, also increased telomerase activity (Figure 1F; supplemental Figure 3A) and telomere length (Figure 1G-H; supplemental Figure 3B) in DKC1_A353V_shPAPD5 and DKC1_A353V_shEXOSC3 hESCs. Cells with silenced *PAPD5* or *EXOSC3* show reduced γH2AX (Figure 1I), indicating lower levels of DNA damage signaling, a common phenotype of DC.[20] Thus, post-transcriptional modulation of *TERC* restores major defects of DKC1_A353V mutants.

We next examined if modulation of *PAPD5* and *EXOSC3* could restore the hematopoietic output of DKC1_A353V cells. Impaired definitive hematopoietic potential in DKC1_A353V cells can be rescued by overexpression of *TERC*.[10] We hypothesized that the silencing of *PAPD5* or *EXOSC3* could also rescue definitive hematopoiesis in DKC_A353V hESCs. We performed serum-free differentiations to independently derive primitive and definitive hematopoietic progenitors by stage-specific modulation of WNT (supplemental Figure 4A).[16,17] Silencing of *PAPD5* and *EXOSC3* does not affect early stages of primitive or definitive hematopoietic development in WT cells (supplemental Figure 4B-I). Likewise, colony-forming potential of both hematopoietic programs is normal (Figure 2A-B), indicating that a reduction in exosome-mediated RNA degradation is not detrimental to hematopoiesis in WT settings.

We next examined the consequences of *EXOSC3* and *PAPD5* silencing specifically during the primitive hematopoietic specification of DKC1_A353V hESCs. Analysis of mesoderm ($KDR^+$ $CD235a^+$) on day 3 of differentiation showed that all hESC lines behaved similarly at this stage (supplemental Figure 5A-B). However, confirming our previous data,[10] at day 11 (Figure 2C-D; $CD43^+$ cells), as well as at the terminal primitive myeloid and erythroid colony potential assessment (Figure 2E), DKC1_A353V cells displayed increased differentiation capacity relative to WT and DKC1_A353V_shPAPD5 cells. This increased primitive hematopoietic potential of DKC1_A353V hESCs, which we hypothesize is a reflection of stress erythropoiesis,[21] is also reduced when *TERC* is overexpressed,[10] indicating that modulation of *PAPD5* mimics the functional consequences of *TERC* overexpression during primitive differentiation of DKC1 mutants. On the other hand, unlike WT cells (Figure 2A-B), silencing of *EXOSC3* is detrimental during primitive hematopoiesis of DKC1_A353V hESCs, because these fail to specify into primitive $CD43^+$ progenitors (Figure 2C-D), leading to minimal erythroid and myeloid potential (Figure 2E). We hypothesize the toxicity observed in DKC1_A353V_shEXOSC3 cells is related to the essential role of the exosome in processing and destruction of different RNA classes,[22] which could deter its clinical use in DC.

As bone marrow failure in DC is caused by defective definitive hematopoietic specification, we analyzed the consequences of *PAPD5* and *EXOSC3* silencing in DKC1_A353V cells during that developmental program. Although day 3 mesoderm (supplemental Figure 5C-D; $KDR^+CD235^{--}$ cells) and day 8 $CD34^+$ $CD43^-$ cells (Figure 2F-G) were similar in all samples, definitive colony potential analysis showed compromised colony-forming potential in DKC1_A353V cells (Figure 2H). However, silencing of *PAPD5* (but not *EXOSC3*) significantly increased the hematopoietic potential in DKC1_A353V_shPAPD5 cells, to levels similar to WT (Figure 2H). Globin expression patterns confirm these populations were derived from definitive, and not primitive, hematopoiesis (supplemental Figure 5E). In addition, although DKC1_A353V cells had a compromised ability to give rise to $CD4^+CD8^+$ T-cell progenitors, DKC1_A353V_shPAPD5 cells displayed a clear increase in $CD4^+CD8^+$ cellularity (Figure 2I). These observations provide compelling evidence that silencing *PAPD5* increases definitive, multilineage, hematopoietic potential in DKC1_A353V mutants. Finally, consistent with *PAPD5* rescuing differentiation by affecting the oligoadenylation of

**Figure 2. *PAPD5* silencing restores defective hematopoiesis in DKC1_A353V cells.** (A) Colony-forming cell (CFC) potential of primitive hematopoietic progenitors in WT, WT_shEXOSC3, and WT_shPAPD5 cells from day 11 of IWP2-derived specification. (B) CFC potential of definitive hematopoietic progenitors in WT, WT_shEXOSC3, and WT_shPAPD5 cells from day 8 sorted $CD34^+CD43^-$ populations, as described in supplemental Figure 3A. (C) Representative flow cytometric analysis of CD34 and CD43 expression on day 11 of differentiation, following IWP2 treatment in WT, DKC1_A353V, DKC1_A353V_shEXOSC3, and DKC1_A353V_shPAPD5 cells. (D) Quantification of $CD43^+$ population obtained from day 11 differentiation cultures treated with IWP2, as in panel C. (E) Primitive CFC potential in day 11 differentiation cultures, as in panel C. (F) Representative flow cytometric analysis of CD34 and CD43 expression on day 8 of definitive differentiation, following CHIR99021 and SB-431542 treatment in WT, DKC1_A353V, DKC1_A353V_shEXOSC3, and DKC1_A353V_shPAPD5 cells. (G) Quantification of $CD34^+CD43^-$ population obtained from day 8 differentiation cultures treated with CHIR99021 and SB-431542, as in panel F. (H) CFC potential of definitive hematopoietic progenitors, generated as shown in supplemental Figure 3A. (I) T-cell potential of $CD34^+CD43^-$ populations derived from WT, DKC1_A353V, DKC1_A353V_shEXOSC3, and DKC1_A353V_shPAPD5, obtained following CHIR99021 and SB-431542 treatment. (J) Relative abundance of oligoadenylated reads at mature 3′ end of *TERC* in WT, DKC1_A353V, DKC1_A353V_shEXOSC3, and DKC1_A353V_shPAPD5 hESC. n = 2. (K) Relative abundance of nonadenylated reads at mature 3′ end of *TERC* as in panel J. All experiments were conducted using n = 3, mean ± standard error of the mean, $*P \leq .05$, unless otherwise indicated. Statistical analysis was performed using 1-way analysis of variance followed by Tukey's post hoc test or Student *t* test (J-K). In red, population of interest. In all panels, LP denotes late passage (passage number >55).

*TERC*, 3′-end sequencing from day 8 definitive $CD34^+CD43^-$ populations shows that *PAPD5* silencing leads to a reduction in oligo(A) species in mature *TERC* (Figure 2J; supplemental Figure 6), with a concomitant increase in the total number of nonadenylated *TERC* reads (Figure 2K) in $CD34^+$ cells.

Our data provide molecular and functional evidence that modulation of *PAPD5* restores in vitro hematopoiesis in DKC1_A353V mutants, through direct regulation of the 3′-end maturation of *TERC*. Likely, a similar strategy could be employed to rescue hematopoiesis in cells with different mutations in DKC1, or harboring mutations in other genes that also lead to reduced levels of mature *TERC*, a hypothesis that should be further tested experimentally. In addition, although our data have not indicated any toxicity associated with the silencing of *PAPD5* during hematopoiesis in WT or DKC1 mutants, future studies aiming at the identification of potential targets of *PAPD5* in the hematopoietic system, as well as their implication for blood development, should be performed. As current therapeutic alternatives for bone marrow failure in DC remain largely ineffective, the posttranscriptional regulation of *TERC* by *PAPD5* might represent a novel avenue for the management of this disease.

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## Authorship

Contribution: W.C.F., S.S., A.T.V., R.P., C.M.S., and L.F.Z.B. designed the experiments and analyzed the data; W.C.F., S.S., A.T.V., and K.A.B. performed the experiments; and W.C.F., S.S., R.P., C.M.S., and L.F.Z.B. wrote the manuscript.

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## Footnotes

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