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14. ABSTRACT

The experiments performed for our DOD BMFRP grant are in line with the original proposal and have significantly increased our knowledge on telomere dysfunction induced BMF. Likewise, we have significantly improved our ability to generate *in vitro* hematopoietic lineages that can be targeted for future replacement therapies for BMF patients. In brief:

Aim 1) We have successfully demonstrated that we can significantly increase blood output in DC settings by altering the endogenous processing of *TERC* in these cells. Silencing of PAPD5 during the hematopoietic differentiation of DKC1 mutant cells increased TERC levels, altered TERC 3' processing, increased telomerase activity, elongated telomeres and improved definitive hematopoietic specification.

Aim 2) We have utilized state-of-the-art single cell RNA sequencing technologies to significantly improve our ability to obtain, from human pluripotent stem cells, an entirely new population of definitive hematopoietic progenitors which recapitulate the signal requirements for hematopoietic stem cell specification during embryogenesis. Our analysis identified unappreciated signal pathways relevant to the specification of CDX4+ hemogenic mesoderm, such as IL2/STAT5, TNF, PI3K, ERK, and NOTCH signaling.

15. SUBJECT TERMS

- Telomerase; telomeres; definitive hematopoiesis; bone marrow failure; dyskerin, RNA decay, miRNA, human pluripotent stem cells, development, signal pathways

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1. INTRODUCTION

Telomeres, the physical ends of eukaryotic chromosomes, are progressively shortened upon each cellular division. Telomere shortening can be prevented by telomerase, a reverse transcriptase that synthesizes telomeres from an RNA template, *TERC*. As hematopoietic stem cells and their downstream progenitors are required to generate functional blood cells throughout life, telomerase activity is essential for blood cell homeostasis. The consequences of impaired telomerase in the circulatory system are well exemplified in patients suffering with aplastic anemia (AA) and dyskeratosis congenita (DC), two bone marrow failure syndromes (BMFs) that are associated with mutations in different telomerase components. The goal of this proposal is to use the *in vitro* hematopoietic differentiation of genetically engineered human pluripotent stem cells (hPSCs), harboring clinically relevant mutations in telomerase, as a tractable system to decipher the molecular pathways able to revert BMF in patients (Aim 1), and as a source of immunologically matched HSCs for transplantation therapy (Aim 2). The experiments proposed here will significantly improve hematopoietic output in cells with mutations in telomerase, generating novel alternatives that can be pursued for the clinical management of DC and AA patients.

2. KEYWORDS

Telomerase; telomeres; hematopoiesis; bone marrow failure; dyskerin, RNA decay

3. OVERALL PROJECT SUMMARY

The goal of this project was to use the targeted differentiation of genome edited human embryonic stem cells as a platform for the development of novel therapies aimed at restoring the function and viability of bone marrow cells, in settings of mutant telomerase. A combination of technologies was used to successfully achieve this goal, including genetic engineering, stem cell manipulation and targeted hematopoietic differentiation. Patients with AA and DC have very short telomeres and carry mutations in genes related to telomere homeostasis. Telomerase is the multi-enzymatic complex responsible for telomere synthesis in mammalian cells. In the absence of telomerase, telomeres progressively shorten, which has been linked to impaired stem cell function in mice and humans. Thus, the tissue defects observed in bone marrow failure patients likely result from the loss of self-renewal in their adult stem cells compartments, caused by accelerated telomere shortening in the presence of mutant telomerase. With the support from the BMFRP from the DOD, in this current project we have 1) identified a novel molecular pathway able to restore haemopoietic output in cells harboring telomerase mutations found in BMF patients (our Aim 1) and, through single cell RNA sequencing, identified several novel pathways that will improve our ability to generate functional hematopoietic cell lines *in vitro* (our Aim 2).

4. ACCOMPLISHMENTS

a. Major goals of project (as stated in the approved SOW)

1) Modulation of miRNA-34a and miRNA-145 levels during hematopoietic differentiation of telomerase mutant cells (part of Specific Aim 1).
   Percentage of completion: 50%

2) Inhibition of *TERC* degradation during hematopoietic differentiation of DKC1_A353V cells (part of Specific Aim 1).
   Percentage of completion: 100%

3) To understand the signal requirements for HSC specification from hPSCs (part of Specific Aim 2).
   Percentage of completion: 95%

4) To identify an hPSC-derived HSC from DC hPSCs (part of Specific Aim 2).
   Current percentage of completion: 0%

Overall, the project developed according to the described original plan and we already have 1 published manuscript directly related to this proposal (attached). Subtask 1 (Aim 1A) was performed slower than expected, to allow for full completion and publication of Subtask 2 (Aim 1B). Subtask 3 has also been completed (see sub-section 3.4 for a detailed description of activities). Studies performed on Subtask 3 yielded new insights into the molecular regulation of definitive hematopoietic specification, and our single cell studies revealed that these definitive hematopoietic progenitors represent a rare subset of total differentiation cultures. As such, we have focused our attention on understanding the genetic regulation of this rare subset, and identifying the critical signal pathways that lead to its specification, which will greatly improve our future ability to perform Subtask 4 in the near future.

b. Major Activities

During the period when our DOD grant was active we worked on all the original goals proposed in our application. Throughout these 2 years we had 4 trainees whose projects were directly related to this grant. Wilson Fok and Alexandre Vessoni, postdoctoral fellows in the Batista Lab, actively worked on the experiments proposed on Specific Aim 1 of this grant, while John Creamer (PhD candidate) and Stephanie Luff (postdoctoral fellow) from the Sturgeon Lab conducted the experiments described on Specific Aim 2.

c. Specific Objectives

The main objectives of our proposal included depicting in detail the molecular consequences of p53 pathway stabilization (in particular the role of miRNA-34a and miRNA-145 activation) in bone marrow failure in DC settings (Aim 1A of the proposal), mediating *TERC* decay during hematopoiesis of DKC1_A353V mutant cells (Aim 1B of the proposal) and finally, increasing the efficiency of definitive hematopoietic specification from hPSCs, through a detailed study of the critical regulators of hemogenic endothelium specification. These insights build on our previously published work, describing the WNT-mediated expression of *CDX4* within nascent mesoderm, as essential for efficient definitive hematopoietic specification from hPSCs (Creamer, Dege et al. 2017). With this insight, we have identified the first differentially expressed gene family, the para-Hox CDX group, as differentiating between definitive and primitive hematopoietic progenitors. As such, we have extensively characterized the expression of *CDX4* within hPSC differentiation cultures, established candidate signal pathway regulators of its expression through bioinformatics predictions, and developed genetic tools to better understand the role of CDX4 in definitive hematopoietic development. As will be described in detail in the "Significant Results" section, we have made significant progress on all of our objectives, with Aim 1B recently being published in *Blood*. In addition, we have created many cellular and molecular tools that will allow us to continue to investigate the consequences of telomerase abnormalities in the development of BMF in DC and AA patients.

d. Significant Results

Specific Aim 1: The major objectives of this Aim were to understand the role that p53, miRNA-34a and miRNA-145 play in the development of hematopoietic failure in patients with dysfunctional telomerase (Aim 1A), as well as the modulation of RNA decay as a strategy to rescue telomerase activity and hematopoietic differentiation in patients DC and AA harboring mutations in DKC1 (Aim 1B).

To start answering the questions proposed in Aim 1A, during the first reporting period we successfully created a novel hPSC model, where we can turn the expression of *TERT on* or *off* at any given point during blood development, through a doxycycline-inducible system (Figure 1A). These cells were engineered using zinc finger nucleases (ZFN), by introducing the two vectors that comprise the TET-ON system (CAG_rtTA and TRE-TERT) into the adeno-associated virus site 1 (AAVS1) "safe harbor" locus. These cells work as expected, and we can easily control telomerase activity and telomere length by addition of DOX to the culture media (see previous report). Using this system (as well as our previously generated DKC1 mutant hESCs) we were able to show p53 is significantly up-regulated with progressive telomere shortening in pluripotent stem cells, and that its activation significantly compromises blood differentiation over time. We focused on modulating specific targets of p53 to try to rescue this phenotype. Over the initial reporting period we started development of new cellular systems, where telomeres are progressively shorter, but these miRNA-34a and miRNA-145 are not activated. This system will allow us to understand the contribution of these non-coding RNAs to the hematopoietic failure observed in

AA and DC patients harboring mutations in telomerase. We have successfully tested and cloned specific antagomir sequences against both miRNA-34a and miRNA-145 that efficiently reduce their activation after DNA damage (data not shown). Our goal is to perform *in vitro* hematopoietic differentiation experiments in cells that retain short telomeres, efficient p53 pathway stabilization, but have an impaired miRNA-34a and miRNA-145 response. These experiments will allow us to directly interrogate the role of these miRNAs in hematopoietic failure in cells harboring mutations commonly found in DC and AA. Construction of these cells took longer than anticipated and combined with a more directed focus on Aim 1B (see next paragraph), we were not able to finish this Aim during the anticipated time frame. We remain however, committed to this question and will continue to pursue this Aim until we have definitely established if targeting of miRNA-34a and miRNA-145 can be a viable route to ameliorate bone marrow failure in DC and AA patients.

In Aim 1B our objective was to understand if the modulation of *TERC* degradation can improve the hematopoietic output of cells harboring clinically relevant mutations in *DKC1*. As reduced levels of TERC also cause DC and AA in patients harboring mutations in TERC, PARN, NOP10, NHP2, NAF1 (in addition to DKC1), these experiments are of broad interest to the field. Recent data has indicated that inhibition of the non-canonical 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. In this Aim, we used our DKC1_A353V hESCs (which have reduced TERC levels, defective telomere maintenance and reduced definitive hematopoietic potential), to understand the effects of reducing EXOSC3 activity, or silencing PAPD5-mediated *TERC* oligoadenylation, on hematopoietic progenitor specification and function in DC. Initially, we treated genetically engineered DKC1_A353 hESCs with siRNAs against *EXOSC3* or *PAPD5*. Transient silencing of *EXOSC3* or *PAPD5* increased *TERC* levels (data not shown), prompting us to constitutively silence these genes. We targeted the AAVS1 safe-harbor locus of both WT and DKC1_A353V hESCs with shRNA hairpins 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* mRNAs (Figure 1B) and protein (Figure 1C). Moreover, *TERC* levels were significantly increased by constitutive silencing of *PAPD5* or *EXOSC3* in DKC1_A353V but not in WT hESCs (Figure 2A). 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 2B) and extended (data not shown) forms of *TERC*, when compared to 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. Modulation of 3' oligoadenylation by *PAPD5*, as well as the inhibition of *EXOSC3*, also increased telomerase activity (Figure 3A) and telomere length, (Figure 3B-C) in DKC1_A353V_shPAPD5 and DKC1_A353V_shEXOSC3 hESCs. Cells with silenced *PAPD5* or *EXOSC3* show reduced γH2AX (Figure 3D), indicating lower levels of DNA damage signaling, a common phenotype of DC. Thus, posttranscriptional 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. We initially 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 (data not shown). However, at Day 11 (Figure 4A-B; $CD43^+$ cells), as well as at the terminal primitive myeloid and erythroid colony potential assessment (Figure 4C), 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, is also reduced when *TERC* is overexpressed (data not shown), indicating that modulation of *PAPD5* mimics the functional consequences of *TERC* overexpression during primitive differentiation of DKC1 mutants. On the other hand, silencing of *EXOSC3* is detrimental during primitive hematopoiesis of DKC1_A353V hESCs, as these fail to specify into primitive $CD43^+$ progenitors (Figure 4A-B), leading to minimal erythroid and myeloid potential (Figure 4C). 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, 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. While Day 3 mesoderm (data not shown) and Day 8 $CD34^+CD43^-$ cells (Figure 5A-B) were similar in all samples, definitive colony potential analysis showed compromised colony-forming potential in DKC1_A353V cells (Figure 5C). However, silencing of *PAPD5* (but not *EXOSC3*) significantly increased the hematopoietic potential in DKC1_A353V_shPAPD5 cells, to levels similar to WT (Figure 5C). Additionally, while 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 6). 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 *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 7), with a concomitant increase in the total number of non-adenylated *TERC* reads (Figure 2K) in $CD34^+$ cells.

Specific Aim 2: In Aim 2, our goal was to identify additional signal pathways that are required for definitive hematopoietic specification from hPSCs. While we are currently able to specify definitive hematopoietic progenitors through stage-specific WNT signaling (Sturgeon, Ditadi et al. 2014), the overall efficiency of this method is low, yielding inconsistent and low-level engraftment in a murine xenograft model (not shown). As such, improving our understanding of definitive hematopoietic specification from hPSCs is a necessary "first step" in the treatment of BMF patients with hPSC-derived hematopoietic progenitors. This Aim was divided into two parts: 1) understanding the specific requirements for specification of definitive hematopoiesis (Aim 2A), and 2) to efficiently generate gene-corrected DC HSCs in vitro (Aim 2B). As our studies in Aim 2A have been yielding new molecular insights into definitive hematopoietic development, and are still ongoing, we have no murine xenograft data to report.

We recently identified that WNT-mediated expression of CDX4 in nascent mesoderm is required for definitive hematopoietic development from hPSCs (Creamer, Dege et al. 2017). CDX4 is expressed during a very narrow window of time, during WNT signal manipulation (Figure 8A), and its expression precedes the specification of any hemato-endothelial progenitors. Therefore, for the remainder of this project we focused our studies on this stage-specific role for CDX4 in definitive hematopoietic specification, with the goal to better understand how to increase CDX4 expression, so as to improve overall definitive hematopoietic output from hPSCs.

We performed scRNA-seq on our WNT-manipulated hPSC differentiation cultures, using the 10X Genomics platform. >6,000 cells from each culture were processed, and analysis using the Seurat platform revealed that these cultures were highly heterogeneous, with 14 transcriptionally distinct sub-populations detected (Figure 8B). Critically, and surprisingly, CDX4 was only expressed within a small subset of these cultures, despite efficient WNT signal manipulation by CHIR99021 treatment (Figure 8A,C). Across the 14 clusters, only cluster #4 exhibited consistent CDX and HOXA gene expression (Figure 8D), both of which are necessary for definitive hematopoietic specification (Ng, Azzola et al. 2016). Collectively, these observations confirmed our original hypothesis, as it provided us with strong evidence that hPSC-derived definitive hematopoietic specification is an inefficient process, with only a small subpopulation of cells expressing the genes required for definitive hematopoietic development. This is a major hurdle to our goal of treating DC and BMF with hPSC-derived hematopoietic progenitors.

We next focused our attention on understanding how this population is specified, and how it matures towards definitive hemogenic endothelium. Using the recently described prediction algorithm "RNA Velocity", described in (La Manno, Soldatov et al. 2018), we observed that CDX4 transcription is in a steady-state, exhibiting no differences in the ratio of unspliced:spliced transcripts (Figure 9A). However, a possible downstream target of CDX4, HOXA3, was clearly in an active transcriptional state within this cluster, as there were significantly more

unspliced:spliced transcipts detected (Figure 9A). Clearly, this mesodermal cluster is actively upregulating the expression of genes important to definitive hemogenic endothelium (Iacovino, Chong et al. 2011). Therefore, we focused our attention on this transcriptional axis as the target for additional signal manipulation.

Using CRISPR/Cas9 technology, we engineered an hPSC line to lack CDX4 expression (CDX4-/Y), while simultaneously harboring inducible CDX4 expression from a tet-responsive locus (not shown). This CDX4 /Y;iCDX4 hPSC line (a "rescue" expression line) was then used to better understand the transcriptional target(s) of CDX4 during definitive hematopoietic specification. With this insight, we will be able to better design differentiation strategies for hPSC-derived definitive hematopoietic differentiation. At the time of this progress report preparation, we are awaiting CDX4-ChIPseq and RNASeq data on these mesodermal differentiation cultures, which will 1) delineate the direct CDX4 transcriptional targets, and 2) provide novel insight into the genetic regulation of definitive hematopoietic development. These target(s) may be relevant in cases of hematopoietic failure, such as DC.

Concurrent to these genetic studies, we next performed pseudotime lineage trajectory analyses to better understand the development of this CDX4+ mesodermal population (Trapnell, Cacchiarelli et al. 2014, Qiu, Hill et al. 2017, Qiu, Mao et al. 2017). These analyses similarly revealed that there was significant heterogeneity within each culture, ranging from undifferentiated hPSCs (SOX2), ectoderm (DLX5/PAX6), endoderm (FOXA2), and mesoderm (KDR; Figure 10A). Critically, these analyses revealed that WNT-specified mesoderm is actually comprised of 2 distinct populations, and that these differ in their relative expression levels of CDX4 (Figure 10B). Furthermore, consistent with our previous studies (Sturgeon, Ditadi et al. 2014), this analysis revealed there was no expression of any hematopoietic, endothelial or HE markers (not shown), indicating that each population is nascent mesoderm, and is not yet committed to a hemato-endothelial fate.

We next sought to better understand, via these analytical tools provided by scRNAseq technology, to refine our candidate set of signal pathways to enhance definitive hematopoietic specification. Analysis of the branchpoint between these populations revealed that these cells were originally mesendoderm-like, expressing primitive streak markers such as T, MIXL1, and FOXA2 (not shown). BEAM analysis (Trapnell, Cacchiarelli et al. 2014, Qiu, Hill et al. 2017, Qiu, Mao et al. 2017) on the bifurcation between these two mesodermal populations was used to identify signal pathways regulating this lineage specification event. This led to the identification of 279 receptor/ligand pairs, 182 of which corresponded with 10 signal pathways (Figure 10C). Several of these pathways were consistent with candidates identified in our original RNAseq studies (not shown), such as HH, AhR, and mTOR. However, these analyses provided new, unappreciated signal pathways that may be relevant to the specification of CDX4+ hemogenic mesoderm, such as IL2/STAT5, TNF, PI3K, ERK, and NOTCH signaling. NOTCH was particularly intriguing, as current models predict that NOTCH is only required at later stages of hematopoietic development, within hemogenic endothelium (Ditadi, Sturgeon et al. 2015).

We assessed increases in definitive hematopoietic specification purely from a functional perspective – ie., did additional signal pathway manipulation lead to increases in overall definitive hematopoietic output from hPSCs, similar to our original observation, that simultaneous treatment with CHIR99021 and SHH yields an increase in overall hematopoietic output (Figure 10D). We systematically combined candidate signal pathway agonists or antagonists at the time of WNT signal manipulation, and then assessed overall definitive hematopoietic potential, using definitive BFU-E as a surrogate, quantitative indicator. As shown in Figure 10D, simultaneous treatment of CHIR99021 with the AhR agonist FICZ did not lead to any increase in hematopoietic potential. However, treatment with the AhR antagonist CH223191 did yield an increase in overall potential. Similarly, simultaneous treatment with the TGFb inhibitor SB431542 yielded an increase in overall output. Moving forward, we will combine all identified signal pathways that yield increases in overall hematopoietic output, and assess for synergistic enhancement of definitive hematopoietic potential. This optimized combination of signal pathway manipulation will then be assessed for its ability to give rise to an HSC-like progenitor, using a murine xenograft model system.

Figures

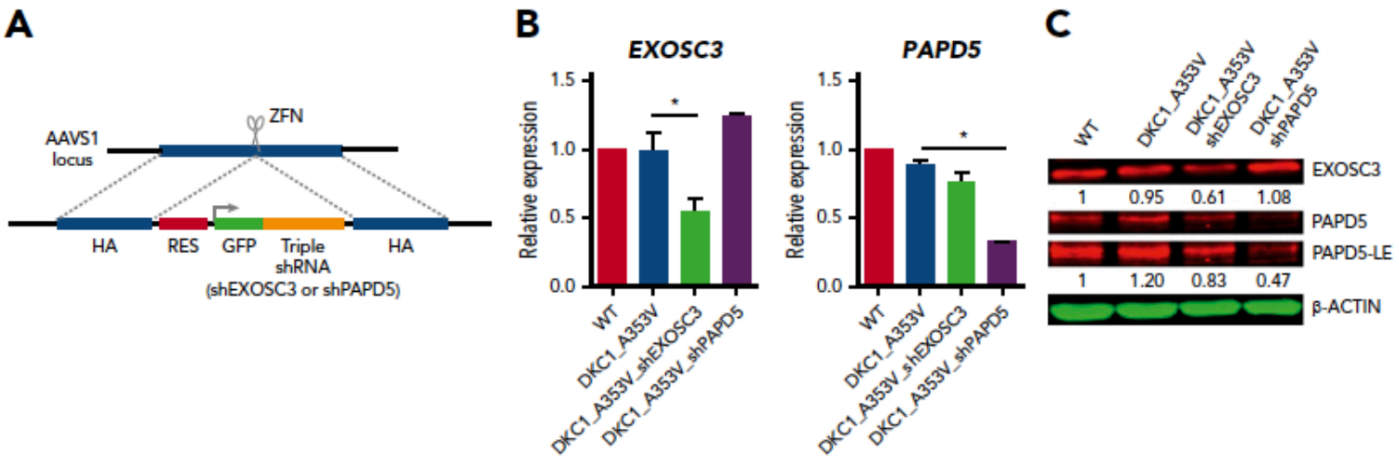

Figure 1: Modulation of *EXOSC3* and *PAPD5* rescue telomere integrity in DKC1_A353V hESCs. (A) Schematic depicting shRNA cassette insertion into the AAVS1 locus of hESCs. shRNA sequences utilized for each cassette are described in Supplemental Methods and Supplemental Table 1. HA: Homology Arm; RES: Resistance Cassette **(B)** Quantification of *EXOSC3* (left) and *PAPD5* (right) levels in WT, DKC1_A353V, DKC1_A353V_shEXOSC3, and DKC1_A353V_shPAPD5 by qRT-PCR. **(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).

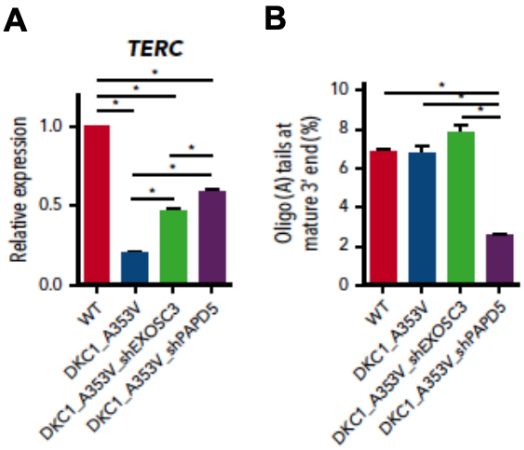

Figure 2: TERC levels and processing. A) Quantification of *TERC* in WT, DKC1_A353V, DKC1_A353V_shEXOSC3, and DKC1_A353V_shPAPD5 by qRT-PCR. **(B)** Relative abundance of oligoadenylated reads at mature 3' end of TERC in WT, DKC1_A353V, DKC1_A353V_shEXOSC3, and DKC1_A353V_shPAPD5 hESC.

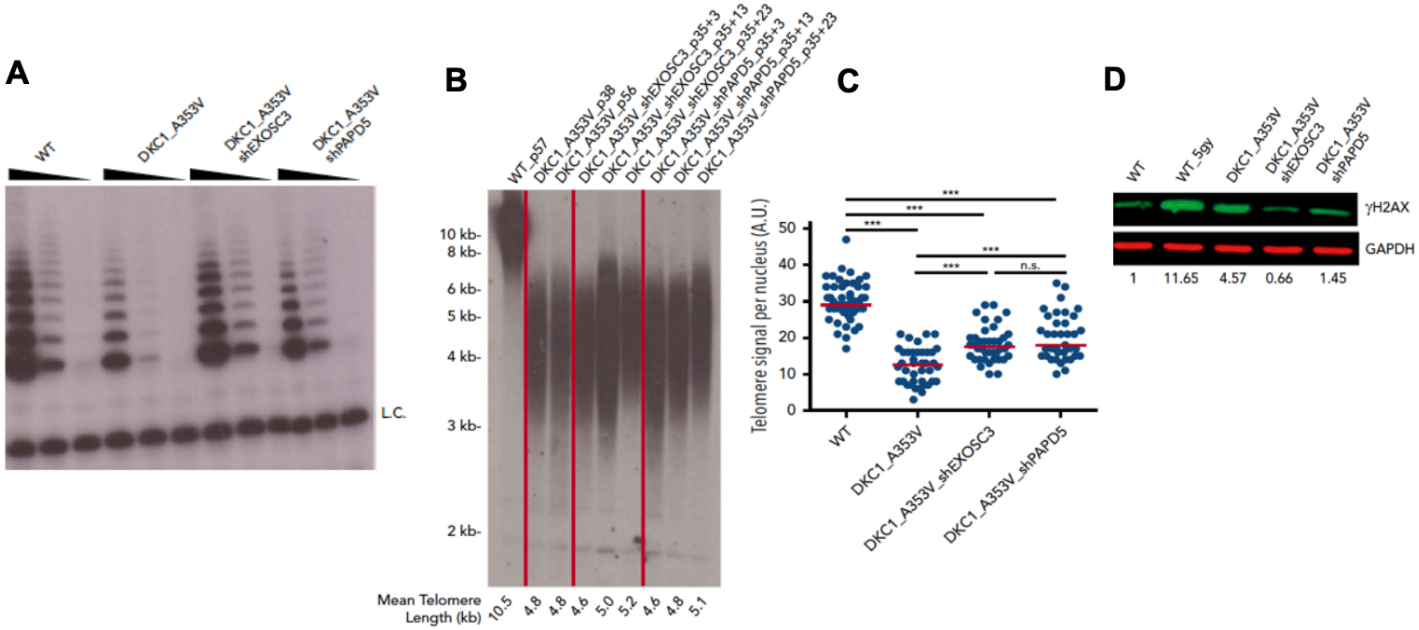

Figure 3: Telomerase levels, telomere length and DNA damage signaling. (**A)** Telomerase activity by TRAP in WT, DKC1_A353V, DKC1_A353V_shEXOSC3, and DKC1_A353V_shPAPD5 hESCs. Range of concentrations represent four-fold serial dilutions. L.C: loading control. **(B)** Telomere length analysis by Telomere 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. **(C)** Quantification of interphase Q-FISH analysis, cells at same passage number as (G). At least 40 nuclei were analyzed in each cell line. **(D)** 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. GAPDH is shown as a loading control. Numbers indicate band intensity relative to GAPDH.

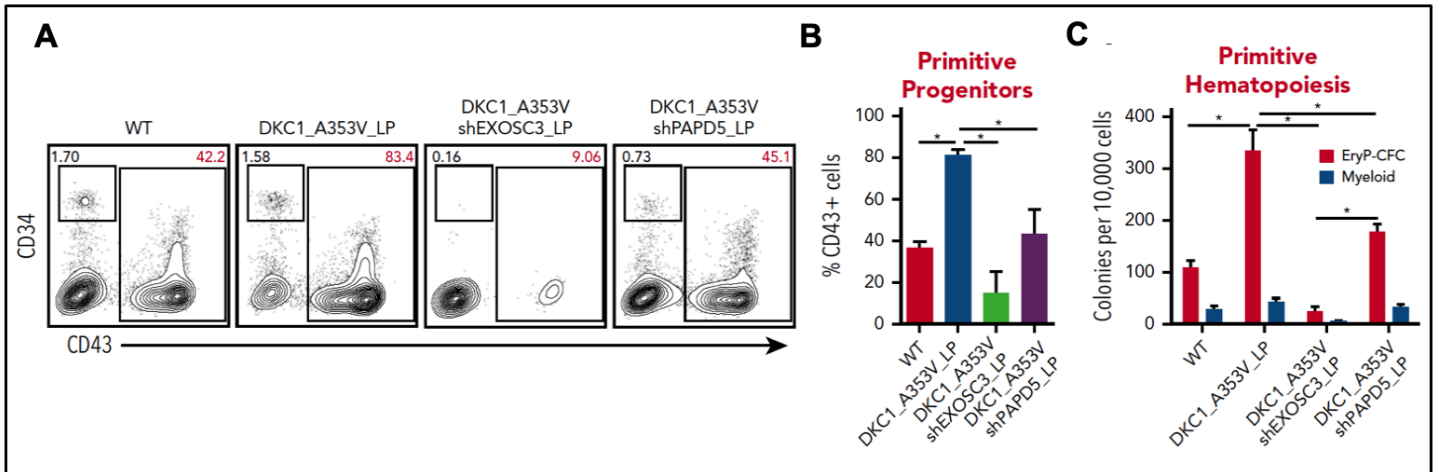

Figure 4: Primitive hematopoietic differentiation. (A) Representative flow cytometric analysis of CD34 and CD43 expression on Day 11 of differentiation, following IWP2 treatment in WT, DKC1_A353V, DKC1_A353V_shEXOSC3, and DKC1_A353V_shPAPD5 cells. **(B)** Quantification of $CD43^{+}$ population obtained from Day 11 differentiation cultures treated with IWP2. **(C)** Primitive CFC potential in Day 11 differentiation cultures.

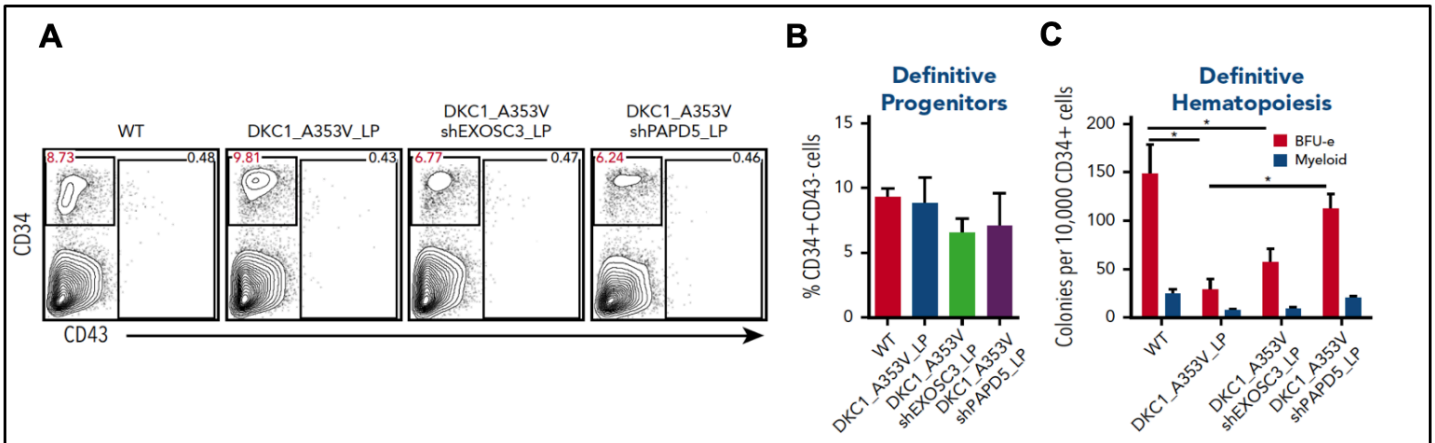

Figure 5: Definitive hematopoietic differentiation (A) Representative flow cytometric analysis of CD34 and CD43 expression on Day 8 of definitive differentiation, following CHIR99021 and SB-431542 treatment in WT, DKC1_A353V, DKC1_A353V_shEXOSC3, and DKC1_A353V_shPAPD5 cells. **(B)** Quantification of $CD34^{+}CD43^{-}$ population obtained from Day 8 differentiation cultures treated with CHIR99021 and SB-431542. **(C)** CFC potential of definitive hematopoietic progenitors.

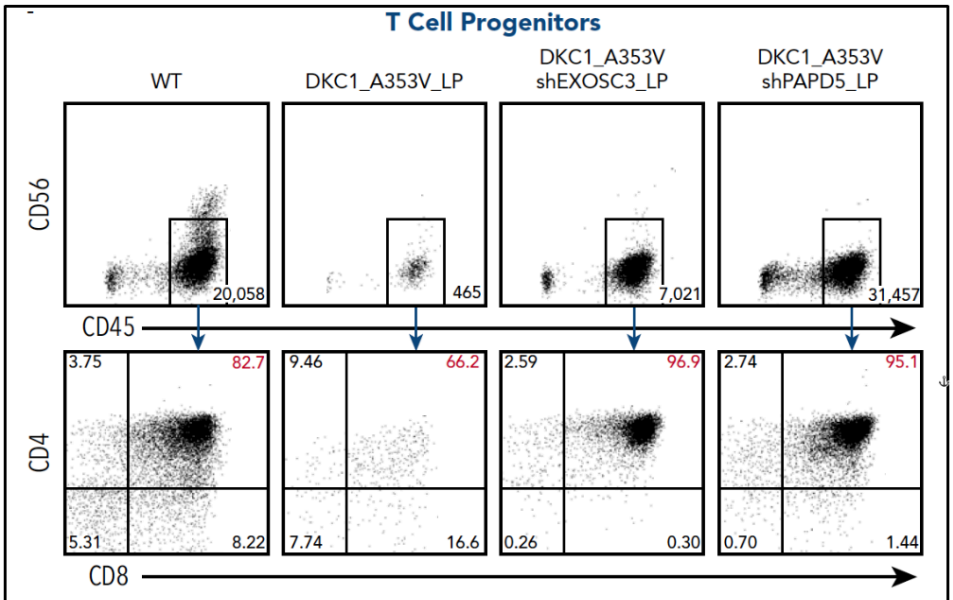

Figure 6: T-cell generation. T-cell potential of CD34^{+}CD43^{-} populations derived from WT, DKC1_A353V, DKC1_A353V_shEXOSC3, and DKC1_A353V_shPAPD5, obtained following CHIR99021 and SB-431542 treatment.

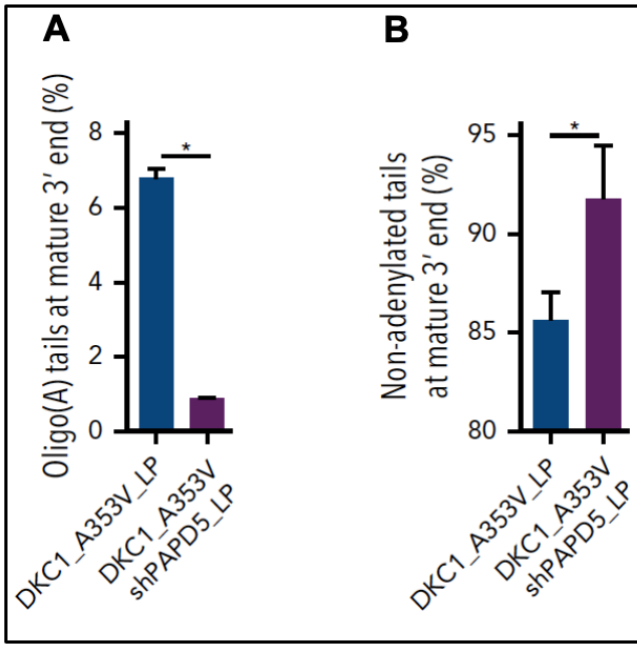

Figure 7: TERC oligoadenylation in CD34+ cells. (A) Relative abundance of oligoadenylated reads at mature 3' end of *TERC* in WT, DKC1_A353V, DKC1_A353V_shEXOSC3, and DKC1_A353V_shPAPD5 hESC. *n*=2. **(B)** Relative abundance of non-adenylated reads at mature 3' end of *TERC*. All experiments were

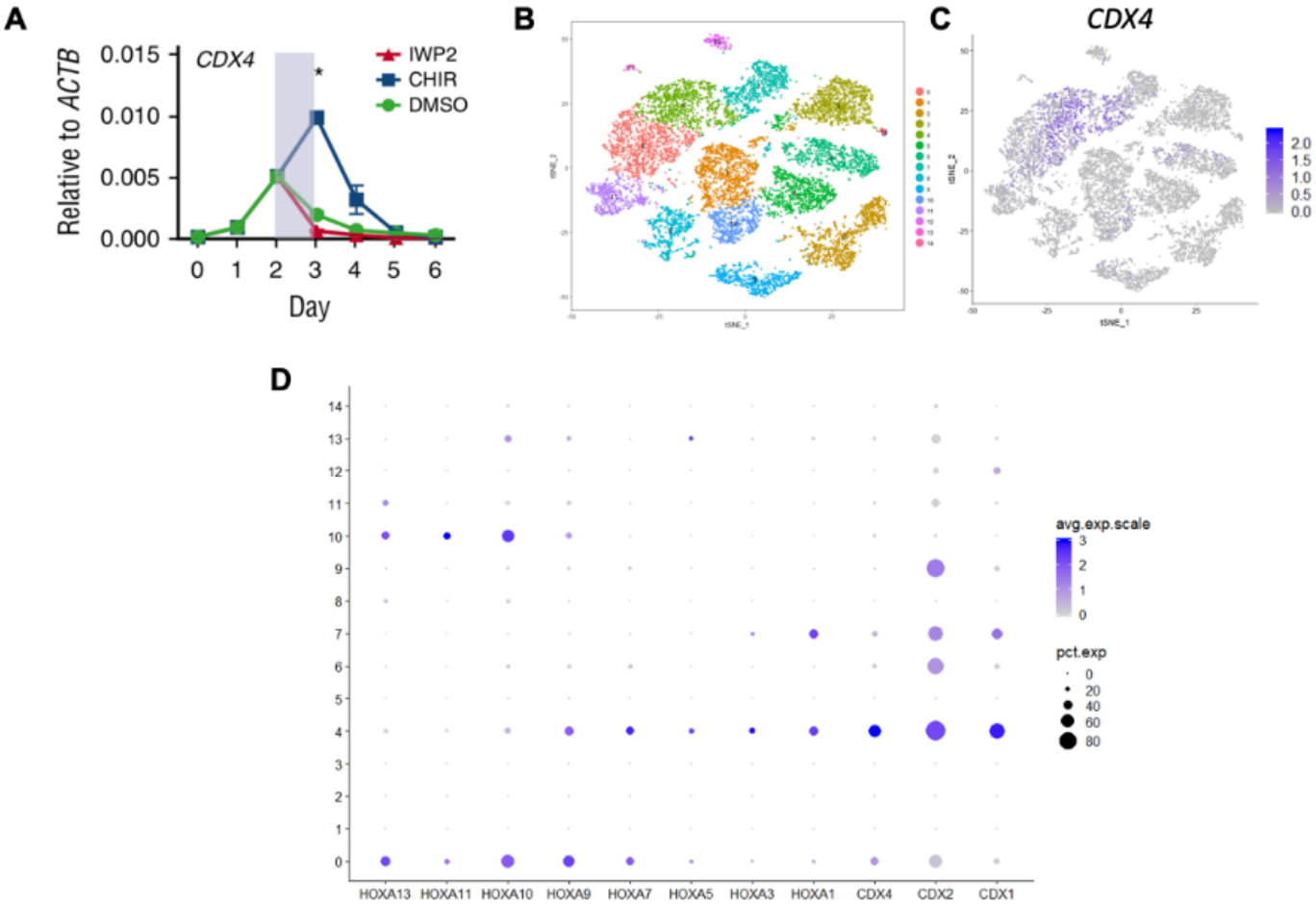

Figure 8: hPSC-derived definitive hematopoietic specification is a relatively inefficient process. (A) qRT-PCR of *CDX4* expression during the specification of hemogenic mesoderm from hPSCs. Shaded in grey is the window of time where WNT signal manipulation is performed to obtain either primitive or definitive hematopoietic specification. Treatment with the WNT agonist CHIR99021 efficiently upregulates *CDX4* within this early mesoderm. Adapted from Creamer et al., 2017. **(B)** tSNE plot generated from single cell RNAseq of Day 3 definitive and primitive specified cultures after filtering out cells with low gene expression. The Seurat R package was used to determine clusters numbered 0-14 as distinct based on differential gene expression. **(C)** Heatmap of *CDX4* expression projected onto the tSNE plot generated by Seurat, where darker blue indicates higher expression by individual cells. Scale is normalized to the highest expressing cells. **(D)** Dot plot of the expression of *HOXA* and *CDX* genes in each of the clusters show in B, where increased physical size of the plot correlates with the percentage of cells expressing the gene in the cluster and darker blue shading indicates a higher level of expression within the cells. Scale is normalized to the highest expressing cells.

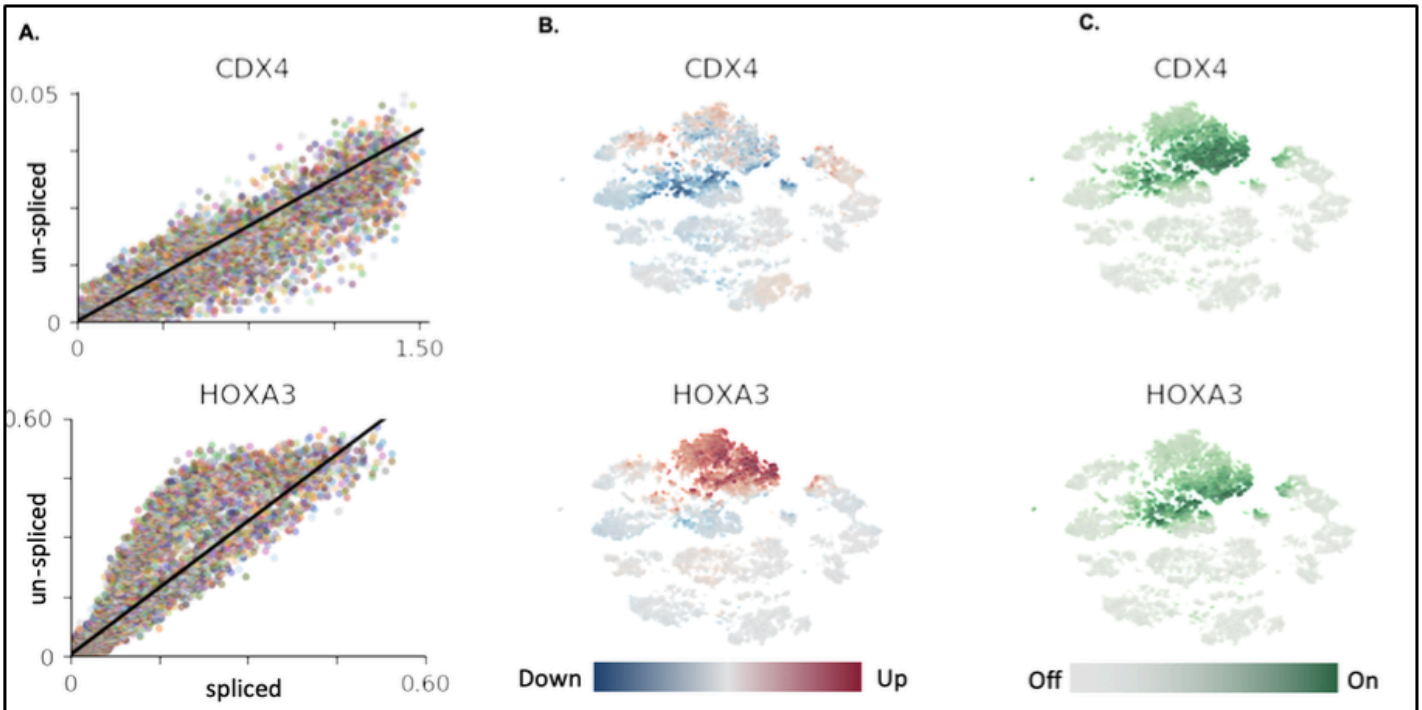

Figure 9: Utilizing RNA velocity in single cell RNAseq to examine *CDX4+* mesoderm. (A) Phase portraits generated by the Velocyto package of *CDX4* and *HOXA3* expression in individual cells, showing the relative distribution of spliced (x-axis) to un-spliced (y-axis) mRNA transcripts detected in each cell after filtering and normalization. Cells above the generated line are considered to be upregulating that particular gene, because of a high proportion of un-spliced transcripts vs spliced transcripts, while cells below the line are downregulating the gene. Scale corresponds to highest and lowest ratio of un-spliced/spliced for each gene **(B)** RNA velocity heatmap projected onto tSNE plot, where red indicates upregulation of the gene, because of a higher proportion of un-spliced to splice transcripts and blue indicates downregulation. **(C)** RNA expression heatmap projected onto tSNE plot where the highest expressing cells for a particular gene are dark green.

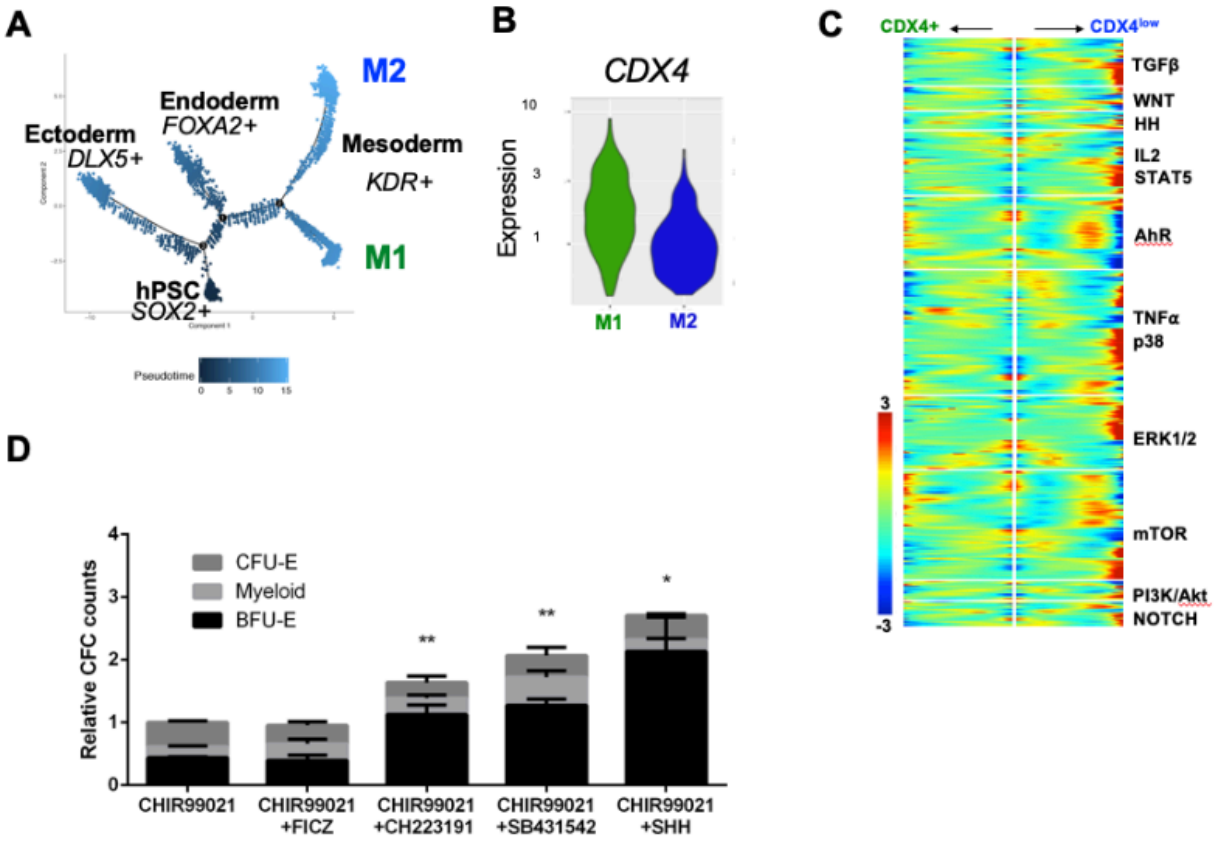

Figure 10: CDX4 expression as a target to enhance definitive hematopoietic specification from hPSCs. (A) Pseudotime analysis of scRNA-seq from unsorted WNTd mesodermal cultures. Expression of germ layer markers in branching populations is consistent with progression of gastrulation. M1: mesoderm branch 1, M2: mesoderm branch 2 **(B)** Expression of CDX4 within each mesodermal branch shows enrichment within M1. **(C)** Heatmap of branchpoint analysis illustrasting gene expression in key signaling pathways before (center) and after (left and right ends) the branching event, demonstrating differential signaing during the patterning of CDX4+ and CDX4- mesoderm. (**D)** Addition of various chemical modulators during mesodermal patterning increases definitive hematopoietic progenitors and/or *CDX4* expression. 5000 CD34+CD184-CD73- hemogenic endothelial cells were cultured with 5000 CD34+CD184+CD73+ arterial endothelial cells sorted at T8 for an additional nine days to undergo the endothelial to hematopoietic transition (EHT) and the resulting floating and adherent cells were plated onto methylcellulose for Colony Forming Cell (CFC) counts. Colony forming unit – erythroid (CFU-E), Myeloid colonies, and burst forming unit – erythroid (BFU-E) were quantified across three biological replicates and normalized to the CHIR99021 sample. Two-way ANOVA statistical analysis was performed across drug treatments, n=3, ** is $p \leq 0.01$, * is $p \leq 0.05$.

e. Opportunities for training and professional development

During this final reporting period we had 4 trainees and 1 staff member who work exclusively on the experiments proposed in this project:

Wilson Fok: 5th year Postdoctoral Fellow (now Research Scientist at Millipore-Sigma)
Alexandre Vessoni: 3rd year Postdoctoral Fellow
Stephanie Luff: 2rd year Postdoctoral Fellow
John Creamer: 4th year PhD candidate
Kendra Sturgeon: Sr. Research Technician, Sturgeon lab

These trainees have participated in several opportunities for training and professional development. They actively participate and have the opportunity to present their data at the following seminar series:

- Division of Hematology Seminar Series: Weekly, (Thursday's at 12pm), students and postdocs from the Divisions of Hematology and Oncology present their recent data to the faculty from those same Divisions.
- "hPSC Work-in-Progress Group": Monthly, members from labs at Washington University who work on human embryonic stem cell biology differentiation meet and present their most recent data in a very informal setting.

Trainees also actively participate in different "journal clubs" where faculty, students and postdocs present papers of interest that were recently published in top tier journals. These include:

- Hematology Division Journal Club: Meets every Tuesday at 12pm and discusses papers on a variety of subjects related to hematopoiesis, stem cell biology, telomere maintenance, and general hematology.
- Hematopoietic Development and Malignancy Program: Meets every Wednesday at 12pm and discusses papers related to hematopoiesis and leukemia.

Trainees are also exposed to the latest research developments around the world, through a series of seminar series at Washington University that bring renowned world-experts to present their recent findings on our campus. These include our own Division of Hematology Seminar series, the Department of Genetics and the Department of Developmental Biology Seminar Series, which run from October through June. Wilson, Alexandre, Stephanie and John have also had the chance to meet personally with various visiting speakers, in an informal setting over lunch.

Finally, both the Batista and Sturgeon groups have their own lab meetings (on Wednesday afternoon and Friday mornings), where trainees present their data and discuss how to improve results and the direction of their projects. In addition, our trainees also have additional training on Responsible Conduct of Research by attending the year-long series provided by our Division, where different faculty members discuss specific topics of relevance, including "Human Experimentation", "Collection, Recording, and Analysis of Data", "Accusation of Fraud", "Student-Mentor Relationships" and "Authorship, Peer Review, and Confidentiality".

f. Dissemination to communities of interest

Results originated from this grant have been disseminated to communities of interest by:

- **Invited talks at different research institutions:**
- Institute of Molecular Medicine (IMM); Lisbon, Portugal (Batista)
- University of California, San Diego (Batista)

- **Presentations at different scientific meetings/conferences:**
- EMBO Meeting on "Telomere Biology in Health and Disease" 2019 (Batista)

- International Society for Stem Cell Research 2019 (Batista)
- American Society of Hematology (Sturgeon)
- International Society of Experimental Hematology (Sturgeon)

5. **CONCLUSION:** Data generated from this DOD award provides 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. 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.

6. **PUBLICATIONS, ABSTRACTS AND PRESENTATIONS**

1) **Journal publications:** Fok WC, Shukla S, Vessoni AT, Brenner KA, Parker R, Sturgeon CM and **Batista LF (2019)**. Posttranscriptional regulation of TERC by PAPD5 inhibition rescues hematopoietic development in dyskeratosis congenita. **Blood**,133(12): 1308-1312.

2) **Books or other non-periodical, one-time publications:** *nothing to report*

3) **Presentations**

- Institute of Molecular Medicine (IMM); (International)
- University of California, San Diego (National)

7. **INVENTIONS, PATENTS AND LICENSES:** nothing to report

8. **REPORTABLE OUTCOMES:** publication attached (Fok W. et al, Blood, 2019)

9. **OTHER ACHIEVMENTS:** nothing to report

10. **REFERENCES**

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Trapnell, C., D. Cacchiarelli, J. Grimsby, P. Pokharel, S. Li, M. Morse, N. J. Lennon, K. J. Livak, T. S. Mikkelsen and J. L. Rinn (2014). "The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells." Nature Biotechnology **32**: 381.

11. APPENDICES: attached publication (Fok et al, 2019)

12. PARTICIPANTS

a. What individuals have worked on the project

a. Luis Batista, PI, 1.86 calendar months
b. Christopher Sturgeon, PI, 1.80 calendar months
c. Kendra Sturgeon, Sr. Research Technician, 1.96 calendar months
d. Ho-Chang Jeong, Post Doc Associate, 6 calendar months

b. Change in active support of the PI

a. Batista

a. **New:** Washington University Micro CDI, PI, 01/01/19 – 12/31/19 (NCE) (supplies only), no overlap
b. **New:** Barnes Jewish Hospital/Siteman Cancer Center, PI, 07/01/19 – 06/30/21, $99,308/yr., no overlap
c. **Completed:** American Federation for Aging Research, PI, 07/01/17 – 06/30/19, $50,000/yr., no overlap
d. **Completed: Mallinckrodt Early Career Development**, PI, 10/01/15 – 06/30/19

b. Sturgeon

a. **New:** American Society of Hematology; PI, 03/01/19 – 02/29/20, $150,000, no overlap
b. **New: NIH R01,** PI, 06/01/19 – 04/30/24, PI, $250,000/yr no overlap
c. **New:** Universal Cells, PI, 11/24/18 - 11/25/19, $200,000, no overlap
d. **Completed:** NIH R01, Co-Investigator, 08/01/17 – 05/31/18
e. **Completed:** Washington University CRM Seed Grant, Co-PI, 02/01/18 – 1/31/19

c. Other organizations involved as partners: *nothing to report*

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Brief Report

HEMATOPOIESIS AND STEM CELLS

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

Wilson Chun Fok,[1] Siddharth Shukla,[2] Alexandre Teixeira Vessoni,[1] Kirsten Ann Brenner,[1] Roy Parker,[2,3] Christopher M. Sturgeon,[1,4,5] and Luis Francisco Zirnberger Batista[1,4,5]

[1]Division of Hematology, Department of Medicine, and [2]Department of Biochemistry, University of Colorado Boulder, Boulder, CO; [3]Howard Hughes Medical Institute, Chevy Chase, MD; and [4]Department of Developmental Biology and [5]Center of Regenerative Medicine, Washington University in St. Louis, St. Louis, MO

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

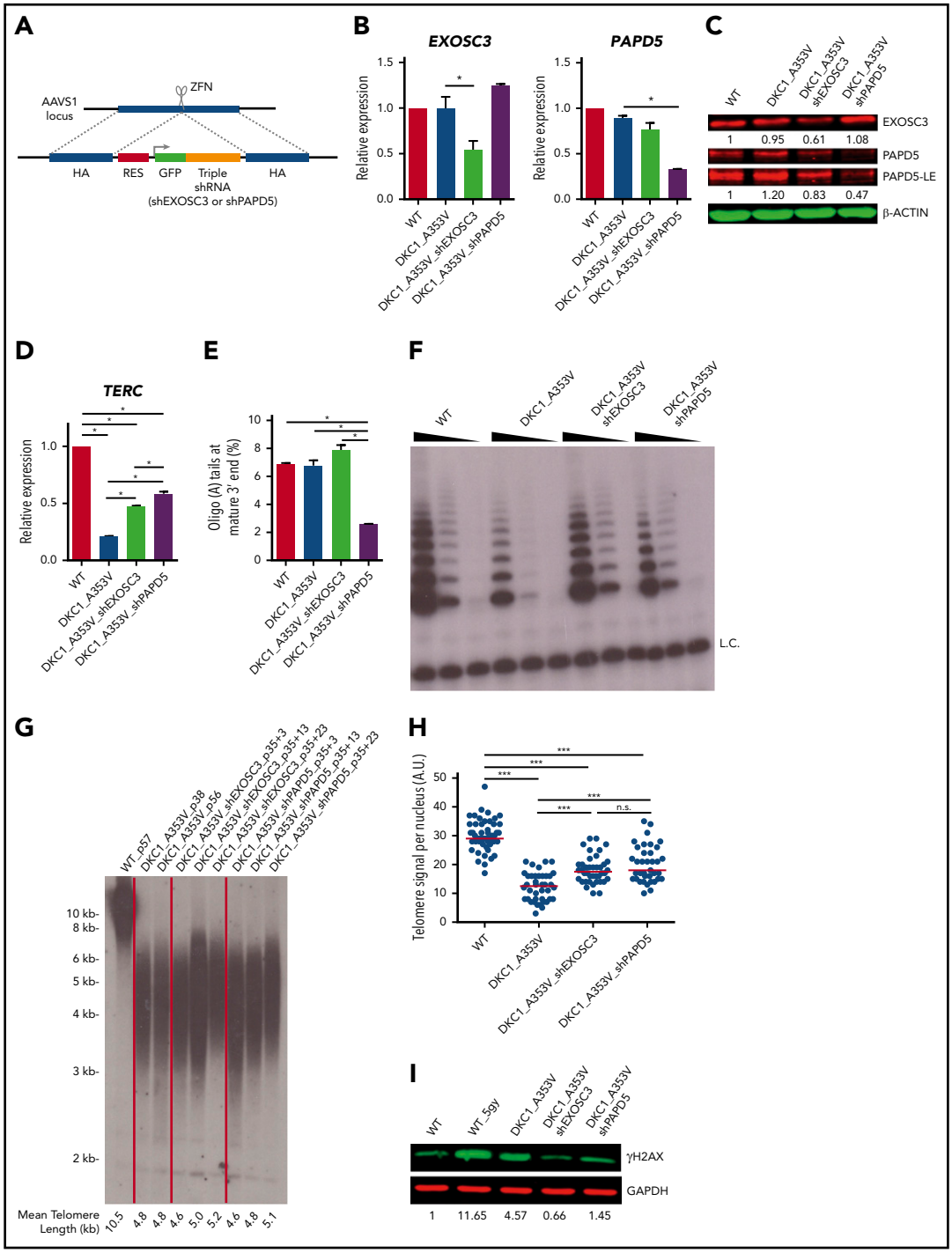

Figure 1. Modulation of *EXOSC3* and *PAPD5* rescue telomere integrity in DKC1_A353V hESCs. (A) Schematic depicting shRNA cassette insertion into the AAVS1 locus of hESCs. shRNA sequences used for each cassette are described in supplemental Methods and supplemental Table 1. HA, homology arm; RES, resistance 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.

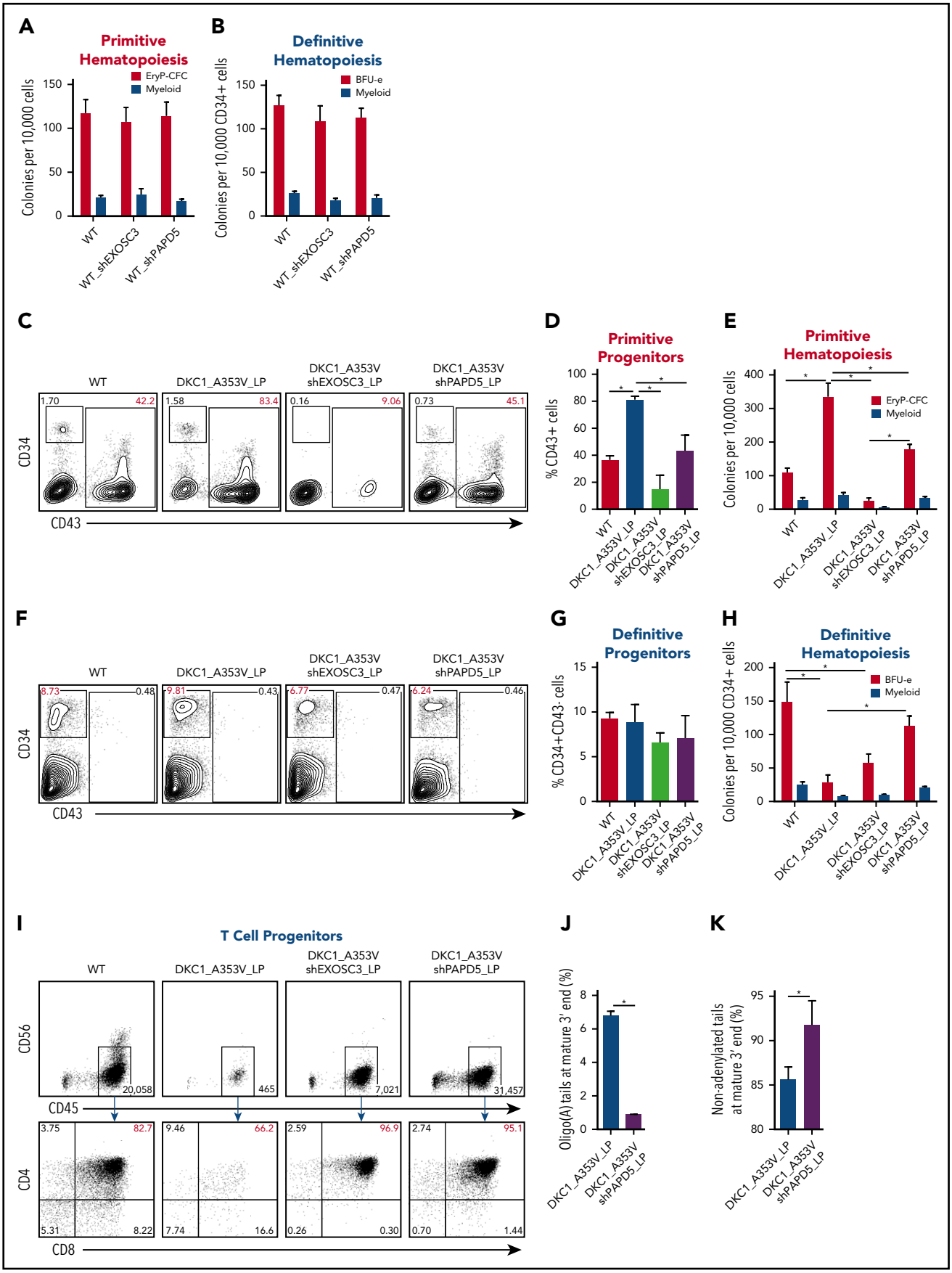

Figure 2.

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

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